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REVIEW

Uncovering the Hidden Credentials of *Brucella* Virulence

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SUMMARY Bacteria in the genus *Brucella* are important human and veterinary pathogens. The abortion and infertility they cause in food animals produce economic hardships in areas where the disease has not been controlled, and human brucellosis is one of the world's most common zoonoses. *Brucella* strains have also been isolated from wildlife, but we know much less about the pathobiology and epidemiology of these infections than we do about brucellosis in domestic animals. The brucellae maintain predominantly an intracellular lifestyle in their mammalian hosts, and their ability to subvert the host immune response and survive and replicate in macrophages and placental trophoblasts underlies their success as pathogens. We are just beginning to understand how these bacteria evolved from a progenitor alphaproteobacterium with an environmental niche and diverged to become highly host-adapted and host-specific pathogens. Two important virulence determinants played critical roles in this evolution: (i) a type IV secretion system that secretes effector molecules into the host cell cytoplasm that direct the intracellular trafficking of the brucellae and modulate host immune responses and

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(ii) a lipopolysaccharide moiety which poorly stimulates host inflammatory responses. This review highlights what we presently know about how these and other virulence determinants contribute to *Brucella* pathogenesis. Gaining a better understanding of how the brucellae produce disease will provide us with information that can be used to design better strategies for preventing brucellosis in animals and for preventing and treating this disease in humans.

KEYWORDS *Brucella*, pathogenesis, virulence determinants

INTRODUCTION

Members of the bacterial genus *Brucella* colonize a variety of mammals (1, 2). They have also been found in reptiles (3, 4) and fish (5, 6). There are currently 12 recognized *Brucella* species (https://www.bacterio.net/brucella.html), and although these bacteria are highly related at the genetic level, they display a remarkable degree of host specificity (Fig. 1). Comparative genomic analyses have provided us with a fascinating picture of how these bacteria evolved from an ancestral alphaproteobacterium to become host-adapted pathogens, and several excellent reviews describe this evolutionary path (7–11).

The best characterized *Brucella* species, *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, and *B. ovis*, cause abortion and infertility in goats and sheep, cattle, pigs, dogs, and sheep, respectively. These strains have long been recognized as economically important pathogens by the agricultural and veterinary communities (1, 2). *B. melitensis*, *B. abortus*, *B. suis*, and *B. canis* can also cause a chronic debilitating febrile illness in humans exposed to infected animals or animal products (12), and human brucellosis is one of the world's leading zoonotic diseases (13, 14). *B. melitensis*, *B. suis*, and *B. abortus* are also of considerable concern from a biodefense perspective, because they have biological characteristics that make them attractive for use as biowarfare or bioterrorism agents (15).

The *Brucella* strains that infect domestic animals are highly host adapted and do not survive for prolonged periods in the external environment (1, 2). The infections caused by these strains also have distinctive characteristics. One is the highly infectious nature of these bacteria for their natural hosts and their high degree of host specificity (2, 16, 17). Another is the chronic nature of these infections, which in some cases can be lifelong. The remarkable capacity of the brucellae to survive and replicate for prolonged periods in host macrophages underlies this persistence (18). *Brucella* infections predominantly cause abortion and infertility in their natural hosts, where these bacteria replicate prolifically in association with placental trophoblasts (Fig. 2). Exposure to aborted fetuses and vaginal secretions serve as major routes of transmission, and venereal transmission is also important in some of these hosts. Offspring can also be infected *in utero* or by consumption of contaminated milk. Although some *Brucella* strains are highly infectious for humans, humans are an incidental host, and human-to-human transmission is extremely rare (19).

Wildlife also serve as natural hosts for *Brucella* strains (Fig. 1), but we know much less about the interactions of these strains with their hosts than we do about the *Brucella* strains that infect domestic animals. *Brucella ceti*, *B. microti*, *B. papionis*, and the unnamed *Brucella* species in the so-called BO2 clade appear to be overt pathogens (3, 4, 20–24), for instance, but whether *B. neotomae*, *B. pinnipedialis*, or *B. vulpis* cause disease in their natural hosts is presently unclear (25–27). *B. ceti*, *B. neotomae*, and BO2 strains including *B. inopinata* (28–35) have also been isolated from human infections, which suggests that wildlife represent a potential zoonotic source of human brucellosis.

Over the past century, the importance of the *Brucella* spp. as veterinary and human pathogens has prompted a considerable amount of research aimed at determining how these bacteria produce disease. The implementation of molecular biology-based approaches in the 1980s led to an acceleration of these efforts (36), and we now have almost 800 complete genome sequences of *Brucella* strains from a wide range of hosts

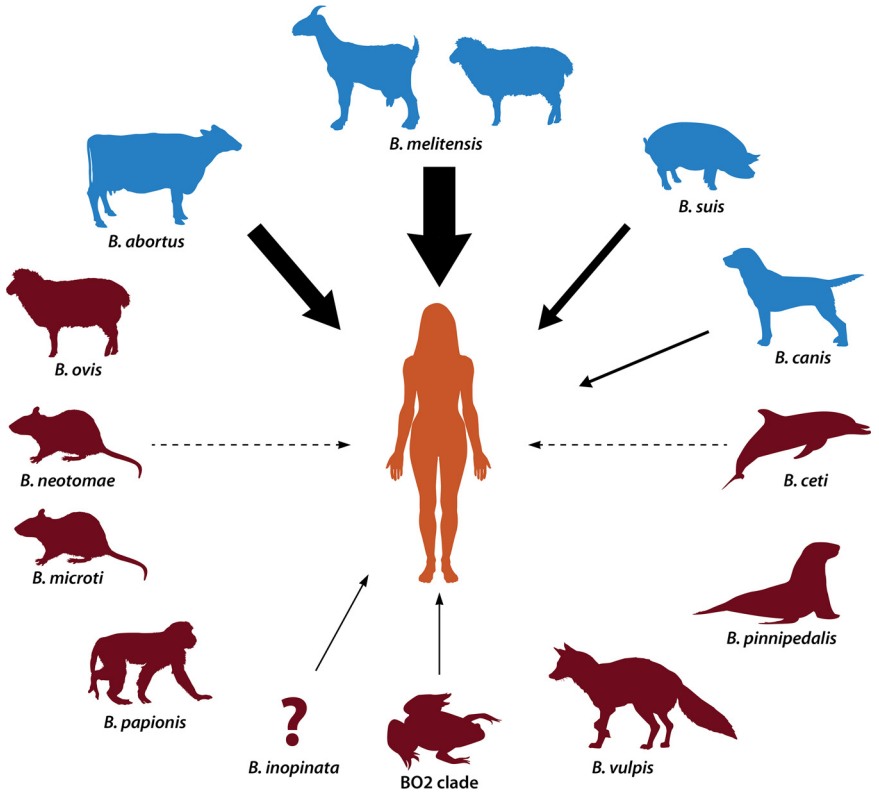

FIG 1 Natural hosts and zoonotic potential of *Brucella* strains. The *Brucella* species shown in blue are recognized zoonotic pathogens, and the thickness of the solid arrows represents the relative frequency with which these hosts serve as sources of human infection. The dashed arrows indicate that these *Brucella* strains have been isolated from human disease but direct transmission from the corresponding natural host to humans has not been documented. The question mark indicates that the natural host for *B. inopinata* is unknown.

available for study. One thing that has become clear as we have examined the molecular basis of *Brucella* pathogenesis is that these bacteria have followed an evolutionary pathway to virulence that is different from the pathways followed by other well-characterized mammalian pathogens (8, 9, 37–39). Ignacio Moriyón and Edgardo Moreno articulated this unique evolutionary pathway very well in a commentary entitled "*Brucella melitensis*: a nasty bug with hidden credentials for virulence" (40) that accompanied the paper describing the completion of the *B. melitensis* 16M genome sequence

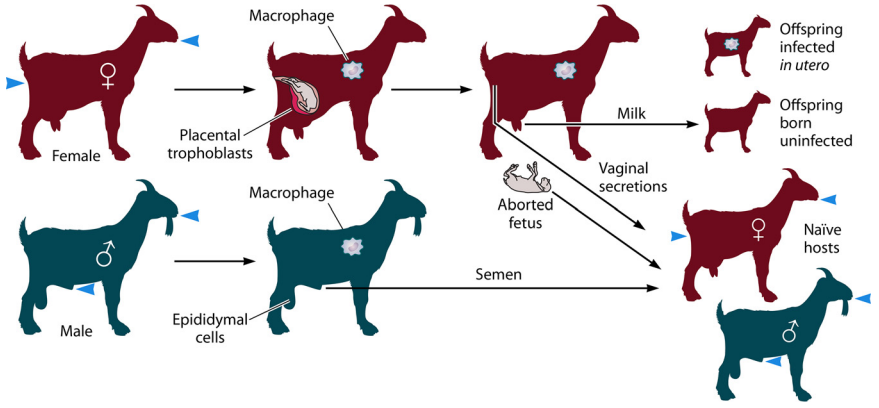

FIG 2 Natural disease cycle of brucellosis in domestic animals. The blue triangles denote the oral, nasal, and venereal routes of infection.

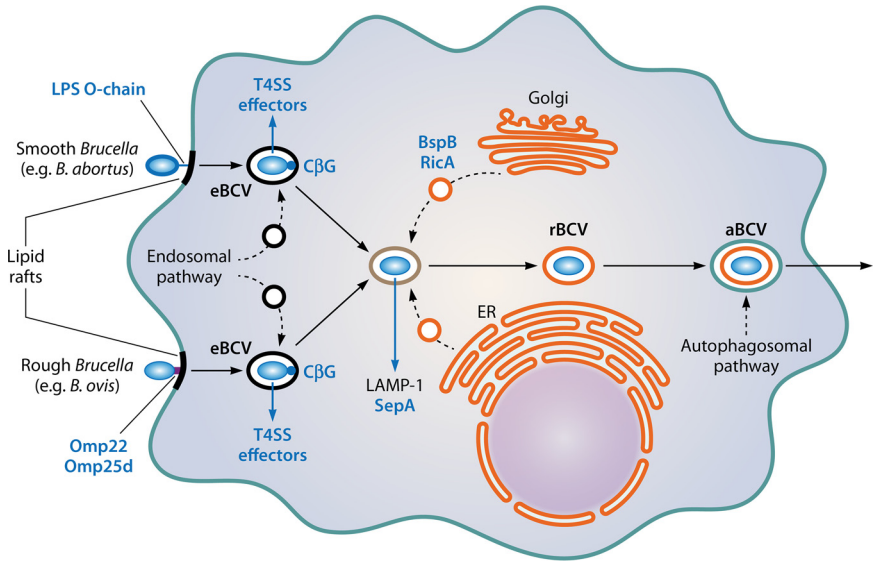

FIG 3 Contributions of the T4SS effectors, the LPS O-chain, Omp22, Omp25d, and cyclic β-1,2-D-glucan (CβG) to the development of the replicative *Brucella*-containing vacuole in host macrophages. The empty black and orange circles represent membrane vesicles trafficking from the endolysosomal pathway, endoplasmic reticulum, and Golgi apparatus to the *Brucella*-containing vacuoles (BCVs). The change in the colors of the BCV membranes represents their change in composition as they transition from eBCVs to rBCVs. The outermost blue membrane of the aBCV represents engulfment of the rBCV by the host cell autophagosomal pathway. eBCV, endosomal BCV; rBCV, replicative BCV; aBCV, autophagosomal BCV.

in 2002 (41). The intent of this review is to inform the reader of the progress that has been made over the past 2 decades toward uncovering some of these "hidden credentials" of *Brucella* virulence.

VIRULENCE DETERMINANTS ACTING AT THE HOST-PATHOGEN INTERFACE

Type IV Secretion System

The most extensively studied *Brucella* virulence determinant has been their type IV secretion system (T4SS) (42). The *Brucella* T4SS consists of 11 proteins, 8 of which make up the core of the transporter (VirB2, VirB3, and VirB5 through VirB10), 2 ATPases (VirB4 and VirB11) that provide energy to drive effector secretion, and a lytic transglycosylase (VirB1) that remodels the bacterial cell peptidoglycan layer during T4SS assembly (43–52). The genes encoding the T4SS reside in an operon (42, 53) that is conserved across *Brucella* strains, and *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, *B. ovis*, *B. microti,* and *B. neotomae virB* mutants are highly attenuated in cultured mammalian cells and experimental and natural hosts (42, 53–75). *Brucella* proteins that assist in the assembly and function of the T4SS are also encoded by genes residing outside the *virB* operon. The secretor activator gene A (SagA) protein, for instance, is a muramidase that is thought to play an important role in remodeling the peptidoglycan layer during assembly of the T4SS (76). The precise function of VirJ is unknown, but this periplasmic protein interacts directly with T4SS substrates that have a periplasmic intermediate during their export and is also required for proper assembly of the T4SS (77). *B. abortus sagA* and *virJ* mutants both display significant attenuation in cultured mammalian cells, but only the *virJ* mutant has been shown to be attenuated in the mouse model (76, 77).

The brucellae maintain predominantly an intracellular residence in their mammalian hosts (18), and one of the primary functions of the T4SS is to control the intracellular trafficking of the *Brucella*-containing vacuoles in host macrophages so that these bacteria avoid killing and degradation in phagolysosomes (56–59). Upon entry into host cells, the brucellae reside in acidified phagosomal compartments known as endosomal

TABLE 1 *Brucella* T4SS effectors and their proposed functions

Effector	ORF[a]	Proposed function	Reference(s)
VceA	BAB1_1652	Unknown	81
VceC	BAB1_1058	Modulation of host immune response	81, 95–97
RicA	BAB1_1279	Intracellular trafficking of BCVs	82, 89
BPE005	BAB1_2005	Modulation of collagen deposition by hepatocytes	83, 106, 107
BPE043	BAB1_1043	Unknown	83
BPE123	BAB2_0123	Modulation of host cell enolase activity	83, 108
BPE275	BAB1_1275	Unknown	83
BtpA	BAB1_0279	Modulation of host immune response, stabilization of host cell microtubules, depletion of host cell NAD	84, 98–100, 102–105
BtpB	BAB1_0756	Modulation of host immune response, stabilization of host cell microtubules, depletion of host cell NAD	84, 103, 105
BspA	BAB1_0678	Unknown	85
BspB	BAB1_0712	Intracellular trafficking of BCVs	85, 87, 89
BspC	BAB1_0847	Unknown	85
BspE	BAB1_1675	Unknown	85
BspF	BAB1_1948	Unknown	85
BspJ	BAB2_0119	Inhibition of host cell apoptosis	85, 110
SepA	BAB1_1492	Intracellular trafficking of BCVs	86

[a]Open reading frame (ORF) designation in *B. abortus* 2308 genome sequence.

Brucella-containing vacuoles (eBCVs), which undergo limited interaction with the lysosomal pathway (78, 79). The intracellular brucellae do not replicate in the eBCVs, but the acidic pH of these compartments serves as a signal for the induction of the genes encoding the T4SS (80). T4SS-mediated secretion of effectors into the host cell then orchestrates a series of events that redirects the intracellular trafficking of the eBCVs, diminishing their interactions with the endolysosomal pathway and initiating their extensive interaction with the host cell endoplasmic reticulum (ER) (Fig. 3). This series of events leads to the formation of the so-called replicative BCVs (rBCVs), which are slightly acidic/neutral pH compartments where the intracellular brucellae sustain their chronic intracellular persistence in the host (78). These rBCVs eventually interact with components of the host cell's autophagy pathway, resulting in the formation of autophagic BCVs (aBCVs), which are thought be important for bacterial egress and cell-to-cell spread in the host (79).

The recent identification and characterization of several T4SS effectors have helped us begin to understand how the T4SS controls the intracellular trafficking of *Brucella* strains (81–86) (Table 1). BspB, for example, directly interacts with the conserved oligomeric Golgi (COG) complex in host cells (87). This multimeric protein complex controls vesicle trafficking between the Golgi apparatus and the ER, and the interaction of BpsB with the COG complex redirects Golgi apparatus-derived vesicles to the rBCV. The T4SS effector RicA directly interacts with Rab2, a small GTPase that also modulates ER-Golgi apparatus interactions in mammalian cells (82, 88). The exact mechanism by which RicA contributes to rBCV development is unclear, but it has recently been shown that the BCV traffic-altering function of BspB is totally dependent upon the activity of RicA (89). Another T4SS effector, SepA, plays a role in exclusion of the lysosomal marker LAMP-1 from eBCVs during their conversion to rBCVs (86), but the host cell target of this effector has not been identified.

Another way that the *Brucella* T4SS contributes to virulence is by modulating the host immune response (90–94) (Fig. 4 and 5). The T4SS effector VceC, for instance, interacts with the host cell ER chaperone BiP (95). This interaction causes ER stress and induces an unfolded protein response (UPR) in *Brucella*-infected cells, which stimulates production of the inflammatory cytokines interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α). VceC-induced production of these cytokines by macrophages induces granuloma formation, which facilitates chronic infection. VceC-mediated inflammatory cytokine production by placental trophoblasts also leads to host cell death and fetal pathology in the pregnant mouse model, which has led investigators to postulate that this T4SS effector may play an important role in transmission in natural hosts (96, 97).

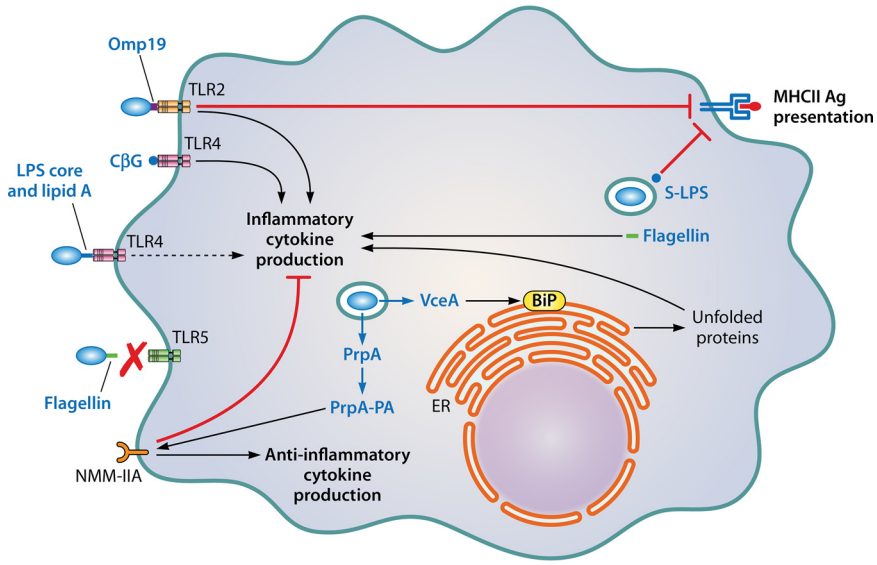

FIG 4 *Brucella* virulence determinants that influence the capacity of macrophages to modulate the host immune response. →, activation; ⊣, inhibition. The dashed arrow indicates that the *Brucella* LPS does not signal strongly through the TLR4 pathway and stimulates a diminished inflammatory response. The red X indicates that the *Brucella* flagellin is not recognized by TLR5.

Unlike VceC, the T4SS effectors BtpA and BtpB inhibit inflammatory cytokine production by dendritic cells by interfering with the TLR-Myd88-MAL signaling pathway (84, 98–100). This ability of the T4SS effectors to both stimulate and inhibit host immune responses has been proposed to allow the brucellae to stimulate enough of a response to benefit their long-term intracellular persistence in the host and sufficient immunopathology to facilitate their spread to other hosts but not elicit a strong enough immune response in the host to result in sterilizing immunity and resolution of the infection (101).

Evidence suggests that the *Brucella* T4SS effectors may also contribute to virulence in other ways. BtpA and BtpB, for instance, stabilize microtubules, which likely has impacts on host cell physiology beyond their ability to directly interfere with Myd88-

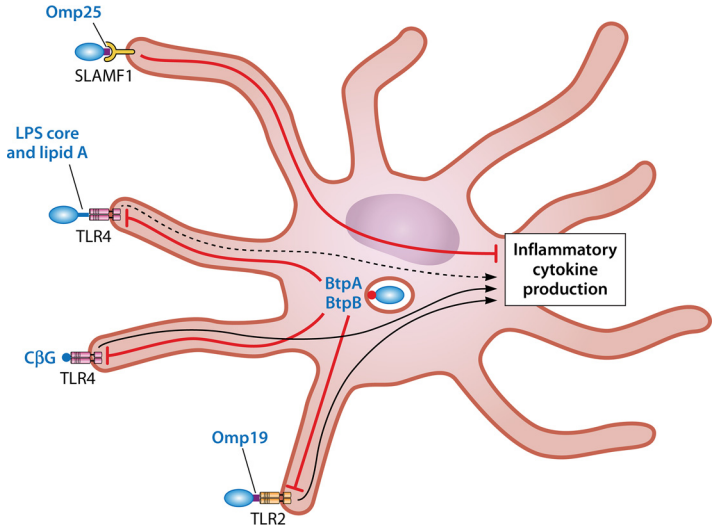

FIG 5 *Brucella* virulence determinants that impact the ability of dendritic cells to modulate the host immune response. →, activation; ⊣, inhibition. The dashed arrow indicates that the *Brucella* LPS does not signal strongly through the TLR4 pathway and stimulates a diminished inflammatory response.

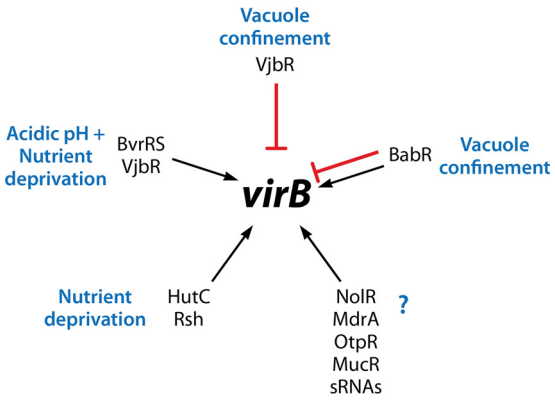

FIG 6 Genetic regulators and the corresponding stimuli that control expression of the genes encoding the T4SS in *Brucella*. →, activation; ⊣, repression. The question mark indicates that the environmental stimuli recognized by these regulators have not been determined. Both activation and repression of *virB* expression have been reported for BabR.

MAL signaling (102, 103), and BtpA induces the unfolded protein response in infected macrophages by an undefined mechanism (104). BtpA and BtpB have recently been shown to have NAD^+ hydrolase activities that allow the brucellae to reduce NAD^+ levels in host cells during infection (105). Because NAD^+ plays such a broad-based role in the physiology of eukaryotic cells, the NAD^+ hydrolase activities of BtpA and BtpB may help explain the multiple contributions that these T4SS effectors appear to be making to *Brucella* virulence. Studies with a human hepatic cell line and mice experimentally infected with *B. abortus* 2308 have also established a link between the T4SS effector BPE005 and collagen deposition and liver fibrosis (106, 107). In addition, the T4SS effector BPE123 directly interacts with the host cell glycolytic enzyme α-enolase during HeLa cell infection and stimulates its activity (108). Normal levels of host cell α-enolase are required for the wild-type replication of *B. abortus* 2308 in HeLa cells. This raises the intriguing possibility that by modulating the activity of a host cell enzyme involved in carbohydrate metabolism, the intracellular brucellae may be able to improve their access to glucose. Experimental evidence suggests that glucose is the preferred carbon source for these bacteria when they reside in alternatively activated macrophages during the chronic stage of infection in mice (109). Finally, recent studies suggest that BspJ may inhibit host cell apoptosis by directly interacting with NME/NM23 nucleoside diphosphate kinase 2 and creatine kinase B in the nucleus of these cells (110).

The genes encoding the T4SS are tightly regulated (111, 112), and proper temporal and stochiometric expression of these genes is essential for virulence (113, 114). The same is presumably true for the T4SS effectors, although the expression patterns of the individual genes encoding these effectors have not been as well characterized as those of the *virB* genes. Strict temporal regulation of the genes encoding the T4SS and its effectors makes sense, considering the orderly progression of the intracellular life cycle of the brucellae in host cells (78, 79) and the observation that the T4SS effectors contribute to the virulence of *Brucella* strains by means other than directing their intracellular trafficking. Not surprisingly, multiple regulators control *virB* expression in *Brucella* (115) (Fig. 6). The two-component regulator BvrRS, for instance, allows the brucellae to recognize the acidic pH and nutrient deprivation they encounter in the eBCV and to activate expression of the gene encoding the quorum-sensing (QS) regulator VjbR, which in turn activates *virB* expression (112). The transcriptional regulator HutC and stringent response regulator Rsh are also thought to allow *Brucella* strains to modulate *virB* expression in response to their nutritional status (116, 117). Other regulators, including MdrA (118), BabR (119–122), LovhK (123), MucR (124, 125), NolR (126), OtpR (127), and small regulatory RNAs (sRNAs) (122, 128), have been implicated in regulating *virB* expression in *Brucella*, but precisely how these regulators contribute to

coordinating the proper expression of these genes and the environmental stimuli to which they are responsive remains to be determined. There is also evidence that the individual contributions of specific regulators to *virB* regulation may differ between the *Brucella* species (129). *B. ovis*, for example, does not require acidic conditions to elicit expression of its *virB* operon (70).

Lipopolysaccharide

Like most Gram-negative bacteria, *Brucella* strains produce a lipopolysaccharide (LPS) molecule that plays an important role in maintaining the integrity of their cell envelope (130, 131). With two notable exceptions, they have a smooth LPS (S-LPS) consisting of a polysaccharide O-chain, core, and lipid A. The exceptions are *B. ovis* and *B. canis,* which naturally produce a rough LPS lacking the O-chain. The importance of the O-chain for the virulence of naturally occurring smooth *Brucella* strains is well documented (132–145). One way that the LPS O-chain contributes to virulence is by protecting smooth *Brucella* strains from the bactericidal activities of complement (139, 143, 146) and the antimicrobial peptides they encounter during their interactions with host phagocytes (147, 148). Another is by serving as an adhesin (Fig. 3). The interaction of the O-chain with lipid rafts on the surface of mammalian cells allows smooth *Brucella* strains to enter these cells by an endocytic pathway that avoids extensive fusion of the BCVs with lysosomes (149). This route of entry is the first required step for the formation of the replicative BCV by smooth strains that occurs prior to the participation of the T4SS effectors (150, 151).

O-chain-mediated uptake of smooth *Brucella* strains also plays an important role in immune evasion, because this route of entry stimulates low levels of proinflammatory cytokine production in macrophages and dendritic cells (152, 153) (Fig. 4 and 5). Smooth LPS shed by *Brucella* strains into BCVs is also resistant to degradation by macrophages (154) and forms complexes with major histocompatibility complex class II (MHC-II), inhibiting the capacity of these phagocytes to present antigens to T lymphocytes (155, 156) (Fig. 4). Still another way that O-chain-mediated entry of smooth *Brucella* strains into macrophages contributes to virulence is by inhibiting caspase 2-mediated apoptosis in these phagocytes (157–161). The precise mechanisms behind this inhibition have not been determined, but the capacity of smooth strains to extend the life span of macrophages likely enhances their ability to avoid immune clearance and disseminate to different organs in their mammalian hosts.

Lipid A is often referred to as "endotoxin" because it is the component of LPS that is recognized by the host pattern recognition receptor Toll-like receptor 4 (TLR4), and the lipid A's of many Gram-negative bacteria induce strong inflammatory responses (162). It has long been known that *Brucella* strains produce an LPS with low endotoxin activity (163–167). One reason that the lipid A of *Brucella* strains does not stimulate a strong inflammatory response is that unlike its enteric counterparts, the *Brucella* lipid A contains very-long-chain fatty acids (VLCFAs) (168, 169). These VLCFAs presumably prevent the *Brucella* lipid A from forming the same strong interactions that other bacterial lipid A molecules form with TLR4 (170) (Fig. 4 and 5). This poor recognition of the *Brucella* lipid A by the innate immune response plays a critical role in the so-called stealthy mode of virulence employed by these bacteria (167). Interestingly, the *Brucella* lipid A also induces premature cell death in human neutrophils (171). Dead neutrophils carrying intracellular brucellae are subsequently engulfed by macrophages and dendritic cells via mechanisms that do not stimulate an inflammatory response. This has been proposed to be yet another strategy that *Brucella* strains can employ to avoid detection by the host immune system during the early stages of infection.

In many bacteria, the LPS core serves primarily as a structural link between the O-chain and the lipid A. It also performs this function in *Brucella* (142), but in addition, recent studies have shown that the LPS core plays an essential role in allowing these bacteria to evade detection by the host immune response (172–175). Specifically, the brucellae produce a core structure containing a lateral oligosaccharide side chain that sterically shields the lipid A and inhibits its binding to TLR4 on host macrophages and

dendritic cells (Fig. 4 and 5). This lateral side chain and the positive charge it imparts on the LPS core also appear to be important for the resistance of both smooth and rough *Brucella* strains to killing by complement and bactericidal peptides (172, 173, 175, 176).

The essential role that the LPS O-chain plays in the virulence of smooth *Brucella* strains raises the intriguing question of why the naturally occurring rough strains are such virulent pathogens in their natural hosts. *Brucella ovis*, for instance, is a major veterinary pathogen in sheep-producing countries, where it causes epididymitis and infertility in rams (177, 178). *Brucella canis* causes abortion and infertility in dogs, and *B. canis* infections can have a severe impact on commercial breeding operations (179). *B. canis* is also being increasingly recognized as a source of human infection (180). A considerable amount of work has been done over the past 20 years to address how the naturally occurring rough *Brucella* species compensate for the lack of an LPS O-chain. One particularly informative finding is that despite their lack of an O-chain, *B. ovis* and *B. canis* strains are almost as resistant to complement as smooth *Brucella* strains (181). The pattern of expression of the Omp25/Omp31 proteins (which will be discussed later) in the outer membrane of *B. ovis* and *B. canis* is different than that observed in smooth strains (182), and experimental evidence suggests that it is this distinctive Omp25/Omp31 composition that makes the rough strains resistant to complement and other antimicrobial peptides (183). Unlike rough mutants derived from naturally occurring smooth strains, *B. ovis* and *B. canis* strains are also fully competent for intracellular replication in macrophages (70, 184, 185), and both establish and maintain chronic spleen infections in experimentally infected mice (70, 183, 186–188). One observation that might explain this difference is that *B. ovis* and *B. canis* employ a lipid raft-mediated route of entry into macrophages that follows a different endocytic pathway than smooth strains (189) (Fig. 3), and this unique route of entry appears to be important for allowing the BCVs containing *B. canis* and *B. ovis* strains to avoid fusion with lysosomes (150, 190). This alternative pathway of entry into macrophages may also explain why *B. ovis* and *B. canis* strains induce limited and ineffective inflammatory responses in their natural hosts (71, 191–193).

The organization and composition of the LPS biosynthesis genes are highly conserved across the *Brucella* species that infect domestic animals and many of the strains that infect wildlife, and the absence of the O-chain from the *B. ovis* and *B. canis* LPS is due to well-described genomic deletions (194–197). But a subset of the so-called early diverging *Brucella* strains found in amphibians and isolated from human disease employ an operon consisting of four genes, *rmlABCD*, to produce an LPS with a rhamnose-based O-chain instead of the perosamine O-side chain that is found universally in other smooth *Brucella* strains (198). Based on the documented role of the perosamine O-chain in virulence, it has been postulated that acquisition of the genes encoding this latter type of LPS O-chain played an important role in the evolution of *Brucella* strains as mammalian pathogens (7, 199). Interestingly, serologic studies suggest that the LPS core of some of the early diverging strains may also differ from that of the classical *Brucella* strains (200).

A couple of gene products that play auxiliary roles in LPS production and are important for *Brucella* virulence have also been identified. *B. abortus ba14k* (201) and *romA* (202) mutants produce LPS with altered levels of O-chain, and both mutants display significant attenuation in mice. RomA is a periplasmic protein thought to stabilize the components of the LPS biosynthetic complex. BA14K is lectin-like protein, but the precise role that it plays in modulating LPS O-chain content has not been determined.

Omp25/Omp31

Brucella strains produce a family of highly conserved outer membrane proteins (OMPs) known as Omp25, Omp25b, Omp25c, Omp25d, Omp31, Omb31b, and Omp22 that play important roles in maintaining the integrity of their cell envelope (203, 204). These 25- to 30-kDa β-barrel OMPs work in conjunction with the LPS O-chain to protect these bacteria from complement and other antimicrobial peptides encountered in

the host, and their contributions to virulence appear to be especially important for the naturally occurring rough strains such as *B. ovis* (181, 183, 205–207). This distinction, however, is not absolute, as *B. abortus* and *B. melitensis omp25* mutants have been shown to be attenuated in mice (205) and natural hosts (208, 209), and a *B. melitensis omp31* mutant is attenuated in cultured mammalian cells and mice (210, 211).

Beyond their structural roles in maintaining cell envelope integrity, the *Brucella* Omp25/Omp31 proteins have also been shown to mediate direct interactions between the brucellae and mammalian cells that are important for virulence. These latter functions may help explain some of the disparate phenotypes reported for *Brucella omp25* and *omp31* mutants in virulence assays. Omp25d and Omp22, for instance, play important roles in the entry of *B. ovis* into mammalian cells (207) (Fig. 3), but there is no evidence that they perform this function in smooth strains (206), where the LPS O-chain seems to be the predominant determinant in mammalian cell entry, as described in the previous section. The Omp25 protein of *B. abortus*, a smooth strain, also directly interacts with the SLAMF1 protein on the surface of dendritic cells and inhibits their maturation and capacity to produce inflammatory cytokines (212) (Fig. 5). The capacity of Omp25 to inhibit TNF-α production during *Brucella* infection was first reported by Jubier-Maurin et al. (213), but only recently has the molecular basis for this activity been clearly defined. There is also evidence suggesting that the *Brucella* Omp25 and Omp31 proteins have the capacity to modulate other aspects of host cell function during infection (214–219), but precisely how these other proposed Omp25/Omp31 functions contribute to virulence remains to be determined.

One of the interesting features of the Omp25/Omp31 proteins is that they are highly conserved across the *Rhizobiaceae*, and the Omp25/Omp31 orthologs are also important for the successful interactions of other alphaproteobacteria with their respective eukaryotic hosts. The Omp25 ortholog AopB, for instance, is required for the wild-type virulence of *Agrobacterium tumefaciens* in plants (220). The *Bartonella* heme-binding proteins (Hbps) are also Omp25/Omp31 orthologs (221), and *Bartonella henselae hbp* knockdown strains are attenuated in endothelial cell cultures (222). Notably, the *Brucella* Omp31b has also been shown to bind hemin *in vitro*, and the gene encoding this protein is iron regulated in *B. suis* (223). This raises the possibility that in addition to their other roles, the Omp25/Omp31 proteins may also contribute to virulence by working in concert with the heme transporter BhuA (224) to allow the brucellae to use heme as an iron source during residence in their mammalian hosts (225).

No single *Brucella* species has been shown to produce all seven of the Omp25/Omp31 proteins (182). Some species, like *B. abortus* and *B. ovis*, lack genes encoding specific Omp25/Omp31 proteins due to large genomic deletions (194, 226). Smaller genetic disruptions appear to be responsible for the distinctive patterns of Omp25/Omp31 production in other *Brucella* species (182). The global regulators BvrRS, VjbR, and CtrA tightly coordinate the expression of the *omp25* and *omp31* genes in response to both environmental stimuli and physiological cues (227–230), which is consistent with the critical role that this family of OMPs plays in the basic physiology and virulence of *Brucella* strains.

Omp10, Omp16, and Omp19

Brucella strains produce three outer membrane lipoproteins: Omp10, Omp16, and Omp19 (231). Omp16 is a homolog of the peptidoglycan-associated lipoprotein (Pal), which is highly conserved in Gram-negative bacteria (232). These proteins interact with the components of the Tol complex and play critical roles in maintaining the structural integrity and function of the outer membrane. The fact that *omp16* is an essential gene in *Brucella* is consistent with Omp16 playing the same role as other Pal homologs (233–235). Omp19, on the other hand, is the most well characterized of the *Brucella* lipoproteins. Purified Omp19 has strong immunomodulatory activities affecting a wide variety of host cells mediated by its interactions with TLR2, and these activities have been proposed to be important not only for the capacity of the brucellae to evade host immune responses but also their ability to induce immunopathology in selected

tissues such as bone and the central nervous system (236–252) (Fig. 4 and 5). In addition, Omp19 shares significant amino acid homology with bacterial protease inhibitors (253), and phenotypic analysis of a *B. abortus omp19* mutant indicates that Omp19 protects the parental strain from proteases encountered in the intestinal tract after oral infection and lysosomal proteases encountered during intracellular residence in host macrophages (254). In addition, Omp19 also protects another immunomodulatory protein, Omp25, from degradation by proteases. Unlike Omp16 and Omp19, which show homology to other bacterial proteins, Omp10 homologs only appear to be present in *Brucella* and a limited number of other alphaproteobacteria (255) and the biological function of this Omp is unknown. Interestingly, while *B. abortus omp19* and *omp10* mutants exhibit significant attenuation in mice (254, 256, 257), the corresponding *B. ovis* mutants do not (233). But the fact that *B. ovis omp10 omp19* double mutants cannot be constructed suggests that these proteins may have a shared physiologic function (233).

Autotransporter Adhesins

Autotransporter (AT) adhesins play important roles in the attachment of many bacterial pathogens to mammalian cells (258). Five AT adhesins have been described in *Brucella*. OmaA and BmaC are type I monomeric ATs (259, 260), BtaE and BtaF are type II trimeric ATs (261–263), and BigA (264) shares structural domains with the *Escherichia coli* adhesin intimin, which is considered an inverse AT adhesin (265). BmaC binds fibronectin on the surface of host cells (260), and BtaE and BtaF bind hyaluronic acid (261, 262), but the receptors for OmaA and BigA have not been described. Interestingly, *Brucella* mutants lacking these AT adhesins exhibit reduced attachment to epithelial cells but display wild-type intracellular replication in cultured macrophages. These mutants are also attenuated in mice when delivered by the intragastric or nasal routes but not when administered intraperitoneally. These experimental findings suggest that the function of the AT adhesins is to facilitate attachment of the brucellae to the host at mucosal surfaces during the early stages of infection. Notably, BigA appears to have a tropism for eukaryotic cell junctions (264), and adhesion to and disruption of host cell junctions is a strategy that other bacterial pathogens employ to cross mucosal barriers in the host (266). A *B. suis btaE btaF* double mutant is also considerably more attenuated in mice than a *btaE* or *btaF* mutant, indicating that BtaE and BtaF play complementary roles in virulence (262). In addition to its role in attachment, there is also evidence that BtaF protects *B. suis* from the bactericidal activity of serum (262).

BmaC, BtaE, and BtaF are localized at one specific pole of the bacterial cell (260–262), and the location of these AT adhesins at the new pole, coupled with the observation that *Brucella* cells in the G_1 phase of the cell cycle are the predominant infectious form (267), has led investigators to propose that these AT adhesins form an adhesive pole on the *Brucella* cell (268, 269). Only a small proportion of the *Brucella* cells in planktonic cultures produce these adhesins, suggesting that the corresponding genes may be optimally expressed only upon exposure to a host-specific stimulus such as contact with mammalian cells. This is consistent with the fact that several AT-encoding genes appear to be regulated by the quorum-sensing regulator VjbR (119) and the global regulator MucR (270, 271) and that a complex regulatory network has been shown to control *btaE* expression in *B. abortus* (272, 273). There appears to be considerable heterogeneity in the *Brucella* genes that encode AT-type adhesins (273), and as noted previously, there is evidence indicating that these adhesins have overlapping functions (262). Consequently, it will be important to study the contributions of the *Brucella* AT adhesins in different species and strains and use mutants with multiple disrupted genes to gain an accurate assessment of how these proteins contribute to virulence.

Cyclic β-1,2-D-Glucan

Many Gram-negative bacteria produce polysaccharide polymers and secrete them into their periplasm, where they perform a variety of physiologic functions (274).

Brucella spp. and other alphaproteobacteria secrete a cyclic polymer consisting of 17 to 20 glucose residues known as cyclic β-1,2-glucan (CβG) into their periplasmic space (275). In *Sinorhizobium* and *Agrobacterium*, the production of CβG is regulated by osmotic conditions, suggesting a role for this molecule in osmoprotection (276). But CβG production is not osmoregulated in *Brucella* (277, 278), and experimental evidence suggests that this polysaccharide plays only a minor role as an osmoprotectant in these bacteria (279). CβG does, however, play an essential role in *Brucella* virulence (275, 280). Specifically, studies employing *B. abortus* CβG synthase mutants and purified CβG indicate that this molecule disrupts lipids rafts on the *Brucella*-containing vacuoles, which is thought to prevent these vacuoles from sustaining their interactions with lysosomes (281) (Fig. 3). CβG has also been shown to have a strong influence on the capacity of macrophages and dendritic cells to produce both proinflammatory and anti-inflammatory cytokines (279, 282–284) (Fig. 4 and 5). It is presently unclear how CβB is released from its periplasmic location in *Brucella* cells to perform these biological functions *in vivo*. Nevertheless, CβG appears to have dual functions in virulence. It plays a critical role in the intracellular trafficking of the brucellae to their replicative niche in host macrophages, and it fine-tunes the host immune response to allow their chronic intracellular persistence. Given the proposed polar nature of the interaction of *Brucella* cells with their mammalian hosts (268, 269), it is also notable that the CβG synthase (Cgs) and transporter (Cgt) display polar localization on the bacterial cell (285). Whether this polar localization of the CβG biosynthesis and transport machinery plays any role in its contributions to virulence remains to be determined.

Flagella

Despite the fact that most *Brucella* strains are nonmotile, Halling reported the discovery of flagellar biosynthesis genes in *B. abortus* in 1998 (286). It was subsequently determined that most, if not all, *Brucella* strains have the genetic capacity to produce flagella (41, 287–289), but they lack chemotaxis genes and only a limited number of strains in the BO2 clade appear to be able to use the flagella for motility (290). Nevertheless, the isolation of *B. melitensis fliF* and *flgF* mutants in signature-tagged mutagenesis screens for attenuation in mice (291) and pregnant goats (62) suggested that these genes might play a role in virulence. The Letesson group demonstrated that *B. melitensis* 16M does in fact produce a single sheathed polar flagellum that is covered by an extension of the outer membrane (289, 292), and studies have confirmed that flagellar biosynthesis genes are required for the wild-type virulence of *B. melitensis* and *B. abortus* strains in the mouse model (289, 293). Interestingly, *B. ovis* mutants lacking flagellar biosynthesis genes are fully virulent in mice, suggesting that the contributions of flagella to pathogenesis may be strain and possibly host dependent (294).

One mechanism by which the flagella appear to contribute to virulence is by modulating the host immune response (295). Unlike flagella from some other Gram-negative pathogens, the *Brucella* flagellin is not recognized by TLR5. This contributes to the so-called stealthy nature of *Brucella* infections. But experimental evidence indicates that the *Brucella* flagellin ends up in the cytoplasm of infected host cells and stimulates an inflammasome-mediated inflammatory response that is important for "limiting" the extent of *Brucella* replication. Thus, the flagellum has been proposed to be yet another virulence determinant that allows the brucellae to fine-tune the host immune response in a manner that facilitates the establishment and maintenance of chronic infections. The possibility also exists that these appendages have yet undiscovered roles in pathogenesis, such as serving as adhesins and/or as sensors of surface attachment as they do in other closely related alphaproteobacteria (296, 297). In addition, sheathed flagella are relatively rare in bacteria, but studies have shown that they play a role in the release of outer membrane vesicles (OMVs) (298), which is an interesting relationship considering the roles that have been proposed for OMVs in host-pathogen interactions during *Brucella* infections (299).

The flagellar biosynthesis genes are tightly regulated in *Brucella*, and while production of the polar flagellum in *B. melitensis* 16M has been observed only *in vitro* in

bacterial cells grown to early exponential phase in a rich medium, these genes are readily expressed when this strain is replicating in mammalian cells (289). Subsets of the *Brucella* flagellar biosynthesis genes are expressed in stages, but the regulatory networks that coordinate the orderly temporal expression of these genes differ from those found in other bacteria (300, 301). The quorum-sensing regulators VjbR and BabR (also known as BlxR), the light-sensing regulator LovhK, the general stress response regulator RpoE1, and cyclic di-GMP-mediated signaling also control flagellar gene expression in *Brucella* (119, 120, 123, 302–305).

Bacteria that employ flagella for motility almost universally have chemotaxis genes that allow them to control the direction of their flagellar rotation in response to gradients of environmental stimuli (306). This allows them to swim toward nutrients and away from toxic compounds. The fact that no chemotaxis genes have been detected in *Brucella* strains is not too surprising given that most of these bacteria do not appear to be using their flagella for locomotion. But it does raise some interesting questions about how the motile BO2 strains direct their movements in their natural environments.

Phosphatidylcholine

The phospholipid phosphatidylcholine (PC) is a major component of eukaryotic cell membranes but is not commonly found in prokaryotes (307). Almost 40 years ago, it was reported that *Brucella* strains contains PC in their cell envelope (308), which led investigators to suspect that the presence of this "eukaryotic" phospholipid in the OM might contribute to virulence. *Brucella* strains produce PC by two different biochemical pathways—the Pcs pathway, which directly produces PC from choline, and the Pmt pathway, which produces PC by methylating the phospholipid phosphatidylethanolamine (309–311). Independent studies performed in two different laboratories have confirmed that PC plays an essential role in *Brucella* virulence. However, these studies provided conflicting results regarding the relative contributions of the Pcs and Pmt pathways to virulence, and further studies will be needed to get a clear picture of how PC contributes to virulence. But, in general, the experimental evidence at hand suggests that the PC/PE ratio in the outer membrane of *Brucella* strains impacts the ability of these bacteria to resist killing by antimicrobial peptides and complement and that PC may also play a role in modulating the host response to infection (309, 310, 312). It is also notable that the incorporation of PC into the outer membrane is a distinctive feature of the alphaproteobacteria in general and that this phospholipid also plays an important role in the interactions of some of the other members of this group (e.g., *Agrobacterium* and the rhizobia) with their respective plant hosts (313).

Exopolysaccharides

Exopolysaccharides (EPSs) are polysaccharide polymers secreted by bacteria that are either (i) attached tightly to the cell surface and form a capsule or (ii) loosely associated with bacterial cells forming an amorphous "slime layer" (314). EPSs play multiple roles in bacterial pathogenesis. They can serve as adhesins and facilitate the attachment of bacteria to eukaryotic cells (315). They can also protect bacteria from the bactericidal activities of complement, neutrophils, and macrophages (316–318) and/or allow them to evade recognition by host innate and acquired immune responses (316, 319). In addition, these polymers allow bacteria to form biofilms, which contributes to their persistence in the environment and in mammalian hosts (320–322).

Brucella strains have the genetic capacity for EPS production (229, 270, 323), but experimental evidence suggests that the corresponding genes are tightly regulated. *B. melitensis* 16M, for instance, does not produce a readily detectable EPS during routine *in vitro* cultivation, but disruption of a putative quorum-sensing pathway causes this strain to produce an apparent EPS detected by calcofluor staining and form "biofilm-like" bacterial cell aggregates in liquid culture (229). EPS production and cellular aggregation have also been described in a *B. melitensis virB* mutant (324) and a *B. abortus* strain overexpressing the glycosyltransferase WbkA (325). Reports of "biofilm"

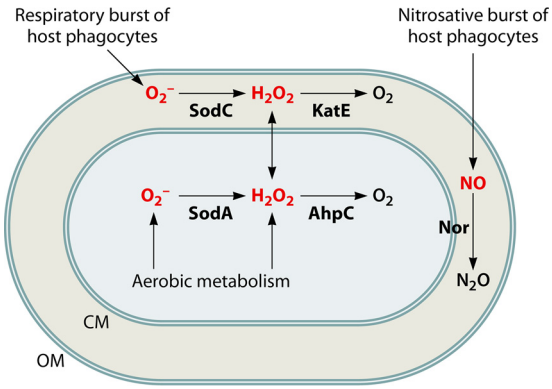

FIG 7 Virulence determinants that allow the brucellae to resist the reactive oxygen and nitrogen species they encounter during their intracellular residence in host phagocytes. Reactive oxygen and nitrogen species are shown in red. OM, outer membrane; CM, cytoplasmic membrane.

formation by *Brucella* strains (326–328) and enhanced Congo red staining of a *B. melitensis mucR* mutant (329) are also consistent with EPS production.

Because the specific genes required for EPS production have not been clearly defined, we do not currently know if EPS plays an important role in *Brucella* virulence. But EPS production is essential for the pathogenic and symbiotic interactions of the agrobacteria and rhizobia with their respective plant hosts (330, 331). Considering the conserved strategies that the alphaproteobacteria employ to sustain successful interactions with their eukaryotic hosts (9), it will be important to determine if EPS production also plays an important role in *Brucella* virulence.

PHYSIOLOGIC ADAPTATION TO THE HOST ENVIRONMENT

Microaerobic Respiration and Denitrification

Brucella strains rely upon a strictly respiratory type of metabolism for growth (332). Within the tissues of their mammalian hosts, these bacteria reside in microaerobic environments, and *bd*- and *cbb*$_3$-type cytochrome oxidases with very high affinities for O_2 play important roles in *Brucella* virulence (333–338). Interestingly, *B. abortus* 2308 requires the *bd*-type cytochrome oxidase for wild-type virulence in mice (334). In contrast, a *B. suis* 1330 mutant lacking the *cbb*$_3$-type cytochrome oxidase displays marked attenuation in mice, but a derivative of this strain lacking the *bd*-type cytochrome displays wild-type virulence (337). The basis for this differential requirement for the *bd*- and *cbb*$_3$-type cytochrome oxidases for the virulence of these two strains is not known. But what is clear from both studies (334, 337) is that a respiratory chain with a high affinity for O_2 is required for successful replication and persistence of the brucellae in mammalian tissues.

Denitrification is a process that allows bacteria to reduce NO_3 and use it instead of O_2 as a terminal electron acceptor for respiration (339). Most *Brucella* strains have the four enzymatic pathways needed for the complete reduction of NO_3 to N_2 (340), and experimental evidence indicates that the denitrification pathway contributes to virulence (335, 340–342). One way it contributes is by allowing the brucellae to employ NO_3 as an alternative electron acceptor to sustain the respiration they need for chronic intracellular persistence in the microaerobic environment of host tissues. Experimental evidence also suggests that the denitrification enzymes provide *Brucella* strains with a defense against the bactericidal activity of NO generated by host macrophages (340, 341, 343) (Fig. 7). In addition, the capacity of the intracellular brucellae to degrade NO protects infected macrophages from apoptosis (343). Interestingly, the degree to which denitrification contributes to virulence appears to differ between *Brucella* species that have different natural hosts (344).

Expression of the genes encoding the *bd*- and *cbb*$_3$-type cytochrome oxidases and

denitrification enzymes is tightly coordinated by the two-component regulators PrrAB (also known as RegAB) and NtrYX (345–348). Not surprisingly, PrrB and NtrY are redox sensors, and in conjunction with their cognate regulators, they activate these genes in response to low levels of ambient O_2.

Carbon and Nitrogen Metabolism

Until recently, when genes associated with central metabolism were identified in genetic screens for bacterial genes linked to virulence, these genes were often assigned to the "housekeeping" category and not examined further. But it is now widely appreciated that the capacity of bacterial pathogens to adapt their central metabolism to take advantage of the carbon, nitrogen, and energy sources they encounter in the host plays an essential role in their virulence (349). It has also become evident that extensive metabolic reprogramming takes place in bacterial pathogens, the host cells they inhabit, and the cells that modulate the host immune response during the course of bacterial infections and that these metabolic adaptations in both the pathogen and host often dictate the outcome of host-pathogen interactions (350–353). Studies reported by Xavier et al. (109), Czyż et al. (354), and Kerrinnes et al. (355) provide striking examples of how metabolic reprogramming in host cells can influence the course of *Brucella* infections.

Studies suggest that most *Brucella* strains rely on a balance of glucose catabolism (55, 109, 356, 357) and the utilization of gluconeogenic substrates such as glutamate and lactate (354, 358) to sustain their intracellular persistence in their mammalian hosts. Selective use of the pentose phosphate pathway for glucose catabolism also appears to have played an important role in the evolution of these bacteria as mammalian pathogens (359). Glucose catabolism appears to be especially important for these bacteria when they reside in alternatively activated macrophages during chronic infection in mice, when these host phagocytes shift from a glycolytic catabolism to one that relies on fatty acid oxidation, which makes glucose more readily available to the intracellular brucellae (109). The enzymes pyruvate kinase (PykM) and pyruvate phosphate dikinase (PpdK) play critical roles in glucose metabolism in *Brucella* (357, 358). PykM is critical for glucose catabolism, PpdK is required for gluconeogenesis, and both are essential for the wild-type virulence of *Brucella* strains in mice. But ongoing studies examining the role of glucose metabolism in *Brucella* virulence indicate that we still have a lot to learn in this area. Experimental evidence, for instance, indicates that the brucellae possess novel enzymes for converting fructose 1,6-bis-phosphate to fructose-6-phosphate during gluconeogenesis in addition to the well-characterized bacterial fructose bis-phosphatases Fbp and GlpX (358, 360). These bacteria also appear to be capable of employing an atypical pathway for converting phosphoenolpyruvate (PEP) to pyruvate during the catabolism of hexoses in the absence of a functional pyruvate kinase (357).

Erythritol is a four-carbon sugar alcohol that serves as a preferred carbon and energy source for many *Brucella* strains (361–363). Substantial amounts of this carbohydrate are present in the placentas of ruminants during the latter stages of pregnancy, and the capacity to catabolize erythritol has been proposed to be an important virulence determinant for the *Brucella* spp., e.g., *B. abortus* and *B. melitensis*, that cause abortion and infertility in cattle, sheep, and goats (364). Recent studies have better defined the enzymes required for erythritol catabolism and shown that this carbohydrate is metabolized by a pathway that feeds into the pentose phosphate pathway (365). At least one erythritol-responsive transcriptional regulator, EryD, has also been identified (366), and this carbohydrate stimulates the expression of a variety of genes in *Brucella*, including those involved in the production of the T4SS and flagella (367, 368). But gaining a clear picture of whether erythritol catabolism contributes to *Brucella* virulence has remained elusive. Genetic studies, for instance, demonstrated a link between production of the siderophore brucebactin, erythritol utilization, and the virulence of *B. abortus* 2308 in pregnant cattle (369, 370), but a direct link between erythritol catabolism and the virulence of this strain in cattle has not been confirmed

experimentally. Erythritol catabolism genes have also been reported to be required for the virulence of *B. abortus* and *B. suis* strains in mice and cultured murine and human macrophages (234, 335, 371). But a recent comparative study of the virulence properties of *B. abortus* mutants blocked at different steps in the erythritol catabolism pathway suggests that the attenuation reported for *B. abortus eryC, eryH,* and *eryI* mutants and *B. suis eryB* and *eryC* mutants was likely due to erythritol toxicity rather than their inability to catabolize this four-carbon sugar alcohol (372). More importantly, this study showed that a *B. abortus eryA* mutant which lacks the first enzyme in the erythritol catabolism pathway and cannot catabolize this sugar alcohol displays only limited and early attenuation in nonpregnant mice and wild-type virulence in bovine and human macrophages and trophoblasts and pregnant mice. More extensive studies of the virulence properties of *eryA* mutants in nonpregnant and pregnant natural hosts will be required before we have a clear picture of the role that erythritol catabolism plays in *Brucella* virulence.

Evidence suggests that amino acids and NH_4^+ provide the bulk of the nitrogen the brucellae need to meet their physiologic requirements during residence in their mammalian hosts (373), although host-derived polyamines may serve as an important N source for these bacteria in alternatively activated macrophages (355). Glutamate can serve as both a sole C and N source for *Brucella* strains (374), and as observed in other bacteria, the enzymes that interconvert α-ketoglutarate, glutamate, and glutamine and the proteins that regulate these enzymes tightly coordinate NH_4^+ assimilation in the brucellae (373). The importance of this regulatory network for virulence is evidenced by the fact that mutants lacking many of these enzymes (e.g., GlnA, GltB, GltD, and GdhZ) and regulators (e.g., GlnD and GlnE) exhibit attenuation in cultured mammalian cells and mice (54, 55, 234, 335, 342, 375). But one distinctive feature of N metabolism in *Brucella* is that the two-component regulator NtrBC and alternative sigma factor RpoN, which play central roles in regulating nitrogen metabolism in many other bacteria (376–379), do not appear to be required for wild-type virulence in *Brucella* (380–382). One caveat here, however, is that the *Brucella* NtrBC and RpoN regulons have not been extensively characterized, so it is not known if these regulators make significant contributions to nitrogen metabolism or if the typical regulatory functions of these proteins have been supplanted by other regulators.

Classical PEP-phosphotransferase (PTS) systems play both catalytic and regulatory roles in bacterial carbohydrate transport and catabolism (383). The *Brucella* spp. do not have a classical PTS, but they employ a related PTS known as the nitrogen-related PTS (PTS^{Ntr}) (384) to coordinate their C and N metabolism (385), and *Brucella* mutants with a disrupted PTS^{Ntr} are attenuated (234, 335, 375). But determining the precise role that the *Brucella* PTS^{Ntr} plays in virulence is complicated by the fact that the *hprK*, *ptsM*, and *ptsO* genes which encode principal components of the *Brucella* PTS^{Ntr} are cotranscribed with the genes that encode the two-component regulator BvrRS. The observation that *Brucella* mutants with a disrupted PTS^{Ntr} are defective in *virB* expression (385) suggests that in addition to coordinating C and N metabolism in *Brucella*, PTS^{Ntr} may also be influencing the regulatory activity of BvrR.

Antioxidants

Brucella strains utilize O_2 as a terminal electron acceptor during their replication in host tissues (334, 337). This puts these bacteria in a precarious position, because it predisposes them to oxidative damage from both external and internal sources (Fig. 7). During their interactions with neutrophils and macrophages, for instance, the brucellae are exposed to the reactive oxygen species (ROS) generated by the respiratory burst of these phagocytes (386, 387). In addition, they must also detoxify the superoxide (O_2^-) and hydrogen peroxide (H_2O_2) generated as by-products of their own aerobic metabolism (388, 389).

Superoxide anion (O_2^-) does not typically cross the cytoplasmic membrane due to its negative charge (390), and two different superoxide dismutases (SODs) with different cellular locations play distinct roles in detoxifying the exogenous O_2^- the brucellae encounter during their interactions with host phagocytes and the endogenous O_2^-

they generate via their aerobic metabolism. The periplasmic Cu/Zn SOD SodC (391) is responsible for detoxifying exogenous O_2^-, and this enzyme plays an important role in protecting the brucellae from the respiratory burst of host macrophages (392). The cytoplasmic Mn SOD SodA, on the other hand, protects these bacteria from the O_2^- generated by their aerobic metabolism (389). Both enzymes are required for the wild-type virulence of *Brucella* strains in mice (389, 392, 393).

In contrast to O_2^-, H_2O_2 moves freely across cellular membranes (390), and the periplasmic catalase KatE (394) and the cytoplasmic peroxiredoxin AhpC (388) work in concert to ensure that the cellular levels of H_2O_2 generated by the aerobic metabolism of *Brucella* strains are maintained below toxic levels. It should be noted, however, that neither of these enzymes plays a major role in protecting the brucellae from the respiratory burst of host macrophages (388). *Brucella* strains lacking KatE exhibit wild-type virulence in mice (388, 395) and pregnant goats (396), and *Brucella ahpC* mutants are also fully virulent in mice (388). On the other hand, *B. abortus ahpC katE* double mutants are extremely attenuated in mice. These experimental findings coupled with the attenuation observed with a *B. abortus sodA* mutant in mice indicate that the brucellae must be able to protect themselves from ROS of both exogenous and endogenous origin to produce infection in their mammalian hosts.

The transcriptional activator OxyR directly regulates *katE* expression in *Brucella* in response to exposure to H_2O_2 (397, 398). But how *ahpC*, *sodC*, and *sodA* are regulated is less clear. The redox-responsive two-component regulator RegAB and the stringent response regulator Rsh have been reported to regulate *sodC* expression (348, 399), Hfq (and presumably sRNAs) have been implicated in *ahpC* and *sodC* regulation (392, 400, 401), and VjbR has been linked to *sodA* regulation (119). Whether these regulatory links are direct or indirect, however, remains to be determined.

Metal Acquisition and Detoxification

Depriving pathogens of iron (Fe), manganese (Mn), and zinc (Zn) is a strategy employed by the host to resist microbial infections (402, 403). The vast majority of the Fe in mammalian tissues, for instance, is incorporated into cellular proteins or heme and is not directly available for use by bacterial pathogens. Neutrophils also release the metal binding proteins calprotectin and lactoferrin into the extracellular environment during the inflammatory response (404–406), and activated macrophages employ the divalent cation transporter Nramp1 to pump Fe^{2+}, Mn^{2+}, and Zn^{2+} out of phagosomes containing intracellular pathogens (407). In addition, the liver produces the hormone hepcidin during the inflammatory response, which inhibits the release of Fe from the liver and spleen into the bloodstream (408).

Brucella strains rely on the siderophore brucebactin (409), the Fe^{2+} transporter FtrABCD (410), the heme transporter BhuA (224), the Mn transporter MntH (411), and the Zn transporter ZnuA (412, 413) to overcome the Fe, Mn, and Zn deprivation they encounter in their mammalian hosts (Fig. 8). Interestingly, the need for brucebactin appears to be host dependent. This siderophore is not required for the virulence of *B. abortus* 2308 in mice (409, 414, 415) but is essential for the virulence of this strain in pregnant goats (416) and cattle (369). The basis for this differential requirement for brucebactin in these hosts is unknown, but experimental evidence suggests that it may be linked to erythritol catabolism (370).

Because Fe, Mn, or Zn can be toxic to bacterial cells when accumulated in excess, the brucellae employ metal-responsive transcriptional regulators, storage proteins, and exporters to prevent metal toxicity. The transcriptional regulators Irr, RirA, Mur, and Zur ensure that the genes encoding high-affinity metal importers are selectively expressed only when bacterial levels of specific metals fall below certain thresholds (417–422). The Mn exporter EmfA (423) and Zn exporter ZntA also transport excess Mn and Zn out of *Brucella* cells, and the Zn-specific transcriptional regulator ZntR tightly regulates ZntA-mediated Zn export (422). The fact that *Brucella irr* (419), *rirA* (234, 424), *emfA* (423), and *zntR* (422) mutants exhibit attenuation in mice strongly suggests that

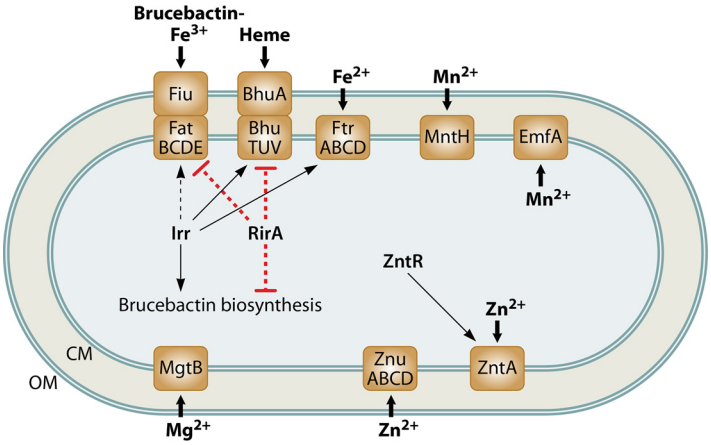

FIG 8 Fe, Mn, Zn, and Mg acquisition systems and defenses against metal toxicity that have been shown to play a role in the virulence of *Brucella*. The solid arrows represent direct regulatory links that have been documented experimentally, and the dashed arrows and lines denote suspected regulatory links. OM, outer membrane; CM, cytoplasmic membrane.

in addition to overcoming the metal deprivation that they encounter with the host, the brucellae must also avoid metal toxicity to be effective pathogens.

Nickel (Ni) and magnesium (Mg) transport also play important roles in *Brucella* virulence. Ni is required for urease activity (see below), and Mg is the most abundant metal found in bacterial cells (425) and an essential micronutrient for *Brucella* (426). Two high-affinity Ni transporters, NikABCDE and NikKLMQO, play functionally redundant roles in supplying *Brucella* strains with the Ni required to support their urease activity (427, 428). Although the Mg transporters in *Brucella* have not been well characterized, a gene predicted to be involved in Mg transport (*mgtB*) was identified in a genetic screen for *B. melitensis* mutants displaying attenuation in cultured mammalian cells and mice (429). A *B. suis* mutant lacking a homolog of a *Salmonella* virulence determinant involved in maintaining cellular Mg/ATP balance (MgtC) (430) is also attenuated in cultured mammalian cells (431).

Urease

Most *Brucella* strains produce urease (432), and this enzyme is thought to protect the brucellae from the extremely acidic conditions they encounter during passage through the gastrointestinal tract following ingestion (63, 433, 434). *Brucella ovis* is an exception, and the absence of urease activity in this bacterium has been proposed to be one of the reasons for its limited host range and lack of transmission via the oral route (196). There are two different genetic loci, *ure1* and *ure2*, in *Brucella* strains containing urease biosynthesis genes, but only one of these loci, *ure1*, encodes a functional urease (433, 434). The *ure2* locus encodes urea and Ni transporters that are thought to be important for maintaining maximum urease activity at the low urea concentrations and acidic pH encountered in the mammalian gastrointestinal tract (428).

PrpA

Proline racemase protein A (PrpA) is a B cell mitogen secreted by *Brucella* into the cytoplasm of infected macrophages, where it is palmitoylated by the host cell and transported to the plasma membrane, where it interacts with the surface receptor NMM-IIA (435–438) (Fig. 3). This interaction stimulates the production of the anti-inflammatory cytokine IL-10 by these macrophages and represses their ability to produce proinflammatory cytokines. The B cell mitogen activity of PrpA also increases the production of *Brucella*-specific IgG2a, which is thought to increase the opsonic uptake of *Brucella* by host macrophages. The enzymatic activity of PrpA is essential for its immune modulatory functions, but the mechanism behind this link is unknown. It is

also unknown how PrpA is secreted into the host cell cytoplasm since this protein has not been described as a T4SS substrate. The late-stage attenuation of a *prpA* mutant in mice suggests that the immunomodulatory functions of PrpA may play a role in the capacity of *Brucella* strains to sustain chronic infections in their mammalian hosts.

BacA

The plant symbiont *Sinorhizobium meliloti* is a close phylogenetic relative of *Brucella*. During its symbiotic interactions with its plant host, *S. meliloti* imports small cysteine-rich peptides known as NCRs (nodule-specific C-rich peptides) from the plant into its cytoplasm via an ABC transporter known as BacA (439). NCR import serves two beneficial functions for the rhizobia during symbiosis. It prevents the potentially bactericidal NCRs from damaging the bacterial cell envelope (440), and the NCRs serve as signaling molecules that drive the intracellular rhizobia to differentiate into N_2-fixing bacteroids (441).

Brucella strains produce a BacA homolog, and a *B. abortus bacA* mutant is attenuated in cultured murine and human macrophages and mice (39, 442, 443). The basis for the attenuation of this mutant is presently unknown. The *B. abortus bacA* mutant has reduced levels of very-long-chain fatty acids in its lipid A (169) and induces stronger inflammatory responses in mice than its parent strain, which likely explains one aspect of its attenuation (443). But the plant NCRs are also considered to be homologs of mammalian defensins (444), and the *Brucella bacA* gene can restore NCR import and symbiotic capacity to an *S. meliloti bacA* mutant (445). This raises the possibility that BacA may also be able to protect *Brucella* strains from the antimicrobial peptides they encounter during their interactions with host phagocytes in a manner similar to that by which this transporter protects *S. meliloti* from NCRs in plants.

GadB, GadC, and GlsA

Many foodborne pathogens like *E. coli* and *Salmonella* rely on acid resistance (AR) systems to help them overcome the low pH they encounter during passage through the stomach (446). One of these is the AR2/AR2_Q system, which is comprised of a glutamate carboxylase (GadB), a glutaminase (GlsA), and a γ-aminobutyric acid (GABA)/glutamate-glutamine antiporter (GadC). GlsA converts glutamine to glutamate, and GadB converts glutamate into GABA. The latter reaction consumes a proton which deacidifies the cytoplasm. GadC exports GABA to the exterior of the bacterial cell in exchange for the import of glutamate or glutamine, which allows Gls and GadB to continue the deacidification cycle in the cytoplasm.

gadB, *gadC*, and *glsA* genes are present in all *Brucella* species, but they encode functional AR2/AR2_Q systems only in *B. microti*, *B. ceti*, *B. pinnipedialis*, *B. inopinata*, and the "atypical" *Brucella* strains isolated from amphibians (447–449). AR2/AR2_Q systems allow these strains to withstand exposure to very acidic pH, and a *B. microti gadBC* mutant is attenuated in mice infected by the oral route. It has been proposed that the genes encoding a functional AR2/AR2_Q system have been retained only in the so-called "ancestral" *Brucella* strains because these bacteria reside for prolonged periods in the external environment, unlike the classical *Brucella* strains, which are highly host adapted.

REGULATION OF VIRULENCE GENE EXPRESSION

BvrRS

The two-component regulator BvrRS (37) controls the expression of genes required at multiple stages of the infectious process, and *Brucella bvrR* and *bvrS* mutants are highly attenuated (37, 234, 335). BvrRS directly controls the expression of *omp25*, *omp22*, and genes involved in LPS lipid A modification (174, 227). The altered cell envelopes of *Brucella bvrR* and *bvrS* mutants make them extremely sensitive to killing by serum (450), less invasive for mammalian cells than their wild-type counterparts (451), and highly susceptible to the bactericidal activities of macrophages and dendritic cells (452, 453). BvrS recognizes the acidic pH and nutrient deprivation encountered by the

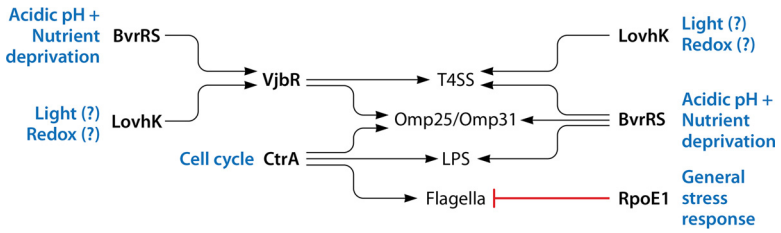

FIG 9 Overlapping regulation of genes encoding major virulence determinants in *Brucella* by BvrRS, VjbR, RpoE, CtrA, and LovhK. BvrRS and LovhK are shown twice to reflect the fact that these regulators have been shown to have both direct and indirect effects on genes encoding these virulence determinants.

brucellae in the endososomal BCVs and via BvrR directly activates the expression of the genes encoding the T4SS (454). BvrRS also indirectly activates the expression of these genes by activating the expression of the gene encoding the quorum-sensing regulator VjbR (112) (Fig. 9). Experimental evidence suggests that many other *Brucella* genes are regulated by BvrRS (228, 455–457), but deciphering the nature of these regulatory links is complicated by the fact that BvrR lies upstream of VjbR in a regulatory network that controls the expression of >200 *Brucella* genes (112, 119, 121). The *bvrR* and *bvrS* genes are also transcribed in an operon with genes that encode a regulatory network that coordinates C and N metabolism in these bacteria (385).

BvrRS is a member of a family of highly conserved two-component regulators that play critical roles in the interactions of alphaproteobacteria with their respective eukaryotic hosts (458). The BvrRS homologs ChvIG and ChvI-ExoS, for example, are essential for the pathogenic and symbiotic interactions of *Agrobacterium tumefaciens* and *Sinorhizobium meliloti* with their plant hosts (459, 460), and BatRS is required for the virulence of *Bartonella henselae* in cultured human endothelial cells (461) and *Bartonella tribocorum* in rats (462). Although the sensor components of these regulators appear to have evolved to recognize different host-relevant environmental stimuli, the genes under the control of these regulators have highly conserved functions directed at overcoming the innate immune responses of these hosts and establishing long-term pathogenic or symbiotic relationships. Together with a periplasmic protein known as ExoR, which modulates the regulatory function of the BvrRS homologs in *Sinorhizobium* and *Agrobacterium*, these proteins comprise a regulatory circuit known as the ExoR-ExoS-ChvI (RSI) invasion switch (458). This regulatory circuit is found only in the alphaproteobacteria that infect eukaryotic hosts and is thought to have played an important role in their evolution to their host-adapted lifestyles. Interestingly, *Brucella* strains possess an *exoR* gene, but whether the corresponding gene product plays a role in modulating the regulatory activity of BvrRS has not been reported.

N-Dodecanoyl Homoserine Lactone, VjbR, and BabR

Quorum sensing (QS) is a cell-to-cell signaling pathway that bacteria use to assess their population density and trigger the density-dependent expression of genes affecting a wide range of cellular behaviors (463, 464). A possible link between QS and *Brucella* virulence was uncovered when the QS signaling molecule *N*-dodecanoyl homoserine lactone (C_{12}-HSL) was detected in the supernatant of *B. melitensis* cultures, and it was found that the addition of exogenous C_{12}-HSL to this strain repressed the expression of its T4SS and flagellar biosynthesis genes (465). Subsequent studies identified the LuxR-type regulator VjbR as being responsible for this regulatory link (302), and >150 *Brucella* genes are now known to be regulated by VjbR (81, 119, 121, 302, 466, 467). *Brucella vjbR* mutants are highly attenuated (65, 188, 234, 302, 468–470), and one of the major reasons for this attenuation is that VjbR is required for the expression of the *virB* operon which encodes the T4SS (Fig. 6 and 9). But VjbR also directly controls the expression of genes encoding other important virulence determinants, including Omp25, the autotransporter adhesin BtaE, the T4SS effector VceC, the cbb_3-type

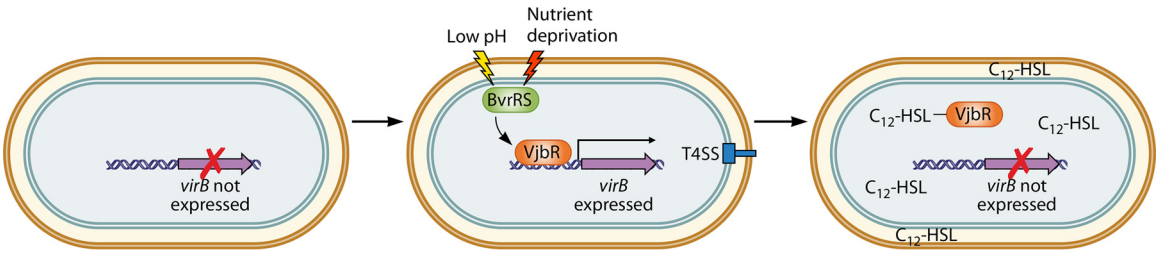

FIG 10 Proposed role of C_{12}-HSL signaling and VjbR in temporal regulation of *virB* expression during development of the *Brucella*-containing vacuoles in host cells. It is thought that accumulation of C_{12}-HSL in the spatial confines of the BCVs prevents *virB* expression during the later stages of BCV development. The phagosome membrane is depicted in brown.

cytochrome oxidase, and FtcR, the master regulator of flagellar gene expression in *Brucella* (81, 272).

Interestingly, the impact that C_{12}-HSL binding has on the regulatory activity of VjbR is the opposite of what is observed for canonical LuxR-type QS regulators in other bacteria. Specifically, the latter regulators generally turn on the expression of virulence genes when they bind the QS signal (463, 464). In contrast, VjbR serves as an activator of *virB* expression in *Brucella*, and binding of C_{12}-HSL to VjbR causes it to disengage from the *virB* promoter (466). Moreover, global studies of gene expression in wild-type *Brucella* strains and isogenic *vjbR* mutants exposed to C_{12}-HSL have also shown that this QS signaling molecule has a negative impact on the regulatory activity of VjbR on many other genes (119, 121, 234, 302). Based on these experimental findings, it has been postulated that C_{12}-HSL and VjbR do not comprise a classical QS pathway but rather a regulatory circuit that allows the intracellular brucellae to sense the spatial confines of the *Brucella*-containing vacuole within host cells and turn off the expression of *virB* and other genes in a temporal fashion (119, 272, 302) (Fig. 10).

Another C_{12}-HSL-responsive LuxR-type transcriptional regulator known as BabR (also known as BlxR) also controls the expression of a subset of the VjbR-regulated genes in *Brucella*, including the *virB* operon and flagellar biosythesis genes, and in some cases these two regulators have opposite effects on these coregulated genes (119–122, 471). BabR also regulates *vjbR* expression and *vice versa*. But in contrast to *vjbR* mutants, *Brucella babR* mutants display little or no attenuation in experimental models (119–121), leaving the importance of the regulatory overlap between VjbR and BabR presently unclear.

It is clear that we still have a lot to learn about the roles that C_{12}-HSL, VjbR, and BabR play in the basic biology and virulence of *Brucella*. For instance, although a lactonase that degrades C_{12}-HSL and modulates its regulatory activity on VjbR has been identified (472), the enzyme(s) responsible for the synthesis of the signaling molecule has not. In addition, the regulatory activity of VjbR at some promoters is dependent on the activities of other coregulators (272), but in only a few cases have these coregulators been identified (111, 115, 116, 118, 273). There are also many genes involved in basic physiologic processes such as metabolism and transport that are regulated by VjbR and/or BabR (119–121), but the importance of these particular regulatory links to virulence have not been determined. Finally, the fact that the *vjbR* and *babR* genes regulate each other (119–121) and are regulated by other global regulators (112, 123, 473) often makes it difficult to pinpoint precisely where VjbR and BabR lie in the regulatory pathways that control specific genes.

General Stress Response and LovhK

The general stress response (GSR) is a global change in gene expression that allows the alphaproteobacteria to withstand a wide variety of environmental stresses (474). Because some of these stresses, such as nutrient deprivation and exposure to reactive oxygen species and acidic pH, are those that the brucellae encounter during their intracellular life cycle in the host, the GSR has been proposed to play an important role

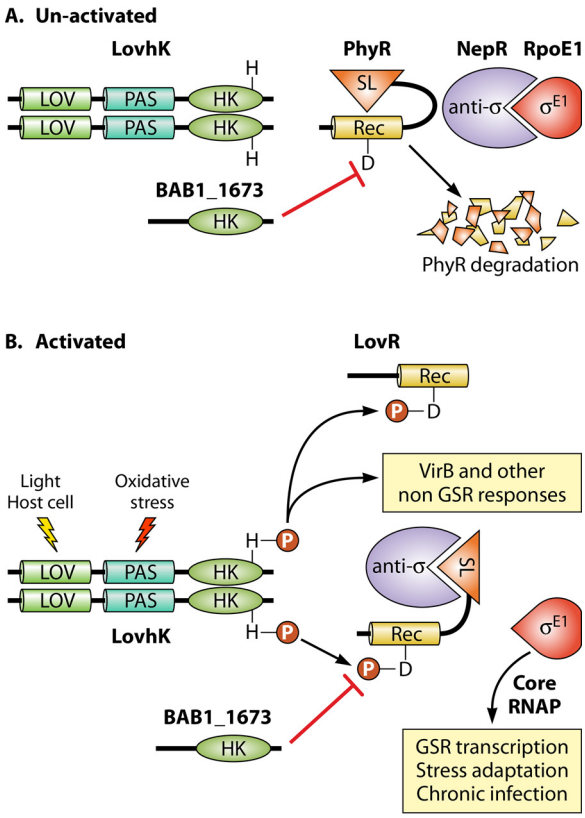

FIG 11 Regulation of the general stress response (GSR) and other virulence determinants in *Brucella* by LovhK. The yellow and red lightning bolts in panel B denote the stimuli thought to activate the histidine kinase activity of Lovhk through its LOV and PAS domains. LovR has been proposed to function as a phosphate sink and to modulate LovhK signaling. HK, histidine kinase domain; SL, sigma factor-like domain; Rec, response regulator receiver domain.

in *Brucella* virulence (400). The alternative σ factor RpoE1 controls the GSR in *Brucella*, and the activity of RpoE1 is modulated by the anti-σ factor NepR, the anti-anti-σ factor PhyR, and the HWE-type sensor histidine kinase (HK) LovhK (305, 475, 476) (Fig. 11). Under nonstress conditions, NepR binds RpoE1 and prevents it from interacting with RNA polymerase (RNAP). In response to undefined environmental or physiologic stimuli (see below), LovhK phosphorylates PhyR and PhyR-P titrates NepR away from RpoE1. This allows RpoE1 to engage RNAP and regulate the >80 genes that comprise the *Brucella* GSR regulon (303, 305, 381). Regulated proteolysis of PhyR and the activity of another uncharacterized HWE-type sensor HK encoded by the gene immediately downstream of *rpoE1* also play important roles in fine-tuning the GSR in *Brucella* (304, 475).

Brucella rpoE1 and *phyR* mutants are more sensitive to oxidative stress and acidic pH than wild-type strains (305, 474), and these mutants exhibit limited attenuation in cultured mammalian cells and delayed clearance in the mouse model of chronic infection (305, 381, 475). These phenotypes are consistent with the proposed role of the GSR in supporting the long-term intracellular persistence of the brucellae in host macrophages. Specific genes in the *Brucella* GSR regulon that have individually been linked to virulence include those encoding the flagella, urease, the *bd*-type cytochrome oxidase, and the lectin-like protein BA14K (303, 305, 381) (Fig. 9).

Attempts to determine the environmental and/or physiologic stimuli that elicit the GSR in *Brucella* have been complicated by the fact that LovhK has two sensory domains that respond to different stimuli (475) (Fig. 11). One of these domains responds to blue light, and the other is thought to be responsive to an undefined redox stimulus. The

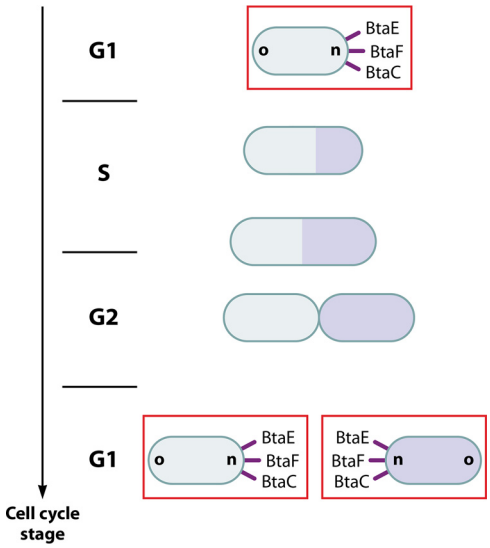

FIG 12 Unipolar cell division and coordination of cell division with the cell cycle in *Brucella*. The polar locations of the AT adhesins BtaE, BtaF, and BmaC are shown, and the red boxes indicate that *Brucella* cells in the G_1 phase of the cell cycle are the most infectious for mammalian cells.

light-sensing domain is required for the wild-type intracellular replication of *Brucella* strains in mammalian cells, but the redox-sensing domain is not (475, 477). But exposure to light does not evoke the GSR in *Brucella* (476). The biological relevance of light as a stimulus for LovhK is also unclear considering the intracellular nature of *Brucella* infections. One proposal that has been put forth is that recognition of light by LovhK may occur during transmission between natural hosts, where exposure to aborted fetuses in the external environment represents an important source of infection for naive animals (476, 477) (Fig. 2).

LovhK also plays an important role in the regulation of virulence genes independent of its role in inducing the GSR (123, 475–477) (Fig. 11). The *virB*, *vjbR*, and *babR* genes require LovhK for their wild-type expression, but these genes are not members of the RpoE1 regulon, and *Brucella lovhK* mutants are considerably more attenuated in mammalian cells and mice than *rpoE1* and *phyR* mutants. The nature of the regulatory links between LovhK and the *virB*, *vjbR*, and *babR* genes is currently unknown.

CtrA and GcrA

Like the other alphaproteobacteria, *Brucella* strains display a distinctive cell cycle characterized by asymmetric cell division, cell polarity, and defined developmental changes in gene expression (268, 269, 478, 479) (Fig. 12). Disruption of the cell cycle has a negative impact on virulence (230, 480, 481), and as noted previously, *Brucella* cells in the G_1 phase of the cell cycle appear to be the most infectious for mammalian cells (267, 482). The transcriptional regulator CtrA serves as the master regulator of the cell cycle in *Brucella* (230, 481, 483), and the histidine kinase PdhS (484), CckA-ChpT phosphorelay, and protease adaptor CpdR (481) modulate the regulatory activity of CtrA is response to endogenous cell cycle signals (Fig. 13). Consistent with its role in regulating the cell cycle, CtrA controls multiple genes involved in DNA replication, chromosome segregation, and cell division in *Brucella* (230), and proper coordination of the expression of these genes is undoubtedly important for sustaining the intracellular persistence of these bacteria in mammalian cells. But CtrA also controls the expression of *omp25* and genes involved in LPS biosynthesis, so it is apparent that its contributions to virulence in *Brucella* go beyond its role as a cell cycle regulator.

GcrA is another cell cycle regulator that allows σ^{70}-bound RNA polymerase to recognize the promoters of genes that have been epigenetically "marked" by the DNA methyltransferase CcrM (485). Recent studies have identified a GcrA ortholog in

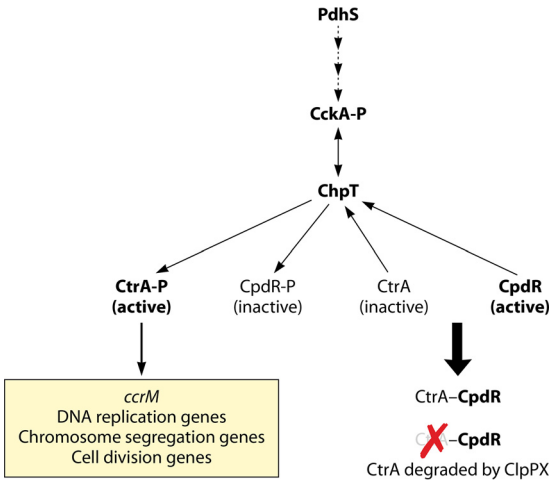

FIG 13 Regulation of CtrA and CpdR activity by PdhS and the CckA-ChpT phosphorelay in *Brucella*. Arrows indicate the direction of phosphate transfer. The red X denotes CtrA degradation by ClpPX. The multiple dashed arrows between PdhS and CckA indicate that the exact number and nature of the regulatory steps between these two regulators have not been determined.

Brucella and shown that this gene is essential for wild-type virulence in cultured mammalian cells (486). But the specific contributions of individual GcrA-regulated genes to virulence remains to be determined.

MucR

The Zn finger protein MucR is a global regulator that plays an important role in the virulence of *Brucella* strains (124, 270, 329, 375, 487). MucR serves predominantly as a transcriptional repressor and has been shown to regulate the expression of a multitude of *Brucella* genes required for virulence, including the *virB* operon, *omp25*, *babR*, and genes involved in LPS, CβG, and flagellar biosynthesis, iron acquisition, and c-di-GMP signaling. Experimental evidence suggests that one of the primary functions of MucR is to work in concert with antagonistic transcriptional activators to ensure the proper temporal expression of these genes during infection (271) (Fig. 14), and similar host-specific regulatory functions have been proposed for MucR homologs in *Agrobacterium tumefaciens* (488) and *Sinorhizobium meliloti* (489). MucR also works in concert with CtrA to regulate the cell cycle in *Caulobacter* (490), and given the conservation of the cell cycle genes across the alphaproteobacteria, it is likely that the *Brucella* MucR performs the same function. Thus, it will also be important to determine how misregulation of the cell cycle contributes to the attenuation of *Brucella mucR* mutants.

GntR

The GntR family is one of the most common types of transcriptional regulators found in bacteria (491). Twenty-one GntR-type regulators have been described in *Brucella* (126), and remarkably, 7 of these have been linked to virulence (126, 492). The regulatory activities of GntR-type regulators are typically modulated by the allosteric binding of metabolites, and correspondingly, most of the genes controlled by these regulators are involved in metabolism (493). Notably, one of the GntR-type regulators in *Brucella* is HutC, which regulates both histidine catabolism and expression of the genes encoding the type IV secretion machinery (116). This relationship is intriguing, because three other GntRs (GntR4, GntR12, and GntR17) have also been linked to proper expression of the *virB* genes in *Brucella* (126, 473, 492, 494). GntR17 is also required for wild-type expression of *omp25* and the genes encoding the quorum-sensing regulators VjbR and BabR in *B. abortus* (473, 494), and GntR12 is required for the capacity of *B. suis* to elicit the unfolded protein response in cultured caprine alveolar macrophages (492). Considering the clear role of the *Brucella* GntRs as virulence

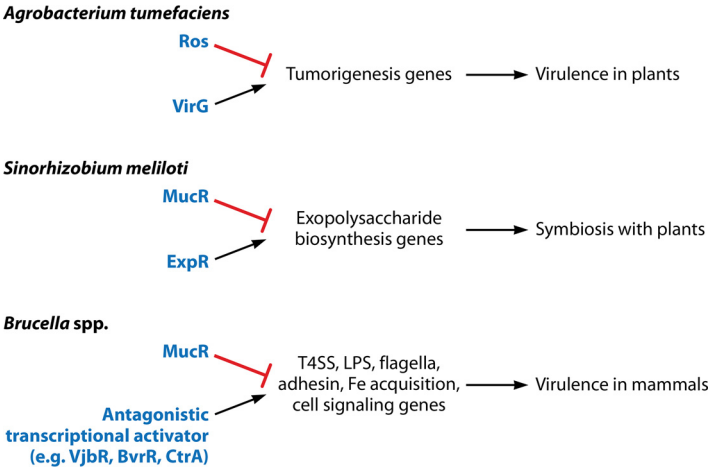

FIG 14 Conserved roles that MucR/Ros-based transcriptional modules are proposed to play in controlling host-specific gene expression in *Agrobacterium*, *Sinorhizobium*, and *Brucella*.

determinants, it will be important to better characterize the individual contributions of these regulators to virulence, define the specific genes they regulate, and identify the metabolites that control the regulatory activities of these GntRs.

OtpR, CpbB, NodVW, TceSR, and PrlSR

The histidine kinase CenK and response regulator CenR form a two-component regulator that plays an essential role in regulating cell growth and division in *Caulobacter* (495). The *Brucella* OtpR is a CenR homolog, and although *otpR* does not appear to be an essential gene in *Brucella*, a *B. melitensis otpR* mutant displays growth and morphologic defects and misregulation of peptidoglycan biosynthesis and cell division genes. This suggests that the *Caulobacter* CenR and *Brucella* OtpR have similar physiologic functions (496, 497). A *B. melitensis otpR* mutant is also extremely attenuated in both cultured mammalian cells and mice. OtpR has been proposed to play an important role in protecting *Brucella* from acidic pH, and indeed, over 500 genes display differential expression in *B. melitensis* 16M and an isogenic *otpR* mutant when these strains are exposed to acidic pH (127, 128). But how many of these genes are directly regulated by OtpR remains to be determined. It is also presently unclear if a CenK ortholog works in concert with OtpR to comprise a two-component regulator in *Brucella*. Interestingly, *otpR* resides upstream and in an operon with a gene encoding the putative cAMP-binding protein CbpB, which also plays a role in the virulence of *B. melitensis* 16M. Moreover, experimental evidence suggests that like OtpR, CbpB may also play an important role in regulating genes involved in peptidoglycan biosynthesis and cell division (498).

NodVW and TceSR are two-component regulators that are highly conserved in *Brucella* strains (499), but neither is found in their close phylogenetic relative, *Ochrobactrum anthropi*, a soil bacterium that only infrequently causes disease in humans. This observation led to the proposition that these regulators played an important role in the evolution of the brucellae as intracellular pathogens. This proposition is supported by the fact that *Brucella nodVW* and *tceSR* mutants are highly attenuated in cultured mammalian cells and mice (500–502). Although the environmental stimuli that the *Brucella* NodVW and TceSR respond to are unknown, it is interesting that NodVW allows the closely related bacterium *Bradyrhizobium japonicum* to recognize small molecules produced by its plant host and express genes required for symbiosis (503, 504).

PrlSR is a two-component regulator that allows *B. melitensis* 16M to form multicellular aggregates when exposed to environments of high ionic strength (505). Although *B. melitensis prlS* and *prlR* mutants are not attenuated in cultured mammalian cells,

these mutants cannot sustain chronic spleen infections in BALB/c mice. The genes that are subject to PrlSR regulation have not been described, but the phenotypes reported for the *prlS* and *prlR* mutants suggest that they are likely involved in exopolysaccharide production or cell envelope modifications.

Stringent Response

When faced with intense nutrient deprivation, bacteria produce the alarmones guanosine 3′-diphosphate 5′-triphosphate (pppGpp) and guanosine 3′-diphosphate 5′-diphosphate (ppGpp), known collectively as (p)ppGpp. These signaling molecules bind to RNA polymerase and cause programmatic changes in gene expression that allow bacteria to undergo a physiologic transition known as the stringent response (506). This transition shifts their metabolism from one directed toward cell growth and division to a maintenance metabolism geared toward survival. Induction of the stringent response also provides bacteria with resistance to a variety of different environmental stresses other than nutrient deprivation. In addition, many bacterial pathogens rely on the stringent response to induce the virulence determinants they need for successful colonization of their mammalian hosts.

The (p)ppGpp synthetase/hydrolase Rsh controls the stringent response in *Brucella* (117). Microarray analysis indicates that approximately 12% of the genes in the *B. suis* 1330 genome are regulated by the stringent response (399). Consistent with its expected role in allowing the brucellae to adapt their physiology to nutrient deprivation, Rsh represses the expression of rRNA genes and induces the expression of methionine biosynthesis genes in *B. suis* 1330 when this strain is exposed to nutrient-deprived conditions. *B. abortus*, *B. suis*, and *B. melitensis rsh* mutants are also more sensitive to nutrient deprivation than their parent strains (117, 507, 508), and depriving a CO_2-dependent *B. ovis* strain of CO_2 elicits the stringent response (509).

B. abortus, *B. melitensis*, and *B. suis rsh* mutants are highly attenuated in mice and cultured mammalian cells (60, 117, 335, 507, 508), but evidence suggests that the contributions of Rsh to virulence go beyond its capacity to allow the brucellae to withstand the nutrient deprivation they encounter in their mammalian hosts. The genes encoding the type IV secretion system, cbb_3-type high-affinity cytochrome oxidase, nitrate reductase, and Cu/Zn superoxide dismutase SodC, for instance, are regulated by Rsh (117, 399). Constitutive production of (p)ppGpp also arrests *Brucella* cells in G_1 of the cell cycle (508), which as noted previously, is their most infectious form. It is obvious that we still have a lot to learn about precisely how the stringent response contributes to *Brucella* virulence.

c-di-GMP Signaling

The signaling molecule bis-(3′–5′)-cyclic diguanylic acid (c-di-GMP) regulates bacterial gene expression at multiple levels. c-di-GMP can bind transcriptional regulators and modulate their regulatory activity, bind to riboswitches in mRNAs and control the efficiency with which these transcripts are transcribed or translated, or bind to proteins and regulate their function (510, 511). c-di-GMP-regulated genes and gene products play critical roles in the virulence of many bacterial pathogens (512, 513), and pioneering studies from the Splitter and Smith laboratories have provided convincing evidence that the same holds true for *Brucella* (304, 327). *Brucella* mutants lacking the diguanylate cyclase CgsB or the c-di-GMP-specific phosphodiesterases BpdA or BpdB, for instance, display significant alterations in their virulence profiles in cultured macrophages and mice, and microarray and proteomic analyses of *B. melitensis cgsB* and *bpdA* mutants indicate that genes encoding important virulence determinants such as the flagella and the type IV secretion system are regulated by c-di-GMP. The mechanism(s) by which c-di-GMP regulates the expression of these genes, however, has not yet been determined, as no c-di-GMP-responsive transcriptional regulators, c-di-GMP-responsive riboswitches, or proteins with catalytic or structural functions responsive to this signaling molecule have been identified in *Brucella*. The phenotypic properties displayed by the *B. melitensis bpdA* mutant

suggest that c-di-GMP signaling controls many aspects of the basic biology of *Brucella* strains, including their central metabolism and progression through the cell cycle (327). This is consistent with the function of c-di-GMP as a global regulator in bacteria in general (510, 511), and in the alphaproteobacteria in particular, where c-di-GMP plays a major role in regulating cell cycle progression and cellular differentiation in *Caulobacter*, *Sinorhizobium*, and *Agrobacterium*, as well as the symbiotic and pathogenic interactions of the last two bacteria with their respective eukaryotic hosts (514–517).

The innate immune sensor known as stimulator of interferon genes (STING) resides in the ER of mammalian cells (518). This pattern recognition receptor recognizes c-di-GMP produced by bacterial pathogens, and STING-induced inflammatory responses can have both positive and negative impacts on bacterial infections (519). Therefore, it is not surprising that recent studies indicate that one way that c-di-GMP production contributes to *Brucella* virulence is by modulating the host immune response (520, 521). Specifically, c-di-GMP binding to STING stimulates the unfolded protein response in the ER of infected cells, and the resulting inflammatory response facilitates granuloma formation, which is thought to be important for the persistence of chronic infections.

sRNAs and Hfq

Small regulatory RNAs (sRNAs) play important roles in regulating prokaryotic gene expression at the posttranscriptional level (522). sRNAs perform their regulatory functions by binding to the mRNA transcripts of genes and blocking their translation or causing folding changes in these transcripts that uncover ribosome binding sites and enhance their translation. sRNAs binding to their cognate mRNA transcripts can also enhance the degradation of these mRNAs or enhance their stability, and this feature of sRNAs also plays an important role in their regulatory function. Most sRNAs (i) range in size from 50 to 300 nucleotides in length, (ii) are rich in secondary structure, (iii) regulate genes that reside remotely from the gene encoding the sRNA, and (iv) have limited patches of nucleotides known as regulatory motifs that share imperfect complementarity with their mRNA targets. The limited complementarity of sRNAs with their target transcripts allows them to regulate multiple genes, but it also means that most sRNAs require the RNA chaperone Hfq to facilitate productive interactions with their target mRNAs (523).

Hfq is required for the wild-type expression of many *Brucella* genes (122, 388, 392, 400, 524, 525), and *B. abortus* and *B. melitensis hfq* mutants exhibit pleiotropic phenotypes and are highly attenuated in cultured mammalian cells and mice (400, 442, 443, 525–527). These experimental findings indirectly provide evidence for the importance of sRNAs in the basic biology and virulence of *Brucella* strains. But the first sRNAs to be identified and characterized in *Brucella* were the 110-nt sRNA AbcR1 and the 116-nt sRNA AbcR2, and studies of the interactions of these sRNAs with their target transcripts has provided us with important insight into how *Brucella* sRNAs function at the molecular level (528, 529). AbcR1 and AbcR2, for example, play redundant roles in controlling the expression of genes encoding ABC transporters involved in amino acid and polyamine transport, and a *B. abortus* mutant lacking both AbcR1 and AbcR2 is highly attenuated. AbcR1 and AbcR2 orthologs are also found in other members of the *Rhizobiales* order of the alphaproteobacteria along with a conserved LysR-type regulator known as VtlR in *Brucella*, which coordinates the expression of these sRNAs. Importantly, these sRNAs control multiple genes required for the successful interactions of these bacteria with their respective hosts (530–532).

Since the discovery of AbcR1 and AbcR2, bioinformatics-based approaches and RNA sequencing (RNA-seq) analysis have been used to identify other sRNA candidates in *Brucella* genomes, and the existence of over 100 potentially authentic *Brucella* sRNAs have been confirmed by reverse transcription PCR (RT-PCR) and/or Northern blot analysis (128, 533–540). Some of these *Brucella* sRNAs are required for virulence in cultured mammalian cells and/or mice, including BSR0602 (535), BSR0441 (537),

BASI74 (538), Bmsr1 (539), and BSR1141 (540). Experimental evidence also suggests that an sRNA designated BsrH plays a role in *Brucella* iron metabolism (534). More extensive studies of these and other newly discovered sRNAs and the auxiliary proteins such as Hfq and RNase E (541) that modulate their regulatory functions will help us better understand how these genetic regulators contribute to the basic biology and virulence of *Brucella*.

CONCLUDING REMARKS

Over the past 2 decades, we have uncovered many of the "hidden credentials" of *Brucella* virulence. But there is obviously a lot more to learn about how these remarkable bacteria produce disease in their natural hosts. We do not know, for instance, how many of the T4SS effectors contribute to virulence. There are also unanswered questions about how the brucellae coordinate the expression of genes encoding virulence determinants in response to external and endogenous signals received in the host and how specific genetic regulators contribute to these patterns of gene expression. How the brucellae adjust their cellular metabolism to adapt to shifts in host cell metabolism is also an area that needs more investigation (109, 354, 355). More experimental work also needs to be done with natural hosts using routes of inoculation that mimic those that occur naturally. Mice have been indispensable for gaining a better understanding of the basic features of *Brucella* virulence (542), but studies suggest that virulence determinants required in natural hosts may not always be identified using the murine model (369). Specific virulence determinants required for *Brucella* strains in their natural hosts may differ, and it is likely that the requirement for individual virulence determinants may vary during different stages of the disease cycle in these hosts (e.g., chronic infection versus abortion and fetal pathology in ruminants). From an evolutionary standpoint, it will be interesting to determine what genetic changes allowed the brucellae to evolve from environmental bacteria with phenotypic characteristics resembling those of their close present-day phylogenetic relative *Ochrobactrum* to become the highly host-adapted and host-specific pathogens they are today (7). This review does not cover studies focused specifically on host immune responses to *Brucella* infection, but this topic is covered by other reviews (543–548). Moreover, it is obvious that a better understanding of these responses is needed before we can truly gain a clear picture of the hidden credentials of *Brucella* virulence.

From a translational perspective, gaining a better understanding of the hidden credentials of *Brucella* virulence provides us with molecular targets that can be exploited in our efforts to design better *Brucella* vaccines and novel antimicrobials that can be used to prevent and treat brucellosis. Vaccines have been integral components of programs that have been successfully used to control food animal brucellosis worldwide. But the currently available vaccines have their limitations and drawbacks, and widespread efforts are under way to develop better *Brucella* vaccines for use in both food animals and wildlife (549–552). There is also presently no safe and effective vaccine that can be used to prevent human brucellosis, and this disease is notoriously difficult to treat (553, 554). The development of new and better chemotherapeutic strategies for treating human brucellosis is an area that deserves greater attention because it has the potential to have a tremendous impact on the status of this disease as one of the world's leading zoonoses and on the threat of *Brucella* strains as agents of bioterrorism.

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