AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

CELLULAR MICROBIOLOGY:
PATHOGEN-HOST CELL MOLECULAR INTERACTIONS

Brucella abortus Senses the Intracellular Environment through the BvrR/BvrS Two-Component System, Which Allows *B. abortus* To Adapt to Its Replicative Niche

Pamela Altamirano-Silva,[a] Jazmín Meza-Torres,[a]* Amanda Castillo-Zeledón,[b] Nazareth Ruiz-Villalobos,[b] Ana Mariel Zuñiga-Pereira,[b] Carlos Chacón-Díaz,[a] Edgardo Moreno,[b,c] Caterina Guzmán-Verri,[a,b] Esteban Chaves-Olarte[a,b]

[a]Centro de Investigación en Enfermedades Tropicales, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

[b]Programa de Investigación en Enfermedades Tropicales, Escuela de Medicina Veterinaria, Universidad Nacional, Heredia, Costa Rica

[c]Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

ABSTRACT *Brucella abortus* is a facultative extracellular-intracellular pathogen belonging to a group of *Alphaproteobacteria* that establishes close interactions with animal cells. This bacterium enters host cells in a membrane-bound compartment, avoiding the lysosomal route and reaching the endoplasmic reticulum through the action of the type IV secretion system, VirB. In this work, we demonstrate that the BvrR/BvrS two-component system senses the intracellular environment to mount the transcriptional response required for intracellular life adaptation. By combining a method to purify intracellularly extracted bacteria with a strategy that allows direct determination of BvrR phosphorylation, we showed that upon entrance to host cells, the regulatory protein BvrR was activated (BvrR-P) by phosphorylation at aspartate 58. This activation takes place in response to intracellular cues found in early compartments, such as low pH and nutrient deprivation. Furthermore, BvrR activation was followed by an increase in the expression of VjbR and VirB. The *in vitro* activation of this BvrR-P/VjbR/VirB virulence circuit rescued *B. abortus* from the inhibition of intracellular replication induced by bafilomycin treatment of cells, demonstrating the relevance of this mechanism for intracellular bacterial survival and replication. All together, our results indicate that *B. abortus* senses the transition from the extracellular to the intracellular milieu through BvrR/BvrS, allowing the bacterium to transit safely to its replicative niche. These results serve as a working model for understanding the role of this family of two-component systems in the adaptation to intracellular life of *Alphaproteobacteria*.

KEYWORDS two-component system, type IV secretion system, brucellosis

Brucella abortus is a relevant pathogen that induces abortion in cattle and a debilitating zoonotic disease in humans (1). The pathogenesis of brucellosis resides in the ability of *B. abortus* to enter and multiply within host cells, since several mutants with defects in its intracellular lifestyle are highly attenuated in animal models (2–4). One of the key *Brucella* virulence factors involved in intracellular replication is the type IV secretion system (T4SS) VirB. This system belongs to a family of prokaryotic molecular machineries that injects proteins and/or nucleic acids into eukaryotic cells in order to modulate the interaction of bacteria with their host. The protein effectors delivered by VirB into host cells are only beginning to be discovered, and their function in modulating intracellular trafficking is largely unknown (5–9). In spite of this, it is widely accepted that the partial and transient interactions of the *Brucella*-containing vacuole

Received 17 October 2017 **Returned for modification** 10 November 2017 **Accepted** 16 January 2018

Accepted manuscript posted online 29 January 2018

Citation Altamirano-Silva P, Meza-Torres J, Castillo-Zeledón A, Ruiz-Villalobos N, Zuñiga-Pereira AM, Chacón-Díaz C, Moreno E, Guzmán-Verri C, Chaves-Olarte E. 2018. *Brucella abortus* senses the intracellular environment through the BvrR/BvrS two-component system, which allows *B. abortus* to adapt to its replicative niche. Infect Immun 86:e00713-17. https://doi.org/10.1128/IAI.00713-17.

Editor Guy H. Palmer, Washington State University

Address correspondence to Esteban Chaves-Olarte, esteban.chaves@ucr.ac.cr.

* Present address: Jazmín Meza-Torres, Institut Pasteur, Paris, France.

P.A.-S. and J.M.-T. contributed equally to this article.

(BCV) with lysosomes allow the expression of VirB, which in turn plays a crucial role in redirecting the intracellular trafficking to the endoplasmic reticulum (ER) (10, 11). The *in vitro* expression of VirB in culture media is variable and low (12–14), in contrast to the consistent and robust expression detected during the first hours of the *B. abortus* intracellular trafficking (13). These observations indicate that the bacterium possesses sophisticated mechanisms to control the expression of this injection machinery. Several transcription factors have been demonstrated to control the regulation of VirB (2, 15–17). Among those, the quorum-sensing transcriptional regulator VjbR has been shown to be crucial for the timely coordination of VirB expression during the *B. abortus* life cycle (2). VjbR is able to bind several promoters, including the *virB* promoter, when autoinducers like homoserine lactone (HSL) are absent. In contrast, the HSL interaction with VjbR inhibits its DNA-binding activity, allowing the expression of genes repressed by VjbR and turning off the expression of genes positively regulated by this transcriptional regulator (2). In addition to the regulation exerted on VirB, VjbR controls, both positively and negatively, a vast range of genes with functions varying from metabolism to virulence (18).

Another key element required for *B. abortus* intracellular replication is the BvrR/BvrS two-component system (TCS). BvrR/BvrS mutants are attenuated in cells and animals with impaired intracellular trafficking and increased susceptibility to bactericidal peptides (4). This system is composed of a sensor histidine kinase protein, BvrS, located in the plasma membrane and a cytoplasmic response regulator, BvrR, controlling genes important for the homeostasis of the outer membrane. The TCS impacts the expression of proteins like Omp25 and Omp22 and the structure of the lipopolysaccharide (LPS), specifically the fatty acids of lipid A (19, 20). Furthermore, proteomics and transcriptomics analyses of BvrR/BvrS mutants indicate the involvement of this TCS in nitrogen and carbon metabolism and the expression of VirB and its transcriptional regulator, VjbR (12, 21, 22). In this context, a direct interaction of BvrR with the *virB* promoter has been demonstrated, and furthermore, constitutive expression in *trans* of BvrR in a mutant with a transposon insertion on *bvrR* restores the expression of VjbR and VirB (12).

In this work, we demonstrate that BvrR/BvrS senses the transition of *B. abortus* from an extracellular to an intracellular environment at early stages of the intracellular life cycle. This response allows *B. abortus* to trigger the transcriptional response required to redirect its trafficking to the ER.

RESULTS

BvrR/BvrS is activated upon entrance to eukaryotic cells. Before analyzing the activation of BvrR/BvrS, we first measured the expression of the system. By Western blotting, we determined that both BvrS and BvrR are highly expressed during early times in the *in vitro* growth curve (Fig. 1A). The level of both proteins decreased at late exponential phase, being almost undetectable at stationary phase (Fig. 1A). This result is consistent with the high activity of the BvrR/BvrS promoter at early exponential phase and its low activity at stationary phase, measured as a *luxAB* transcriptional fusion (Fig. 1B). Thus, we used bacteria grown to exponential phase to determine the phosphorylation status of BvrR.

To analyze the activation state of BvrR/BvrS, we measured the proportion of BvrR phosphorylation by Phos-tag SDS-PAGE (23). Recombinant BvrR separated by this method migrated as a single band, whereas recombinant BvrR previously incubated with carbamoyl phosphate migrated as two bands, indicating that the upper band corresponds to BvrR-P (Fig. 2A). The same two-band pattern was observed in lysates derived from *B. abortus* in exponential phase (Fig. 2A). To confirm that the upper BvrR band observed in *B. abortus* lysates corresponds to BvrR-P, alanine or glutamic acid was substituted for aspartate 58 (the putative phosphorylated amino acid). The wild-type BvrR version and the two aspartate 58 mutants were introduced into a mutant *B. abortus* strain that does not express BvrR (4). When lysates from the corresponding strains were separated by Phos-tag SDS-PAGE, wild-type BvrR migrated as two bands,

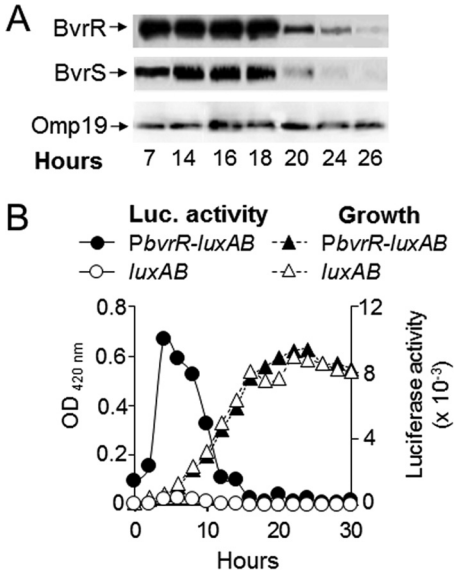

FIG 1 BvrR/BvrS expression varies throughout the *in vitro* growth curve. (A) *B. abortus* 2308 was grown in TSB, and samples were taken at the indicated times. Bacterial lysates were separated by 10% SDS-PAGE, transferred to PVDF membranes, and probed with anti-BvrR, anti-BvrS, or anti-Omp19 antibodies (loading control). (B) *B. abortus* containing an in *trans* transcriptional fusion of the *bvrR* promoter (P_{bvrR}) with *luxAB* (black symbols) or a promoterless *luxAB* as a negative control (white symbols) were grown on TSB for the indicated times, and the optical density (OD) was determined at 420 nm (triangles). The transcription of P_{bvrR} (black circles) was determined at the indicated times by measuring the luciferase activity (circles). These results are representative of at least three independent experiments.

following the same pattern observed in *B. abortus* 2308 lysates (Fig. 2B). On the other hand, both aspartate 58 mutants migrated only as a single band, with the upper band being absent (Fig. 2B). This result indicates that this upper band indeed corresponds to BvrR-P and that aspartate 58 is the phosphorylated residue in BvrR. Following this, two macrophage cell lines, J774 and RAW 264.7, were infected for 2 h with *B. abortus* grown to exponential phase. Intracellular bacteria were then extracted and purified (24). BvrR was phosphorylated upon entrance to both cell lines (Fig. 3A). BvrR phosphorylation was dependent on *B. abortus* cell entrance, since BvrR-P was barely observed in bacteria recovered from macrophages treated with cytochalasin (Fig. 3B), a treatment that inhibits phagocytosis (Fig. 4). The highest BvrR phosphorylation was achieved at 2 h after internalization (Fig. 3C and F). After this time, BvrR-P gradually diminished and was

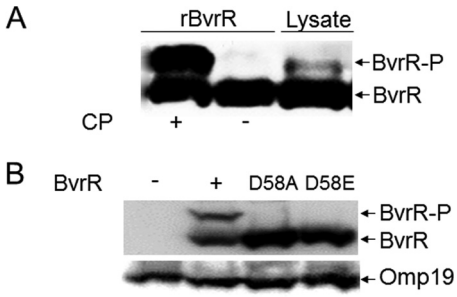

FIG 2 BvrR activation can be determined by Phos-tag SDS-PAGE. (A) Recombinant BvrR (rBvrR) was phosphorylated (+), or not (−), with carbamoyl phosphate (500 mM) for 20 min. As a control, a total lysate from *B. abortus* 2308 grown to exponential phase was used. Samples were separated by 10% SDS-PAGE containing Phos-tag, transferred to PVDF membranes, and probed with anti-BvrR antibodies. (B) A *bvrR*-negative *B. abortus* mutant containing an empty vector (−), a plasmid encoding wild-type *bvrR* (+), or mutated versions of BvrR with substitutions for aspartate 58 by either alanine (D58A) or glutamic acid (D58E) was grown in TSB to exponential phase. Bacterial lysates were prepared and separated by 10% SDS-PAGE containing Phos-tag, transferred to PVDF membranes, and probed with anti-BvrR or anti-Omp19 antibodies (loading control).

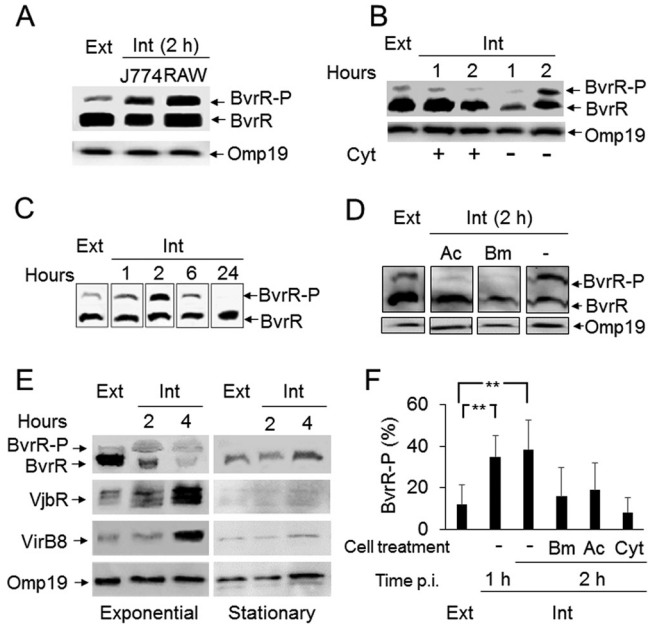

FIG 3 BvrR/BvrS is activated upon entrance to host cells. (A) RAW 264.7 or J744 macrophages were infected with *B. abortus* in exponential phase. After 2 h, intracellular bacteria were purified. Bacterial lysates were prepared and separated by 10% SDS-PAGE containing Phos-tag, transferred to PVDF membranes, and probed with anti-BvrR or anti-Omp19 antibodies (loading control). Bacteria grown *in vitro* in TSB were used as a control for extracellular (Ext) *B. abortus*. (B) RAW 264.7 macrophages were treated with cytochalasin D (Cyt) for 2 h before infection or were left untreated. Cells were then infected with *B. abortus* in exponential phase for the indicated times. After infection, intracellular bacteria were purified and processed as described for panel A. (C) RAW 264.7 macrophages were infected with *B. abortus* in exponential phase for the indicated times. After infection, intracellular bacteria were purified and processed as described for panel A. Since the amount of total BvrR varied significantly throughout the intracellular curve, the intensity of each lane was adjusted to obtain the same signal of unphosphorylated BvrR in all the samples. (D) RAW 264.7 macrophages were treated with 30 μM ammonium chloride (Ac) or 50 nM bafilomycin (Bm) or were left untreated (−). Cells were then infected with *B. abortus* 2308 at exponential growth phase. After 2 h, intracellular bacteria were purified (Int) and processed as described for panel A. All the lanes correspond to duplicates of the same condition and belong to the same gel. (E) RAW 264.7 macrophages were infected with *B. abortus* in exponential or stationary growth phase for the indicated times. After infection, intracellular bacteria were purified and processed as described for panel A for the detection of BvrR-P or for the detection of VjbR and VirB8 by Western blotting. (F) The percentage of BvrR-P from total BvrR was calculated for each indicated condition by densitometry from at least three independent experiments. **, $P < 0.005$, compared to results for extracellular (Ext) bacteria (Student's *t* test). p.i., postinfection.

absent at 24 h postinfection (Fig. 3C). Endosome acidification is essential for BvrR intracellular phosphorylation, since its inhibition with either ammonium chloride or bafilomycin abrogates BvrR/BvrS activation (Fig. 3D and F). The intracellular activation of BvrR was followed by an increase in the expression of VjbR and VirB8 (Fig. 3E). In agreement with the differential expression of BvrR/BvrS during the growth curve, there was no increase in VjbR and VirB expression when the bacteria were at stationary phase, in which the levels of BvrR/BvrS are low (Fig. 3E).

BvrR/BvrS is activated at low pH and under nutrient-limited conditions. To explore the signals that might activate the BvrR/BvrS TCS, we exposed *B. abortus* grown to exponential phase to different pHs (5.0 and 7.0) and different nutrient concentrations, rich medium (tryptic soy broth [TSB]), and minimal medium (MM). It was possible to activate BvrR only when bacteria were exposed to the combination of low pH and limited nutrient availability (Fig. 5A), conditions that mimic the environment found by *B. abortus* during its early interaction with eukaryotic host cells. None of the other combinations tested induced activation of BvrR/BvrS (Fig. 5A). The *in vitro* conditions that induced BvrR phosphorylation also increased VjbR and VirB8 expression (Fig. 5B). *In vitro* incubation of *B. abortus* under low-nutrient conditions and at different pHs indicated that the strongest activation of the system was achieved under acidic

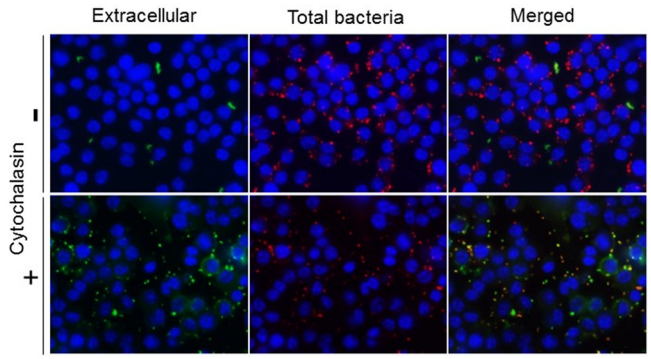

FIG 4 Cytochalasin inhibits phagocytosis of macrophages. Macrophages incubated with (10 μM) or without cytochalasin were infected with *B. abortus*. After 2 h, cells were extensively washed and living nonpermeabilized macrophages were incubated with an FITC-conjugated anti-*B. abortus* antibody for 30 min at 4°C. Cells were then fixed and permeabilized, and bacteria were further detected with a rabbit-anti-*Brucella* antibody and an anti-rabbit antibody–Texas Red conjugate. Nuclei were stained with DAPI. Intracellularly located bacteria are visible in red, whereas extracellular bacteria are green and red.

conditions (pH 4.0 and pH 5.0) with negligible activation at neutral pHs (pH 6.0 and pH 7.0) (Fig. 6A). Direct acidification of TSB with citric acid did not induce BvrR phosphorylation (Fig. 6B). When bacteria grown at stationary phase, expressing low levels of BvrR/BvrS, were exposed to low-nutrient conditions and acidic pH, we did not detect activation of the BvrR/BvrS nor an increase in the expression of VjbR or VirB8 (Fig. 7A and B). The expression of VjbR and VirB8 was not induced in a *bvrR*-negative strain grown to exponential phase and exposed to a low-nutrient medium in acidic pH (Fig. 8).

Activation of the BvrR-P/VjbR/VirB virulence circuit impacts the ability of *B. abortus* to survive intracellularly. As shown above, activation of BvrR/BvrS both *ex*

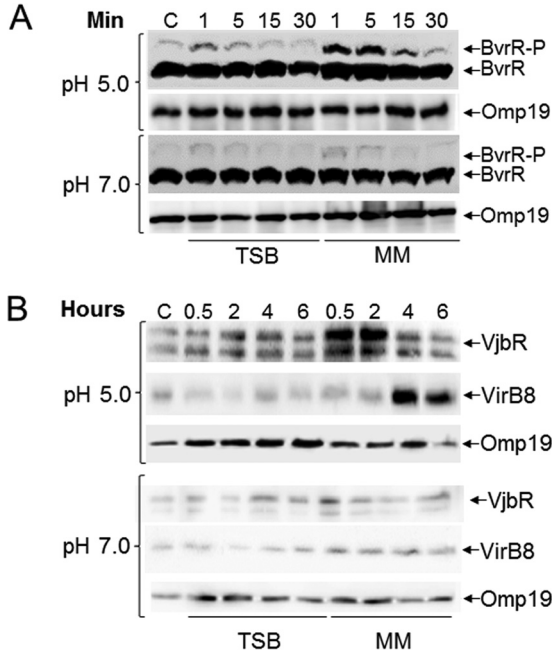

FIG 5 *In vitro* conditions mimicking the intracellular environment induce activation of BvrR/BvrS and expression of VjbR and VirB. *B. abortus* 2308 in exponential phase was incubated in minimal medium (MM) or rich medium (TSB) at pH 5.0 or pH 7.0 for the indicated times. After incubation, bacterial lysates were prepared and separated by 10% SDS-PAGE containing Phos-tag to evaluate BvrR phosphorylation (A) or by 10% SDS-PAGE to determine VjbR and VirB expression by Western blotting (B). Omp19 detection was used as a loading control. These results are representative of at least three independent experiments.

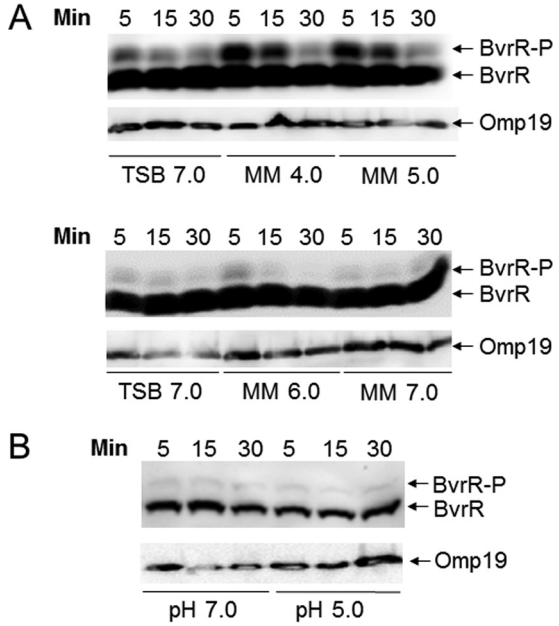

FIG 6 Acidification is required but not sufficient to induce BvrR phosphorylation. (A) *B. abortus* 2308 in exponential growth phase was incubated *in vitro* in minimal medium (MM) at the indicated pH for the indicated times or in TSB at pH 7.0. After incubation, bacterial lysates were prepared, separated by 10% SDS-PAGE containing Phos-tag, transferred to PVDF membranes, and probed with anti-BvrR or anti-Omp19 antibodies (loading control). (B) *B. abortus* 2308 was grown in TSB to exponential growth phase. The pH of the culture medium was then decreased to 5.0 by the addition of citric acid. Control bacteria were not treated, and the pH of the medium remained at pH 7.0. Samples were taken at the indicated times after treatment. Bacterial lysates were prepared, separated by 10% SDS-PAGE containing Phos-tag, transferred to PVDF membranes, and probed with anti-BvrR or anti-Omp19 antibodies (loading control). These results are representative of at least three independent experiments.

vivo and *in vitro* was followed by an increase in the expression of VjbR and VirB. Whereas the direct VirB regulation by VjbR has been well documented (25), no direct interaction has been reported between BvrR and the *vjbR* promoter (P_{vjbR}). To test this possibility, electrophoretic mobility shift assay assays (EMSAs) were performed. Recombinant BvrR was *in vitro* phosphorylated (BvrR-P) using carbamoyl phosphate. When BvrR-P was incubated with a labeled P_{vjbR} probe, a clear retardation in the migration was observed, in contrast to an internal region of the ribosomal L7/L12 gene used as a negative control (Fig. 9A). This interaction was specific, since unlabeled P_{vjbR} outcompeted labeled P_{vjbR} (Fig. 9B). In contrast, unlabeled $P_{L7/L12}$ was unable to outcompete P_{vjbR} (Fig. 9B). To determine the sequence of events, BvrR/BvrS was activated *in vitro* by low pH and nutrient-limited conditions in the presence or absence of homoserine lactone (HSL), a molecule that binds to VjbR and inhibits its DNA-binding ability (26). In the presence of HSL, BvrR/BvrS was still activated (Fig. 9C). However, under these conditions, VirB expression did not increase (Fig. 9C). All together, these results indicate the existence of a virulence circuit in which BvrR/BvrS activation leads to the expression of VjbR and subsequently to increased levels of VirB.

To analyze whether the activation of this virulence circuit plays a role in the intracellular survival of *B. abortus*, we determined the fate of the bacteria in cells whose endosomal acidification was inhibited. BvrR was not phosphorylated in cells previously treated with bafilomycin (Fig. 3D and F), in agreement with the requirement of a low pH for the activation of BvrR/BvrS. Consistent with previous reports (27), *B. abortus* was unable to replicate in bafilomycin-treated cells (Fig. 10A). However, if *B. abortus* was previously exposed to low pH in nutrient-limited medium, then the bacterium was able to replicate in bafilomycin-treated cells (Fig. 10A). In contrast, bacteria exposed to a neutral pH and nutrient-rich medium were rapidly cleared (Fig. 10A). Exposure to low pH under nutrient-limited conditions was not able to rescue a *bvrR*-negative mutant

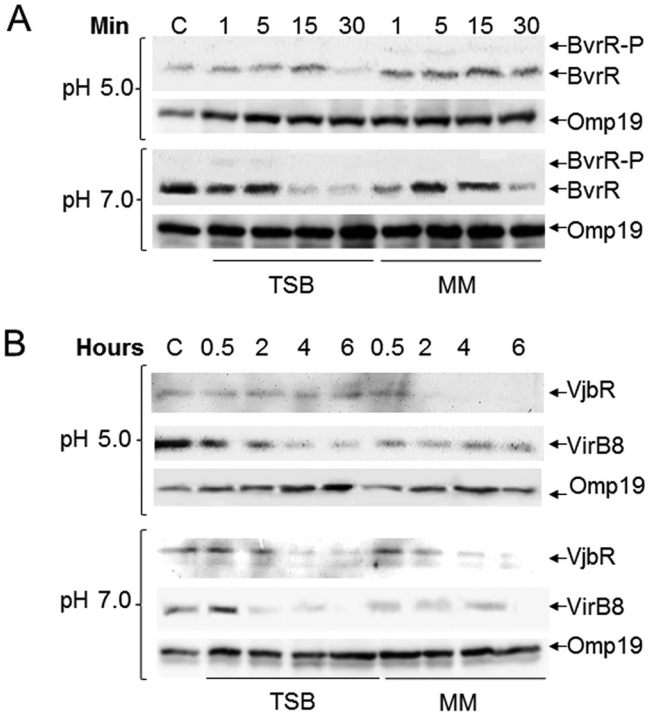

FIG 7 *In vitro* conditions mimicking the intracellular environment do not activate BvrR/BvrS or induce the expression of VjbR and VirB when *B. abortus* is in stationary growth phase. *B. abortus* 2308 in stationary growth phase was incubated in minimal medium (MM) or rich medium (TSB) at pH 5.0 or pH 7.0 for the indicated times. Untreated bacteria grown in TSB were used as a control (lane C). After incubation, bacterial lysates were prepared and separated by 10% SDS-PAGE containing Phos-tag to evaluate BvrR phosphorylation (A) or 10% SDS-PAGE to determine VjbR and VirB expression by Western blotting (B). Omp19 detection was used as a loading control. These results are representative of at least three independent experiments.

(Fig. 10B), indicating that this phenomenon is dependent on a functional BvrR/BvrS system. In agreement with the high activation of BvrR/BvrS in bacteria grown to exponential phase and the low activation in bacteria grown to stationary phase, the former achieved a significantly higher intracellular replication in RAW 264.7 macrophages (Fig. 10C). Thus, induction of the virulence circuit formed by BvrR/BvrS, VjbR, and VirB positively modulated the ability of *B. abortus* to survive intracellularly.

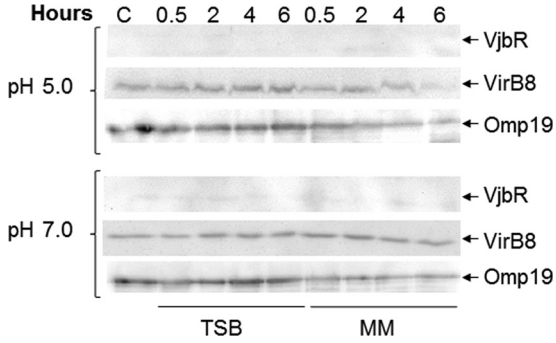

FIG 8 BvrR controls the expression of VjbR and VirB in response to low pH and minimal medium. A *bvrR*-negative *B. abortus* strain in exponential phase was incubated in minimal medium (MM) or rich medium (TSB) at pH 5.0 or pH 7.0 for the indicated times. Untreated bacteria grown in TSB were used as a control (lane C). After incubation, bacterial lysates were prepared and separated by 10% SDS-PAGE to determine VjbR and VirB expression by Western blotting. Omp19 detection was used as a loading control.

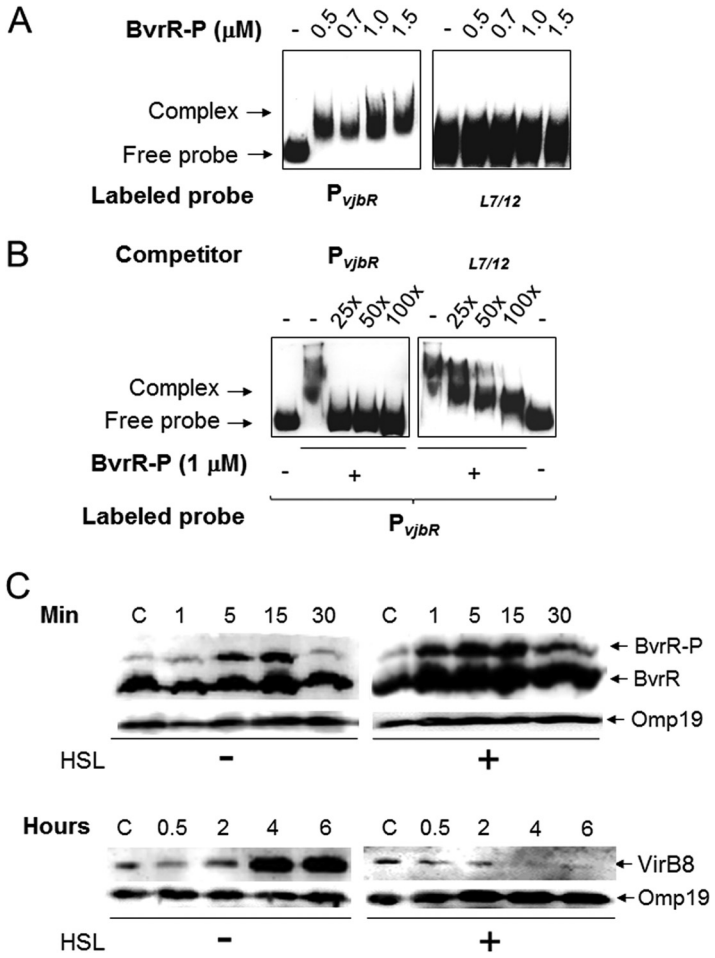

FIG 9 BvrR-P, VjbR, and VirB form a virulence circuit. (A) Digoxigenin-labeled probes containing the promoter region of *vjbR* (P_{vjbR}) or the negative-control L7/L12 were incubated with increasing concentrations of phosphorylated BvrR (BvrR-P). Samples were then separated in nondenaturing gels, transferred to a membrane, and developed by chemiluminescence using anti-digoxigenin antibodies. (B) Digoxigenin-labeled probes of P_{vjbR} were incubated with BvrR-P (1 mM) and an excess of the indicated nonlabeled probes. Samples were then processed as described for panel A. (C) *B. abortus* 2308 in exponential phase was incubated in minimal medium at pH 5.0 for the indicated times in the presence (50 μM) or absence of homoserine lactone. After incubation, bacterial lysates were prepared and separated by 10% SDS-PAGE containing Phos-tag to evaluate BvrR phosphorylation or by 10% SDS-PAGE to determine VjbR and VirB expression by Western blotting. Omp19 detection was used as a loading control. These results are representative of at least three independent experiments.

DISCUSSION

Previous evidence indicates that BvrR/BvrS impacts the expression level of VirB and its transcriptional regulator, VjbR. Furthermore, a direct interaction of BvrR and VjbR with the *virB* promoter has been previously demonstrated (12, 25). Since these three systems are crucial for *B. abortus* virulence and intracellular survival, we postulate the existence of a virulence transcriptional circuit in which BvrR/BvrS induces the expression of VjbR and, in turn, VirB is expressed. Considering the existence of this virulence circuit and that VirB expression is significantly induced upon entrance to the eukaryotic host cell (28), we hypothesized that BvrR/BvrS is responsible for sensing the intracellular location of the bacterium. In the present work, and by combining a method to extract and purify intracellular bacteria with the direct detection of the phosphorylation state of BvrR, we unambiguously determined the activation of BvrR/BvrS during the early stages of the interaction of *B. abortus* with its eukaryotic host cell. Thus, our direct experimental evidence indicates that BvrR/BvrS functions as a sensor that detects the

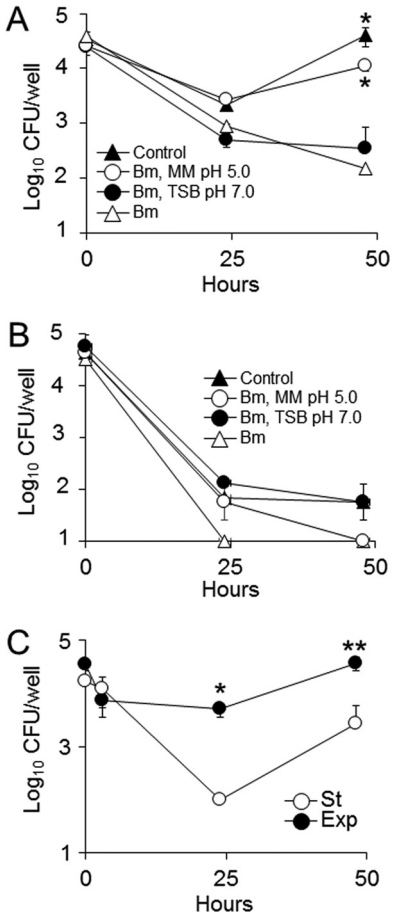

FIG 10 Activation of the BvrR-P/VjbR/VirB virulence circuit promotes intracellular survival of *B. abortus*. *B. abortus* 2308 (A) or a *bvrR*-negative strain (B) in exponential phase was incubated *in vitro* with MM at pH 5.0 (white circles) or TSB at pH 7.0 (black circles) for 3 h. Untreated bacteria grown in TSB were used as a control. Before infection of RAW 264.7 macrophages, the acidification of endosomes was inhibited with 50 nM bafilomycin (Bm). Control cells (black triangles) remained untreated. The intracellular growth was determined at the indicated times using a gentamicin protection assay. *, $P < 0.05$, compared to results with nontreated *B. abortus* replicating on Bm-treated cells (white triangles) (Student's *t* test). (C). Macrophages (RAW 264.7) were infected with *B. abortus* in exponential (Exp) or stationary (St) phase and incubated in the presence of gentamicin (5 μg/ml) for the indicated times. Cells were then lysed, and intracellular bacteria were determined by plate counting. *, $P < 0.05$, and **, $P < 0.005$, compared to the results for the corresponding time in stationary phase (Student's *t* test). These results are representative of at least three independent experiments.

transition of *B. abortus* from the extracellular milieu to the intracellular niche. This activation during the early stages of the *B. abortus* intracellular cycle results in the expression and assembly of the T4SS system, allowing the escape of the bacterium from the lysosomal route and the redirection of its trafficking to the endoplasmic reticulum (Fig. 11). Interestingly, we observed a high expression of BvrR/BvrS in exponential phase and an almost negligible expression in stationary phase, correlating with the ability to switch on, or not, the virulence circuit described above. This difference might be responsible for the higher intracellular replication achieved by bacteria in exponential phase than that achieved by bacteria in stationary phase.

Several two-component system orthologues to BvrR/BvrS have been postulated to mediate the interaction of *Alphaproteobacteria* with their corresponding eukaryotic hosts. For instance, the BatR/BatS TCS from *Bartonella henselae* controls the adaptive response during its life cycle by being activated at a neutral pH upon entrance to eukaryotic host cells to trigger the expression of a T4SS crucial for intracellular replication (29). The ChvG/ChvI TCS from *Agrobacterium tumefaciens* controls the expression of acid-induced genes essential for the formation of plant tumors (30, 31). Finally, the

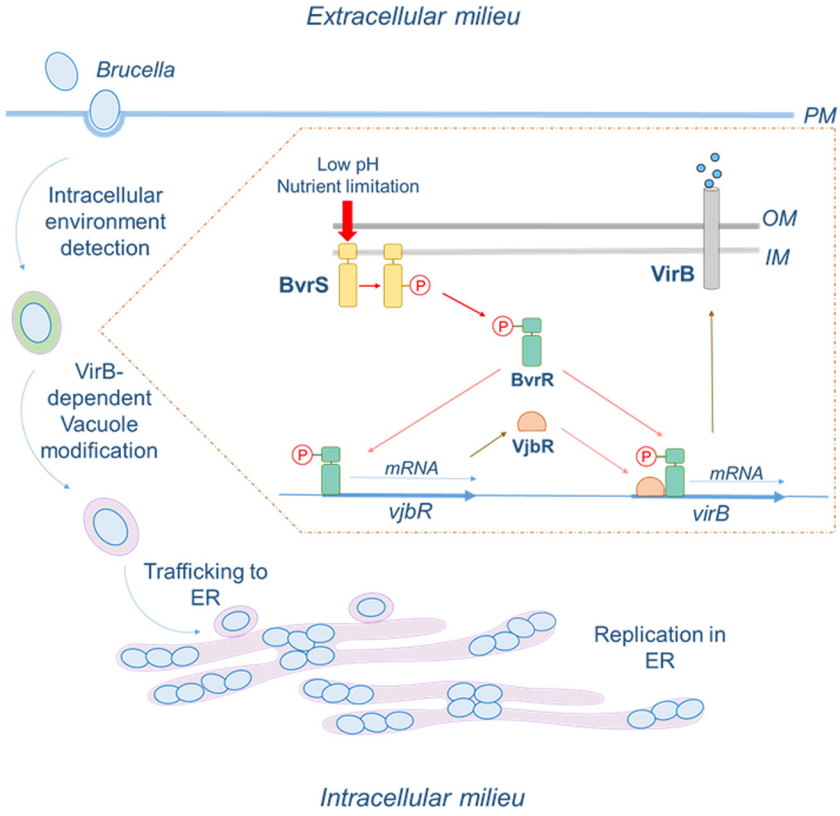

FIG 11 Model showing the coordination by BvrR/BvrS of the intracellular response of *B. abortus*. Once *B. abortus* enters the cells, BvrS detects intracellular environment cues such as low pH and a nutrient-limited medium that trigger its autophosphorylation. The phosphate group is then transferred to BvrR, which in turn will bind its regulated promoters. Among those, the transcription of *vjbR* will be induced and both VjbR and BvrR will cooperate to induce the expression of the *virB* operon. The assembly of this T4SS will allow *B. abortus* to modify its intracellular trafficking in order to reach the ER, its replicative niche. PM, plasma membrane; IM, inner membrane; OM, outer membrane.

ExoS/ChvI TCS from *Sinorhizobium meliloti* is essential for symbiosis with host plants (32, 33). The role of these systems has been mainly deduced from indirect evidence, since mutants in these TCSs are defective in establishing an interaction with host cells (4, 31). The activation of the systems by host-derived signals has also been determined indirectly by measuring the expression of target genes (30, 34–36). Thus, the direct results presented here contribute to the understanding of the interaction of *Alphaproteobacteria* with their eukaryotic hosts.

The precise nature of the signal sensed by BvrS remains to be determined. Our results indicate that acidity is essential but not sufficient for the activation of BvrR, since treatment of cells with bafilomycin or ammonium chloride partially abrogated the activation of the system but acidification alone did not induce BvrR phosphorylation. We could activate *in vitro* BvrR/BvrS by combining low pH with a nutrient-limiting environment. Furthermore, this *in vitro* activation was functional since it rescued *B. abortus* from the inhibition of intracellular replication exerted by bafilomycin. However, the intracellular milieu encountered by the bacterium is far more complex, and other factors may contribute to a complete activation of the system. In this context, it is tempting to speculate that rather than sensing a single signal, BvrS responds to a biophysical environment composed by several factors. This environment would induce conformational changes in BvrS, leading to its autophosphorylation and the subsequent activation of BvrR. Alternatively, as demonstrated for related alphaproteobacteria, the periplasmic protein ExoR could sense the intracellular environment leading to its degradation. In *S. meliloti*, ExoR physically interacts with ExoS, the sensor of the ExoS/ChvI TCS, keeping it in an off state. Upon proteolysis of ExoR within the periplasm,

TABLE 1 Plasmids and vectors used in this study

Plasmid or vector	Characteristics or relevant features	Source or reference
pJET1.2	Cloning vector with BamHI and XhoI restriction sites; size, 2,974 bp; Amp^r	ThermoScientific, 2013
pJET1.2 P_{bvrR}	pJET1.2 plasmid containing a fragment with *bvrR* promoter (P_{bvrR}) cloned in BamHI/XhoI restriction sites. The fragment contains 225 bp upstream from the *bvrR* initiation codon, including the intergenic region between *pckA* (BAB1_2090) and *bvrR* (BAB1_2092); size, 3,199 bp; Amp^r	This study
pJD27	Expression vector containing *luxAB* (luciferase activity) and BamHI and XhoI restriction sites; size, 4,935 bp; Amp^r	2
pJD27 P_{bvrR}	pJD27 with *luxAB* activity controlled by the P_{bvrR} which contains a 225-bp fragment upstream from the *bvrR* initiation codon cloned in BamHI/XhoI restriction sites; size, 5,160 bp; Amp^r	This study
pCR8/GW/TOPO vector	Cloning vector, with recombination sites *att*L1 and *att*L2; size, 2,817 bp; Spc^r	Life Technologies Corporation, 2012
GWEntrybvrR	pCR8/GW/TOPO with a 1,093-bp insert which includes 198 bp that corresponds to P_{bvrR} located from nucleotide 2026638 to nucleotide 2026835, 720 bp that corresponds to *bvrR* sequence from nucleotide 2026836 to nucleotide 2027555, and 175 bp of the *bvrR-bvrS* intergenic region located from nucleotide 2027556 to nucleotide 2027730 of the *B. abortus* 2308 genome; size, 3,910 bp; Spc^r	This study
GWEntrybvrRD58A	GWEntrybvrR with *bvrR* mutation that substitutes an alanine residue for an aspartate residue in position 58; size, 3,910 bp; Spc^r	This study
GWEntrybvrRD58E	GWEntrybvrR with *bvrR* mutation that substitutes a glutamic acid residue for an aspartate residue in position 58; size, 3,910 bp; Spc^r	This study
pTOPObvrR	pCR8/GW/TOPO with a 738-bp insert corresponding to *bvrR*; size, 3,555 bp; Spc^r	This study
pTOPObvrRD58A	pCR8/GW/TOPO with a 738-bp insert corresponding to *bvrR*D58A; size, 3,555 bp; Spc^r	This study
pTOPObvrRD58E	pCR8/GW/TOPO vector with a 738-bp insert corresponding to *bvrR*D58E; size, 3,555 bp; Spc^r	This study
prH002	Gateway-compatible vector useful for complementation and for constitutive overexpression, with a pBBR1 replication origin that contains the toxic cassette ccdB flanked by *attR1* and *attR2* recombination sites; size, 6,420 bp; Cm^r	41
prH002bvrR	prH002 with a 738-bp insert corresponding to *bvr*; Cm^r	This study
prH002bvrRD58A	prH002 with a 738-bp insert corresponding to *bvrR*D58A; Cm^r	This study
prH002bvrRD58E	prH002 with a 738-bp insert corresponding to *bvrR*D58E; Cm^r	This study

the negative effect on ExoS/ChvI is released, and this system then upregulates crucial phenotypes as succinoglycan production (37). In *A. tumefaciens*, the ChvG/ChvI TCS is repressed by ExoR at neutral pH, but when the bacterium encounters acid pH, ExoR is proteolyzed, allowing the transcription of motility and chemotaxis genes, succinoglycan biosynthesis genes, a type VI secretion system (T6SS), and various virulence genes (35).

It would be interesting in the future to monitor the state of BvrR phosphorylation at late stages of the intracellular life cycle, when the bacteria have moved from the ER to autophagosome-like compartments and are ready to exit the cell (38). In fact, a VirB requirement in postreplication events has recently been demonstrated (39). Thus, it is likely that under these circumstances, bacteria again increase their competence for intracellular survival and would then activate the virulence circuit formed by BvrR/BvrS, VjbR, and VirB to initiate another cycle of replication within host cells.

MATERIALS AND METHODS

Bacterial culture and strain construction. The plasmids, strains, and primers used in this study are detailed in Tables 1, 2, and 3, respectively. *B. abortus* strains were maintained and grown using previously described protocols (40). When needed, *B. abortus* strains were supplemented with antibiotics to maintain plasmid selection. *Escherichia coli* strains used for cloning were grown in LB (37°C) supplemented with 50 $\mu g \cdot ml^{-1}$ kanamycin (Kan), 100 $\mu g \cdot ml^{-1}$ ampicillin (Amp), or 30 $\mu g \cdot ml^{-1}$ chloramphenicol (Cm) when required. *B. abortus* strains were grown in tryptic soy broth (TSB) at 37°C supplemented with 5% CO_2.

Construction of plasmids prH002bvrR, prH002bvrRD58A, and prH002bvrRD58E. To determine BvrR phosphorylation site and to validate SDS-PAGE containing Phos-tag as a technique that allows the detection of phosphorylation of this response regulator, we generated two point mutations in the BvrR putative phosphorylation aspartate residue. To generate the point mutations in the *bvrR* gene, the GeneArt site-directed mutagenesis system kit (Invitrogen) was used in accordance with the manufacturer's specifications. Briefly, in order to amplify a 1,093-bp fragment containing 198 bp of *bvrR* promoter, 720 bp of *bvrR*, and 175 bp of the *bvrR-bvrS* intergenic region, a PCR was carried out using primers 3-200bvrR3 and revtrSinterg1.5 (Table 3). The PCR product was cloned into plasmid pCR8/GW/TOPO

TABLE 2 Strains used in this study

Brucella strains	Characteristics or relevant features	Source or reference
B. abortus 2308	WT *Brucella* strain, virulent, smooth LPS; Nal^r	40
B. abortus luxAB	WT *B. abortus* 2308 strain harboring plasmid pJD27 (*luxAB* gene-luciferase activity); Amp^r	This study
B. abortus P*bvrR-luxAB*	WT *Brucella* strain harboring pJD27 P_{bvrR} plasmid ($P_{bvrR\text{-}luxAB}$ transcriptional fusion); Amp^r	This study
B. abortus bvrR-pbvrR	*B. abortus bvrR* mutant harboring prH002bvrR with a 738-bp insert corresponding to *bvrR*; Cm^r	This study
B. abortus bvrR-pbvrRD58A	*B. abortus bvrR*-negative mutant harboring plasmid prH002bvrRD58A with a 738-bp insert corresponding to *bvrR*D58A; Cm^r	This study
B. abortus bvrR-pbvrRD58E	*B. abortus bvrR*-negative mutant harboring plasmid prH002bvrRD58E with a 738-bp insert corresponding to *bvrR*D58E; Cm^r	This study

(Table 1), generating plasmid GWEntrybvrR. GWEntrybvrR was methylated, and its *bvrR* region was mutated specifically in aspartate residue 58 (BvrR phosphorylation site). The products were transformed into the *E. coli* One Shot MAX Efficiency DH5α-T1^R strain. In order to confirm the mutations, the plasmids were sequenced according to the manufacturer's instructions (BigDye Terminator v.3.1 cycle sequencing kit; Applied Biosystems), using primers 3-200bvrR3 and revtrRSinterg1.5. The plasmid with the *bvrR* point mutation in the putative phosphorylation site in which a glutamate residue was substituted for the aspartate residue at position 58 was named GWEntrybvrRD58E, and when an alanine residue was substituted for the aspartate residue, it was named GWEntrybvrRD58A. Using primers HisbvrRF and HisbvrRR, *bvrR*, *bvrR*D58A, and *bvrR*D58E (738-bp fragment), sequences were subcloned into the entry plasmid pCR8/GW/TOPO according to the manufacturer's instructions (pCR8/GW/TOPO TA cloning kit; Invitrogen). Chemically competent cells of *E. coli* One Shot Mach1-T1 (Invitrogen) were transformed with the plasmids pTOPObvrR, pTOPObvrRD58A, and pTOPObvrRD58E. To confirm the correct sense of the inserts, M13F and M13R primers were hybridized, respectively, with upstream and downstream regions from the insert. Additionally, M13F and M13R primers were used with bvrR-bv1R and bvrR-bv1F primers, respectively, to determine the orientation of the insert. An amplicon from each allele with the right sense of insertion was selected and sequenced (BigDye Terminator v.3.1 cycle sequencing kit; Applied Biosystems) using primers M13F and M13R. The obtained plasmids, pTOPObvrR, pTOPObvrRD58A, and pTOPObvrRD58E, were subcloned into the vector prH002 by site-specific recombination using the Gateway LR recombination cloning procedure in accordance with manufacturer's instructions (Invitrogen Life Technologies) (41, 42). The vectors prH002bvrR, prH002bvrRD58A, and prH002bvrRD58E were transformed and selected with chloramphenicol into chemically competent *E. coli* TOP10F′ cells to preserve the plasmid frozen. The plasmids were then extracted, transformed in the chemically competent *E. coli* S17-1 cells, and selected with chloramphenicol. The resulting plasmids were introduced into the *B. abortus bvrR*-negative (4) strain by mating with *E. coli* S17-1 as previously described (43). Conjugants carrying the plasmid were selected by plating the mating mixture onto tryptic soy agar (TSA)-Nal-Cm plates, which were incubated at 37°C for 3 days.

Construction of plasmid pBvrR-luxAB transcriptional fusion (pJD27-P_{bvrR}). To study the *bvrR* promoter (P_{bvrR}) activity, a luciferase transcriptional fusion approach was used. Briefly, in order to amplify

TABLE 3 Primers used in this study

Primer	Sequence 5′→3′	Characteristics or relevant features	Source
bvrR_D58A_F	AATCTTGCGATCTTCGCTATCAAGAT	Primers used for substituting an alanine residue for the BvrR aspartate residue in position 58	This study
bvrR_D58A_R	GCCGCGCTTAGAACGCTAGAAGCGATAGTTCTACGGCGCG		
bvrR_D58E_F	AATCTTGCGATCTTCGAAATCAAGATGCCGCGC	Primers used for substituting a glutamic acid residue for the BvrR aspartate residue in position 58	This study
bvrR_D58E_R	TTAGAACGCTAGAAGCTTTAGTTCTACGGCGCG		
3-200bvrR3	CACTGATTCGCTTGAGGACG	Primers used to confirm the BvrR aspartate residue substitution in position 58 in the GWEntrybvrR vector	This study
revtrRSinterg1.5	CGTTGCCGCCGTGCTCTG		
HisbvrRF	ATGAAGGAAGCTTCGGCAAC	Primers used to subclone *bvrR*, *bvrR*D58A, and *bvrR*D58E (738-bp) sequences into the plasmid pCR8/GW/TOPO	This study
HisbvrRR	CGCAGAAAGCGGCAGATCTTA		
M13F	GTAAAACGACGGCCAG	Primers used to sequence and confirm the orientation of the *bvrR* sequence insert in pTOPObvrR, pTOPObvrRD58A, and pTOPObvrRD58E	Life Technologies Corporation, 2012
M13R	CAGGAAACAGCTATGAC		
bvrR-bv1F	CATGGACGGTATGGAGCTTCTG	Primers used to confirm insert orientation in pTOPObvrR, pTOPObvrRD58A, and pTOPObvrRD58E	This study
bvrR-bv1R	GAGGAAGATGACCGGCAGATC		
3-200bvrR3	CACTGATTCGCTTGAGGACG	Primers used to clone a *bvrR* promoter (P_{bvrR}) into pJET1.2 and pJD27 plasmids	This study
3-200bvrR5	CGTCTGCGTTGCCGAAGC		
pjd27seqF	AAAGGGAACAAAAGCTGGAG	Primers used to sequence and confirm the insert orientation of pJD27-P_{bvrR}	This study
pjd27seqR	CGTAGGCGAAAATACGACTCA		

a 225-bp fragment containing P_{bvrR} genomic DNA, primers 3-200bvrR3 and 3-200bvrR5 were used (Table 3). The PCR product was cloned into plasmid pJET1.2 in accordance with the manufacturer's instructions (CloneJET PCR cloning kit; Thermo Scientific). The resulting plasmid was named pJET1.2 P_{bvrR}. Plasmid pJET1.2 P_{bvrR} was electroporated into *E. coli* XL1-Blue electrocompetent cells, digested with BamHI and XhoI restriction enzymes, and ligated into the pJD27 BamHI and XhoI restriction sites (R. C. Essenberg, Department of Biochemistry, Oklahoma State University). Primers pjd27seqF and pjd27seqR were used to sequence and confirm the insert orientation of pJD27-P_{bvrR} (Table 3). Plasmid pJD27-P_{bvrR} was then electroporated in *B. abortus* 2308 (40), and the resulting strain was named *B. abortus* $P_{bvrR\text{-}luxAB}$. For a negative control, *B. abortus* 2308 was electroporated with the empty vector pJD27 and the strain was named *B. abortus luxAB*.

BvrR and BvrS detection and determination of BvrR phosphorylation. The *in vitro* growth curve was made as described elsewhere (12). The bacteria were concentrated by centrifugation (10,000 $\times$ *g*, 10 min), resuspended in Laemmli sample buffer, and heated at 100°C for 20 min. The protein concentration was determined by the Bio-Rad DC method according to the manufacturer's instructions. Equal amounts of protein (20 μg) were loaded onto a 10% gel for SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with the indicated antibodies. To analyze the phosphorylated status of BvrR, samples were solubilized in Laemmli sample buffer without heating, and equal amounts of protein (20 μg) were loaded onto a 10% gel for SDS-PAGE containing Phos-tag (100 mM) and $MnCl_2$ (0.2 mM). Recombinant BvrR phosphorylated with the phosphate universal donor carbamoyl phosphate was used as a positive control (44).

Determination of *bvrR* promoter activity. The *bvrR* promoter (P_{bvrR}) activity was determined by a luciferase transcriptional fusion construct introduced in *trans* in *B. abortus* 2308. A promotorless *luxAB* construct was used as a negative control. The strains were grown in TSB at 37°C, and at the times indicated in the figures, the optical density at 420 nm (OD_{420}) was measured, the bacterial load was calculated by plate counting, the luciferase activity was determined by mixing 0.1 ml of samples with 10 μl of ethanol-decanal, and, after 10 min, light production (relative luminescence units [RLU]) was measured for 5 s using a BioTek Synergy HT luminometer. Luciferase activity is expressed as the number of RLU per CFU/0.1 ml at a given time point.

***In vitro* BvrR/BvrS activation assay.** The different *B. abortus* strains at the growth phases indicated in the figures were centrifuged (10 000 $\times$ *g*, 3 min) and exposed to minimal medium (33 mM KH_2PO_4, 60.3 mM K_2HPO_4, and 0.1% yeast extract) or nutrient-rich medium (TSB) at pH 5.0 or 7.0 (adjusted with citric acid) for the times indicated in the figures at 37°C at 200 rpm. After incubation, bacteria were concentrated by centrifugation (10,000 $\times$ *g*, 3 min) and resuspended in Laemmli sample buffer. Total lysates were then processed for electrophoretic and immunochemical analyses as described above. To inhibit VjbR DNA-binding activity, *B. abortus* 2308 in exponential phase had been previously incubated in the presence (50 μM) of homoserine lactone for 1 h. Bacteria were then processed as described above.

Cell culture and gentamicin protection assay. Murine macrophage-like J774.A1 cells (ATCC, TIB-67), murine RAW264.7 macrophages (ATCC TIB-71), or HeLa epithelial cells (ATCC clone CCL-2) were cultivated and infected with *B. abortus* in exponential or stationary phase as previously described (24). For the intracellular replication experiments, 2 days before infection, cells were seeded in 24-well tissue culture plates to obtain a final concentration of 5×10^5 cells per well and multiplicities of infection (MOI) of 500 and 100 were used for HeLa cells and macrophages, respectively. For the intracellular bacterial isolation experiments, 2 days before infection, cells were seeded in 6-well tissue culture plates to obtain a final concentration of 3×10^6 cells per well and an MOI of 2,000 was used (24). Drugs to inhibit phagosome acidification were used as described by Porte (27). To inhibit macrophage phagocytosis, cytochalasin was used at concentrations and incubation times described by Palmer et al. (45).

Intracellular replication quantitation. The number of intracellular viable *B. abortus* CFU was determined at different hours postinfection. Cells were washed twice with phosphate-buffered saline (PBS) and treated for 10 min with Triton X-100 (0.1%). Lysates were serially diluted and plated on tryptic soy agar dishes for quantitation of CFU.

Immunofluorescence microscopy. In order to differentiate extracellular bacteria from internalized bacteria, at 2 h postinfection, a double immunofluorescence was performed. To determine extracellular bacteria, infected cells were washed three times with PBS and incubated with a cow anti-*B. abortus* antibody (conjugated with fluorescence isothiocyanate [FITC]) for 30 min at 4°C. Then, to stain all bacteria, cells were fixed and permeabilized, and bacteria were stained with a rabbit anti-*Brucella* antibody and anti-rabbit antibody–Texas Red. Nuclei were stained with DAPI, and slides were mounted using ProLong gold medium (Invitrogen). Intracellularly located bacteria are exclusively red, whereas extracellular bacteria are stained red and green.

Purification of intracellular bacteria. Intracellular *B. abortus* bacteria were purified at the hours postinfection in the figures using a previously standardized protocol based on sucrose gradients and ultracentrifugation (24). Afterwards, the pellet was stored at −70°C for electrophoretic and immunochemical analyses described above.

Statistical analysis. Statistical analyses were performed using SPSS software. All results are presented as means ± SD from at least three independent experiments, unless otherwise stated. Statistical significance was determined by Student's *t* test.

ACKNOWLEDGMENTS

This work was funded by Fondos de Recursos del Sistema FEES/CONARE, Red Temática de Brucelosis, Vice Presidency for Research, University of Costa Rica (project 803-B3-761) (www.vinv.ucr.ac.cr/), the National Council of Science and Technology of

Costa Rica through FORINVES (grant FV-0004-13) (www.conicit.go.cr/), and the International Center for Genomic Engineering and Biotechnology (contract CRP/12/007) (www.icgeb.trieste.it).

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

1. Moreno E, Moriyon I. 2006. The genus *Brucella*, p 315–456. *In* Dworkin M, Falcow S, Rosenberg E, Schleifer K-H, Stackebrandt E (ed), The prokaryotes. Springer, New York, NY.
2. Delrue RM, Deschamps C, Léonard S, Nijskens C, Danese I, Schaus JM, Bonnot S, Ferooz J, Tibor A, De Bolle X, Letesson JJ. 2005. A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *Brucella melitensis*. Cell Microbiol 7:1151–1161. https://doi.org/10.1111/j.1462-5822.2005.00543.x.
3. Sieira R, Comerci DJ, Sánchez DO, Ugalde RA. 2000. A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. J Bacteriol 182:4849–4855. https://doi.org/10.1128/JB.182.17.4849-4855.2000.
4. Sola-Landa A, Pizarro-Cerdá J, Grilló MJ, Moreno E, Moriyón I, Blasco JM, Gorvel JP, López-Goñi I. 1998. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol Microbiol 29:125–138. https://doi.org/10.1046/j.1365-2958.1998.00913.x.
5. de Jong MF, Starr T, Winter MG, den Hartigh AB, Child R, Knodler LA, van Dijl JM, Celli J, Tsolis RM. 2013. Sensing of bacterial type IV secretion via the unfolded protein response. mBio 4:e00418-12. https://doi.org/10.1128/mBio.00418-12.
6. Döhmer PH, Valguarnera E, Czibener C, Ugalde JE. 2014. Identification of a type IV secretion substrate of *Brucella abortus* that participates in the early stages of intracellular survival. Cell Microbiol 16:396–410. https://doi.org/10.1111/cmi.12224.
7. Ke Y, Wang Y, Li W, Chen Z. 2015. Type IV secretion system of *Brucella* spp. and its effectors. Front Cell Infect Microbiol 5:72. https://doi.org/10.3389/fcimb.2015.00072.
8. Marchesini MI, Herrmann CK, Salcedo SP, Gorvel JP, Comerci DJ. 2011. In search of *Brucella abortus* type IV secretion substrates: screening and identification of four proteins translocated into host cells through VirB system. Cell Microbiol 13:1261–1274. https://doi.org/10.1111/j.1462-5822.2011.01618.x.
9. Myeni S, Child R, Ng TW, Kupko JJ, Wehrly TD, Porcella SF, Knodler LA, Celli J. 2013. *Brucella* modulates secretory trafficking via multiple type IV secretion effector proteins. PLoS Pathog 9:e1003556. https://doi.org/10.1371/journal.ppat.1003556.
10. Celli J, de Chastellier C, Franchini DM, Pizarro-Cerda J, Moreno E, Gorvel JP. 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J Exp Med 198:545–556. https://doi.org/10.1084/jem.20030088.
11. Comerci DJ, Martínez-Lorenzo MJ, Sieira R, Gorvel JP, Ugalde RA. 2001. Essential role of the VirB machinery in the maturation of the *Brucella abortus*-containing vacuole. Cell Microbiol 3:159–168. https://doi.org/10.1046/j.1462-5822.2001.00102.x.
12. Martínez-Núñez C, Altamirano-Silva P, Alvarado-Guillén F, Moreno E, Guzmán-Verri C, Chaves-Olarte E. 2010. The two-component system BvrR/BvrS regulates the expression of the type IV secretion system VirB in *Brucella abortus*. J Bacteriol 192:5603–5608. https://doi.org/10.1128/JB.00567-10.
13. Sieira R, Comerci DJ, Pietrasanta LI, Ugalde RA. 2004. Integration host factor is involved in transcriptional regulation of the *Brucella abortus virB* operon. Mol Microbiol 54:808–822. https://doi.org/10.1111/j.1365-2958.2004.04316.x.
14. Starr T, Ng TW, Wehrly TD, Knodler LA, Celli J. 2008. *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. Traffic 9:678–694. https://doi.org/10.1111/j.1600-0854.2008.00718.x.
15. Caswell CC, Gaines JM, Roop RM. 2012. The RNA chaperone Hfq independently coordinates expression of the VirB type IV secretion system and the LuxR-type regulator BabR in *Brucella abortus* 2308. J Bacteriol 194:3–14. https://doi.org/10.1128/JB.05623-11.
16. Sieira R, Arocena GM, Zorreguieta A, Comerci DJ, Ugalde RA. 2012. A MarR-type regulator directly activates transcription from the *Brucella abortus virB* promoter by sharing a redundant role with HutC. J Bacteriol 194:6431–6440. https://doi.org/10.1128/JB.01007-12.
17. Sieira R, Arocena GM, Bukata L, Comerci DJ, Ugalde RA. 2010. Metabolic control of virulence genes in *Brucella abortus*: HutC coordinates *virB* expression and the histidine utilization pathway by direct binding to both promoters. J Bacteriol 192:217–224. https://doi.org/10.1128/JB.01124-09.
18. Uzureau S, Lemaire J, Delaive E, Dieu M, Gaigneaux A, Raes M, De Bolle X, Letesson JJ. 2010. Global analysis of quorum sensing targets in the intracellular pathogen *Brucella melitensis* 16 M. J Proteome Res 9:3200–3217. https://doi.org/10.1021/pr100068p.
19. Guzman-Verri C, Manterola L, Sola-Landa A, Parra A, Cloeckaert A, Garin J, Gorvel JP, Moriyon I, Moreno E, Lopez-Goni I. 2002. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the *Rhizobiaceae*. Proc Natl Acad Sci U S A 99:12375–12380. https://doi.org/10.1073/pnas.192439399.
20. Manterola L, Moriyón I, Moreno E, Sola-Landa A, Weiss DS, Koch MH, Howe J, Brandenburg K, López-Goñi I. 2005. The lipopolysaccharide of *Brucella abortus* BvrS/BvrR mutants contains lipid A modifications and has higher affinity for bactericidal cationic peptides. J Bacteriol 187:5631–5639. https://doi.org/10.1128/JB.187.16.5631-5639.2005.
21. Viadas C, Rodríguez MC, Sangari FJ, Gorvel JP, García-Lobo JM, López-Goñi I. 2010. Transcriptome analysis of the *Brucella abortus* BvrR/BvrS two-component regulatory system. PLoS One 5:e10216. https://doi.org/10.1371/journal.pone.0010216.
22. Lamontagne J, Forest A, Marazzo E, Denis F, Butler H, Michaud JF, Boucher L, Pedro I, Villeneuve A, Sitnikov D, Trudel K, Nassif N, Boudjelti D, Tomaki F, Chaves-Olarte E, Guzmán-Verri C, Brunet S, Côté-Martin A, Hunter J, Moreno E, Paramithiotis E. 2009. Intracellular adaptation of *Brucella abortus*. J Proteome Res 8:1594–1609. https://doi.org/10.1021/pr800978p.
23. Barbieri CM, Stock AM. 2008. Universally applicable methods for monitoring response regulator aspartate phosphorylation both in vitro and in vivo using Phos-tag-based reagents. Anal Biochem 376:73–82. https://doi.org/10.1016/j.ab.2008.02.004.
24. Chaves-Olarte E, Altamirano-Silva P, Guzmán-Verri C, Moreno E. 2014. Purification of intracellular bacteria: isolation of viable *Brucella abortus* from host cells. Methods Mol Biol 1197:245–260. https://doi.org/10.1007/978-1-4939-1261-2_14.
25. de Jong MF, Sun YH, den Hartigh AB, van Dijl JM, Tsolis RM. 2008. Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the *Brucella* type IV secretion system. Mol Microbiol 70:1378–1396. https://doi.org/10.1111/j.1365-2958.2008.06487.x.
26. Arocena GM, Sieira R, Comerci DJ, Ugalde RA. 2010. Identification of the quorum-sensing target DNA sequence and *N*-acyl homoserine lactone responsiveness of the *Brucella abortus virB* promoter. J Bacteriol 192:3434–3440. https://doi.org/10.1128/JB.00232-10.
27. Porte F, Liautard JP, Köhler S. 1999. Early acidification of phagosomes containing *Brucella suis* is essential for intracellular survival in murine macrophages. Infect Immun 67:4041–4047.
28. Boschiroli ML, Ouahrani-Bettache S, Foulongne V, Michaux-Charachon S, Bourg G, Allardet-Servent A, Cazevieille C, Liautard JP, Ramuz M, O'Callaghan D. 2002. The *Brucella suis virB* operon is induced intracellularly in macrophages. Proc Natl Acad Sci U S A 99:1544–1549. https://doi.org/10.1073/pnas.032514299.
29. Quebatte M, Dehio M, Tropel D, Basler A, Toller I, Raddatz G, Engel P, Huser S, Schein H, Lindroos HL, Andersson SG, Dehio C. 2010. The BatR/BatS two-component regulatory system controls the adaptive response of *Bartonella henselae* during human endothelial cell infection. J Bacteriol 192:3352–3367. https://doi.org/10.1128/JB.01676-09.

30. Li L, Jia Y, Hou Q, Charles TC, Nester EW, Pan SQ. 2002. A global pH sensor: *Agrobacterium* sensor protein ChvG regulates acid-inducible genes on its two chromosomes and Ti plasmid. Proc Natl Acad Sci U S A 99:12369–12374. https://doi.org/10.1073/pnas.192439499.
31. Charles TC, Nester EW. 1993. A chromosomally encoded two-component sensory transduction system is required for virulence of *Agrobacterium tumefaciens*. J Bacteriol 175:6614–6625. https://doi.org/10.1128/jb.175.20.6614-6625.1993.
32. Cheng HP, Walker GC. 1998. Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. J Bacteriol 180:20–26.
33. Yao SY, Luo L, Har KJ, Becker A, Rüberg S, Yu GQ, Zhu JB, Cheng HP. 2004. *Sinorhizobium meliloti* ExoR and ExoS proteins regulate both succinoglycan and flagellum production. J Bacteriol 186:6042–6049. https://doi.org/10.1128/JB.186.18.6042-6049.2004.
34. Wu CF, Lin JS, Shaw GC, Lai EM. 2012. Acid-induced type VI secretion system is regulated by ExoR-ChvG/ChvI signaling cascade in *Agrobacterium tumefaciens*. PLoS Pathog 8:e1002938. https://doi.org/10.1371/journal.ppat.1002938.
35. Heckel BC, Tomlinson AD, Morton ER, Choi JH, Fuqua C. 2014. *Agrobacterium tumefaciens* ExoR controls acid response genes and impacts exopolysaccharide synthesis, horizontal gene transfer, and virulence gene expression. J Bacteriol 196:3221–3233. https://doi.org/10.1128/JB.01751-14.
36. Yuan ZC, Liu P, Saenkham P, Kerr K, Nester EW. 2008. Transcriptome profiling and functional analysis of *Agrobacterium tumefaciens* reveals a general conserved response to acidic conditions (pH 5.5) and a complex acid-mediated signaling involved in *Agrobacterium*-plant interactions. J Bacteriol 190:494–507. https://doi.org/10.1128/JB.01387-07.
37. Lu HY, Luo L, Yang MH, Cheng HP. 2012. *Sinorhizobium meliloti* ExoR is the target of periplasmic proteolysis. J Bacteriol 194:4029–4040. https://doi.org/10.1128/JB.00313-12.
38. Starr T, Child R, Wehrly TD, Hansen B, Hwang S, López-Otin C, Virgin HW, Celli J. 2012. Selective subversion of autophagy complexes facilitates completion of the *Brucella* intracellular cycle. Cell Host Microbe 11:33–45. https://doi.org/10.1016/j.chom.2011.12.002.
39. Smith EP, Miller CN, Child R, Cundiff JA, Celli J. 2016. Postreplication roles of the *Brucella* VirB type IV secretion system uncovered via conditional expression of the VirB11 ATPase. mBio 7:e01730-16. https://doi.org/10.1128/mBio.01730-16.
40. Sangari F, Agüero J. 1991. Mutagenesis of *Brucella abortus*: comparative efficiency of three transposon delivery systems. Microb Pathog 11:443–446. https://doi.org/10.1016/0882-4010(91)90040-H.
41. Hallez R, Letesson JJ, Vandenhaute J, De Bolle X. 2007. Gateway-based destination vectors for functional analyses of bacterial ORFeomes: application to the Min system in *Brucella abortus*. Appl Environ Microbiol 73:1375–1379. https://doi.org/10.1128/AEM.01873-06.
42. Dricot A, Rual JF, Lamesch P, Bertin N, Dupuy D, Hao T, Lambert C, Hallez R, Delroisse JM, Vandenhaute J, Lopez-Goñi I, Moriyon I, Garcia-Lobo JM, Sangari FJ, Macmillan AP, Cutler SJ, Whatmore AM, Bozak S, Sequerra R, Doucette-Stamm L, Vidal M, Hill DE, Letesson JJ, De Bolle X. 2004. Generation of the *Brucella melitensis* ORFeome version 1.1. Genome Res 14:2201–2206. https://doi.org/10.1101/gr.2456204.
43. Bukata L, Altabe S, de Mendoza D, Ugalde RA, Comerci DJ. 2008. Phosphatidylethanolamine synthesis is required for optimal virulence of *Brucella abortus*. J Bacteriol 190:8197–8203. https://doi.org/10.1128/JB.01069-08.
44. Lynch AS, Lin EC. 1996. Transcriptional control mediated by the ArcA two-component response regulator protein of *Escherichia coli*: characterization of DNA binding at target promoters. J Bacteriol 178:6238–6249. https://doi.org/10.1128/jb.178.21.6238-6249.1996.
45. Palmer LM, Reilly TJ, Utsalo SJ, Donnenberg MS. 1997. Internalization of *Escherichia coli* by human renal epithelial cells is associated with tyrosine phosphorylation of specific host cell proteins. Infect Immun 65:2570–2575.