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The Two-Component System BvrR/BvrS Regulates the Expression of the Type IV Secretion System VirB in *Brucella abortus*▽

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The pathogenesis of *Brucella* is related to the ability to multiply intracellularly, an event controlled by the two-component system BvrR/BvrS (TCS BvrRS) and the type IV secretion machinery VirB (T4SS VirB). We have hypothesized that the TCS BvrRS transcriptionally regulates the T4SS VirB. To test this hypothesis, we have compared the levels of VirB proteins in the wild-type strain *Brucella abortus* 2308 and mutant strains devoid of the sensor and regulator genes (*bvrS* and *bvrR* mutants, respectively). While the *bvrR* and *bvrS* mutants showed low levels of the VirB1, VirB5, VirB8, and VirB9 proteins, the same proteins were overexpressed in the *bvrR* mutant complemented with a plasmid carrying a functional *bvrR* gene. Quantitation of *virB5* mRNA confirmed these data and indicated that the influence of the TCS BvrRS on the T4SS VirB occurs at the transcriptional level. The expression of the transcriptional activator VjbR also depended on the TCS BvrRS. In addition, we demonstrate a direct interaction between the promoter region of the VirB operon and the response regulator BvrR. Altogether these data demonstrate that the TCS BvrRS controls the expression of the T4SS VirB through direct and indirect mechanisms.

Brucella organisms are intracellular bacteria infecting animals and humans (21, 23). The pathogenesis exerted by members of the genus is critically dependent on the establishment of chronic intracellular infections (3, 21). Among the various *Brucella* systems and molecules known to participate in virulence, the two-component regulatory system BvrR/BvrS (TCS BvrRS) and the type IV secretion system VirB (T4SS VirB) are critical. The TCS BvrRS, composed of a histidine kinase sensor located in the cell membrane (BvrS) and a cytoplasmic regulator (BvrR), participates in the homeostasis of the outer membrane (OM) controlling the structure of the lipopolysaccharide (LPS) and the expression of periplasmic and OM proteins (Omp) (12, 15, 20). Mutants with mutations in this regulatory system are nonvirulent in mice, displaying increased sensitivity to bactericidal peptides and complement, deficient cell invasion, and altered intracellular trafficking (30). The T4SS VirB is devoted to the control of *Brucella* intracellular trafficking; as a consequence, bacterial mutants defective in this system are impaired in their ability to multiply within cultured cells or to persist in mice (6, 22, 29). It has been proposed that the T4SS VirB extends from the inner membrane to the OM and delivers effectors into the host cell in order to control the biogenesis of the intracellular compartment where the bacteria will eventually reside (9). Although there has been some controversy, the VirB mutants do not show altered cell invasion (7, 11). The expression of the T4SS VirB is tightly regulated both *in vitro* and *in vivo*. *In vitro*, the expression of this system is detectable only when bacteria are at late logarithmic growth phase (10, 29), while *in vivo*, the expression increases dramatically 3 h after entry into the eukaryotic host cell when the bacteria are trafficking to the endoplasmic reticulum. Then, the system rapidly decreases, reaching undetectable levels when the bacterium starts its replication (5, 28). Several molecular systems have been reported to modulate or impact VirB expression. Among them, a direct interaction with the promoter region of VirB has been demonstrated only for integration host factor (IHF), VjbR, and HutC (2, 9, 27, 28).

Both the *Brucella* T4SS VirB and the TCS BvrRS have homologs present in alphaproteobacterial endosymbionts and pathogens of plants and animals such as *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Agrobacterium*, and *Bartonella* (4, 19, 30). Indeed, ChvG/ChvI and ExoS/ChvI are also two-component systems devoted to the control of critical functions during *Agrobacterium tumefaciens* parasitism and *Sinorhizobium meliloti* endosymbiosis, respectively. The ChvG/ChvI system regulates the acid-induced expression of the *aopB* gene, coding for an Omp, and the expression of the *virB* and *virE* genes, coding for T4SS proteins responsible for the transfer of *A. tumefaciens* transfer DNA (T-DNA) to host cells (13, 16, 33). ChvG/ChvI mutants are nonvirulent and display increased sensitivity to detergents, antibiotics, and low pH (8). Similarly, the *S. meliloti* ExoS/ChvI system controls the expression of the flagellum and the production of succinoglycan, components required for the invasion of legume plants, and the TCS BatR/BatS of *Bartonella henselae* regulates in a pH-dependent manner several virulence genes of this intracellular pathogen (25).

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Due to the negative impact that mutations in the TCS BvrRS exert in intracellular trafficking, we have hypothesized that this system controls the expression of the T4SS VirB. Indeed we have found that the TCS BvrRS exerts a direct transcriptional control on the expression of VirB.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Brucella abortus* 2308 Nal^r is a virulent smooth-LPS strain described elsewhere (26). Nonvirulent *B. abortus bvrS* and *B. abortus bvrR* mutants are smooth-LPS strains derived from *B. abortus* 2308 Nal^r with a mini-Tn*5* insertion in the *bvrS* and *bvrR* genes, respectively. The *bvrR* mutant transformed with the *bvrR*-carrying plasmid pBBR2.13 generated the reconstituted *bvrR* p*bvrR*$^+$ strain of *B. abortus* (30). *B. abortus* strains were grown in tryptic soy broth (TSB), and *Escherichia coli* BL21 was grown on Luria-Bertani (LB) or 2× yeast extract-tryptone (YT) medium supplemented with 50 μg/ml ampicillin or 30 μg/ml chloramphenicol when required.

Expression of the VirB promoter in β-galactosidase assay. To analyze the direct effect of BvrR on the transcription of the VirB promoter (P_{VirB}), a β-galactosidase transcriptional fusion approach was used (9). Briefly, *E. coli* strain BL21 (*lacZ*$^+$) was transformed with plasmid pSURS2 (P_{VirB}-*lacZ* Cm^r) and with plasmid pBBR2.13 (*bvrR*$^+$ Amp^r) (30) or the empty vector pBBR1-MCS4 (Amp^r). *E. coli* BL21 not transformed or transformed only with pSURS2 was used as a control. The expression of BvrR by bacteria transformed with plasmid pBBR2.13 was confirmed by Western blotting. Bacteria were grown to logarithmic growth phase in the presence of the corresponding antibiotics. The β-galactosidase activity was determined and expressed in Miller units as previously described (12).

VirB5 mRNA quantitation by real-time PCR. For RNA extraction bacterial cultures were adjusted to 10^9 CFU/ml and disrupted with 0.5% Zwittergent 3-16 at 37°C for 1 h. Total RNA was extracted using the RNeasy midikit (Qiagen) according to the manufacturer's instructions. Eluted RNA was treated with Ambion Turbo DNase according to the robust protocol. RNA samples were retrotranscribed using the Applied Biosystems high-capacity cDNA kit and random primers. Serial dilutions of each cDNA from 8 to 0.5 ng were used for *virB5* amplification with primers 5′GAGCGGCGGCTACCT3′ and 5′GCAAAAGCC AGGTATACGATCC3′ along with TaqMan probe 5′AAACGGCCAACCTCT TAC3′ or primers 5′GCTGTCCAAGCTTCTCGAAGA3′ and 5′CCGGCAGC AGCAACAG3′ together with TaqMan probe 5′TTCGGCTGCTGCTCCG3′, amplifying the 50S ribosomal protein L7/L12 gene used as an endogenous control and 2× TaqMan universal PCR kit according to the manufacturer's instructions. Nontemplate controls contained water instead of cDNA, and genomic DNA contamination controls contained nonretrotranscribed RNA. All samples were run in triplicate under standard conditions on an Applied Biosystems 7500 real-time PCR machine. Data analysis was carried out as described previously (24).

Purification of fusion proteins and generation of antibodies. BvrR was expressed as a glutathione *S*-transferase (GST) tag fusion protein using vector pGEX-2KT (GE Healthcare). Cloning and purification of the GST fusion protein were done according to the manufacturer's instructions. Briefly, a PCR product from *B. abortus* genomic DNA carrying *bvrR* was obtained using the following primers: 5′-GGTCCGCGAATTCATATGAAGGAAGCTTC-3′ and 5′-GCGGCAGATCGAATTCCTTACGCTTC-3′, containing an EcoRI restriction site. Vector and PCR product were digested, ligated, and transformed into *E. coli* BL21 competent cells for propagation of the generated plasmid, pGEX-2KT-BvrR. Expression of GST-BvrR was achieved by growing *E. coli* BL21 harboring pGEX-2KT-BvrR in 2-fold YT medium with aeration at 37°C to mid-exponential growth phase (A_{600} of 0.5). Cultures were then induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 30°C for 2 h. Bacteria were collected by centrifugation at 7,700 × *g*, resuspended in phosphate-buffered saline (PBS), and lysed by sonication. After centrifugation at 12,000 × *g*, the supernatant was incubated with glutathione-Sepharose beads (GE Healthcare) for 2 h at room temperature. Beads were washed twice with PBS, and GST-BvrR was eluted with 10 mM glutathione (Sigma). The suspension was stored for further use at −70°C. Expression of His-tagged VjbR was achieved as previously reported (9). Rabbit anti-BvrR and anti-VjbR antibodies were produced by four intramuscularly applied boosts of the proteins (250 μg) in complete (first boost) or incomplete (second to fourth boost) Freund adjuvant (Sigma). Antibodies from 2 ml of serum were adsorbed to GST-BvrR-Sepharose beads or His-tagged VjbR-Sepharose beads; eluted with 0.2 M glycine, pH 2.5; and collected in 1 M Tris, pH 9.0. The antibodies were concentrated by ultrafiltration and stored at −20°C in 50% glycerol.

Electrophoretic and immunochemical analysis. The different *Brucella* or *E. coli* strains were grown to the indicated growth phases in TSB at 37°C. The bacteria were concentrated by centrifugation (10,000 × *g*, 10 min), resuspended in Laemmli sample buffer (14), 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, and equal amounts of protein (20 μg) were loaded onto a 12.5% gel for SDS-PAGE. Separated proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and probed with the indicated antibodies. Membranes were further incubated with peroxidase-conjugated anti-mouse or anti-rabbit antibodies, and the detected bands were visualized by a chemiluminescence reaction.

P_{VirB} pulldown experiment. *B. abortus* 2308 lysates were obtained using a procedure described elsewhere (28). Briefly, bacteria were grown at 37°C on TSB to late logarithmic growth phase and then concentrated by centrifugation (10 min at 12,800 × *g*). The resulting pellet was resuspended in protoplasting buffer containing 15 mM Tris-HCl (pH 7.6), 0.45 M sucrose, 8 mM EDTA, and lysozyme to a final concentration of 0.4 mg/ml. The suspension was incubated for 15 min on ice and then centrifuged for 10 min at 5,000 × *g*. The pellet was resuspended in disruption buffer (20 mM Tris-HCl, pH 7.6, 3 mM β-mercaptoethanol) supplemented with protease inhibitor cocktail (Roche Applied Bioscience). The bacteria were disrupted by sonication on ice and centrifuged at 18,500 × *g*. The DNA region encoding the *virB* promoter was amplified by PCR from *B. abortus* 2308 genomic DNA with the primers 5′-biotin–GATCGTCTC CTTCTCAGAG-3′ and 5′-CGCATACCACTTGTATATAAG-3′ under the cycling parameters of 94°C for 4 min and 35 cycles of 94°C for 30 s, 48°C for 1 min, and 68°C for 2 min and continued with a 68°C incubation for 10 min. As a negative control, the promoter region of the *dhbCEBA* operon was used. This region was amplified with the primers 5′-biotin-CGGCATCCAGCCATCTGC AAAA-3′ and 5′-ATCACCTCACGCATGCCGCT-3′ under the conditions previously described (1). The PCR products were purified using the QIAquick PCR purification kit (Qiagen). The biotinylated amplicons were coupled to streptavidin-Sepharose beads (GE Healthcare). Briefly, the beads were washed twice in wash buffer (10 mM Tris, pH 7, 1 mM EDTA, 2 M NaCl). The beads were then incubated with 1 μg of the biotinylated amplicons and incubated for 15 min at room temperature with occasional shaking. The resulting DNA-coated beads were washed in washing and incubation buffer (50 mM Tris, pH 7, 1 mM EDTA, 100 mM KCl, 5% glycerol, 0.1% Triton X-100). For the pulldown experiment, the DNA-coated beads were resuspended in incubation buffer with *B. abortus* 2308 lysate at 37°C for 30 min under agitation. Alternatively, the DNA-coated beads were incubated with purified BvrR. After incubation the beads were washed and the bound proteins were eluted with SDS-PAGE lysis buffer and separated by 12.5% SDS-PAGE. Separated proteins were transferred to a PVDF membrane and probed with anti-BvrR or anti-VjbR polyclonal antibodies. Membranes were further incubated with peroxidase-conjugated anti-rabbit antibodies, and the detected bands were visualized by chemiluminescence reaction.

RESULTS AND DISCUSSION

The absence of a functional TCS BvrRS impacts the growth curve of *B. abortus*. Since the T4SS VirB expression is highly dependent on the replication phase (29), the growth curves of the strains used in this study were carefully analyzed. *Brucella* was inoculated at a density of 5×10^9 CFU in TSB, and samples were taken during a 30-h period. In all the experiments *B. abortus* 2308 reached significantly higher absorbances than did the *bvrR* and *bvrS* mutants (Fig. 1). The *B. abortus bvrR* p*bvrR*$^+$ strain reached absorbances similar to those displayed by the parental *B. abortus* 2308 (Fig. 1). In spite of the absorbance differences, all the strains entered the different phases of the growth curve at approximately the same time. The logarithmic phase started between 16 and 18 h, and the late logarithmic phase occurred between 24 and 26 h. After this time all the strains were clearly in stationary phase (Fig. 1). These curves were used to take samples in order to analyze the expression of the T4SS VirB and the transcription regulator VjbR.

T4SS VirB proteins are diminished in TCS BvrRS mutants. In order to determine if the absence of a functional TCS

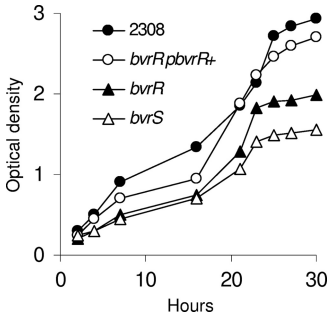

FIG. 1. Growth curves of *B. abortus* 2308 and TCS BvrRS mutant strains. The indicated strains were inoculated in TSB at 5×10^9 CFU, and the absorbance was measured at 420 nm at the indicated times. Representative curves are shown.

BvrRS influences the expression of the VirB operon, the different strains were grown to early and late growth phases and samples were tested accordingly. These samples were probed by Western blotting with antibodies against VirB1, VirB5, VirB8, and VirB9. At early logarithmic phase, these proteins were nearly absent in *B. abortus* 2308 and in the *B. abortus bvrR* and *B. abortus bvrS* mutants (Fig. 2A). Interestingly, all of them were detected in lysates from the *B. abortus bvrR* p*bvrR*$^+$ strain (Fig. 2A). At late logarithmic phase the four proteins were detected in *B. abortus* 2308 lysates, in agreement with previous reports (10, 29). In contrast, VirB1, VirB5, VirB8, and VirB9 were clearly diminished in lysates from *B. abortus bvrR* and *B. abortus bvrS* mutants (Fig. 2B). Again, lysates from the reconstituted *B. abortus bvrR* p*bvrR*$^+$ strain showed levels of VirB proteins equal to or higher than those of *B. abortus* 2308 (Fig. 2B). In order to correlate these results with the level of expression of the TCS BvrRS, the lysates were probed with anti-BvrR antibodies. These antibodies recognized a conspicuous band corresponding to BvrR in *B. abortus* 2308 lysates. This band was severely diminished in lysates from the *B. abortus bvrS* mutant and completely absent, as expected, in lysates from the *B. abortus bvrR* mutant (Fig. 2C). On the other hand, BvrR was overexpressed in the *B. abortus bvrR* p*bvrR*$^+$ strain, demonstrating that the complementation was functional (Fig. 2C). Anti-Omp19 antibodies were used as a loading control, while anti-Omp25 (also known as anti-Omp3a) antibodies were used to corroborate the previously reported phenotype of these strains (Fig. 2C) (12, 15).

Since we found an overexpression of T4SS VirB proteins in the *B. abortus bvrR* p*bvrR*$^+$ strain, we decided to thoroughly analyze the expression of this T4SS during the growth curve and compare it with that of *B. abortus* 2308. From 16 to 20 h the level of VirB8 was negligible in both strains. VirB8 was detected in *B. abortus* 2308 lysates at 23 h, reaching a maximum expression at 24 h and then disappearing for the rest of the experiment (31 h) (Fig. 2D). In the *B. abortus bvrR* p*bvrR*$^+$ strain, on the other hand, VirB8 was already detected at 22 h, reached a maximum expression at 23 h, and then maintained a fairly constant level until the end of the experiment, well into stationary phase (Fig. 2D). Similar results were obtained when the lysates were probed with the anti-VirB5 antibody (data not shown). An analysis of BvrR expression on the same samples indicated that this protein is present in *B. abortus* 2308

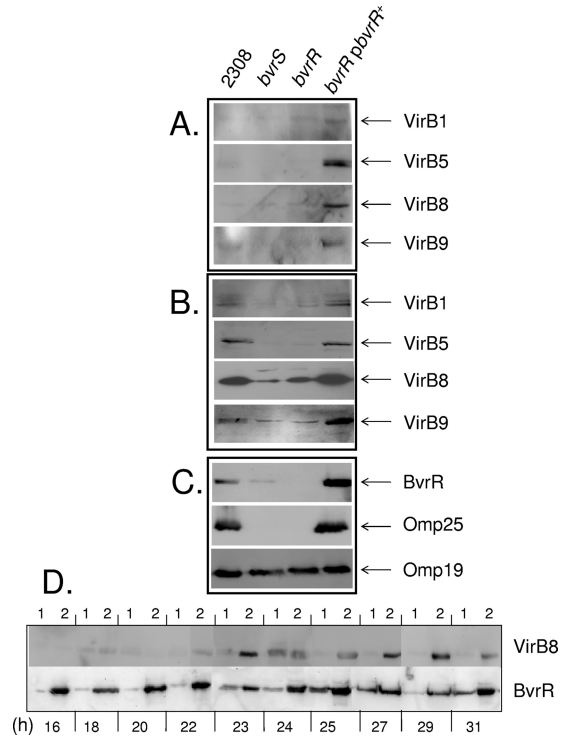

FIG. 2. Levels of VirB1, VirB5, VirB8, and VirB9 in *B. abortus* 2308 and in TCS BvrRS mutants. (A and B) The indicated strains were grown in TSB to early logarithmic (A) and late logarithmic (B) phases. Equal amounts (20 μg) of whole-bacterium lysates were then separated by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with the indicated antibodies. After incubation with peroxidase-conjugated anti-rabbit or anti-mouse antibodies, immune complexes were detected by chemiluminescence reaction. (C) Samples taken as described for panels A and B were probed with anti-BvrR, anti-Omp25, and anti-Omp19 (loading control) antibodies. (D) *B. abortus* 2308 (lanes 1) and the *B. abortus bvrR* p*bvrR*$^+$ strain (lanes 2) were grown in TSB, and samples were taken at the indicated times. Equal amounts (20 μg) of whole-bacterium lysates were then separated by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with the indicated antibodies. After incubation with peroxidase-conjugated anti-rabbit antibodies, immune complexes were detected by chemiluminescence reaction. These experiments are representative of at least three performed.

throughout the growth curve with a maximum expression at late logarithmic phase (Fig. 2D). BvrR was constitutively overexpressed in the *B. abortus bvrR* p*bvrR*$^+$ strain (Fig. 2D). Altogether these data indicate that overexpression of BvrR induces a higher and more prolonged expression of T4SS VirB proteins.

***virB5* mRNA is diminished in the *bvrS* mutant.** To corroborate the data observed at the protein level, we measured the *virB5* transcriptional levels in the wild-type *B. abortus* 2308 and the *bvrS* mutant. The strains were grown to different growth phases, and total RNA was prepared. *virB5* mRNA was quantified by real-time PCR using *l7/l12* ribosomal gene transcript as an internal control and normalized against the value obtained at 18 h for *B. abortus* 2308. At 18 h the two strains contained similar amounts of the *virB5* mRNA. In 2308, there was a dramatic increase in the expression of this gene, reaching a peak at 24 h (Fig. 3). In the *bvrS* mutant the levels remained

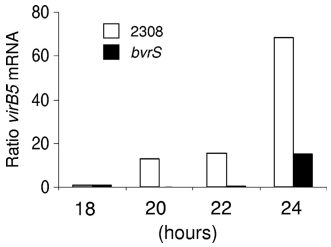

FIG. 3. *virB5* mRNA level in *B. abortus* 2308 and the *B. abortus bvrS* mutant. *virB5* and *l7/l12* cDNAs were amplified by quantitative real-time PCR from RNA samples collected at the indicated times. Relative *virB5* mRNA quantification after normalization with *l7/l12* mRNA is shown. The percentage of error was in all cases less than 0.3. Results representative of three independent experiments are shown.

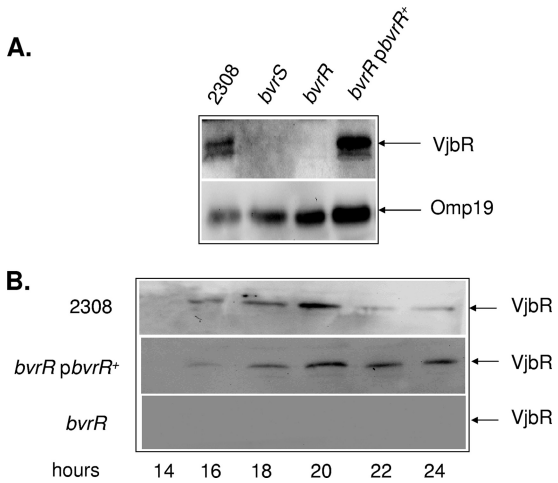

FIG. 4. VjbR expression during the growth curve in *B. abortus* 2308 and TCS BvrRS mutants. (A) The indicated strains were grown in TSB to log phase. Equal amounts (20 μg) of whole-bacterium lysates were then separated by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with anti-VjbR antibodies or anti-Omp19 antibodies. (B) The strains were grown in TSB, and samples were taken at the indicated times. Whole-bacterium lysates were processed as described for panel A. These results are representative of at least three independent experiments.

low until the 24-h point, where a moderate increase was detected. At this point, the *bvrS* mutant contained 5 times less *virB5* transcript than did *B. abortus* 2308.

The transcriptional regulator VjbR is diminished in the TCS BvrRS mutants. It has been shown that the transcriptional regulator VjbR is directly involved in the regulation of the T4SS VirB (2, 9, 10). Thus, the level of this protein in the context of the BvrRS mutants was determined. For this, we raised polyclonal antibodies against purified VjbR that recognized a single band in *B. abortus* 2308 lysates of the molecular weight expected for this protein. We then analyzed the expression of VjbR in lysates of *B. abortus* 2308 and the TCS BvrRS mutants. Samples were collected at early logarithmic growth phase, when VjbR has been reported to achieve its maximum expression (10). While there was a clear band detected in *B. abortus* 2308, this band was absent in the *B. abortus bvrR* and *B. abortus bvrS* mutants (Fig. 4A). VjbR was reconstituted to a normal level in the *B. abortus bvrR* p*bvrR*$^+$ strain (Fig. 4A). The expression of VjbR was analyzed throughout the growth curve in *B. abortus* 2308 and the *B. abortus bvrR* and *B. abortus bvrR* p*bvrR*$^+$ strains. In *B. abortus* 2308 VjbR was detectable in early logarithmic phase at 16 h, it reached maximum expression at 20 h, and then the level diminished but was still detectable (Fig. 4B). In the *B. abortus bvrR* p*bvrR*$^+$ strain the VjbR expression pattern followed a similar dynamic with a slight increase at some time points with respect to the parental *B. abortus* 2308 (Fig. 4B). Under these conditions, VjbR was not detected in the *B. abortus bvrR* mutant (Fig. 4B).

BvrR binds directly to the VirB promoter. The possibility that the transcription of the T4SS VirB could be under the direct control of the TCS BvrRS or could occur through the indirect effect mediated by VjbR was investigated by a promoter pulldown approach. The region encoding the VirB promoter was amplified using biotinylated primers, and the PCR product was coupled to streptavidin-Sepharose beads. These beads were incubated with *B. abortus* 2308 whole lysates or a suspension of purified BvrR, and the pulled-down proteins were analyzed by Western blotting. BvrR was detected in the pulldown experiment when the lysate was incubated with streptavidin-Sepharose beads coated with the VirB promoter region; in contrast, BvrR was absent when the lysate was incubated with streptavidin-Sepharose beads loaded with a biotinylated amplicon comprising the promoter region of the operon *dhbCEBA* (negative control) (1) (Fig. 5A). The biotinylated VirB probe, but not the *dhbCEBA* probe, was able to bind VjbR (positive control) in this pulldown experiment, thus confirming the approach (Fig. 5A). To further demonstrate, from a functional perspective, a direct interaction between BvrR and the *virB* promoter, we used a strategy that demonstrated the role of VjbR on the transcription of this T4SS (9). The *E. coli* BL21 strain was transformed with a plasmid carrying *bvrR* (pBBR2.13), a plasmid containing the *virB* promoter fused to *lacZ* (pSURS2), or both. By Western blotting we confirmed that the *E. coli* strains transformed with pBBR2.13 expressed BvrR, indicating an appropriate transcription of the TCS in this heterologous setting (Fig. 5B). Under these conditions, the β-galactosidase activity, driven by the *virB* promoter, was significantly enhanced by the presence of BvrR (Fig. 5C). The direct induction of the *virB* promoter in *E. coli* by VjbR (9) or BvrR (this report) indicates that the system is able to use the transcriptional machinery of the heterologous host. This is in contrast to the expression of *Agrobacterium tumefaciens* virulence genes in *E. coli*. These genes, in addition to the TCS VirA/VirG, also required the *A. tumefaciens rpoA* gene encoding the α subunit of the RNA polymerase (17, 18).

The regulation of VirB expression by *Brucella* is a complex process both in bacteriological cultures and within cells. In cultures, VirB proteins and transcripts are only transiently detected at the late logarithmic phase (10, 29), whereas within cells, the VirB expression seems to be restricted to the first hours of infection (28). This restricted window of expression is probably the reason why no differences in VirB levels were detected in previous proteomic and transcription comparative studies between wild-type *B. abortus* and the BvrRS TCS mutants (12, 15, 32). Several molecular systems have been reported to impact VirB expression, and some of them have been demonstrated to directly bind the *virB* promoter (2, 9, 27, 28).

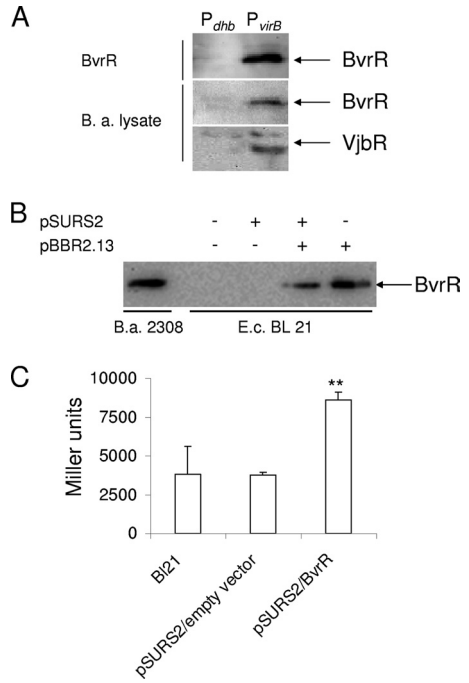

FIG. 5. Direct interaction of the VirB promoter with BvrR. (A) Purified BvrR protein or whole-bacterium lysates of *B. abortus* 2308 were incubated with streptavidin-Sepharose beads preloaded with a biotin-labeled amplicon comprising the *virB* promoter region or with streptavidin-Sepharose beads preloaded with the *dhb* promoter region (negative control). Beads were then washed, and bound proteins were resuspended in SDS-containing buffer. Proteins were then separated by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with anti-BvrR or anti-VjbR antibodies. After incubation with peroxidase-conjugated anti-rabbit antibodies, immune complexes were detected by chemiluminescence reaction. (B) Equal amounts (20 μg) of lysates of *E. coli* BL21 transformed with the indicated plasmids or *B. abortus* 2308 were separated by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with anti-BvrR antibodies. (C) *E. coli* BL21 was electroporated with a plasmid containing the *virB* promoter fused to *lacZ* (pSURS2), with a plasmid containing *bvrR* (pBBR2.13), or both (pSURS2/pBBR2.13). β-Galactosidase activity expressed in Miller units was calculated in logarithmically growing bacteria. These results are representative of at least three independent experiments. **, $P \leq 0.005$.

In this work we have included the TCS BvrRS in the list of transcriptional regulators that directly control VirB expression. Besides the direct control exerted over the VirB system, it is clear that the TCS BvrRS also impacts the transcriptional regulator VjbR, a well-recognized determinant of VirB expression. This is consistent with a recent microarray analysis of the *B. abortus bvrR* mutant where this transcriptional regulator was found to be underexpressed (32). Moreover, a proteomic characterization of a VjbR mutant presented a striking resemblance to the corresponding characterization of BvrRS mutants (15, 31). Based on all these results, we put forward the hypothesis that the TCS BvrRS positively affects the transcription of VjbR and then this transcriptional factor regulates VirB transcription, together with direct participation by BvrR. Thus, the BvrRS control of VirB expression occurs by direct and indirect means. Due to the prominent role of VirB and VjbR in virulence control and intracellular replication, this may explain in part the deep attenuation of the *bvrS* and *bvrR* mutants (30). Within this context, it is tempting to speculate that the TCS BvrRS is, then, a master regulator of virulence genes that senses when *B. abortus* enters a eukaryotic host cell and thus switches on several pathways important to this part of the life cycle. It is clear, however, that BvrRS is necessary but not sufficient for VirB expression, since even if overexpressed throughout the growth curve, it did not induce a constitutive overexpression of VirB at early times. At this phase, probably, the negative regulators acting on the VirB promoter dominate over the positive input added by BvrR. It would be interesting in the future to correlate the level of phosphorylation of BvrR with the actual expression of VirB intracellularly in order to support this hypothesis.

The results presented here are not exclusive to *Brucella* but instead seem reminiscent of those with other alphaproteobacteria. For instance, in *A. tumefaciens* the ChvG/ChvI system (orthologous to BvrR/BvrS) controls the expression of the *virB* and *virE* genes required for the transference of T-DNA to target plants (16). Similarly, the TCS BatR/BatS from *Bartonella henselae* regulates its corresponding T4SS VirB in a pH-dependent manner (25). Thus, the relationship between this family of TCSs important for the interaction with plant and animal cells and the T4SSs that deliver the effectors that allow this interaction may represent an evolutionarily conserved strategy among alphaproteobacteria.

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REFERENCES

1. **Anderson, E. S., J. T. Paulley, and R. M. Roop II.** 2008. The AraC-like transcriptional regulator DhbR is required for maximum expression of the 2,3-dihydroxybenzoic acid biosynthesis genes in *Brucella abortus* 2308 in response to iron deprivation. J. Bacteriol. **190:**1838–1842.
2. **Arocena, G. M., R. Sieira, D. J. Comerci, and R. A. Ugalde.** 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.
3. **Barquero-Calvo, E., E. Chaves-Olarte, D. S. Weiss, C. Guzman-Verri, C. Chacon-Diaz, A. Rucavado, I. Moriyon, and E. Moreno.** 2007. *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. PLoS One **2:**e631.
4. **Batut, J., S. G. Andersson, and D. O'Callaghan.** 2004. The evolution of chronic infection strategies in the alpha-proteobacteria. Nat. Rev. Microbiol. **2:**933–945.
5. **Boschiroli, M. L., S. Ouahrani-Bettache, V. Foulongne, S. Michaux-Charachon, G. Bourg, A. Allardet-Servent, C. Cazevieille, J. P. Liautard, M. Ramuz, and D. O'Callaghan.** 2002. The *Brucella suis* virB operon is induced intracellularly in macrophages. Proc. Natl. Acad. Sci. U. S. A. **99:**1544–1549.
6. **Celli, J., C. de Chastellier, D. M. Franchini, J. Pizarro-Cerda, E. Moreno, and J. P. Gorvel.** 2003. *Brucella* evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. **198:**545–556.
7. **Celli, J., and J. P. Gorvel.** 2004. Organelle robbery: *Brucella* interactions with the endoplasmic reticulum. Curr. Opin. Microbiol. **7:**93–97.
8. **Charles, T. C., and E. W. Nester.** 1993. A chromosomally encoded two-component sensory transduction system is required for virulence of *Agrobacterium tumefaciens*. J. Bacteriol. **175:**6614–6625.
9. **de Jong, M. F., Y. H. Sun, A. B. den Hartigh, J. M. van Dijl, and R. M. Tsolis.** 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.

10. **Delrue, R. M., C. Deschamps, S. Leonard, C. Nijskens, I. Danese, J. M. Schaus, S. Bonnot, J. Ferooz, A. Tibor, X. De Bolle, and J. J. Letesson.** 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.
11. **Fontes, P., M. T. Alvarez-Martinez, A. Gross, C. Carnaud, S. Kohler, and J. P. Liautard.** 2005. Absence of evidence for the participation of the macrophage cellular prion protein in infection with *Brucella suis*. Infect. Immun. **73:**6229–6236.
12. **Guzman-Verri, C., L. Manterola, A. Sola-Landa, A. Parra, A. Cloeckaert, J. Garin, J. P. Gorvel, I. Moriyon, E. Moreno, and I. Lopez-Goni.** 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.
13. **Jia, Y. H., L. P. Li, Q. M. Hou, and S. Q. Pan.** 2002. An *Agrobacterium* gene involved in tumorigenesis encodes an outer membrane protein exposed on the bacterial cell surface. Gene **284:**113–124.
14. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
15. **Lamontagne, J., H. Butler, E. Chaves-Olarte, J. Hunter, M. Schirm, C. Paquet, M. Tian, P. Kearney, L. Hamaidi, D. Chelsky, I. Moriyon, E. Moreno, and E. Paramithiotis.** 2007. Extensive cell envelope modulation is associated with virulence in *Brucella abortus*. J. Proteome Res. **6:**1519–1529.
16. **Li, L., Y. Jia, Q. Hou, T. C. Charles, E. W. Nester, and S. Q. Pan.** 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.
17. **Lohrke, S. M., S. Nechaev, H. Yang, K. Severinov, and S. J. Jin.** 1999. Transcriptional activation of *Agrobacterium tumefaciens* virulence gene promoters in *Escherichia coli* requires the *A. tumefaciens* RpoA gene, encoding the alpha subunit of RNA polymerase. J. Bacteriol. **181:**4533–4539.
18. **Lohrke, S. M., H. Yang, and S. Jin.** 2001. Reconstitution of acetosyringone-mediated *Agrobacterium tumefaciens* virulence gene expression in the heterologous host *Escherichia coli*. J. Bacteriol. **183:**3704–3711.
19. **López-Goñi, I., L. Manterola, and S. Q. Pan.** 2004. The *Brucella* BvrS/BvrR and related two-component regulatory systems of the a-2 proteobacteria: common regulatory strategies of animal and plant pathogens and endosybionts, p. 213–230. *In* I. López-Goñi and I. Moriyón (ed.), Brucella: molecular and cellular biology. Horizon Bioscience, Norwich, Norfolk, United Kingdom.
20. **Manterola, L., I. Moriyon, E. Moreno, A. Sola-Landa, D. S. Weiss, M. H. Koch, J. Howe, K. Brandenburg, and I. Lopez-Goni.** 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.
21. **Moreno, E., and I. Moriyón.** 2006. The genus *Brucella*, p. 315–456. *In* M. Dworkin, S. Falkow, E. Rosenberg, K. H. Schleifer, and E. Stackebrandt (ed.), The prokaryotes, 3rd ed., vol. 5. Springer, New York, NY.
22. **O'Callaghan, D., C. Cazevieille, A. Allardet-Servent, M. L. Boschiroli, G. Bourg, V. Foulongne, P. Frutos, Y. Kulakov, and M. Ramuz.** 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol. Microbiol. **33:**1210–1220.
23. **Pappas, G., N. Akritidis, M. Bosilkovski, and E. Tsianos.** 2005. Brucellosis. N. Engl. J. Med. **352:**2325–2336.
24. **Pfaffl, M. W.** 2001. A new mathematical model for relative quantification in real time RT-PCR. Nucleic Acids Res. **29:**2002–2007.
25. **Quebatte, M., M. Dehio, D. Tropel, A. Basler, I. Toller, G. Raddatz, P. Engel, S. Huser, H. Schein, H. L. Lindroos, S. G. Andersson, and C. Dehio.** 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.
26. **Sangari, F., and J. Aguero.** 1991. Mutagenesis of *Brucella abortus*: comparative efficiency of three transposon delivery systems. Microb. Pathog. **11:**443–446.
27. **Sieira, R., G. M. Arocena, L. Bukata, D. J. Comerci, and R. A. Ugalde.** 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.
28. **Sieira, R., D. J. Comerci, L. I. Pietrasanta, and R. A. Ugalde.** 2004. Integration host factor is involved in transcriptional regulation of the *Brucella abortus virB* operon. Mol. Microbiol. **54:**808–822.
29. **Sieira, R., D. J. Comerci, D. O. Sanchez, and R. A. Ugalde.** 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.
30. **Sola-Landa, A., J. Pizarro-Cerda, M. J. Grillo, E. Moreno, I. Moriyon, J. M. Blasco, J. P. Gorvel, and I. Lopez-Goni.** 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.
31. **Uzureau, S., J. Lemaire, E. Delaive, M. Dieu, A. Gaigneaux, M. Raes, X. De Bolle, and J. J. Letesson.** 2010. Global analysis of quorum sensing targets in the intracellular pathogen *Brucella melitensis* 16 M. J. Proteome Res. **9:**3200–3217.
32. **Viadas, C., M. C. Rodriguez, F. J. Sangari, J. P. Gorvel, J. M. Garcia-Lobo, and I. Lopez-Goni.** 2010. Transcriptome analysis of the *Brucella abortus* BvrR/BvrS two-component regulatory system. PLoS One **5:**e10216.
33. **Zhu, J., P. M. Oger, B. Schrammeijer, P. J. Hooykaas, S. K. Farrand, and S. C. Winans.** 2000. The bases of crown gall tumorigenesis. J. Bacteriol. **182:**3885–3895.