Microbial Pathogenesis 185 (2023) 106442

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

A sensor histidine kinase from a plant-endosymbiont bacterium restores the virulence of a mammalian intracellular pathogen

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ABSTRACT

Alphaproteobacteria include organisms living in close association with plants or animals. This interaction relies partly on orthologous two-component regulatory systems (TCS), with sensor and regulator proteins modulating the expression of conserved genes related to symbiosis/virulence. We assessed the ability of the $exoS^{+}_{Sm}$ gene, encoding a sensor protein from the plant endosymbiont *Sinorhizobium meliloti* to substitute its orthologous *bvrS* in the related animal/human pathogen *Brucella abortus*. ExoS phosphorylated the *B. abortus* regulator BvrR *in vitro* and in cultured bacteria, showing conserved biological function. Production of ExoS in a *B. abortus bvrS* mutant reestablished replication in host cells and the capacity to infect mice. Bacterial outer membrane properties, the production of the type IV secretion system VirB, and its transcriptional regulators VjbR and BvrR were restored as compared to parental *B. abortus*. These results indicate that conserved traits of orthologous TCS from bacteria living in and sensing different environments are sufficient to achieve phenotypic plasticity and support bacterial survival. The knowledge of bacterial genetic networks regulating host interactions allows for an understanding of the subtle differences between symbiosis and parasitism. Rewiring these networks could provide new alternatives to control and prevent bacterial infection.

1. Introduction

The Alphaproteobacteria encompasses bacteria present in a wide variety of environments and with different lifestyles. Some interact with plants or animals adopting a cell-associated lifestyle, either as symbionts or as pathogens [1]. They confront different environments during their life cycle, coordinating gene expression spatially and temporally, which is critical for survival. This coordination relies mainly on gene regulatory networks, essential for phenotypic plasticity.

Despite noticeable idiosyncratic differences, Alphaproteobacteria members show conservation of gene regulatory networks that reflect on their function [2,3]. Two-component signal transduction systems (TCS) are phosphotransfer pathways used by bacteria to respond to environmental cues. The canonical model describes that a dimer sensor membrane protein binds ATP in response to external stimulation and is autophosphorylated at a conserved histidine residue, hence the name of sensor histidine kinase. The sensor protein transfers the phosphoryl group to its cognate response regulator at a conserved aspartate residue. This homodimer phosphorylated response regulator increases its affinity for DNA target regions thereby regulating transcription. At high

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https://doi.org/10.1016/j.micpath.2023.106442
Received 8 February 2023; Received in revised form 22 July 2023; Accepted 3 November 2023
Available online 8 November 2023
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concentrations, the sensor protein can dephosphorylate the response regulator, limiting the concentration of the phosphorylated response regulator. Accessory proteins that promote kinase and phosphatase activities have been described. Additionally, non-canonical functions have been described highlighting the TCS's complexity [4].

In endosymbionts such as *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, and in pathogens such as *Agrobacterium*, *Bartonella* and *Brucella*, there are orthologous TCS belonging to the EnvZ/OmpR family, composed of a histidine-kinase sensor located in the cell membrane and a cytoplasmic transcriptional regulator. A periplasmic protein named ExoR, interacting with the sensor protein has also been described, and in some cases, it downregulates its function [5–7]. The *S. meliloti* TCS ExoS/ChvI/ExoR was one of the first TCS described within the Rhizobiales. Using loss-of-function and gain-of-function exoS::*Tn5, chvI::Tn5 and exoR::Tn5* mutants its signaling pathway has been characterized [8,9]. ExoS/ChvI/ExoR regulates the expression of genes required during free-living stages, for example, those related to motility, those involved in succinoglycan production, as well as those required during host association stages [10]. Therefore the name "RSI invasion switch" was suggested to refer to orthologous signaling pathways involved in the switch from a free to a cell-associated lifestyle by regulation of gene expression [5]. Likewise, the animal pathogen *B. abortus* relies on the ortholog TCS BvrR/BvrS to invade and survive inside macrophages [11]. Recently, ExoR was also described in *B. abortus* and related to BvrR/BvrS. Notably, a *B. abortus exoR* mutant is capable of infecting Raw macrophages, HeLa cells, and mice, suggesting that its function might be different from that of described orthologs [7].

Several attempts to obtain null mutants in *S. meliloti* and *Brucella* have been described [9,12,13]. Bélanger et *al.* 2009 and Wang et al., 2010 [14,15] reported the construction of *S. meliloti chvI* null mutants, that grow only in minimal medium or minimal medium supplemented with specific carbon sources, suggesting that *chvI* is conditionally essential. Mutations in TCS *A. tumefaciens* ChvG/ChvI, *S. meliloti* ExoS/ChvI, *B. henselae* BatR/BatS and *B. abortus* BvrR/BvrS generate pleiotropic phenotypes [8,10,16–23]. Therefore, it is likely that these systems influence both host-microbe interactions and bacterial physiology in response to changes in the environment. In *B. abortus*, BvrR phosphorylation is observed when bacteria are exposed to the combination of low pH and limited nutrient availability, conditions that mimic the intracellular environment encountered by the bacteria during the early stages of its intracellular trafficking [24]. *B. abortus bvrR::Tn5* and *bvrS::Tn5* mutants have a differential expression at gene and protein levels as compared to the wild-type strain, affecting cell envelope homeostasis and coordination of gene regulation of carbon and nitrogen metabolism [18,22]. Several virulence factors such as the type IV secretion system VirB, its transcriptional regulator VjbR and outer membrane proteins, particularly Omp25, are under the transcriptional control of BvrR/BvrS [24–26].

S. meliloti exoS ($exoS^{+}_{Sm}$) can suppress the mutant phenotype of an *A. tumefaciens chvG* mutant, sensitive to complex growth media [9,27]. Likewise, complementation with *A. tumefaciens chvG* restored the production of succinoglycan to normal levels in a *S. meliloti exoS::Tn5* mutant encoding a defective *exoS* gene. Additionally, *A. tumefaciens chvI* restored the regulated alkaline phosphatase activity in an *Escherichia coli phoB::Tn5* mutant [28]. However, in these cases, the interaction between the histidine kinases and the corresponding response regulators was not demonstrated.

The evolutionary pathway that led to the current Alphaproteobacteria symbionts and host-associated pathogens remains unclear. Very likely their common ancestor relied on sensory systems to respond and eventually adapt to different niches. As a preliminary attempt to understand the role of the RSI bacterial invasion switch within Alphaproteobacteria evolution, we explored whether the ExoS sensor protein belonging to a regulatory circuit from the plant endosymbiont *S. meliloti* could replace the function of the orthologous BvrS from the animal pathogen *B. abortus*, regardless of their differences in cell cycle and living environments. ExoS was able to phosphorylate the *B. abortus* response regulator BvrR and restore virulence of a *B. abortus bvrS* mutant, demonstrating not only that there are functional conserved traits within these bacteria but also that signal cues are probably structurally conserved. Understanding these regulatory circuits contributes to resolving the subtle differences between symbiosis and parasitism.

2. Results

***S. meliloti* sensor protein ExoS can phosphorylate *B. abortus* regulator protein BvrR *in vitro*.** Before evaluating the ability of ExoS to function in a *B. abortus* context, we first determined whether ExoS is capable of phosphorylating BvrR *in vitro*. As previously reported [27], the purified cytoplasmic domain of ExoS ($ExoS_{cyto72}$) exhibited autophosphorylation in the presence of [γ-^{32}P] ATP (Fig. 1a). When purified BvrR was included in this reaction, a lower molecular weight band corresponding to the size of BvrR was observed, indicating that ExoS recognizes and phosphorylates the regulator BvrR *in vitro*. Incubation of BvrR with only [γ-^{32}P] ATP did not result in autophosphorylation. Additionally, this reaction is dependent on the proper ExoS folding and is resistant to acidic conditions (Fig. S1), which is consistent with the fact that Asp58 is probably the phosphorylated acidic residue [27,29]. These results and those previously obtained [27] indicate that the kinase activity of ExoS is exerted *in vitro* on its cognate response regulator ChvI and also on BvrR.

ExoS restores the ability of *B. abortus bvrS* to invade and replicate within epithelial cells and macrophages. Obtaining null mutants in *bvrR/bvrS* and orthologous TCS has proven difficult, and when obtained, they grow on minimal but not in rich media, limiting their phenotype and virulence characterization [14]. On the other hand, the use of transposon mutants led to pioneering studies on the role of ExoS/ChvI in the regulation of gene expression in *S. meliloti* and host-interactions [8,17,30,31].

Hence, in the absence of a null *bvrS B. abortus* mutant, we decided to use a *B. abortus* 2308 W *bvrS::Tn5* mutant (referred to herein as *B. abortus bvrS)* to contribute to the understanding of the evolution of this family of TCS and the role they play in bacterial-host interactions.

It has been described that *B. abortus bvrS* cannot invade and multiply within macrophages and epithelial cells [11]. To test whether *S. meliloti* ExoS could substitute for *B. abortus* BvrS *ex vivo,* we transformed this *B. abortus bvrS* sensor mutant with an *exoS*-containing plasmid.

Exclusive *bvrS* mutation in the *B. abortus* background strain was confirmed by whole-genome sequencing and the absence of downstream polar effects was established by detection of specific mRNA by RT-PCR (Fig. S2). A single colony chimera transformant, hereby denoted as *B. abortus bvrS* p$exoS^{+}_{Sm}$, was chosen for further characterization. The inserted plasmid was isolated from this strain, and the presence of $exoS+_{Sm}$ was confirmed by PCR and sequencing. Moreover, the $exoS+_{Sm}$ mRNA was amplified from *B. abortus bvrS* p$exoS^{+}_{Sm}$ total RNA by RT-PCR but not from *B. abortus bvrS* or *B. abortus* parental strain (Fig. S2). Additionally, an anti-BvrS antibody showed a strong signal corresponding to the molecular weight of ExoS in *B. abortus bvrS* p$exoS^{+}_{Sm}$, suggesting antibody cross-reaction to ExoS (Fig. 1b). This signal was absent in the non-complemented *B. abortus bvrS* mutant and in the same mutant complemented with a plasmid encoding *bvrS*, named *B. abortus bvrS* p$bvrS^{+}_{Ba}$ (Fig. 1b). The reason for no BvrS signal (and for BvrR as shown in Fig. 2a) in *B. abortus bvrS* p$bvrS^{+}_{Ba}$ was investigated. The presence of cloned *bvrS* was detected by PCR however when mRNA *bvrS* expression was analyzed by RT-qPCR, there was no relative difference between *B. abortus bvrS* p$bvrS^{+}_{Ba}$ as compared to the non-complemented strain, *B. abortus bvrS* (relative quantification: 1.00, coefficient of variation C: 32.9). The reason for this remains elusive and precludes the use of this strain as complementation control, as observed in the results obtained to evaluate intracellular replication in HeLa cells (Fig. 1c). Moreover, despite different strategies used, it was not possible to clone

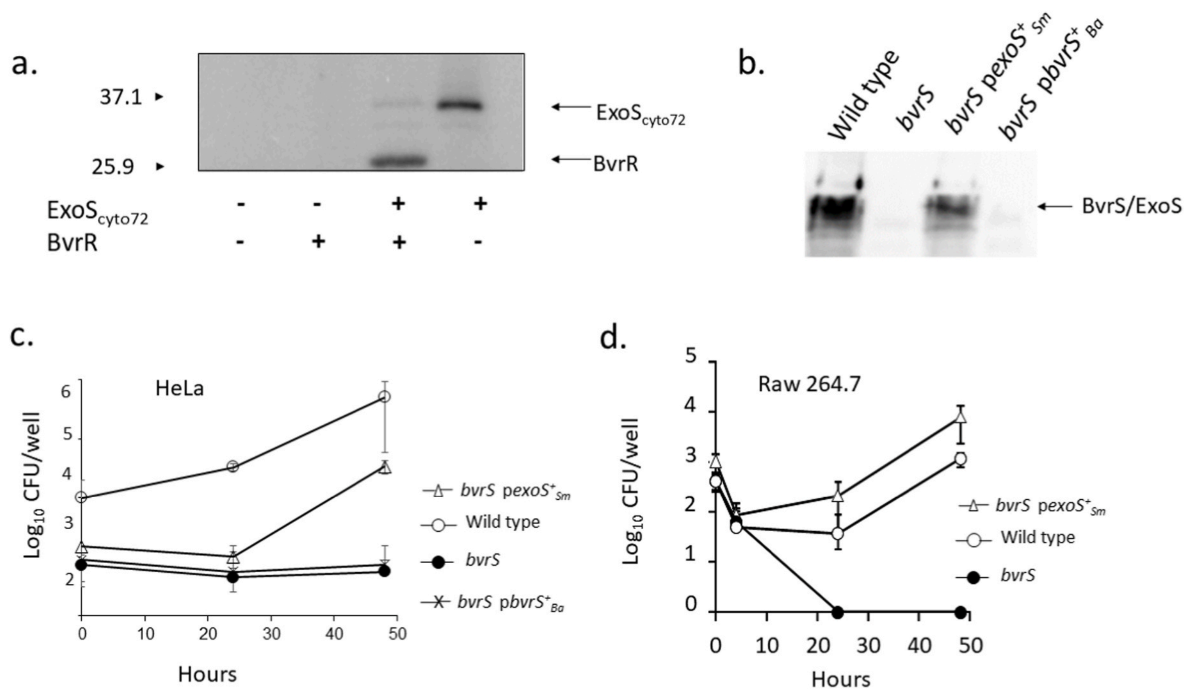

Fig. 1. ExoS phosphorylates BvrR *in vitro* and restores *B. abortus* intracellular replication. a. His-tagged $ExoS_{cyto72}$ and GST-tagged BvrR were purified by affinity chromatography. Purified proteins were incubated alone or in combination in the presence of [$\gamma-^{32}$P] ATP, separated by SDS-PAGE and radiolabeled proteins detected by Phosphorimager. The molecular weight of size markers is indicated. **b**. Cell lysates from exponentially grown *B. abortus*, *B. abortus bvrS, B. abortus bvrS* p*exoS*$^{+}_{Sm}$, and *B. abortus bvrS* p*bvrS*$^{+}_{Ba}$ were separated by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with an anti-BvrS antibody. The expected molecular weight of BvrS is 66.7 KDa and that of ExoS is 63.7 KDa. HeLa cells **(c)** or Raw 264.7 macrophages **(d)** were infected for 30 min with the indicated *B. abortus* strains. After infection, extracellular bacteria were eliminated by adding 100 μg/ml gentamicin for 1h and cells were further incubated in the presence of 5 μg/ml gentamicin. After the indicated times, cells were lysed in 0.1% triton, and CFU determined by plating in TSA. Experiments were repeated at least three times with similar results.

bvrS in the same vector as *exoS*, suggesting that cloning and expression of orthologous genes are influenced by unknown factors. Nevertheless, both plasmids encoding *exoS* and *bvrS* are expected to replicate at low copy numbers.

We then analyzed the phenotype of *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ chimeric strain. Intracellular-survival assays were performed in cell cultures as indicated in Methods section. The *B. abortus* parental strain and *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ displayed similar intracellular replication dynamics in HeLa cells and Raw 264.7 macrophages (Fig. 1). In HeLa cells, the intracellular replication rate at 24 h achieved by *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ was lower than that of the parental strain, increasing more drastically than the parental strain, until 48 h of growth. As expected, the *B. abortus bvrS* mutant did not replicate, and as mentioned, neither was the complemented strain *B. abortus bvrS pbvrS*$^{+}_{Ba}$. In macrophages, the intracellular replication dynamic was similar between the parental 2308 W strain and *B. abortus bvrS* p*exoS*$^{+}_{Sm}$, with an intracellular replication rate higher for *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ as compared to the parental strain at 24 and 48h of growth. The ability of *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ to multiply intracellularly was confirmed by differential immunofluorescence microscopy of infected HeLa cells and Raw macrophages 48 h post-infection (Fig. S3).

Altogether, these experiments indicate that the production of ExoS reestablishes the ability of *B. abortus bvrS* to invade, survive and replicate within host cells.

The low levels of VirB8, *virB5*, VjbR and BvrR in *B. abortus bvrS* are restored by ExoS. BvrR/BvrS is one of the systems known to regulate the production of the type IV secretion system (T4SS) VirB, by direct binding of BvrR to the *virB* promoter region and by controlling the expression of its transcriptional activator VjbR, important for intracellular survival [22,26,32,33]. Complementation of *B. abortus bvrS* with p*exoS*$^{+}_{Sm}$ restored the production of VirB8 to higher levels than the parental strain and the production of VjbR was restored to similar levels as those observed in the parental strain (Fig. 2a). Consistent with previously reported data [26] *virB5* mRNA levels were lower in *B. abortus bvrS* by RT-qPCR. At mid-log phase, the expression was a 0.018-fold change (CV = 39), and at stationary phase, 0.22-fold change (CV = 20) relative to the expression levels detected in the parental *B. abortus* strain. Transcription of *virB5* was restored in *B. abortus bvrS pexoS*$^{+}_{Sm}$ to similar or higher levels relative to the parental strain: 1.41 fold change at mid-log phase (CV = 17) and 3 times fold change at stationary phase (CV = 27). Likewise, the levels of BvrR were diminished in *B. abortus bvrS* and restored by p*exoS*$^{+}_{Sm}$ complementation as compared to the parental strain (Fig. 2a and b). On the contrary, the complementation of *B. abortus bvrS* with p*bvrS*$^{+}_{Ba}$ resulted in no detection of BvrR under these conditions. Thus, these experiments confirm that ExoS substitutes BvrS within the *B. abortus* host interaction regulatory network and suggest that the production of BvrR/BvrS is under an autoregulatory loop.

BvrR phosphorylation was assessed at different growth points in cell lysates, using Phos-tag™ SDS-PAGE and anti-BvrR antibodies (Fig. 2b). Two protein bands were detected in the parental *B. abortus* strain at all-time points tested, and with different intensities. The lower molecular weight band was identified as non-phosphorylated BvrR when compared to purified BvrR analyzed in similar conditions. The higher molecular weight band was identified as phosphorylated BvrR (P-BvrR) when compared to purified *in vitro* phosphorylated BvrR using carbamyl phosphate as the phosphate donor. Non-phosphorylated BvrR was detected in *B. abortus bvrS* mutant cell lysates at all-time points. Both, non-phosphorylated BvrR and P-BvrR were detected at all-time points in the chimera strain *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ and at higher intensities than in *B. abortus bvrS* mutant cell lysates. This indicates that *S. meliloti* ExoS can phosphorylate *B. abortus* BvrR in cultured bacteria.

The outer membrane properties of *B. abortus bvrS* are restored by ExoS. Disruption of either *bvrS* or *bvrR* reduces the production of at least two Omps, Omp25 (BAW_10696, BAB1_0722) and Omp22

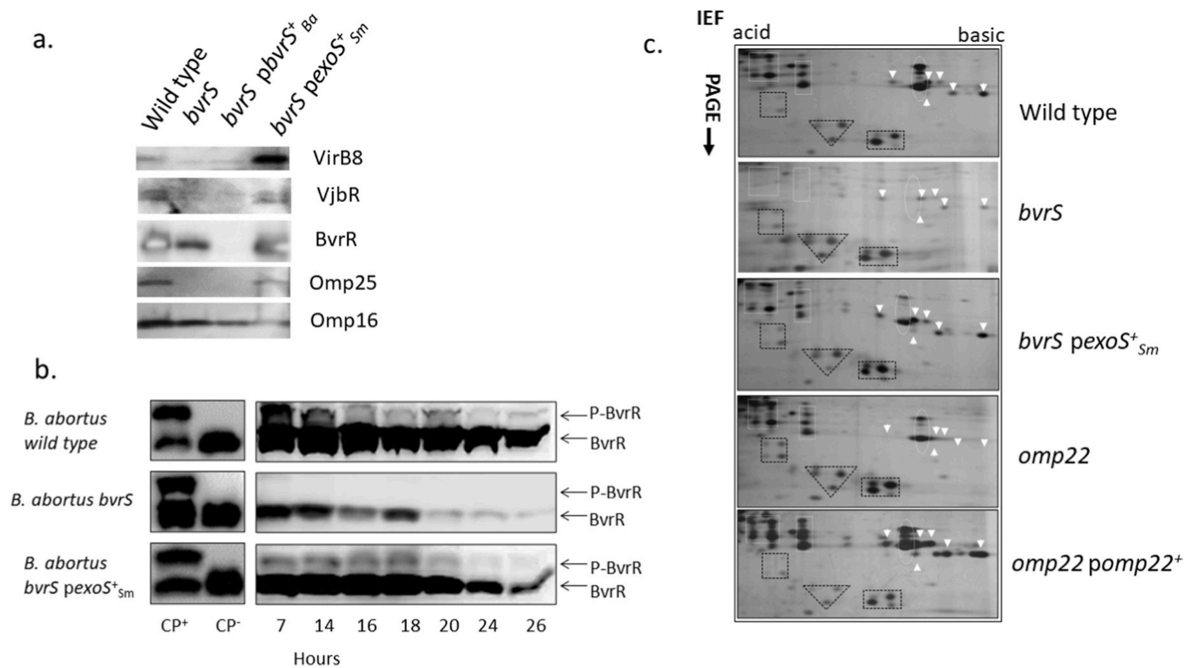

Fig. 2. ExoS restores wild-type production levels of proteins known to be influenced by BvrR/BvrS in a *bvrS* background. a. Cell lysates from exponentially grown *B. abortus*, *B. abortus bvrS,* and *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ were separated by 12.5% SDS-PAGE, transferred to PVDF membranes, and probed with the indicated antibodies. Detection of Omp16 using monoclonal anti-Omp16 antibodies was used as loading control. After incubation with peroxidase-conjugated anti-mouse or anti-rabbit antibodies immune complexes were detected by chemiluminescence reaction. All experiments were repeated at least three times with similar results. **b.** Levels of phosphorylated BvrR in *B. abortus*, *B. abortus bvrS,* and *B. abortus bvrS* p*exoS*$^{+}_{Sm}$. The specified strains were grown in TSB from the log phase to the stationary phase and samples were taken at the indicated times. Equal amounts (25 μg) of whole-bacterium lysates were then separated by 10% SDS-PAGE containing Phos-tag™, transferred to PVDF membranes, and probed with an anti-BvrR antibody. After incubation with peroxidase-conjugated anti-rabbit antibody, immune complexes were detected by chemiluminescence reaction. CP+: *in vitro* phosphorylated recombinant BvrR using carbamyl phosphate as phosphate donor. CP-: non-phosphorylated recombinant BvrR. **c.** OM fragments from *B. abortus*, *B. abortus bvrS, B. abortus bvrS* p*exoS*$^{+}_{Sm}$, *B. abortus omp22,* and *B. abortus omp22* p*omp22*+ were separated by 2D-electrophoresis and detected by silver staining. Black geometrical figures indicate selected groups of proteins present in all strains tested. White geometrical figures indicate groups of proteins absent on *B. abortus bvrS*. White arrowheads point to Omp22 (Omp3b) isoforms.

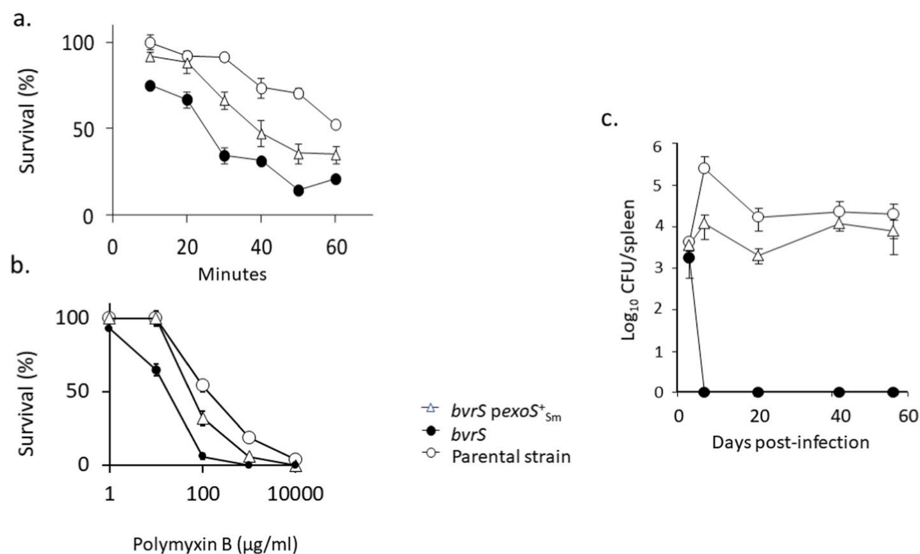

Fig. 3. The complemented strain *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ displays intermediate resistance to polymyxin B and can replicate in mice. a. Triplicate samples of *B. abortus* strains were exposed to polymyxin B (50 μg/ml) at 37 °C for the indicated times. After incubation aliquots were plated in TSA and CFU determined after 72h. The survival rate was calculated using the corresponding CFU obtained for each strain without polymyxin B. The experiment was repeated three times with similar results. Strain *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ was significantly different from the parental strain at all time points tested but at 10 min and 20 min **b.** Triplicate samples of parental *B. abortus* strains were exposed to the indicated concentrations of polymyxin B for 1 h at 37 °C. Thereafter, samples were processed as indicated above. Strain *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ was significantly different from the parental strain at concentrations tested but at 1 μg/ml and 10 μg/ml **c.** Groups of four mice were inoculated intraperitoneally with the indicated *B. abortus* strains as stated in the materials section. The CFU per spleen were calculated at the indicated times. Strain *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ was significantly different from the parental strain at all time points tested but at 3 days and 7 days post-infection. $P < 0,05$ (Anova test).

(BAW_11241, BAB1_1302) [25,34]. Similarly, the production of Omps in other Alphaproteobacteria has been related to BvrR/BvrS orthologs [35–38]. To assess if *S. meliloti* ExoS restores the production of these proteins in the chimeric *B. abortus bvrS* p*exoS*$^{+}_{Sm}$, outer membrane fragments from *B. abortus*, *B. abortus bvrS,* and *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ were analyzed by 2D-electrophoresis. Two groups of acidic proteins and one group of basic proteins were faint or undetectable in the *bvrS* strain as compared to the parental strain (Fig. 2c). The production of these proteins was restored close to the parental strain levels in the *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ chimera (Fig. 2c). Likewise, the presence of *exoS* restored the production of a group of basic proteins of slightly lower molecular weight as compared to the parental strain. These protein isoforms were identified as Omp22 since they were absent in a *B. abortus omp22* strain and overexpressed in the same strain reconstituted with plasmid-borne *omp22* (Fig. 2c) [36]. In addition, total bacterial lysates were analyzed by Western blot using monoclonal anti-Omp25 antibodies. A conspicuous ~25 kDa band was present in *B. abortus* and absent in *B. abortus bvrS* and *B. abortus bvrS pbvrS*$^{+}_{Ba}$, was present in *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ (Fig. 2a). Thus, *S. meliloti* ExoS reestablishes the production of Omp25 and Omp22 proteins in *B. abortus.*

Defects in the outer membrane, particularly the LPS, of the *B. abortus bvrS* mutant, are responsible for an increased sensitivity to polymyxin B as compared to the parental *B. abortus* strain [39]. Similarly, the RSI invasion switch is activated when *S. meliloti* is exposed to sub-lethal doses of antimicrobial peptides [37]. To determine if *S. meliloti exoS*$+_{Sm}$ gene restores the resistance to polymyxin B of the chimeric *B. abortus* strain, time and dose-dependent survival curves were obtained. *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ showed an intermediate polymyxin B sensitivity with 50% survival after 40 min incubation compared to the parental strain (Fig. 3a). This chimeric strain also showed an intermediate behavior in a dose-response curve against increasing concentrations of polymyxin B (Fig. 3b). This result suggests that the LPS molecule on the surface of the *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ is at least partially restored.

ExoS restores the ability of *B. abortus bvrS* to survive in mice. *B. abortus bvrS* is rapidly eliminated when inoculated intraperitoneally in mice [11]. To determine if *exoS*$+_{Sm}$ rescues this phenotype, bacterial replication growth curves were performed in mice (Fig. 3c). While *B. abortus bvrS* was rapidly eliminated, *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ was recovered from the spleens of inoculated mice throughout the whole experiment. The CFUs counts were significantly lower than those obtained for *B. abortus* parental strain during the first weeks. However, later (42 and 56 days) *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ reached the levels achieved by the parental counterpart, confirming that the virulence factors needed for intracellular survival were expressed.

3. Discussion

The results presented here show that the ortholog ExoS was able to phosphorylate BvrR which in course restores the production of the T4SS VirB, the quorum sensing transcriptional regulator VjbR, the response regulator BvrR, and the outer membrane proteins Omp22 and Omp25, similar to wild type levels. The functional and biological properties of the outer membrane in the *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ chimera are also restored. These traits, related to *Brucella* ability to establish an intracellular niche [38], are absent in the highly attenuated *B. abortus bvrS* mutant and in this mutant strain complemented with a plasmid intended to express wild-type *bvrS*. Moreover, the production of ExoS in the *B. abortus bvrS* mutant was able to restore replication in host cells and the capacity to infect mice, indicating that a conserved sensor protein from *S. meliloti*, a plant symbiont, can strengthen an attenuated mutant of an animal pathogen to multiply inside mice.

There are several alternatives to explain the *exoS* complementation. First, it has to be assumed that ExoS is properly assembled in the *B. abortus* cytoplasmic membrane, then it autophosphorylates and transfers the phosphate group to the transcriptional regulator BvrR with its subsequent activation. The ability of ExoS not only to restore BvrR levels similar to those observed in the wild-type strain but also to phosphorylate BvrR *in vivo*, further corroborates the *in vitro* phosphorylation findings. Taken together, these results, along with previous studies [27], strongly indicate that ExoS exerts its kinase activity not only on its cognate response regulator ChvI but also on BvrR. This suggests a conserved evolutionary pathway of the bacterial RSI switch in a plant endosymbiont and a facultative extracellular intracellular animal pathogen, opening possibilities for modulating the capacity of these bacteria to interact with different hosts. In this same line of thought recently, a regulatory pathway functionally analogous to the RSI invasion switch was described as central to the outcome of a host-bacteria interaction in the plant pathogen *Ralstonia solanacearum*. By introducing mutations in this switch, the pathway was rewired to improve intracellular symbiosis and decreased the virulence of the original pathogen, originating legume symbionts [40].

The successful complementation observed could in principle depend on the overexpression of *exoS*$^{+}_{Sm}$ and the dimer formation required for the kinase activity of sensors [41], which could be favored by ExoS overproduction. However, *exoS*$^{+}_{Sm}$ is encoded in a low copy number plasmid. Moreover, in the absence of the cognate signal, the balance between the kinase and phosphatase activities in sensor proteins shifts towards the latter [42]. Thus, even if *exoS*$^{+}_{Sm}$ was overexpressed, in the absence of the appropriate signal one would expect BvrR to remain non-phosphorylated, which is not the case. A second obvious alternative is that the signal recognized by ExoS is also present in the *B. abortus* bacteriological media and within the intracellular milieu of mammalian cells. The signal may be the same or structurally alike as the one detected by BvrS. Likewise, it may be that BvrS and ExoS recognize different signals (e.g. pH and osmolality), and both of them are present in the medium and in cells. Finally, although ExoS would detect one type of signal in *S. meliloti*, in the context of *B abortus* a distinct second signal would be detected as a result of an "emergent" structural property.

Overall, these proteins show 64% identity (Fig. S4) and there is, as expected an almost perfect 3D fit obtained by modeling both proteins using a histidine kinase domain (Fig. S5), like the kinase domain of the ortholog EnvZ [4]. Two regions in ExoS are most variable according to substitution rate analysis [5] located in the periplasmic region (Fig. S4). Despite the relatively low degree of identity (47%) between BvrS and ExoS in this putative sensing periplasmic, hydrophilic domain, these domains have a similar predicted secondary structure (Fig. 4).

This supports the hypothesis that both sensors recognize related signals. Indeed, pH has been suggested as a signal in studied Alphaproteobacteria [19,23,43,44] but probably it is not exclusive [17,24,37,45]. Our previous work on BvrR/BvrS signaling [24,46] has highlighted that the combination of low pH and limited nutrient availability can influence BvrR/BvrS activation, with consequential effects on virulence, depending on the physiological state of the bacteria. Nevertheless, it is crucial to consider that specific biochemical conditions appear to determine the activation/deactivation of TCS [4].

For instance, the sensor protein EnvZ in *E. coli* possesses a histidine residue responsible for autophosphorylation in response to stimuli, situated within a disordered region that is part of an alpha-helix, and flanked by another alpha-helix recognized by its cognate response regulator OmpR. This unique organization allows EnvZ to adopt multiple conformations, enabling interactions with osmolytes and environmental stimuli, even from membrane lipids, and leading to varied signaling outputs [47].

Similarly, the *E. coli* histidine kinase EvgS can sense high concentrations of alkali metals through its periplasmic domain, while its cytoplasmic domain senses low pH [48].

These distinctive biochemical sensing mechanisms, not yet described for BvrR/BvrS or ChvI/ExoS, might be significant for understanding the reasons behind ExoS's ability to fully substitute BvrS function in certain cases, such as regulating BvrR, VirB8, VjbR, and Omps expression, and partially complementing functional properties, such as resistance to polymyxin B and intracellular replication. Certainly, the rate of non-

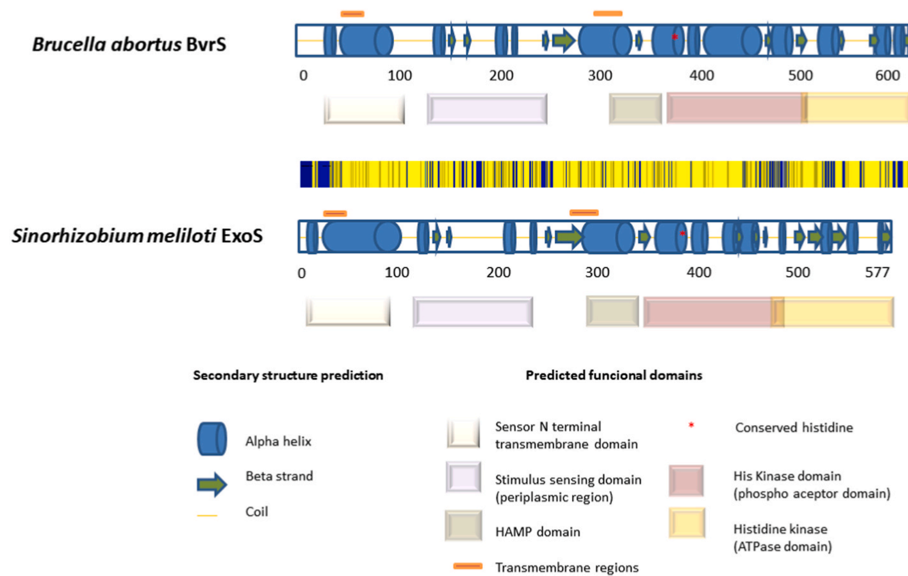

Fig. 4. Comparison of predicted structures of *B. abortus* BvrS and *S. meliloti* ExoS. The bar with colors between both structures represents the primary structure comparison. Yellow: identical amino acid residues, light yellow: conserved amino acid residues, blue: non-conserved amino acid residues. Protein sequence alignment is presented in Fig. S4.

phosphorylated versus phosphorylated response regulator and the genes being regulated deserves further investigation [29,49].

The absence of complementation by the same *B. abortus bvrS* gene remains puzzling. One possible explanation is that the presence of multiple copies of the BvrS sensor gene disrupts an autoregulatory *bvrR/bvrS* loop, while this effect is not observed with copies of the ExoS sensor gene, as indicated by the concomitant absence of BvrR in the *bvrS*-complemented mutant and its presence in the *exoS*-complemented *bvrS* mutant. This hypothesis gains support from the observation that no differences in *bvrS* expression at the transcriptional level were noted between *B. abortus bvrS* and *B. abortus bvrS pbvrS*$+_{Ba}$.

In *B. abortus bvrS*, BvrR is produced but at different levels compared to the wild-type strain across the tested growth time points. Moreover, phosphorylated BvrR was not detected in this strain, suggesting the absence of BvrS kinase activity. It has been documented that null kinase mutants can affect the concentration of the response regulator not bound to the DNA target regions, and autophosphorylation of the response regulator under such conditions can be achieved using phosphodonors to varying degrees. Both BvrR and ChvI have demonstrated the capability to utilize carbamoyl phosphate or acetyl phosphate for *in vitro* autophosphorylation of an aspartate residue (Fig. 2) [24,27].

The introduction of additional BvrR target regions as *bvrS* copies might lead to the sequestration of BvrR and, if present, BvrR-P, subsequently downregulating *bvrR* and *bvrS* expression. It is worth mentioning that three and four BvrR binding sites have been described near *bvrS* and *bvrR*, respectively [50]. However, the influence of BvrR binding to these different sites and potential interactions among them on *bvrR* and *bvrS* transcription remains unknown.

On the other hand, orthologous TCS from different species may be more prone to complement each other, since evolutionary forces favor structural conservation to preserve a specific function [51–53]. In the case of BvrR and ChvI, there are stretches of 15 identical amino acids surrounding the putative phosphorylated aspartates at positions 58 and 52 respectively [5,24,27], explaining the high degree of complementation and the ability of ExoS to readily recognize BvrR as its cognate response regulator.

The general strategies used by cell-associated Alphaproteobacteria for interacting with host cells are often maintained throughout evolution and modulated according to particular requirements [54]. Despite some similarities in these bacteria lifestyles, they must confront different environments both extracellularly and intracellularly. We demonstrate that BvrS and ExoS are capable of phosphorylating BvrR, indicating that adjustment to these alternative environments is more likely achieved by the TCS they belong to. This reinforces the hypothesis that this TCS is a decisive factor influencing the outcome of the liaison established between cell-associated Alphaproteobacteria and their host, either as a symbiont or as a pathogen.

4. Methods

Bacterial strains, growth conditions, and electroporation. *B. abortus* strains were grown in tryptic soy broth (TSB) and *Escherichia coli* BL21 was grown on LB or 2XYT medium supplemented with 50 μg/ml ampicillin when required. Otherwise indicated, all bacterial cultures were collected for analysis at late log growth phase. *B. abortus* 2308 Wisconsin is a virulent smooth-lipopolysaccharide strain described elsewhere [55]. Attenuated *B. abortus bvrS* is a smooth-lipopolysaccharide strain, derived from *B. abortus* 2308 Wisconsin with a mini*Tn5* insertion in *bvrS* [11]. *B. abortus omp22 (omp3b)* is a smooth-lipopolysaccharide strain, derived from *B. abortus* 2308 Wisconsin with a kanamycin-resistance cassette inserted in *omp22 (omp3b)* [56]. This strain was transformed with plasmid pBBR3b containing *omp22 (omp3b)* to generate strain *B. abortus omp3b* p*omp3b*+. Strain *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ was obtained after electroporation of *B. abortus bvrS* with plasmid p*exoS*$^{+}_{Sm}$. This plasmid is a derivative of pT84Ω10 (*chvI*$^{-}$, *exoS*$^{+}_{Sm}$), replicating at low copy numbers [9], and was generated by replacing the tetracycline cassette for a chloramphenicol resistance cassette from vector pBBRMCS-1 [57] by lambda Red [58]. Despite different strategies tested, it was not possible to clone *bvrS* into the same derivative vector. Therefore, *bvrS* was cloned into pCR®8/GW/TOPO® using the Gateway® LR Clonase™ II Enzyme Mix (Invitrogen) and transferred to vector pRH002, also replicating at low copy numbers and with the same chloramphenicol resistance cassette [57,59,60]. The resulting plasmid is named p*BvrS*$^{+}_{Ba}$. Strain *B. abortus bvrS* electroporated with plasmid p*BvrS*$^{+}_{Ba}$ was used for complementation analysis.

All procedures involving live bacteria were carried out according to the "Reglamento de Bioseguridad de la CCSS 39975-0" year 2012, after the "Decreto Ejecutivo #30965-S″ year 2002 and research protocol NFEG06 approved by the National University, Costa Rica.

Whole-genome sequencing. Genome sequencing of *B. abortus bvrS* was performed at Wellcome Trust Sanger Institute, following in-house protocols [61,62] (Quail et al., 2008: Quail et al., 2012). Sequencing reads (n = 3756400) were de novo assembled using Velvet Optimiser [63], returning 13 contigs with a total length of 3260799 with N50 of 364347. Contigs were ordered as described [55] against *B. abortus* 2308 Wisconsin under accession code ERS568782 at the European Nucleotide Archive. The transposon position was inferred from BLAST comparisons and confirmed by mapping against the reference and the ordered contigs, using sequencing (Phred>30) and mapping (>50) quality filters. The reads covered 98.81% of *B. abortus* 2308 Wisconsin. Visualizations were done with Artemis and comparisons with the Artemis Comparison Tool [64]. The sequence data from *B. abortus bvrS* is available at the European Nucleotide Archive under accession number ERS374426.

PCR, RT-PCR and relative quantitation of gene expression by real-time PCR (RT-qPCR). Plasmid DNA from *B. abortus bvrS* $exoS+_{Sm}$ was isolated by alkaline lysis according to standard procedures. Specific amplification of *exoS* was achieved using primers: 5′GTCCGAGCCTCGC CGATGAGT3′ and 5′GCTGATGCGGCCGTCCTTGC3′ under the following conditions: 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 65 °C for 1 min and 68 °C for 30 s and continued with a 68 °C incubation for 10 min. Specific amplification of plasmid-borne *bvrS* from *B. abortus bvrS* $pbvrS^{+}_{Ba}$ was achieved using primers: bvrS-terF 5′ ATGGTCGCAGAGACCCAGAA 3′ and the M13 Universal primer 5′ CAGGAAACAGCTATGAC 3′ under the following conditions: 94 °C for 5 min, 30 cycles at 94 °C for 30 s, 60 °C for 25 s and 68 °C for 30 s, and continued with a 68 °C incubation for 10 min. The PCR products were analyzed on agarose gels using standard procedures. DNA sequencing of the obtained amplicons was performed using the Big Dye terminator kit 3.1 according to manufacturer instructions (Life Technologies). For RNA extraction bacterial cultures at different growth stages were treated according to established protocols [26]. Synthesis of cDNA was carried out using Revert Aid M-MuLVReverse Transcriptase with random hexamers or with primer: 5′CGTGTCGGCGAGATCAGTCT3′ and following manufacturer's instructions (Thermo Fisher Scientific). The obtained cDNA was used as template for mRNA *exoS* detection by PCR using the primer indicated above together with 5′CAACCAGTTCCGGGAAGGTC3′ and under the following conditions: 94 °C for 4 min, 35 cycles at 94 °C for 30 s, 55 °C for 1 min and 68 °C for 30 s and continued with a 68 °C incubation for 10 min. Confirmation of non-polar effects in *B abortus bvrS* was achieved using cDNA as template for PCR and the following primers 5′ GTGCCCGCTTTATCGTTGATC 3′ and 5′ GATGCAGGAGTGTGCGAT-CATC 3′, designed to amplify the downstream *bvrR/bvrS* region. The PCR products were analyzed on agarose gels using standard procedures. DNA sequencing of the obtained amplicons was performed as described above.

For relative quantification of *virB5* expression by real-time PCR the different strains were grown at 37 °C with shaking in TSB, to different growth phases. RNA samples were extracted as described and retro-transcribed using Applied Biosystems high-capacity cDNA kit and random primers. Relative quantification and data analysis were carried out as described [26]. For *bvrS* expression, RNA extraction and cDNA production were carried out as described for *virB5* expression. For the PCR step, the following primers were used: qbvrS_F1 (5′ CCGCCGATA-GAGGATGAGAC 3′) and qbvrS_R1 (5′ ATAGAAAAGGCGCGACAGC 3′), together with SYBR™ Green PCR Master Mix (Thermo Fisher Scientific) under the following conditions: an initial denaturation step at 95 °C for 10 min, followed by 45 cycles at 95 °C for 5 s and 60 °C for 10 s. Fluorescence signals were acquired in the green channel using a Rotor-Gene Q 5plex HRM instrument (Qiagen). Relative quantification and data analysis were carried out as described [26].

Polymyxin B sensitivity. Sensitivity to polymyxin B was tested as described elsewhere with some modifications [7,11]. Strains were grown in TSB until the exponential growth phase and concentration was adjusted to 5×10^6 CFU/ml. For the time-dependent survival curve, triplicates of 100 μl aliquots of each *B. abortus* strain were exposed to polymyxin B (50 μg/ml) in 1 ml tubes at 37 °C. Samples were then taken every 10 min up to 60 min. After incubation, 5 μl of each triplicate were plated in TSA, and CFU determined after 72h. The survival rate was calculated using the corresponding CFU obtained for each strain without polymyxin B. For the dose-dependent survival curve, strains were grown as described above and triplicates of 100 μl aliquots of each *B. abortus* strain were exposed to 100 μl of different polymyxin B concentrations in a microplate for 1 h. Samples were then processed as described above.

Purification of fusion proteins, antibodies and *in vitro* phosphorylation reaction. BvrR was expressed as a GST tag fusion protein and purified as described [26]. Rabbit anti-BvrR, anti-BvrS, anti-Vjbr and anti-*Brucella* antibodies were used as described [24,26,46]. The cytoplasmic domain of ExoS ($ExoS_{cyto72}$) was purified as a His-tagged fusion protein on a nickel column as previously described [27]. For phosphorylation reactions, the purified $ExoS_{cyto72}$ protein was mixed with [γ-32P] ATP in the reaction buffer (30 mM HEPES [pH7.4], 10 mMMgCl2, 5 mMCaCl2, 50 mM KCl) along with unlabeled ATP to give a final concentration of 0.4 mM. The reaction mixture was incubated at room temperature for 10 min. The labeled protein was mixed with or without purified BvrR protein for an additional 5 min before being mixed with an equal volume of loading buffer to stop the reactions. Radioactive proteins separated by 12.5% SDS-PAGE were detected by autoradiography. To investigate the resistance of BvrR phosphorylation to acidic conditions in the presence of ExoS, the reaction was conducted following the aforementioned protocol. However, before adding the loading buffer, HCl (0.1 M) was introduced, and the reaction mixture was incubated for 5 min.

To assess the significance of ExoS folding on BvrR phosphorylation, the ExoS autophosphorylation reaction was carried out using the previously described procedure. Subsequently, 1% SDS was added to the reaction mixture, followed by a 5-min incubation period. After this, BvrR was introduced into the reaction and the process was continued as outlined above.

Detection of *in vivo* BvrR phosphorylation. *B. abortus, B. abortus bvrS,* and *B. abortus bvrS* $pexoS^{+}_{Sm}$ were grown as indicated previously. Samples from the log phase to the stationary phase were centrifuged, resuspended in lysis buffer and centrifuged again to obtain whole-cell lysates. Samples were resuspended in Laemmli sample buffer and equal amounts of protein (25 μg) were loaded without heating onto a 10% gel for SDS-PAGE containing 100 μM Phos-tag and 0.2 mM $MnCl_2$. Western blot was performed as described [24,26]. A positive control for phosphorylation was prepared *in vitro* with carbamoyl phosphate disodium salt. Recombinant purified BvrR (2 mg/ml) was incubated for 20 min with carbamoyl (final concentration 50 mM) and buffer Tris-HCl pH 7.0 (Tris-HCl 200 mM, $MgCl_2$ 20 mM and KCl 200 mM) at room temperature. The reaction was diluted at 1:25 for SDS-PAGE Phos-Tag analysis.

Preparation of outer membrane fragments. The indicated *B. abortus* strains were grown for 48 h in RPMI. The bacteria were discarded by centrifugation at 10000×*g* followed by 0.45 μm filtration. The resulting supernatant was concentrated by ultrafiltration using a 100 kDa cutoff filter. Proteins in outer membrane fragments were further concentrated by centrifugation at 100 000 X G for 1 h [25].

Electrophoretic and immunochemical analysis. The different *Brucella* strains were grown in TSB at 37 °C with shaking, and samples were taken at different time points of growth. Sample processing, SDS-PAGE and Western Blot analysis with the indicated antibodies were carried out as previously described [24,26]. Detection of Omp16 using monoclonal anti-Omp16 antibodies was used as a loading control. For 2D-electrophoresis, proteins were first focused on an electric field according to their isoelectric points using 3-10 immobiline dry strips (GE, Healthcare) and then separated in 12.5% SDS-PAGE. Gels were silver stained using standard protocols. Protein spots were quantified by densitometry using Image J [25].

Structural comparisons. The amino acid sequences of BvrR and ExoS (Accession numbers 068164 and P72292) were retrieved from the

UniProt database (https://www.uniprot.org/) to build a structural model with SWISS-MODEL (http://swissmodel.expasy.org/) using histidine kinase (PDB accession No.4I5S) as a template. The structural model was visualized using Discovery Studio Visualizer. Protein sequences alignment was performed using LALIGN (https://www.ebi.ac.uk/Tools/psa/lalign/). Secondary structure predictions, functional and topology analysis were performed using the following tools: PSIPRED [65], Ali2D (https://toolkit.tuebingen.mpg.de/tools/ali2d) HMMER (http://hmmer.org/), and InterPro [66]. An agreement of more than one prediction was selected for BvrR and ExoS comparison and visualization.

Intracellular replication assays and immunofluorescence. Human cervix carcinoma cells (HeLa; American Type Culture Collection No. CCL-2) were grown to subconfluency at 37 °C under 5% CO_2 in Eagle's minimal essential medium supplemented with 5% fetal bovine serum, 2.5% sodium bicarbonate and 1% glutamine. Murine macrophages (Raw 264.7; American Type Culture Collection No. TIB-71) were grown to subconfluency at 37 °C under 5% CO_2 in Dulbecco's medium supplemented with 10% fetal bovine serum, 2.5% sodium bicarbonate and 1% glutamine. Penicillin (100 units/ml) and streptomycin (100 μg/ml) routinely added, were excluded from cell cultures during *Brucella* infections. Before infection, 24-well-plates monolayers were washed in PBS and kept at 4 °C. Infections were carried out as described [56,67,68]. Briefly, overnight TSB bacterial cultures were diluted in Eagle's minimal essential medium to reach the desired multiplicities of infection (MOI: 500 for HeLa, 100 for macrophages). After adding the diluted bacteria to the monolayers, plates were centrifuged at 300×*g* at 4 °C, incubated for 30 min at 37 °C under 5% CO_2 and washed 3 times with PBS. Extracellular bacteria were killed by adding Eagle's minimal essential medium supplemented with 100 μg/ml gentamicin for 1 h. Cells were further incubated for the indicated times in the presence of 5 μg/ml gentamicin, followed by washes with PBS. Cells were lyzed with 0.1% Triton X-100 for 10 min. The samples were collected, spun and resuspended in 110 μl of tryptic soy broth. Aliquots were diluted and plated in tryptic soy agar and incubated at 37 °C for 3 days to determine CFU. Procedures for immunofluorescence microscopy have been described [69]. Briefly, HeLa cells and Raw 264.7 macrophages grown in 13 mm glass coverslips were infected for 30 min with *B. abortus*, *B. abortus bvrS* or *B. abortus bvrS* p*exoS*$^{+}_{Sm}$ at a MOI of 500 or 100 according to cell type and proceed as stated above. After infection, extracellular bacteria were eliminated adding 100 μg/ml gentamicin for 1h. Cells were further incubated for 48 h with 5 μg/ml gentamicin, fixed with 3.7% paraformaldehyde-PBS, and free aldehyde groups were quenched with 50 mM NH_4Cl-PBS. Monolayers were processed for immunofluorescence using FITC anti-BrLPS antibodies. Cell nuclei were stained with 1 mg/ml 4′,6-diamidino-2-phenylindole and individual cells containing replicating bacteria were photographed.

Virulence assays. To estimate bacterial multiplication rates in spleens, groups of 20 eight-week-old female BALB/c were intraperitoneally inoculated with 0.1 ml of a suspension containing 5×10^5 CFU/ml of each bacterial strain and the CFU in spleens were determined at various times post-infection. Dilutions from this homogenate were seeded on TSA and CFU numbers determined as described [70]. Maintenance of mice and experimental procedures were followed according to the requirements of the Ministry of Science and Technology of Costa Rica and the corresponding National Costa Rican regulations on the use of experimental animals "Ley de bienestar de los animales N° 7451" (http://www.protecnet.go.cr/salud).

CRediT authorship contribution statement

Esteban Chaves-Olarte: Formal analysis, Writing – original draft, Conceptualization, Writing – review & editing, Resources, Visualization, Funding acquisition. **Jazmín Meza-Torres:** Writing – review & editing, Investigation. **Fabiola Herrera-Rodríguez:** Writing – review & editing, Investigation. **Esteban Lizano-González:** Writing – review & editing, Investigation. **Marcela Suárez-Esquivel:** Data curation, Investigation, Visualization, Formal analysis, Writing – review & editing. **Kate S. Baker:** Data curation, Investigation, Visualization, Formal analysis, Writing – review & editing. **Olga Rivas-Solano:** Writing – review & editing, Investigation. **Nazareth Ruiz-Villalobos:** Writing – review & editing, Investigation. **Fabián Villalta-Romero:** Investigation, Data curation, Writing – review & editing, Formal analysis, Software, Visualization, Validation. **Hai-Ping Cheng:** Writing – review & editing, Formal analysis, Resources, Writing – original draft. **Graham C. Walker:** Writing – review & editing, Formal analysis, Writing – original draft, Resources. **Axel Cloeckaert:** Formal analysis, Writing – review & editing, Resources. **Nicholas R. Thomson:** Resources, Writing – review & editing, Funding acquisition, Formal analysis. **Teresa Frisan:** Formal analysis, Funding acquisition, Resources, Writing – review & editing. **Edgardo Moreno:** Writing – original draft, Resources, Formal analysis. **Caterina Guzmán-Verri:** Project administration, Resources, Formal analysis, Investigation, Supervision, Funding acquisition, Validation, Conceptualization, Writing – original draft, Data curation, Visualization, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the International Centre for Genetic Engineering and Biotechnology (CRP/COS11-02 to ECO and CRP/CRI20-02 to CGV), the International Foundation for Science (B/3456-1 to CGV), the National Council of Science and Technology (FORINVESFV-0004-13 to ECO), the National Rector's Council (FEES-CONARE), the Vice-rectory for Research of the University of Costa Rica (program "Redes Temáticas" to ECO), the National Institutes of Health (GM031030 to G.C.W and GM081147 to HP.C), The Swedish Research Council to TF and Wellcome Trust. Authors from the Sanger Institute are supported by Wellcome Trust [098051]. KB was founded by a Wellcome Trust Postdoctoral Training Fellowship for Clinicians [106690/Z/14/Z]. G.C.W. is an American Cancer Society Professor. We are thankful to I. Moriyón and I. López-Goñi from the University of Navarra, Spain for providing the *B. abortus* mutant strains, and C. Baron from the University of Montreal, Canada for anti-VirB8 antibodies. We also thank Reinaldo Pereira for technical assistance and Gordon Dougan for helpful discussions. ECHO, CGV conceived and designed experiments, ECHO, JMT, FHR, ELG, ORS, NRV, FVR, TF, CGV performed the experiments, ECHO, MSE, KSB, FVR, HPC, GCW, AC, NRT, TF, EM, CGV analyzed the data, ECHO, FVR, HPC, GCW, AC, NRT, TF, EM, CGV contributed reagents/materials/analysis tools, ECHO, HPC, GCW, EM, CGV drafted the manuscript. All authors approved the final version of the manuscript. The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2023.106442.

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