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Regulation of *Brucella* virulence by the two-component system BvrR/BvrS

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Abstract

The *Brucella* BvrR/BvrS two-component regulatory system is highly similar to the regulatory and sensory proteins of *Sinorhizobium* and *Agrobacterium* necessary for endosymbiosis and pathogenicity in plants, and very similar to a putative system present in the animal pathogen *Bartonella*. Mutations in the *bvrR* or *bvrS* genes hamper the penetration of *B. abortus* in non-phagocytic cells and impairs intracellular trafficking and virulence. In contrast to virulent *Brucella*, BvrR/BvrS mutants do not recruit small GTPases of the Rho subfamily required for actin polymerization and penetration to cells. Dysfunction of the BvrR/BvrS system alters the outer membrane permeability, the expression of several group 3 outer membrane proteins and the pattern of lipid A acylation. Constructs of virulent *B. abortus* chimeras containing heterologous LPS from the *bvrS*$^{-}$ mutant demonstrated an altered permeability to cationic peptides similar to that of the BvrR/BvrS mutants. We hypothesize that the *Brucella* BvrR/BvrS is a system devoted to the homeostasis of the outer membrane and, therefore in the interface for cell invasion and mounting the required structures for intracellular parasitism.

Keywords: *Brucella* virulence; BvrR/BvrS; Two-component system; Outer membrane proteins

1. Introduction

Bacteria must modulate the expression of specific genes in response to changes in environmental conditions. This adaptive response is mediated in part by two-component regulatory systems. One component of the system acts as an environmental sensor, often located in the cytoplasmic membrane, that transmits a signal to the second component, a cytoplasmatic response regulator, which then mediates changes in gene expression (Stock

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et al., 1995). According to the model that has been proposed for the transduction of environmental signals by these two-component systems, the sensor protein, commonly a histidine kinase, responds to an environmental stimulus by autophosphorylation on a histidine residue. The phosphoryl group is then transferred to an aspartate residue in the regulator kinase protein, which generates a cascade of events leading to transcription factors whose affinity for specific DNA sequence is modulated by phosphorylation (Stock et al., 1995). By these means, bacterial two-component systems mediate the response to a variety of chemicals and physical signals and control functions like cell division, physiology, communication, development, sporulation, taxias, response to stress, and virulence. In addition, it has been observed the existence of a ''cross-talk'' between the sensor of one system and the regulator of another system. However, the mechanism by which different two-component systems are integrated into a coordinated cellular response in not well understood. Facultative intracellular bacteria such as *Brucella* must survive in varied and changing conditions ranging from the open environment to the intracellular medium of professional and non-professional phagocytes (see Moreno and Gorvel in this issue). For this, the bacterium must coordinate an intricate network of factors to generate a suitable adaptive response to the various signals. In this respect, two-component regulatory systems are necessary for controlling the transition from one environment to the other, and in consequence some of them are also implicated in virulence (Mekalanos, 1992; Dziejman and Mekalanos, 1995).

2. The BvrR/BvrS two-component regulatory system

In the absence of obvious and conspicuous classical virulence factors commonly detected in other bacterial pathogens, we propose that *Brucella* virulence depends on the homeostasis and distinctive properties of the outer membrane which allows this bacterium to invade, survive and replicate within host cells (Moriyón and Berman, 1982; Martínez de Tejada et al., 1995; Freer et al., 1996; Moreno and Moriyón, 2002). One of the physiological signs related to this property is the marked resistant of *Brucella* to bactericidal substances present in lysosomes and body fluids and to microbicidal polycations that disrupt the cell envelope of most Gram-negative and Gram-positive bacteria (Martínez de Tejada et al., 1995; Freer et al., 1996). On these grounds, we generated transposon *B. abortus* 2308 mutants sensitive for bactericidal cationic peptides, on the premise that this would generate non-virulent bacteria (Sola-Landa et al., 1998). Indeed, DNA analyses of transposon mutants sensitive to cationic peptides identified two contiguous open reading frames (ORF), which were designated *bvrR* and *bvrS* (for *Brucella* virulence related, see below), with two potential promoter sequences and a potential ribosome-binding site upstream the first ORF (Sola-Landa et al., 1998; GenBank accession numbers AF005157 and AF012539). A database search of the corresponding deduced amino acid sequences revealed a high level of identity with conserved domains of two-component regulatory systems. BvrR (237 amino acids) shows significant similarity to DNA-binding OmpR subfamily of response regulators proteins including PhoP, PhoB, VirG, PmrA (Ronson et al., 1987; Volz, 1995; Mizuno and Tanaka, 1997) (Fig. 1). BvrS (601 amino acids) has conserved regions homologous to members of the histidine protein

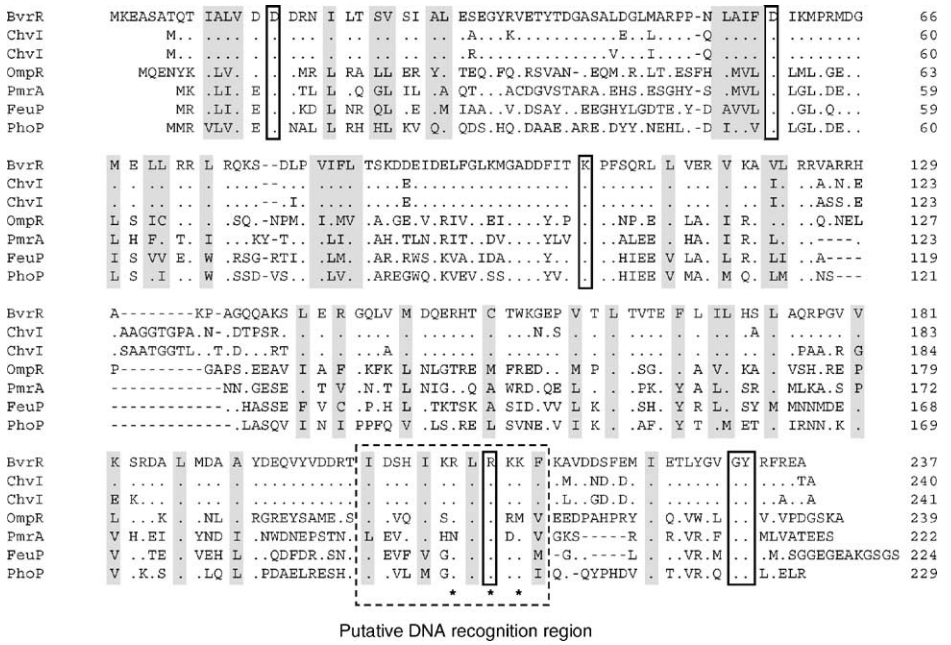

Fig. 1. Alignment of the predicted amino acid sequences of *B. abortus* BvrR and related proteins of the OmpR family: *S. meliloti* ChvI (database accession number P50350), *A. tumefaciens* ChvI (Q07783), *E. coli* OmpR (P03025), *S. typhimurium* PmrA (L13395), *B. melitensis* FeuP (X87324), and *S. typhimurium* PhoP (P14146), respectively. Dots indicate conserved amino acids and dashes indicate gaps inserted to permit a correct alignment. Conserved hydrophobic residues characteristic of the OmpR family are shadowed and highly conserved residues are boxed. The putative DNA recognition region is shown and positive charge conserved residues in this region are indicated by asterisks (Mizuno and Tanaka, 1997). Similarities with BvrR decrease from *S. meliloti* ChvI to *S. typhimurium* PhoP.

kinase superfamily which function as membrane receptors with sensory domains at the external surface of the membrane (Stock et al., 1995). The analysis of the sequence of BvrS (Fig. 2) reveals two hydrophobic regions in the N-terminus that are predicted to be transmembrane domains (Kyte and Doolittle, 1982). These domains delimit a hydrophilic region predicted to contain the periplasmic segment sensing an unidentified environmental stimulus (Sola-Landa et al., 1998).

The analysis of the genomes of *B. suis* and *B. melitensis* (http://tigrblast.tigr.org/ufmg/ and http://www.genome.scranton.edu/WIT2/) demonstrate the presence of the *bvrR/bvrS* two-component system in these species, and few differences are detected between the BvrR and BvrS proteins of *B. abortus*, *B. suis* and *B. melitensis*. For instance, the BvrR of *B. melitensis* and *B. suis* are 100% identical to each other but different from the *B. abortus* regulator in only four amino acids; BvrS of *B. melitensis* and *B. suis* are different in only three amino acids and differ from the *B. abortus* sensor in four and seven amino acids, respectively. Putative genes coding for BvrR and BvrS proteins can also be detected in the genomes of *B. ovis*, *B. neotomae* and *B. canis* by PCR with primers specific for the *bvrR/bvrS* sequence, indicating that this two-component system is highly conserved in the genus

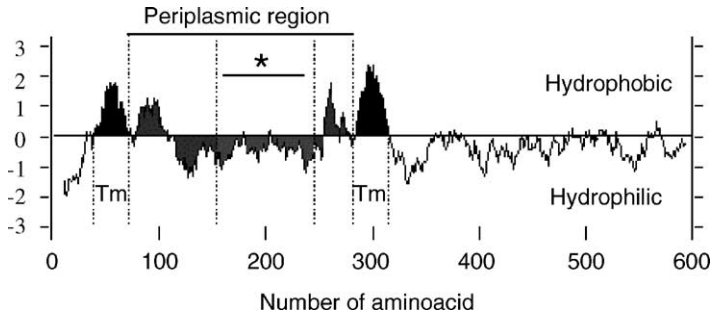

Fig. 2. Kyte–Doolittle hydrophobicity plot of BvrS showing the hydrophobic and hydrophilic domains. Transmembrane domains (Tm) and the periplasmic domain are shown. The asterisk represent the region of the periplasmic domain with the major divergence between the BvrS and other sensor proteins.

Brucella (López-Goñi et al., unpublished results). The BvrR/BvrS possesses a high level of identity with other two-component systems present in cell associated α-2 *Proteobacteria* (Sola-Landa et al., 1998), which are also necessary for endosymbiosis and bacterial parasitism. For instance, BvrR had 86, 81, 81 and 76% of similarity to the *Mesorhizobium loti* ChvI, *Bartonella bacilliformis* BatR, *Sinorhizobium meliloti* ChvI and *Agrobacterium tumefaciens* ChvI, respectively. Similarly, BvrS had 68, 62, 59 and 59% of similarity to the *M. loti* ExoS, *S. meliloti* ExoS, *B. bacilliformis* BatS and *A. tumefaciens* ChvG, respectively (Fig. 3). In addition, BvrS cross-reacts with ExoS sensor protein of *S. meliloti* (Guzmán-Verri et al., 2002). A detailed comparison of BvrS with ExoS, BatS and ChvG sensor proteins reveals that the periplasmic domain involved in environmental sensing possess less similarity than other protein domains (Figs. 2 and 3), suggesting that they were derived for sensing different stimuli (Sola-Landa et al., 1998). This information suggests that all these α-2 *Proteobacteria* two-component systems had a common origin that has evolved to sense different stimuli according to the environment.

3. The BvrR/BvrS system controls the expression of outer membrane proteins and the structure of lipid A

As demonstrated for other two-component systems, multiple genes should be under the control of BvrR/BvrS. Mutants in the BvrR/BvrS system are not only more susceptible to bactericidal polycationic substances, but also display a more hydrophobic outer membrane surface as compared to the parental *B. abortus* 2308 strain (Sola-Landa et al., 1998; Sola-Landa, 2000). Since these two properties have been demonstrated to be related to the integrity of the outer membrane (Martínez de Tejada et al., 1995; Freer et al., 1996), it was likely that the BvrR/BvrS system modulated somehow the structure of the outer membrane. Two dimensional gel electrophoresis showed differences in various outer membrane proteins (Omps) between wild type and both *Brucella bvrR*$^{-}$ and *bvrS*$^{-}$ mutants (Guzmán-Verri et al., 2002). Reactivity with monoclonal antibodies showed that one set of protein spots practically absent in the *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants corresponded to *Brucella* Omp of 25 kDa, known as Omp25 (Sola-Landa, 2000), and renamed as Omp3a

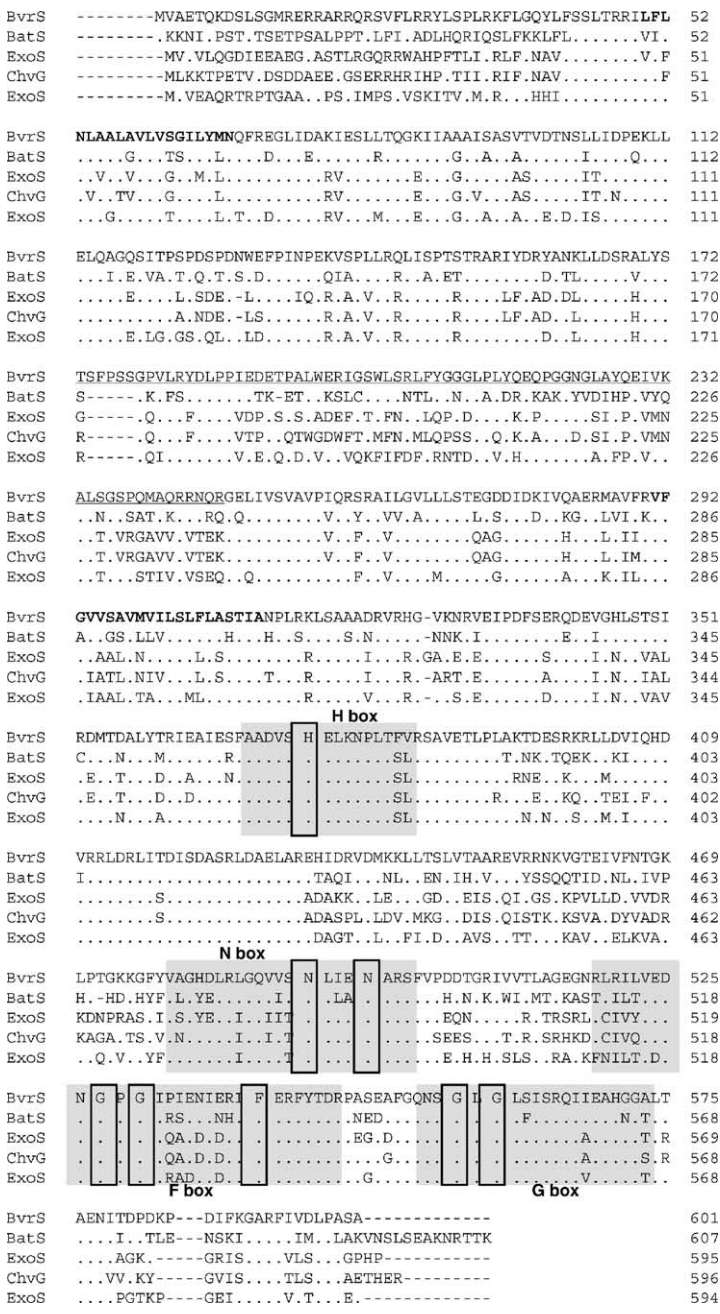

Fig. 3. Alignment of the amino acid sequence of *B. abortus* BvrS with *B. bacilliformis* BatS (database accession number CAC29082), *S. meliloti* ExoS (AF027298), *A. tumefaciens* ChvG (AAL41064) and *M. loti* ExoS (NP105816), respectively. Dots indicate conserved amino acids and dashes indicate gaps inserted to permit a correct alignment. Boxes H, N, F and G of the histidine protein kinase family are shadowed and totally conserved amino acids known to be conserved in other bacteria within those regions are boxed. Bold characters in BvrS represent transmembrane domains and underlined residues represent the major divergence in the periplasmic domains.

according to its original designation as a group 3 Omp (Verstreate et al., 1982). In agreement with the two dimensional gel electrophoresis, the *omp3a* promoter activity was also dramatically decreased in both *bvrR*$^-$ and *bvrS*$^-$ mutants as compared to that of the wild type strain (Guzmán-Verri et al., 2002). In addition, a second set of protein spots all corresponding to a previously unidentified Omp of 22 kDa (Omp3b) was absent in *bvrS*$^-$ mutant. Partial amino acid sequences obtained from Omp3b protein spots from two dimensional gel electrophoresis allowed the cloning and sequencing of the corresponding *omp3b* gene (Guzmán-Verri et al., 2002; GenBank accession number AJ313014). The analysis of the deduced amino acid sequence predicted an outer membrane localization of the protein and revealed homology with the OmpA group of Omps. A database search showed that Omp3b had 47% similarity to RopB, an Omp of unknown function of the plant endosymbiont *Rhizobium leguminosarum* (Roest et al., 1995). Omp3b had also 45% similarity to Omp31 from *B. melitensis* and to Omp3a (Omp25) from different *Brucella* species. The analyses of the genomes of *B. suis* and *B. melitensis* have demonstrated the presence of several putative genes coding for Omps ranging from 25 to 31 kDa, supporting the hypothesis that they constitute a family of Omps (group 3 Omps, herein renamed as Omp3a, Omp3b, etc.). To the present, no specific function has been ascribed to this group 3 Omps. However, it is known that *Brucella* Omp3a deficient mutants display reduced virulence (Edmonds et al., 2001) and are incapable to down regulate TNF-α production after macrophage infection (Jubier-Maurin et al., 2001), as compared to the wild type virulent *Brucella*. It is not known whether Omp3a is directly involved in these events or whether its absence results in pleiotropic effects that eventually conduce to reduced virulence.

Additional differences in at least two other Omps between *bvrR*$^-$ and *bvrS*$^-$ mutants and the wild type strain were detected by two dimensional gel electrophoresis, but sequence analysis has not been possible (Guzmán-Verri et al., 2002). No significant variations in Omp10, Omp16, Omp19, Omp2b, Omp1, periplasmic cyclic glucans, total free lipids and native hapten polysaccharide have been observed between both *bvrR*$^-$ and *bvrS*$^-$ mutants and the wild type strain (Sola-Landa, 2000). Although the structure of the *O*-polysaccharide chain of the LPS seems to be the same by ^{13}C-NMR, differences in the chromatographic pattern of lipid A were observed by HPTLC, indicating that the BvrR/BvrS system regulates either directly or indirectly the degree of lipid A acylation (Manterola et al., unpublished results). Divergence in the LPS between *bvrR*$^-$ and *bvrS*$^-$ mutants and wild type was also revealed by the sensitivity of LPS chimeras to cationic peptides (Moreno et al., unpublished results). Virulent *B. abortus* 2308 chimeras containing LPS of *bvrR*$^-$ or *bvrS*$^-$ mutants became more sensitive to the action of microbicidal peptides; on the contrary, *bvrR*$^-$ and *bvrS*$^-$ mutants containing LPS of wild type *B. abortus* 2308 were more resistant to these peptides. The results of this biological assay open the possibility that other LPS components (e.g. core sugars) in addition to lipid A could diverge from those of the wild type *Brucella*, and structural analyses are necessary to test this hypothesis.

Altogether, all the results obtained so far clearly indicate that the BvrR/BvrS system regulates the structure of outer membrane components that are crucial for the homeostasis and eventually for virulence (Fig. 4). Therefore, despite the fact that the BvrR/BvrS system displays low homology to the *Salmonella* PhoP/PhoQ system, at least with respect to the

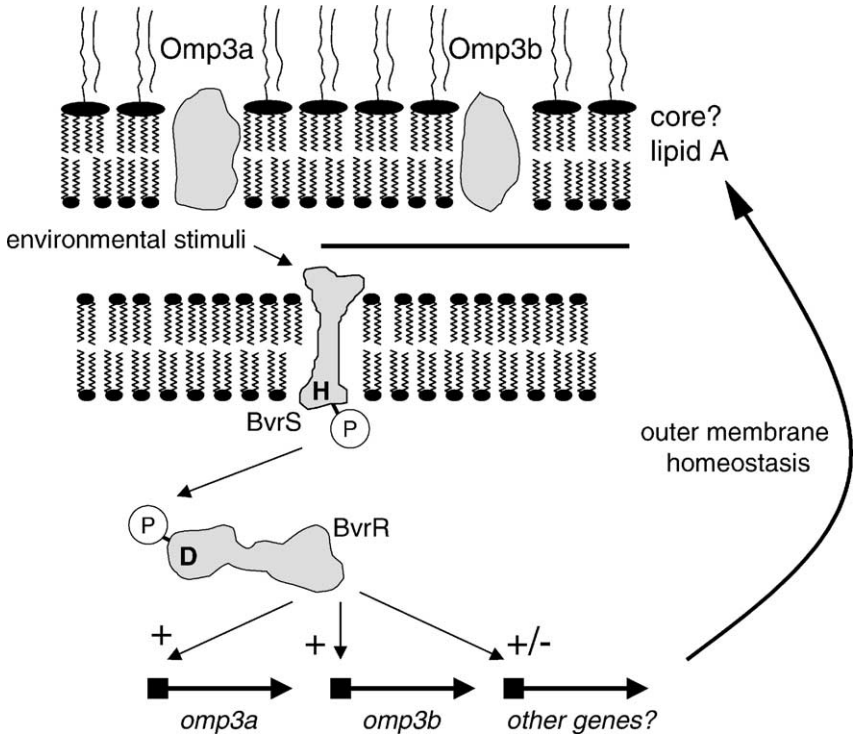

Fig. 4. A model representation of the *Brucella* BvrS/BvrR two-component regulatory system. In response to an unknown environmental stimulus the periplasmic sensor domain of the BvrS protein translocates the signal to a histidine (H) kinase domain which in turn promotes its own phosphorylation. These catalyzes the phosphorylation of the BvrR regulatory protein on an aspartate (D). The phosphorylated BvrR protein activates the transcription of *omp3a* and probably *omp3b* and other genes not yet identified. Upon this, the structure of the lipid A, of possibly the core of the LPS and the expression of Omp3a (Omp25) and Omp3b as well as the presence of other proteins at the *Brucella* outer membrane are directly or indirectly regulated. The consequence of mutating either of these two *bvr* genes in *Brucella* is an altered sensitivity to cationic peptides and outer membrane permeability as well as a hampered cell invasion and intracellular replication. Knockout of both *bvr* genes is lethal (O'Callaghan).

regulation of the expression of Omps and lipid A structure these two systems may be functionally analogous (Miller, 1995; Guo et al., 1997, Guo et al., 1998).

4. The BvrR/BvrS system controls *Brucella* virulence and is essential for the intracellular survival

The parental *B. abortus* 2308 virulent strain efficiently replicates in spleens of mice reaching high numbers that remain for at least 6 weeks (Sola-Landa et al., 1998). In contrast, both *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants are rapidly eliminated by day 12 post infection, with small but significant differences between them (Sola-Landa et al., 1998). Also, whereas the wild type strain replicates within macrophages and epithelial cells, none of the

mutants multiplies in these cell types. Consistent with the small differences observed in mice, the *bvrR*$^{-}$ but not the *bvrS*$^{-}$ mutant had some residual intracellular replication in epithelial cells, suggesting that the lack of an active sensor is a more severe defect that the absence of a functional regulatory protein. Thus, deficient cellular parasitism may be due to two different but connected events: the competence to invade and the ability to replicate within cells. Electron and fluorescent microscopy has clearly shown that mutation in the *bvrR* or *bvrS* genes hampers both the penetration of *B. abortus* to cells and impairs intracellular trafficking and virulence (Sola-Landa et al., 1998). For instance, in contrast to the virulent *B. abortus*, the *bvrS*$^{-}$ mutant does not recruit the small GTPases of the Rho subfamily required for actin polymerization and penetration to cells (Guzmán-Verri et al., 2001). Moreover, despite the fact that *bvrS*$^{-}$ mutant binds in higher numbers to cells than virulent *B. abortus*, Cdc42 GTPase is directly activated only by the latter strain, indicating that the BvrR/BvrS system is required for recruiting those molecular determinants necessary for invasion (Guzmán-Verri et al., 2001).

The *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants internalized by macrophages are readily destroyed within phagolysosomes (Sola-Landa et al., 1998). Similarly, high numbers of *B. abortus bvrR*$^{-}$ and *bvrS*$^{-}$ mutants ingested by epithelial cells displaying phagocytic phenotype (by treatment with the GTPase activator *E. coli* necrotizing factor) are also destroyed within lysosomal compartments (Chaves-Olarte et al., unpublished results). In contrast, wild type *Brucella* pursues its intracellular trafficking from early vacuolar compartments to autophagosomes and finally to the endoplasmic reticulum, where it extensively replicates. This means that, once inside the cells, both *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants are incapable to inhibit lysosome fusion, indicating that the BvrR/BvrS system is involved not only in cell invasion but also in controlling vacuole maturation and intracellular trafficking. Based on all these observations, it is tempting to speculate that the BvrR/BvrS system regulates surface bacterial molecules necessary to recruit host cellular determinants for penetration. In addition, the distorted outer membrane displayed by the mutants could hamper the correct assemblage of secretions systems (e.g. VirB, type IV secretion system) necessary for controlling vacuole maturation and intracellular trafficking. Finally, the higher sensitivity of these mutants to the action of bactericidal substances very likely contributes to its destruction within intracellular vacuoles.

5. *Brucella bvrR/bvrS* mutants as vaccine candidates

Despite the fact that the *bvrR*/*bvrS* mutants show a smooth phenotype and readily growth in regular bacteriological cultures, these strains do not display residual virulence. Experiments performed in mice have demonstrated that the *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants protect mice against virulent *B. abortus* 2308 at levels similar to those obtained with *B. abortus* strain 19 and at higher level than rough *B. abortus* RB51 (Grilló et al., unpublished results). This suggests that the *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants are good vaccine candidates. Moreover, the absence of Omp3b in the *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants could be used for serological discrimination. Finally, their low virulence and the low endotoxic properties characteristic of all *brucellae* make these *bvrR*$^{-}$ and *bvrS*$^{-}$ mutants strains an attractive possibility for the development of human vaccines against brucellosis.

6. Conclusions

The similarity of the *Brucella* BvrR/BvrS system with some chromosomally encoded systems present in plant endosymbionts and plant and animal pathogens of the α-2 *Proteobacteria* is considerably higher that with other well known two-component systems involved in bacterial virulence. The fact that the ChvI/ExoS and ChvI/ChvG systems of *S. meliloti* and *A. tumefaciens* are also involved in plant symbiosis and parasitism (Charles and Nester, 1993; Cheng and Walker, 1998), suggests that these systems are crucial for the adaptation of these bacteria to the pericellular or intracellular environment (Moreno et al., 1990; Sola-Landa et al., 1998) that constitutes an evolutionary trend in cell associated α-2 *Proteobacteria*.

Only three two-component systems have been described in *Brucella*: the FeuP/FeuQ system similar to the *R. leguminosarum* FeuP involved in the regulation of iron uptake (Dorrell et al., 1998); the NtrB/NtrC system involved in the regulation of nitrogen metabolism (Dorrell et al., 1999); and the BvrR/BvrS system. The analysis of the genomes of various *Brucella* spp. has revealed that they encode only eight two-component regulatory systems. This number is significantly smaller than that of *M. loti* (33 systems; Kaneko et al., 2000), *S. meliloti* (36 systems; Galibert et al., 2001), *E. coli* (30 systems; Blattner et al., 1997), *Bacillus subtilis* (34 systems; Kunst et al., 1997), and even smaller than that of some pathogens with narrow host tropism such as *Mycobacterium tuberculosis* (11 systems; Cole et al., 1998). By stressing its essential for role in the biology of *Brucella*, these figures bring into attention the significance of the BvrR/BvrS system and of the outer membrane properties in *Brucella* parasitism.

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