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# The two-component system PrlS/PrlR of *Brucella melitensis* is required for persistence in mice and appears to respond to ionic strength

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Bacterial adaptation to environmental conditions is essential to ensure maximal fitness in the face of several stresses. In this context, two-component systems (TCSs) represent a predominant signal transduction mechanism, allowing an appropriate response to be mounted when a stimulus is sensed. As facultative intracellular pathogens, *Brucella* spp. face various environmental conditions, and an adequate response is required for a successful infection process. Recently, bioinformatic analysis of *Brucella* genomes predicted a set of 15 bona fide TCS pairs, among which some have been previously investigated. In this report, we characterized a new TCS locus called *prlS*/*R*, for probable proline sensor–regulator. It encodes a hybrid histidine kinase (PrlS) with an unusual $Na^+$/solute symporter N-terminal domain and a transcriptional regulator (belonging to the LuxR family) (PrlR). *In vitro*, *Brucella* spp. with a functional PrlR/S system form bacterial aggregates, which seems to be an adaptive response to a hypersaline environment, while a *prlS*/*R* mutant does not. We identified ionic strength as a possible signal sensed by this TCS. Finally, this work correlates the absence of a functional PrlR/S system with the lack of hypersaline-induced aggregation in particular marine *Brucella* spp.

## INTRODUCTION

*Brucella* spp. are Gram-negative facultative intracellular pathogens (Letesson *et al.*, 2002; Moreno & Moriyon, 2002) that cause chronic infections in a variety of mammalian hosts. The key feature of their infectious cycle is the capacity to survive and multiply intracellularly in both phagocytic and non-phagocytic cells. This ability relies mostly on the subversion of the vacuolar traffic of the eukaryotic infected host cells (Gorvel & Moreno, 2002) and on the modulation of the host immune response. During infection of the host and its cells, *Brucella* has to adapt to an array of environmental conditions, necessitating a timely and spatially coordinated expression of virulence genes (Letesson & De Bolle, 2004). In bacteria, two-component systems (TCSs) are the predominant signal transduction mechanisms that allow the cells to sense their environment and to mount an appropriate response. In a typical TCS, a sensor histidine kinase (HK), in response to an internal or external stimulus, becomes autophosphorylated on a conserved histidine residue, and the phosphate is then transferred to a conserved aspartate residue in a cognate response regulator (RR) that mostly acts through regulation of gene expression (Parkinson, 1993).

Before reaching the final output regulator, the phosphate can go through additional partners following a His–Asp–His–Asp route in a more complex pathway called a

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Abbreviations: DIC, differential interference contrast; EPS, extracellular polysaccharide; HRP, horseradish peroxidase; Omp, outer membrane protein; RR, response regulator; SSS, $Na^+$/solute symporter; TCS, two-component system; WT, wild-type.

Two supplementary figures and a supplementary table are available with the online version of this paper.

phosphorelay (Laub *et al.*, 2007; Perraud *et al.*, 1999). Worthy of note is the emerging view of the bacterial TCS as a signalling unit on which auxiliary regulators or adaptors can act to integrate multiple signal and cellular pathways (for reviews, see Buelow & Raivio, 2010; Mascher *et al.*, 2006; Mitrophanov & Groisman, 2008).

Recent bioinformatic analysis of *Brucella* genomes has identified the available set of HKs and RRs with 15 predicted bona fide TCS pairs (Lavín *et al.*, 2010). Several of these systems have been characterized previously in some *Brucella* species: BvrSR (Sola-Landa *et al.*, 1998), FeuQP (Dorrell *et al.*, 1998), NtrBC (Dorrell *et al.*, 1999), NtrXY (Carrica *et al.*, 2012; Foulongne *et al.*, 2000), the flagellar master regulator FtcR (Léonard *et al.*, 2007), the CtrA phosphorelay (Hallez *et al.*, 2007), the blue-light-activated LOV HKs (Swartz *et al.*, 2007) and CenR (Zhang *et al.*, 2009). Some other TCSs have been identified by transpositional mutagenesis during global screening for virulence factors (Lestrate *et al.*, 2000; Wu *et al.*, 2006) but left uncharacterized. This is the case for the *Brucella melitensis* transpositional mutant (G4) that we identified in a previous study (Lestrate *et al.*, 2000). Indeed, a mini-Tn*5* was inserted between genes encoding a sensor (BMEI1606) and a regulator (BMEI1607) of a TCS pair that was later, based on homology, called PrlS and PrlR, for probable proline sensor and regulator (Lavín *et al.*, 2010; Letesson & De Bolle, 2004). Here we report the characterization of this TCS. Mutants in either *prlS* or *prlR* have no defect in cellular models of infection but are highly attenuated in mice at 4 weeks post-infection. We also describe the involvement of this TCS in the formation of bacterial aggregates in response to ionic strength and a possible signal sensed by this TCS. Finally, this work correlates the absence of a functional PrlR/S system with the lack of hypersaline-induced aggregation in particular marine *Brucella* spp.

## METHODS

**Strains, plasmids and culture conditions.** All strains and plasmids used in this study are listed in Table 1. Following the classical method, *Brucella* strains were grown with shaking at 37 °C in 2YT medium (10 g yeast extract $l^{-1}$, 16 g peptone $l^{-1}$, 5 g NaCl $l^{-1}$) containing appropriate antibiotics, from an initial $OD_{600}$ of 0.05. The *Escherichia coli* strains DH10B (Invitrogen) and S17-1 (Simon *et al.*, 1983) were routinely grown in Luria–Bertani (LB) medium at 37 °C with appropriate antibiotics. Chloramphenicol, nalidixic acid and gentamicin were used at 20, 25 and 50 μg $ml^{-1}$, respectively.

**Table 1.** Strains and plasmids used in this study

| Strain or plasmid | Characteristics | Source or reference |
|---|---|---|
| ***Brucella* spp. strains** | | |
| *B. melitensis* 16M | WT, $Nal^R$, parental strain | A. Macmillan, Central Veterinary Laboratory, Weybridge, UK |
| *B. melitensis* Δ*prlS* | $Nal^R$ | This study |
| *B. melitensis* Δ*prlR* | $Nal^R$ | This study |
| *B. melitensis* Δ*vjbR* | Δ*vjbR*::$Kan^R$, $Nal^R$ (formerly CD100) | Delrue *et al.* (2005) |
| *B. abortus* 2308 | WT, $Nal^R$ | Sangari & Agüero (1991) |
| *B. suis* 1330 | Reference strain ATCC 23444, swine isolate | Morgan & Corbel (1976) |
| *B. canis* RM6/66 | Reference strain ATCC 23365, dog | Carmichael & Bruner (1968) |
| *B. ovis* 63/290 | Reference strain ATCC 25840, sheep | Buddle (1956) |
| *B. neotomae* 5K33 | Reference strain NCTC 10084, desert woodrat | Stoenner & Lackman (1957) |
| *B. microti* CCM4915 | Isolated from common vole, Czech Republic | Scholz *et al.* (2008) |
| *B. pinnipedialis* B2/94 | Reference strain NCTC 12890, common seal, Scotland | Foster *et al.* (2007) |
| *B. ceti* B1/94 | Reference strain NCTC 12891, porpoise, Scotland | Groussaud *et al.* (2007) |
| *B. ceti* M13/05/01 | Also named UK3/05, striped dolphin, Scotland | Groussaud *et al.* (2007) |
| *B. ceti* M644/93/1 | Also named B14/94, common dolphin, Scotland | Groussaud *et al.* (2007) |
| ***E. coli* strains** | | |
| DH10B | $F^-$ *mcr*A Δ(*mrr–hsdRMS–mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*) 7697 *galU galK rpsL nupG* $\lambda^-$ ($Str^R$) | Invitrogen |
| S17-1 | *recA*, *thi*, *pro*, *hsdR*$^-$*M*$^-$RP4 : 2-Tc:Mu: Km Tn*7* λpir, allows plasmid mobilization | Simon *et al.* (1983) |
| BL21(DE3) | $F^-$ *ompT gal* (*dcm*) (*lon*) $hsdS_B$ ($r_B^-$ $m_B^-$; *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase | Novagen |
| **Plasmids** | | |
| pBBR1MCS1 | Broad-host-range cloning vector, high copy number, $Cm^R$ | Kovach *et al.* (1994) |
| pGEM-T Easy | $Amp^R$ | Promega |
| pET15b | $Amp^R$ | Novagen |
| pET15bGW | $Amp^R$ $Cm^R$ Gateway-compatible pET15b (Novagen) | V. Van Mullem, unpublished results |
| pDonR201 | $Kan^R$, BP cloning vector | Invitrogen |

An aggregative phenotype was observed only when the strains were grown in rich medium containing 400 mM NaCl (16 g peptone $l^{-1}$, 10 g yeast extract $l^{-1}$, 23.3 g NaCl $l^{-1}$) for 72 h in 10 ml cultures at 37 °C with shaking in 50 ml high-clarity polypropylene conical centrifuge tubes (BD).

**Molecular techniques.** DNA manipulations were performed according to standard techniques (Ausubel *et al.*, 1991). Restriction enzymes were purchased from Roche and primers were purchased from Eurogentec. Primer sequences are listed in Table S1 available with the online version of this paper.

**Construction of mutants.** *B. melitensis* 16M knockout mutants Δ*prlS* and Δ*prlR* were both generated by allelic replacement using a two-step strategy. Briefly, upstream and downstream regions of about 500 bp flanking the *prlS* or *prlR* gene were amplified by PCR from *B. melitensis* genomic DNA using the following primers: (i) F1_D1606 and F2_D1606, (ii) R1_D1606 and R2_D1606; (i) F1_D1607 and F2_D1607, (ii) R1_D1607 and R2_D1607. For each construct, a second PCR was used to associate the two PCR products using the following primer pairs: (i) F1_D1606 and R2_D1606; (ii) F1_D1607 and R2_D1607. Finally, the Δ*prlS* and Δ*prlR* fragments were cloned into pGEM-T Easy (Promega) to generate pGEM-TΔ*prlS* or pGEM-TΔ*prlR* intermediate vectors. The Δ*prlS* and Δ*prlR* fragments excised by *Not*I restriction were subcloned into final vectors and used to construct *Brucella* mutants following the strategy previously described (Mignolet *et al.*, 2010). Gene replacement was confirmed by PCR. It should be noted that the *prlS* CDS (BMEI1606) in the *B. melitensis* 16M genome sequence (GenBank accession no. AE008917) is predicted to encode a 1305 amino acid protein. However, when aligned to orthologous sequences it became obvious that the gene encoding *prlS* in *Brucella abortus* 2308 or *B. melitensis* Rev.1 encoded a shorter protein (composed of 1174 amino acids) with a predicted molecular mass of 128 kDa. This difference was assigned to a sequencing error in the GenBank *B. melitensis* 16M sequence, which was confirmed by resequencing (results not shown).

**Cellular infections.** Evaluation of the intracellular survival of the *B. melitensis* wild-type (WT) and the Δ*prlS*, Δ*prlR* and Δ*vjbR* mutant strains in SV40 bovine macrophages and JAR713 trophoblasts was performed as previously described (Delrue *et al.*, 2001). Briefly, the bacterial strains were grown overnight in 2YT medium and then inoculated at an m.o.i. of 300 to monolayers of cells in 24-well plates. After a 10 min centrifugation at 1000 r.p.m. at room temperature, the plates were placed in a 5 % $CO_2$ atmosphere at 37 °C for 1 h. Afterwards, the cells were washed with PBS and incubated in the presence of medium containing gentamicin (50 μg $ml^{-1}$) at 37 °C under 5 % $CO_2$ until the end of the infection (48 h later). Cells were then washed and lysed with 0.1 % Triton X-100 in PBS (W/o Ca, Mg; Lonza BioWhittaker) for 10 min, and serial dilutions of lysates were plated on 2YT solid medium to enumerate viable bacteria able to develop colonies. We used the Δ*vjbR* strain as a positive control for attenuation in a cellular model of infection (Delrue *et al.*, 2005). Data are expressed as the $\log_{10}$ of the number of c.f.u. per well.

**Mouse infections.** Virulence assays with BALB/c mice were performed as described previously (Fretin *et al.*, 2005). Briefly, 8-week-old female mice were inoculated intraperitoneally with 0.2 ml of a suspension containing $10^5$ c.f.u. of the appropriate bacterial strain. At 5 days and 4 weeks post-inoculation, four mice from each group were sacrificed for spleen collection. Spleens were homogenized in 2 ml PBS/0.1 % Triton X-100, and serial dilutions of the homogenate were plated on 2YT solid medium to determine bacterial survival. Data are expressed as the $\log_{10}$ of the number of c.f.u. per spleen. The animal handling and procedures of this study were in accordance with the current European legislation (directive 86/609/EEC) and in agreement with the corresponding Belgian law '*Arrêté royal relatif à la protection des animaux d'expérience du 6 avril 2010 publié le 14 mai 2010*'. The complete protocol was reviewed and approved by the Animal Welfare Committee of the Facultés Universitaires Notre-Dame de la Paix (FUNDP, Belgium) (permit number 05-558).

**Microscopy.** Strains previously grown for 72 h in 2YT containing 400 mM NaCl were placed on a microscope slide that was covered with a pad of 1 % agarose containing PBS (Jacobs *et al.*, 1999) with a sealed coverslip. These slides were placed on a microscope stage at room temperature. The samples were observed on a Nikon E1000 microscope through a ×100 differential interference contrast (DIC; Normarski) objective with a Hamamatsu Orca-ER LCD camera. Image acquisition and processing were performed with NIS element (Nikon) software.

**Protein purification, generation of antibodies and immunoblot analysis.** *B. melitensis* recombinant PrlR was overexpressed in *E. coli* as fusion proteins with an N-terminal tag comprising a hexahistidine peptide (Novagen). The overexpression vector pET15b*prlR* was constructed by the Gateway technique (Invitrogen) using a sequenced pDonR201-*prlR* from the ORFeome version 1.1 (Dricot *et al.*, 2004) as the entry vector. After overexpression in *E. coli* BL21(DE3), protein purification was performed on a nickel-loaded IMAC Sepharose High Performance Resin (GE Healthcare) in denaturing conditions with 6 M guanidinium chloride. Protein refolding was performed using dialysis with 20 mM Tris/HCl and 500 mM NaCl (pH 8) at 4 °C.

Immunization was performed as described previously (Denoel *et al.*, 1997) with 50 μg of purified recombinant proteins to produce specific antibodies to PrlR. Immunoblot analysis was performed as described previously (Bellefontaine *et al.*, 2002). Immunodetection of the PrlR, FliC and FlgE proteins was performed using anti-PrlR (diluted 1 : 1000), anti-FliC (diluted 1 : 3000) and anti-FlgE (diluted 1 : 5000) rabbit polyclonal antibodies (Fretin *et al.*, 2005), respectively.

Omp89 detection with anti-Omp89 monoclonal mouse antibodies (A53/10B02/A01) (Cloeckaert *et al.*, 1990) was used as a loading control. The secondary antibodies used were horseradish peroxidase (HRP)-conjugated donkey anti-rabbit (Amersham catalogue no. NA934, 1/5000 dilution) and HRP-conjugated goat anti-mouse antibodies (Amersham catalogue no. NA931, 1/10 000 dilution). The ECL system was used for produce chemiluminescence before detection using film.

**Dot blot analysis.** *Brucella* strains were grown overnight in 2YT medium at 37 °C and cells were harvested in stationary phase. Crude extracts were prepared as described previously (Uzureau *et al.*, 2007) and serial twofold dilutions were applied to a nitrocellulose membrane (Amersham). Outer-membrane protein (Omp) immunodetection was performed using the following mAbs: anti-Omp10 (A68/07G11/C10), anti-Omp16 mAb (A68/08C03/G03) and anti-Omp2b (A68/25G05/A05). HRP-conjugated goat anti-mouse antibodies (Amersham) were used at a 1 : 5000 dilution with the ECL system for development before visualization on film.

## RESULTS

### The genomic locus of the *prlS/R* genes is well conserved in α-proteobacteria

The genes *prlS* and *prlR* are in opposite orientation and very well conserved in brucellae (Lavín *et al.*, 2010; Letesson & De Bolle, 2004). This predicted TCS pair is also well conserved in several other α-proteobacteria. In all these bacteria, a gene (*mscL*) encoding a large-conductance mechanosensitive channel is found upstream of *prlS*

(Fig. 1a, c). In *Brucellaceae* only, a gene encoding a proline imidopeptidase is found upstream of *prlR* (Fig. S1).

The *prlS* CDS (BMEI1606) in the *B. melitensis* 16M genome sequence (GenBank accession no. AE008917) is a probable hybrid HK that, in addition to the classical domains found in HK (i.e. PAS fold, HisKA, ATPase) contains an N-terminal sensory domain with 12 transmembrane segments homologous to members of the $Na^+$/solute symporter (SSS) family (Jung, 2001) and a conserved RR domain at its C-terminal part (Fig. 1b). Homologous proteins are found not only in α-proteobacteria but also in γ-proteobacteria [e.g. *Pseudomonas aeruginosa* (40 % identity) and *Vibrio cholerae* (39 % identity)]; in these two cases, the HK is not located close to an RR. The *prlS* gene has been described as a probable pseudogene in *Brucella ovis* (strain ATCC 25840) and in *Brucella microti* (strain CCM4915) (Lavín *et al.*, 2010).

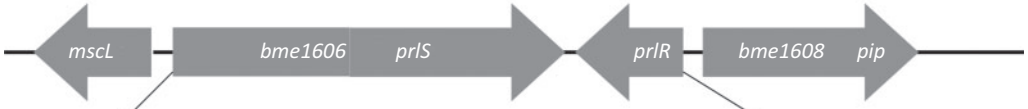
The *prlR* gene (BMEI1607) encodes a 213 amino acids protein with a predicted molecular mass of 23 kDa. PrlR is a typical RR with an N-terminal domain bearing the conserved aspartate residue of the receptor domain (REC) and a C-terminal HTH domain of the LuxR-like (GerE) superfamily (Pfam no. PF00196) (Galperin, 2010).

None of the homologues of this highly conserved TCS has been characterized, and the function of the corresponding proteins is completely unknown as well as the signal that could be perceived by the HK. We described previously (Lestrate *et al.*, 2000) that the G4 mutant bearing a mini-Tn*5* inserted between *prlS* and *prlR* is attenuated in mice. Therefore, we started to characterize this TCS and its putative function further.

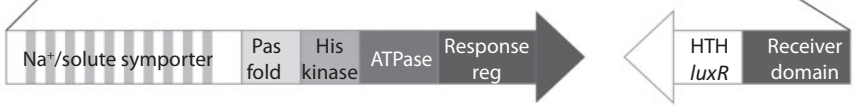
## Mutants in the *prlS* or *prlR* gene are attenuated at 4 weeks post-infection in mice

Transpositional insertions may fail to fully eliminate target gene function (Kang *et al.*, 2004); therefore, we decided to start the characterization from newly constructed mutants. Two deletion mutants of the TCS genes, Δ*prlS* and Δ*prlR*, were constructed by allelic replacement in the *B. melitensis* 16M strain (see Methods). The capacity of the two mutants, Δ*prlS* and Δ*prlR*, to multiply within cultured cells was analysed. As shown in Table 2, neither mutant displayed a growth defect 48 h post-infection, either in bovine SV40 macrophage-like cells or in JAR713 trophoblastic cells. In contrast, the Δ*vjbR* strain, used as a control

(a)




(b)




(c)

| | Hybrid histidine kinase | | | Response regulator | |
|---|---|---|---|---|---|
| **Bacterium** | **Name** | **Identity (%)** | | **Name** | **Identity (%)** |
| *Brucella suis* 1330 | Sensor histidine kinase/response regulator | 100 | | Two-component transcriptional regulator, LuxR family | 100 |
| *Mesorhizobium* sp. BNCI | Periplasmic sensor hybrid histidine kinase | 60 | | Two-component transcripional regulator, LuxR family | 67 |
| *Rhizobium etli* CFN 42 | Probable two-component sensor histidine kinase/response regulator hybrid protein | 52 | | Probable two-component response regulator protein | 65 |
| *Rhodopseudomonas palustris* CGA009 | Sensor histidine kinase, possible proline sensor PrlS | 47 | | Two-component transcriptional regulator, LuxR family | 57 |

**Fig. 1.** The TCS PrlS/PrlR. (a) Genomic organization of the locus (*mscL*, large conductance mechanosensitive channel; *pip*, proline imidopeptidase). (b) Domain organization of the PrlS and PrlR proteins. (c) Conservation of this TCS in several α-proteobacteria.

**Table 2.** Attenuation of *prlS* and *prlR* mutants

Attenuation in cellular models (48 h post-infection) and in BALB/c mice (5 days and 4 weeks post-infection) is expressed as the difference of the log(c.f.u.) between the WT strain and the mutants ± SD, which was calculated from values obtained for each mutant in the different models of infection ($n$=3 for the cellular models and $n$=4 for the mouse model). Cellular infections were performed twice in triplicate, with an m.o.i. of 300 bacteria per cell. The Δ*vjbR* mutant was used as a positive control for attenuation in the cellular models. ND, Not determined; p.i., post-infection.

| Mutation | Attenuation in: | | | |
|---|---|---|---|---|
| | SV40 bovine macrophages | JAR713 trophoblasts | BALB/c mice | |
| | | | 5 days p.i. | 4 weeks p.i. |
| Δ*prlR* | −0.08 ± 0.14 | 0.11 ± 0.21 | −0.13 ± 0.32 | 1.93 ± 0.46 |
| Δ*prlS* | −0.01 ± 0.08 | −0.23 ± 0.09 | −0.21 ± 0.11 | 1.73 ± 0.79 |
| Δ*vjbR* | 2.5 ± 0.2 | 2.2 ± 0.3 | ND | ND |

for attenuation (Delrue *et al.*, 2005), showed an important growth defect in both cell lines (Table 2). Taken together, these results demonstrated that this TCS is not essential for intracellular growth of the pathogen in the cell types tested.

To determine whether the PrlS/R TCS could be involved in adaptation of *B. melitensis* to the *in vivo* conditions, the ability of the mutants to infect BALB/c mice was assessed and compared with the *B. melitensis* 16M parental strain. The number of viable bacteria in the spleen was evaluated at different times post-infection. At 5 days post-infection, Δ*prlS* or Δ*prlR* replication in the spleen was indistinguishable from that of the WT strain (Table 2). However, after 4 weeks, both mutants were markedly attenuated (with almost two logs difference from the control). These results indicated that the TCS PrlS/R is necessary for persistence in mice and is somehow involved in adaptation of *B. melitensis* to the *in vivo* environments.

## Mutants in either *prlR* or *prlS* have unaltered growth in 2YT culture

To rule out any possible effect of an *in vitro* growth defect on the above-reported *in vivo* attenuation, we examined the growth of the Δ*prlR*, Δ*prlS* and WT strains in rich 2YT broth using a Microbiology Reader Bioscreen. No significant growth impairment was discernible for the two mutated strains in this medium (growth rate and $OD_{600}$ at stationary phase were similar to those of the WT) (Fig. 2a). This was indicative that neither PrlS nor PrlR was needed during growth under the tested conditions. To ascertain whether the PrlR protein was produced or not during *in vitro* growth we performed a Western blot on bacterial extracts harvested along the growth curve in 2YT (Fig. 3a). As shown in Fig. 3(b), PrlR is present in quantity when *Brucella* is growing *in vitro*. PrlR was detected at a similar level from the lag phase to the stationary phase. The detection of flagellar proteins (FlgE and FliC) known to be only present in early exponential phase was used as a control for variably expressed proteins. As a loading control, we used the detection of Omp89 (Léonard *et al.*, 2007).

## The WT *B. melitensis* 16M strain but not the Δ*prlS* or Δ*prlR* mutants aggregates in liquid culture containing 400 mM NaCl

The conservation of the *mscL* gene (encoding a large conductance mechanosensitive channel), known to participate in the regulation of osmotic pressure within the cell (Moe *et al.*, 1998) upstream of the *prlS* homologue in several α-proteobacteria (i.e. *Sinorhizobium meliloti*, *Mesorhizobium loti*, *Rhizobium etli* and *Agrobacterium tumefaciens*), prompted us to test whether the mutants in the *prlS/R* genes have altered behaviour in response to osmotic stress. The WT strain and the two mutants Δ*prlS* and Δ*prlR* were grown in liquid culture in a rich medium (2YT) with various amounts of NaCl added (100, 200, 300 and 400 mM). All the strains were able to grow under these conditions up to 400 mM NaCl (in liquid medium but not on solid medium). No significant growth impairment was shown for the mutant strains as compared with the WT in 2YT/400 mM NaCl (Fig. 2b). However in the presence of this concentration of NaCl all the strains appeared to slow down growth earlier than in regular 2YT medium, reaching a slightly lower $OD_{600}$.

With increasing duration of culture in the presence of 400 mM NaCl it became obvious that the WT strain formed bacterial aggregates, while the *prlR* mutant failed to do so (Fig. 4). Similar results were obtained with the *prlS* mutant (not shown). The aggregative phenotype of both mutants was restored by supplying *in trans* a WT copy of the *prlS* or *prlR* gene to generate the respective complemented strains (Fig. 4 and data not shown).

## Aggregation of the WT *B. melitensis* 16M strain is induced by ionic stress but not osmotic stress

To determine whether the aggregative phenotype observed for the WT *B. melitensis* 16M strain in the presence of NaCl was a response to an osmotic stress or to a salt stress we cultivated the strain for 72 h in liquid 2YT containing either 400 mM NaCl or other molecules [namely KCl (314.45 mM) or sucrose (628.9 mM)] to reach an equivalent osmolality (around 800 mosmol $kg^{-1}$), with or without an ionic stress.

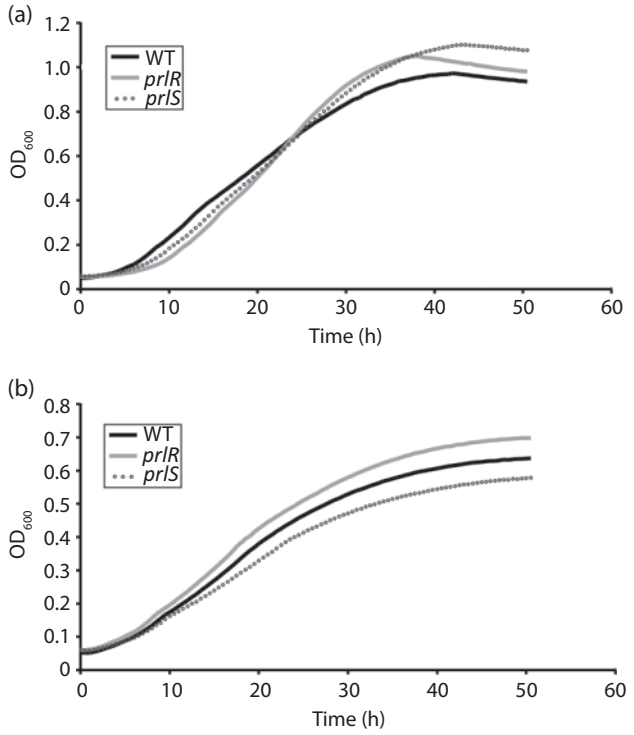



**Fig. 2.** Cultures of *B. melitensis* 16M WT and of the *prlS* and *prlR* mutants in 2YT (a) and 2YT/400 mM NaCl (b). Monitoring of $OD_{600}$ in a Microbiology Reader Bioscreen of triplicate samples.

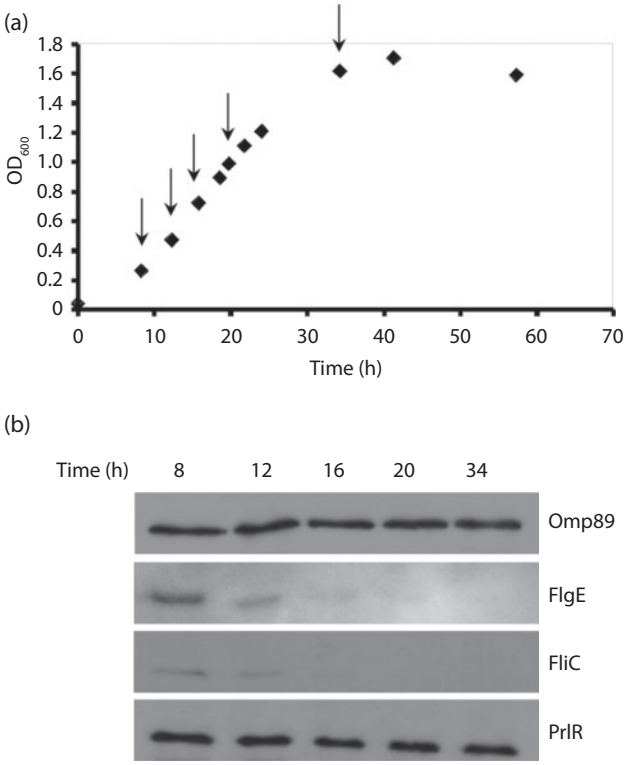



**Fig. 3.** Constitutive expression of the PrlR protein in 2YT medium in *B. melitensis* 16M. (a) Growth of *B. melitensis* 16M in liquid 2YT broth. Arrows indicate samples harvested for Western blotting. (b) Western blot of Omp89, FlgE, FliC and PrlR expression in *B. melitensis* WT. The strain was cultivated in 2YT broth and samples were harvested after 8, 12, 16, 20 and 34 h of culture. Total extracts were separated by electrophoresis, transferred to nitrocellulose membranes and probed with anti-PrlR, anti-FliC and anti-FlgE rabbit sera. A monoclonal antibody against Omp89 was used as a loading control.

As shown in Table 3, both KCl and NaCl were able to induce clumping of the WT strain similarly when added to 2YT medium to reach around 800 mosmol $kg^{-1}$. In contrast, sucrose was unable to induce aggregates in iso-osmolar conditions. This indicates that the clumping phenotype is not an adaptation to hyperosmotic non-ionic stress but is probably a saline stress response.

In order to further test this hypothesis, we used $MgCl_2$ (or $CaCl_2$) to supplement the 2YT. These multivalent salts allowed us to keep the same ionic strength (0.4 M) but almost halve the osmolality (485 mosmol $kg^{-1}$) as compared with the 2YT medium containing 400 mM NaCl. In these conditions aggregates were clearly visible after 72 h of culture (Table 3). In contrast, when NaCl was added to reach 485 mosmol $kg^{-1}$ (corresponding to only 0.24 M ionic strength), no clumping arose.

## Naturally occurring *prlS* or *prlR* mutant strains display the same lack of aggregative phenotype in the presence of 400 mM NaCl

Since an increasing number of sequenced *Brucella* genomes are available [Broad Institute (http://www.broadinstitute.org/annotation/genome/brucella_group/MultiHome.html)], we were able to determine that the TCS PrlS/R is well conserved in most species of the genus *Brucella*, even if there is some amino acid sequence length heterogeneity. Indeed, *prlS* was previously identified as a pseudogene in both *B. ovis* and *B. microti* (Lavín *et al.*, 2010). Moreover, the predicted PrlR protein is 56 amino acids shorter in two *Brucella ceti* strains (M13/05/1 and M644/93/1), while the PrlS protein is 400–500 amino acids shorter in *Brucella neotomae* 5K33 and two *B. ceti* strains (M490/95/1 and B1/94). This offered us a unique opportunity to test whether these natural mutants in either *prlS* or *prlR* display the same phenotype as the mutants that we constructed in *B. melitensis*. PrlR was detected by Western blotting in all the tested strains except the two *B. ceti* strains, which had a truncated version (M13/05/1 and M644/93/1) (not shown). As expected according to the PrlS/PrlR-dependent clumping, these two strains also failed to aggregate in the presence of 400 mM NaCl. Moreover, all the tested strains with a normal-sized PrlS aggregated in the presence of 400 mM NaCl, but the two tested strains with an altered version of PrlS (*B. microti* CCM 4915 and *B. ceti* B1/94) failed to do so (see Table 4).

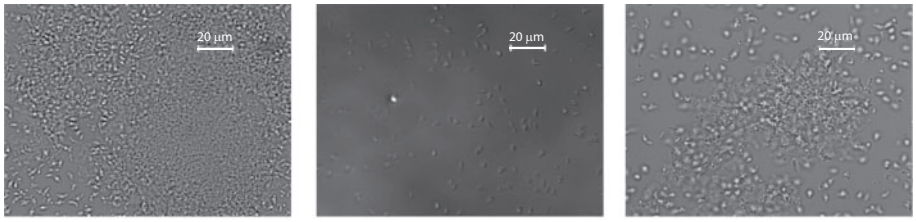



**Fig. 4.** Aggregative phenotype after 72 h of growth in 2YT rich medium supplemented with 400 mM NaCl. Microscopic observation (DIC) of the strains on an agarose pad. Aggregates are present in the WT but absent in the Δ*prlR* mutant. The aggregative phenotype was restored by supplying *prlR in trans* on plasmid pMR10.

## DISCUSSION

In this paper we characterized a novel TCS of *Brucella* called PrlS/PrlR that appears to control stress adaptation but also persistence in the host. *B. melitensis* deletion mutants in either *prlS* or *prlR* share the same phenotype. This reinforces the previous idea (Lavín *et al.*, 2010; Letesson & De Bolle, 2004) that PlrS and PrlR are part of the same bona fide TCS.

PrlS has an N-terminal input domain with 12 predicted transmembrane helices. This N-terminal sensor domain belongs to the SSS family (Jung, 2001), and is homologous but distantly related to the transporter protein PutP of *E. coli*. While the biochemistry and topology of the PutP-like carrier proteins have been extensively studied (Jung, 1998; Pirch *et al.*, 2003; Wegener *et al.*, 2000), there are almost no data on the corresponding domain when it is part of a sensor kinase. It is clear that these domains function as sensors of membrane-associated stimuli, but whether the stimulus is related to ion or electrochemical gradients and transport processes, to the mechanical properties of the cell envelope (such as turgor or mechanical stress) or both remains to be determined (Mascher *et al.*, 2006).

Up to now, no functional data were available for the TCS orthologous to PrlS/PrlR in α-proteobacteria. One clue was the presence of a gene encoding a large conductance mechanosensitive channel (*mscL*) upstream of *prlS* in the highly conserved genomic environment around this TCS in α-proteobacteria. These channels are widely distributed (Kung *et al.*, 2010), and play a critical role in reacting to physical stresses at the cell membrane by the release of cytoplasmic osmolytes (Levina *et al.*, 1999). These stresses could be changes in turgor pressure caused by osmotic perturbation or even when remodelling the cell wall upon entry into stationary phase or during growth and division (Kung *et al.*, 2010). This genomic conservation, as well as the fact that several *prlS* homologues are annotated as 'proline sensor' and that proline is a well-known osmoprotectant (Kunte *et al.*, 1999; Racher *et al.*, 1999), are preliminary indications that the TCS PrlS/PrlR could play a role in the *Brucella* response to changes in osmotic pressure or related membrane stresses. In fact, preliminary studies from our laboratory using microarray technology suggest that PrlR regulates, directly or indirectly, genes involved in cell wall (peptidoglycan biosynthesis and remodelling) and envelope biogenesis [Omps, lipoproteins, LPS, extracellular polysaccharide (EPS)], as well as genes

**Table 3.** Effect of osmolality and ionic strength on the clumping phenotype of *B. melitensis* 16M

+, Macroscopically discernible aggregates; −, no macroscopically discernible aggregates.

| 2YT complemented with: | Osmolality* (mosmol $kg^{-1}$) | Ionic strength (mol $l^{-1}$) | Aggregates observed |
|---|---|---|---|
| – | 85.5 | | − |
| NaCl† | 800 | 0.40 | + |
| KCl† | 800 | 0.40 | + |
| Sucrose | 800 | 0 | − |
| $MgCl_2$‡ | 485 | 0.40 | + |
| NaCl | 485 | 0.24 | − |

*Taking account of only NaCl and added molecules.
†The same results were obtained with LiCl, NaBr and KBr.
‡The same results were obtained with $CaCl_2$.

**Table 4.** Comparison of the amino acid sequence length of PrlS/R from various *Brucella* species and correlation with the detection of PrlR in Western blots (WB) and the aggregative phenotype in the presence of 400 mM NaCl.

ND, Not determined.

| Species | Strain | Length of the PrlR protein (aa) | Length of the PrlS protein (aa) | Aggregation in 2YT/ 400 mM NaCl | PrlR detected by WB |
|---|---|---|---|---|---|
| *B. melitensis* | 16M | 213 | 1174* | + | + |
| *B. abortus* | 2308 | 213 | 1174 | + | + |
| *B. suis* | 1330 | 213 | 1174 | + | + |
| *B. canis* | RM6/66 | 213 | 1174 | † | + |
| *B. ovis* | 63/290 | 213 | Pseudogene ($\psi$) | † | + |
| *B. neotomae* | 5K33 | 213 | 760 | ND | + |
| *B. microti* | CCM 4915 | 213 | Pseudogene ($\psi$) | − | + |
| *B. pinnipedialis* | B2/94 | 213 | 1174 | + | + |
| *B. ceti* | B1/94 | 213 | 641 | − | + |
| *B. ceti* | M13/05/01 | 157 | 1174 | − | − |
| *B. ceti* | M644/93/1 | 157 | 1174 | − | − |

*Based on the *prl*S sequence of the Rev.1 strain. There is a sequencing error in the GenBank *B. melitensis* 16M sequence (Gene ID: 1197317). This was confirmed by resequencing (results not shown).
†Rough strains are self-aggregative even in the absence of NaCl.

involved in metabolism and stress response mechanisms (R. M. Yañez Villanueva, unpublished data). Some Omps also have their patterns of expression modified in the Δ*prlR* background and this effect is complemented (see Fig. S2).

Bacteria adapt to osmotic or related stress through multiple mechanisms leading to the accumulation of $K^+$ and neosynthesis of osmoprotectants (Krämer, 2010; Wood, 2006, 2007). Osmoadaptation also induces remodelling of the cell envelope and cell surface structures i.e. membrane or periplasmic protein composition, lipid composition, periplasmic glucan level and EPS synthesis (Miller & Wood, 1996). Ultimately, some of these modifications could lead to biofilm (or aggregate) formation (Karatan & Watnick, 2009).

In the presence of up to 400 mM NaCl (800 mosmol $kg^{-1}$), neither the *prlS* nor the *prlR* mutant showed a significant growth defect, although both mutants exhibited a lack of aggregation as compared with the WT strain, and this phenotype could be complemented. Moreover, natural *Brucella* strains with truncation or pseudogenization in orthologues of either *prlS* or *prlR* behave similarly in the presence of 400 mM NaCl. Importantly, all the *B. ceti* strains analysed have a defective PrlS/PrlR system. In contrast to a non-ionic hyperosmotic stress, a saline stress involves an ionic component in addition to the osmotic one. The aggregative phenotype was not a response to osmotic stress alone because it was not observed in the presence of sucrose at the same osmolality (800 mosmol $kg^{-1}$) and appears to be linked to the ionic strength of the medium, which reduces the water availability.

This behaviour related to 'biofilm' formation could promote bacterial survival when faced with desiccation by maintaining a hydrated microenvironment, even if inherent resistance linked to biofilms cannot be ruled out (Iibuchi *et al.*, 2010). Importantly, *Brucella* spp. cannot tolerate this osmolality on solid media, probably because water availability is reduced even further on solid medium. Moreover, the lack of aggregation, if generalized to all *B. ceti* strains, could be related to their ecology. Although *Brucella pinnipedialis* (affecting predominantly seals) and *B. ceti* (affecting predominantly porpoises and dolphins) are both reported as marine mammal *Brucella* strains, the lifestyles of their respective hosts are very different. Indeed, in contrast to cetaceans, pinnipeds are able to live out of water, notably for reproduction, birth and breeding. So, in contrast to their close marine mammal neighbours, it is unlikely that *B. ceti* has to cope with hydric stress. In such a context, a reduced selective pressure could explain the loss of the TCS *prlS/R*.

Here we describe for the first time to our knowledge a condition in which all the tested *Brucella* spp. with an intact PrlS/R TCS form an aggregate in rich culture media containing a high concentration of NaCl and yeast extract. We suggest that the TCS PrlS/R is somehow involved in sensing a membrane perturbation signal. This aggregation in the presence of 400 mM NaCl could result from EPS production and/or from a modification of outer-membrane properties. An aggregative phenotype has already been described for quorum-sensing mutants in *B. melitensis* (Godefroid *et al.*, 2010; Uzureau *et al.*, 2007) and was thought to be linked to the production of an EPS. Nevertheless, the *vjbR* mutant also has a lot of other surface modifications that could account for the aggregative phenotype (Delrue *et al.*, 2005; Uzureau *et al.*, 2007). Finally, the lower persistence of the *prlR/S* mutants of *B.*

*melitensis* in mice suggests that this system and, more likely, the genes under its control, are crucial to ensure a successful infection of the eukaryotic host.

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