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FixL-like sensor FlbS of *Brucella abortus* binds haem and is necessary for survival within eukaryotic cells

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ABSTRACT

Replication of *Brucella* inside eukaryotic cells is essential for pathogenesis, and successful infection requires rapid adaptation to the intracellular milieu. Close relatives of *Brucella* use the two-component system FixLJ to survive inside the host. We aimed to identify a homologous sensor in *Brucella abortus*. A predicted protein with transmembrane and conserved histidine kinase domains was identified as the Fix-like *Brucella* sensor, FlbS. Although it lacks the PAS domain, recombinant FlbS binds haem in vitro. An internal in-frame deletion in *flbS* severely decreased *B. abortus* survival inside professional and non-professional phagocytes. This phenotype was reverted by genetic complementation. These results indicate the critical role of this haemoprotein in the intracellular lifestyle of *Brucella*.

1. Introduction

Brucella is a genus of gram-negative facultative intracellular bacteria that cause brucellosis, a widespread zoonotic disease. *Brucella* virulence is dependent on the ability of the organisms to survive and reproduce inside eukaryotic cells to produce chronic infection. When *Brucella* species encounter eukaryotic cells at the mucosa as the first contact with the host, they must be prepared for a timely adaptation for efficient cellular invasion and survival in a compartment facing stress conditions such as low oxygen availability.

Brucella species regulate gene expression in response to low oxygen tension, a phenomenon observed in proteomic analyses [1] and instudies of mutant phenotypes under microaerobic conditions. Thus, it is known that 2 high-oxygen-affinity terminal oxidases, cytochrome c and bd ubiquinol, are induced and required for intracellular survival of *B. abortus* and *B. suis* [2,3]. Another strategy for anaerobic respiration in this pathogen is the use of nitrate as an electron acceptor rather than oxygen. Intracellular replication is inhibited in *Brucella* mutants (*nar*, *nir*, *nor*, and *nos*) that lack components of the denitrification pathway [4,5]. It was recently discovered that the genes involved in this pathway are regulated by the two-component system NtrYX [6]. Interestingly,*ntrY* is not critical for the intracellular survival of *B. abortus* [7]. The induction of *ntrY* under microaerobic conditions [6] suggests that *Brucella* species use another system to sense oxygen limitation.

Brucella is a member of the alpha-2 subgroup of proteobacteria, order Rhizobiales, along with symbionts and plant pathogens *Bradyrhizobium*, *Sinorhizobium*, and *Azorhizobium*. These organisms sense oxygen levels through the two-component system FixLJ, which increases the transcription of genes required for nitrogen fixation and anaerobic respiration during nodulation in leguminous plants [8].

FixL is a homodimeric sensor that coordinates its kinase activity with the status of iron at its haem-binding domain. Under aerobic conditions, oxygen is bound to the haem cofactor and the activity of the histidine kinase domain is inhibited. When the oxygen dissociates, the catalytic domain is activated and the phosphate group at a conserved histidine residue is donated to the FixJ regulator for signal transduction. The homology of FixL family members is centred in the C-terminal domain where histidine kinase and ATPase domains are present. The N-terminus of FixL is usually hydrophobic, anchored to the periplasmic membrane, and differs between homologues. The intermediate region contains the haem attachment site represented by PAS domains [9].

Available *Brucella* genomes do not contain obvious FixL and FixJ orthologues. However, this pathogen interacts with eukaryotic

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cells in a way that resembles endosymbiotic interactions and some regulatory mechanisms are common to the processes [10]. This behaviour led us to hypothesise that *Brucella* may have a similar FixLJ two-component system that could mediate adaptation to the host.

An *in silico* search revealed 2 adjacent genes on chromosome II whose products are homologous to the conserved regions of the C-terminus of *Bradyrhizobium japonicum* FixL and to FixJ. We designated these proteins FlbS (Fix-like *Brucella* sensor) and FlbR (FixJ-like *Brucella* regulator) and performed functional analyses ofFlbS. Recombinant FlbS bound haem in vitro. An isogenic mutant expressing a truncated version of the protein showed reduced survival inside professional and non-professional phagocytes. These results suggest that the *B. abortus* sensor of the FlbSR two-component system is, similar to FixL, a haemoprotein necessary for efficient intracellular survival.

2. Materials and methods

2.1. Bacterial strains, plasmids, and culture conditions

All strains and plasmids are listed in Table 1. All liquid cultures in *Brucella* broth, tryptic soy broth (TSB), or Luria–Bertani medium were incubated at 37 °C in a rotary shaker at 250 rpm. Microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) were generated using CampyGen™ envelopes in a sealed jar (Oxoid, Hampshire, England). Thioglycollate medium with indicator (USP) (Britania Laboratories, Bs As, Argentina) was used as a semi-solid medium. Growth was monitored by measuring optical density at 600 nm (OD_{600}). The number of viable bacteria was determined by plating serial dilutions onto appropriate solid medium. For microaerobic growth, aliquots of the inoculum (OD_{600} of 0.1) were dispensed in several tubes in order to take one tube for each time point. The media were supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin for plasmid selection and 40 μg/mL haemin for protein purification. Procedures involving live *Brucella* wereperformed in a biosafety level 3 laboratory. All reagents were purchased from Sigma, unless otherwise stated.

2.2. Computational analysis

Searches for amino acid sequence similarity were performed using the Blast service provided by NCBI (http://www.ncbi.nlm.nih.gov). Sequence alignment was performed using EMBL-EBI clustalW2 (http://www.ebi.ac.uk) and edited using Jalview version 2 (www.jalview.org) [11]. Hydrophobicity analysis was performed using the TopPred program (http://mobyle.pasteur.fr). Conserved domains were searched using the ScanProsite program (http://www.expasy.org/tools/scanprosite/) and SMART (http://smart.embl-heidelberg.de). Secondary protein structure and fold recognition were predicted using the protein homology/analogy recognition engine (Phyre 2) server (http://www.sbg.bio.ic.ac.u).

2.3. Expression of recombinant protein

To express the predicted cytosolic domain of FlbS, named FlbS*, the sense (5′-GGACTAGTCGATCGAGGCAAACAGGTC-3′) and antisense (5′-CGGGATCCAGATCGATGTGCGGGAG-3′) primers were used in a PCR reaction with *B. abortus* 2308 chromosomal DNA template. The *Spe*I and *Bam*HI sites (underlined) were incorporated for future directional cloning. The PCR product (830 bp) was cloned into pGEM-T Easy (Promega) and replicated in *Escherichia coli* Top10 competent cells to produce pGEM*flbS**. This plasmid was digested with *Bam*HI and *Eco*RI, and the liberated fragment was cloned into pTrcHis C (Invitrogen). The recombinant plasmid, pTrcHis*flbS**, was transformed into and expressed in *E. coli* DH5α. His6-tagged FlbS* was purified from the inclusion bodies as described by the manufacturer but by using a column renaturation step [12].

Protein from soluble extracts was quantified by the Bradford method by using the Bio-Rad kit and BSA standard.

2.4. Haemin binding assay

The interaction of recombinant FlbS* with haem was studied through the spectral properties of haem [13]. Haemin was dissolved in a 50:48:2 mixture of ethanol/water/5 M NaOH and

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotypic or phenotypic description	Reference or source
Strains		
B. abortus 2308	Wild type, smooth, virulent, Nar^R	Laboratory stock
B. abortus 2308*flbS*1	2308 mutant of *flbS*, smooth	This study
B. abortus 2308*flbS*1C	*B. abortus* 2308*flbS* harboring plasmid pBBR*flbS*	This study
B. abortus 2308*flbS*1C*	*B. abortus* 2308*flbS* harboring plasmid pBBR*flbS**	This study
E. coli DH5α $F'I^q$	F′ Φ80*dlacZ*ΔM15(*lasZYA-argF*)U169 *deoR recA1 endA1 hsdR12*(rk^- mk^+)*phoA supE44* λ- thi-1 *gyrA96 relA1*/F′ *proAB*$^+$ *lacI*^{q} *Z*ΔM15 *zzf*::Tn5(Km^R)	[24]
E. coli Top 10	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 recA1araD139* Δ(*ara-leu*) 7697 *galU galK rpsL* (Str^R) *endA1 nupG* λ^-	Invitrogen
E. coli S17λ*pir*	λ lysogenic S17-1 derivative producing the protein for replication of plasmids carrying oriR6K	[25]
Plasmids		
pBlueKS2*SacB/R*	*B. subtilis sacB* cloned into pBluescript-KSII, Amp^R	[14]
pKS2 *SacBflbS*1	*B. abortus* Δ*flbS* cloned into pBlueKS2*SacB/R*. Amp^R	This study
pTrsHis C	Cloning expression vector, Amp^R, N-Terminal 6xHis	Invitrogen
pTrsHis*flbS**	N-terminal truncated *B. abortus* FlbS coding sequence cloned into pTrsHis C, Amp^R	This study
pGEM-T easy	Cloning vector, Amp^R	Promega
pGEM*flbS*	*B. abortus flbS* cloned into pGEM-T easy	This study
pGEM*flbS**	Fragment containig the coding sequence of the N-terminal truncated *B. abortus* FlbS cloned into pGEM-T easy, Amp^R	This study
pGEM*flbS*1	pGEM*flbS** with a 231 bp *Nru*I deletion	[15]
pBBR1MCS-4	Broad host range-cloning vector, Amp^R	This study
pBBR1*flbS*	*B. abortus flbS* cloned into pBBR1MCS-4, Amp^R	This study
pBBR1*flbS**	Fragment containig the coding sequence of the N-terminal truncated *B. abortus* FlbS cloned into pBBR1MCS-4, Amp^R	

binding studies were conducted using an appropriate dilution in 50 mM Tris/HCl pH 8.0. The absorption spectrum of 10 μM haem was recorded in the presence or absence of 10 μM FlbS*. Negative and positive control experiments were conducted using lysozyme and BSA at the same concentrations.

2.5. Construction of an in-frame FlbS deletion mutant

To disrupt the *B. abortus flbS*, an *Nru*I fragment (231 bp) was digested from the coding region of *flbS* in pGEM*flbS** to provide the plasmid pGEM*flbS*1. The in-frame deletion was confirmed by sequence analysis. The *Spe*I and *Bam*HI fragment from the new plasmid was cloned into pBlueKS2SacB/R to obtain pKS2SacB*flbS*1 which was electroporated into *B. abortus* 2308. *Brucella* Amp^R, selected as a result of a single recombination, harbours the *sacB* gene.Excision of the plasmid to generate the mutant was induced by sucrose [14]. Determination of ampicillin sensitivity and colony PCR were performed in wild-type and mutant strains to confirm the double recombination event in *B. abortus flbS*1.

2.6. Complementation of the mutation

For complementation studies, the DNA fragment corresponding to *FlbS** was obtained by digestion of pGEM*flbS** with *Bam*HI and *Spe*I, and cloned into pBBR1MCS-4 [15], giving rise to the plasmid pBBR*flbS**. To clone the entire coding *flbS* sequence, the sense primer(5′-CGGGATCCAGCAGATTGTCGGCCAT-3′) with the *Bam*HI site was used in a PCR reaction with the same antisense primer described in Section 2.3. The amplified fragment (1327 bp) was cloned into pGEM-T Easy to produce pGEM*flbS*. By digestion with *Bam*HI and *Spe*I, the fragment was directionally cloned into pBBR1MCS-4 to produce pBBR*flbS*.

pBBR*flbS* and pBBR*flbS** were transferred to *B. abortus* 2308*flbS*1 by biparental mating using *E. coli* S17λpir as the donor strain [16] to obtain the complemented strains 2308*flbS1*C and 2308*flbS1*C*.

2.7. In vitro infection assay

Infection of HeLa and murine-macrophage-like J774A.1 cell lines was performed in 24-well microplates [17]. Bacterial strains grown aerobically in TSB were used to infect epithelial cells and macrophage-like cells at m.o.i. of 100 and 50, respectively. Briefly, the gentamicin protection assay was performed at 1 h p.i. to eliminate extracellular bacteria. At the indicated times, eukaryotic cells were lysed and the number of viable intracellular bacteria was determined.

2.8. Statistical analysis

All statistical analyses were performed using Student's two-tailed *t*-test. $P \leqslant 0.05$ was considered significant. Results are expressed as the means ± S.D.

3. Results

3.1. In silico identification of FlbS as the sensor kinase of a novel two-component system in Brucella

To investigate whether a sensor kinase similar to FixL was present in *Brucella abortus*, we used the *Bradyrhizobium japonicum* FixL sequence (505 residues) to perform a GenBank Blast search [18] against the translated genome of *Brucella abortus* 2308. The best-homology was found with a predicted protein of 356 amino acids encoded by a sequence (BAB2_0040) in chromosome II. This amino acid sequence does not possess the PAS domain characteristic of FixL; thus, the alignment is mainly produced at the C-terminal domain with 33% identity and 54% similarity. As expected for a two-component system, the contiguous ORF (BAB2_0041) encodes a putative two-component LuxR family transcriptional regulator. Additionally, the coding sequences are located in the same DNA strand, overlapping by a few nucleotides as if they were part of a transcriptional unit. Both genes are highly conserved among the *Brucella* genomes. Thus, we named these *B. abortus* proteins FlbS and FlbR (**F**ix-**l**ike ***B****rucella* **s**ensor and **r**egulator, respectively).

FlbS contains a predicted transmembrane domain of approximately 100 amino-acid residues and contains the conserved histidine kinase and HATPase domains at the C-terminus, as do other two-component signal transduction sensors (Fig. 1A). These results are in agreement with those obtained for secondary structure prediction and homology models at the Phyre 2 server. A PAS-fold domain was not identified, but 226 residues of the C-terminal domain were successfully modelled with 100% confidence by the single highest scoring template. The model was based on c4ew8A (PBD header: transferase; crystal structure of a C-terminal part of tyrosine kinase, *divl*, from *Caulobacter crescentus*) as the template hit.

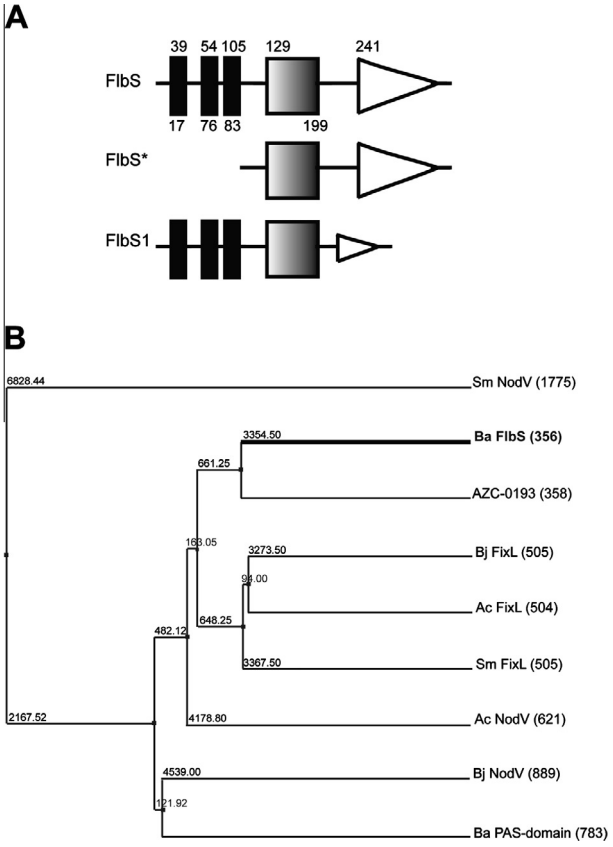

Fig. 1. *In silico* analysis of *B. abortus* FlbS protein. (A) Predicted domain structure of FlbS: transmembrane domains are shown in black, histidine kinase in grey, and HATPase_c in white. Numbers indicate residue positions according to the SMART program. (B) Phylogenetic analysis of FlbS with a selection of FixL and NodV proteins. An average distance tree using BLOSUM62 was constructed with the amino acid sequences (length indicated in brackets) obtained from the NCBI database. *B. abortus* YP_418285.1 (BaFlbS); *B. melitensis* YP_414082.1 (BmPAS-domain containing protein); *S. meliloti* CCNWSX0020 (SmNodV) and CAA798971 (SmFixL); *B. japonicum* CAA40143.1 (BjFixL) and AA6606981 (BjNodV); *A. caulinodans* CAA39979.1 (AcFixL), YP_0015242761 (AcNodV), and YP_0015231091 (AZC-0193).

A new Blastp search using FlbS as the query revealed that the putative *Brucella* sensor was significantly homologous (51% identity, 67% positive) to theputative sensor protein of *Azorhizobium caulinodans* (gene AZC_0193). Moreover, this gene also overlaps with a putative DNA-binding response regulator. The homology between the putative response regulators encoded by *flbR* and AZC_0194 was also high, with 62% identity and 77% similarity along the entire sequences. Although AZC_0193 and AZC_0194 are annotated as a sensor nodulation protein and nodulation protein W, respectively, the function of these proteins has not been demonstrated [19].

NodVW is the two-component system necessary for rhizobials to nodulate leguminous plants [20]. The loci for *A. caulinodans* FixLJ and NodVW are different from those of AZC-0193/AZC-0194. Thus, we performed a phylogenetic analysis of these new sensors (FlbS and AZC-0193) and the related FixL and NodV proteins of *A.caulinodans*, *B. japonicum*, and *S. meliloti*. We also included a *B. abortus* PAS domain-containing protein encoded in chromosome I as a sole putative sensor. These proteins were aligned in ClustalW2, and the phylogenetic tree was based on percent identities as shown in Fig. 1B.

3.2. Recombinant FlbS binds haem

Considering that FixL proteins use haem as a cofactor for gas sensing, and *B. abortus* Irr binds haem in vitro although it lacks the predicted haem-binding domain [21], we decided to investigate whether FlbS could also be a haemoprotein. The cytosolic peptide sequence (FlbS*) of the recombinant protein wasexpressed and purified to perform an in vitro haem-binding assay. The haem absorption spectrum changes upon interaction with haem-binding proteins; therefore, we recorded the absorption spectrum of haem in the presence or absence of FlbS. As shown in Fig. 2, the 385 nm absorption peak of haem shifted to 412 nm in the presence of FlbS. This shift is characteristic of haemoproteins, in which the Soret band shifts to longer wavelengths because of changes of electron distribution in the porphyrin ring [13].

3.3. FlbS is not needed for the extracellular B. abortus growth under microaerobic conditions

To investigate whether this haemoprotein mediates the ability of *B. abortus* to grow under microaerobic conditions, a mutant was constructed. The mutation was planned as an internal in-frame deletion to conserve bacterial membrane integrity and transcription of the downstream gene. The deletion comprises part of the histidine kinase domain and produces a *B. abortus* protein of 279 amino acids (FlbS1) (Fig. 1A).

Once the mutant 2308*flbS1* was obtained, its oxygen preference relative to the wild type was assayed in semi-solid media containing thioglycollate. No significant differences were observed in the patterns displayed by both strains. The wild type and mutant behaved as microaerophiles after 48 h of incubation at 37 °C (data not shown). An evaluation of the mutant growth in low-oxygen atmospheres containing 5% O_2 revealed no modification ingeneration time compared with the wild-type strain (Supplementary Fig. 1A and B). Both strains reached stationary phase in liquid cultures at the same time with similar viable counts in normal-oxygen atmospheres containing 21% O_2 (data not shown). These results indicate that the *flbS1* mutation does not affect wild-type growth in vitro under our experimental conditions.

3.4. FlbS is required for Brucella survival in eukaryotic cells

To determine the requirement of FlbS for adaptation to the intracellular growth of *Brucella*, we tested the ability of *Brucella* to replicate inside non-professional phagocytes in 2308 and 2308*flbS1* in vitro. The number of intracellular bacteria obtained

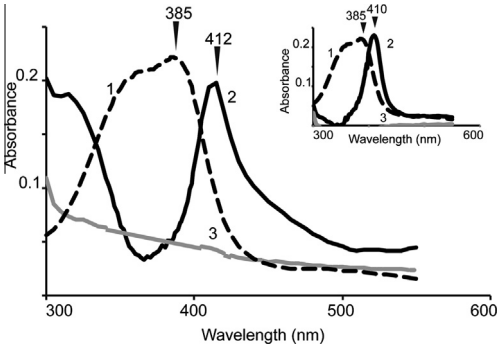

Fig. 2. Characterization of FlbS as a haem-binding protein. Absorption spectrum of 10 μM haem (1) or 10 μM FlbS (3) compared with the resulting haem + FlbS spectra (2) obtained by subtracting the free haem contribution. The inset shows comparative spectra of haem (1), haem + BSA (2), and haem + lysozyme (3) as positive and negative controls, respectively. All mixtures were made by adding each protein at a concentration of 10 μM to the haem solution. Data are representative of four independent experiments. Absorption peak wavelengths are indicated.

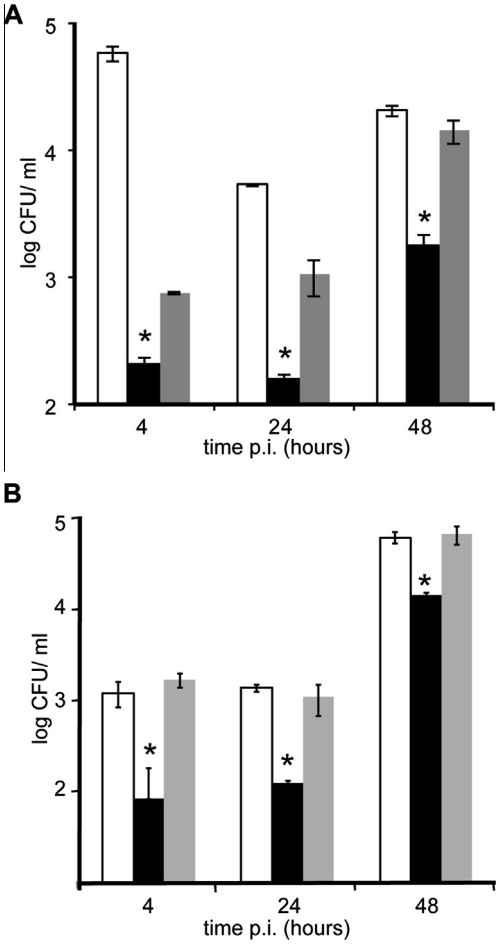

Fig. 3. Intracellular survival of *B. abortus* strains in nonprofessional phagocytes (A) or professional phagocytes (B). HeLa or J774A.1 cells (10^5 cells/well) were infected with *B. abortus* 2308 (white bars), 2308*flbS1* (black bars), and 2308*flbS1C* (grey bars) at an m.o.i. of 100 or 50, respectively. At different times p.i., eukaryotic cells were lysed, and the number of viable intracellular bacteria was determined. Data represent the means and standard deviations from one experiment performed in duplicate and are representative of 3 independent experiments. *$P < 0.05$.

at 4, 24, and 48 h p.i. (Fig. 3A) was significantly lower in the mutant than in the wild-type strain. The mutant phenotype was complemented with a plasmid carrying wild-type *flbS*. No complementation was obtained with a plasmid carrying the sequence for FlbS* expression (data not shown). These results indicate that the entire FlbS protein, with the N terminus connected to the histidine kinase domain, is necessary for successful survival in HeLa cells. After 24 h p.i., the number of intracellular mutants increased exponentially with a growth rate comparable to that of the wild type, indicating that mutant replication occurs inside the host. To determine whether the reduced number of intracellular mutants compared to the wild type at 4 h p.i. resulted from an entry defect, the HeLa infection was determined at 1 h p.i. The number of intracellular wild-type cells expressed as CFU/ml ($1.3 \times 10^3 \pm 0.4 \times 10^3$) was not significantly different from that of intracellular mutant cells ($0.9 \times 10^3 \pm 0.1 \times 10^2$).

To test whether the survival of the mutant was also affected inside professional phagocytes, infection was performed in macrophage-like J774A.1 cells. *B. abortus flbS* was significantly less efficient than the wild type for intracellular survival (Fig. 3B), although it was able to replicate after 24 h p.i.

4. Discussion

We performed an *in silico* search to identify two-component system homologues to FixLJ in*Brucella abortus*. Usually, searches for FixLJ homologues are based on 3 important criteria. First, both genes are located in 2 adjacent coding sequences; second, the response regulator belongs to the LuxR family, and third, the sensor binds haem. Haem binding is provided by the PAS domain. In this study, we identified the loci BAB2_0040 and BAB2_0041 based on the first 2 criteria.

The product of BAB2_0040 contains predicted histidine kinase sensors and does not contain the PAS domain according to protein sequence and structure databases. Regardless, we demonstrated that the recombinant *B. abortus* FlbS* binds haem. The red shift of the haem spectrum displayed by this protein is characteristic of haemoproteins that retain methionine/histidine or bis-histidine coordination to the haem iron [13]. Thus, the third criterion was fulfilled.

The putative *B. abortus* FlbSR two-component system showed higher identity with the products of *A. caulinodans* Azc_0193/Azc_0194 than with FixLJ proteins. Comparative protein analysis from 3 representative rhizobial species including *A. caulinodans* revealed that FlbS with its putative *A. caulinodans* orthologue is located in a different branch than those containing FixL proteins. Comparative sequence analysis using the NCBI Protein Cluster Database showed that FlbSR is not conserved in Rhizobiales but is present in other pathogenic proteobacteria such as *Bordetella bronchiseptica*, *Stenotrophomonasmaltophilia*, and *Xanthomonas albilineans*. Thus, FlbS seems to be the sensor kinase of an uncharacterized two-component regulatory system with functions related to those of FixL, given that they are phylogenetically related.

As indicated for the in vitro infection assays, *B. abortus* FlbS is necessary for efficient survival inside professional and non-professional phagocytes. The adherence and internalization processes of *Brucella* were not affected by the mutation in *flbS*. The increased number of mutant cells inside phagocytes at 48 h compared to 24 h p.i. suggests that FlbS is not critical for replication, as shown during its extracellular growth. Thus, the reduced number of viable intracellular mutants relative to the wild type at 4 h p.i. is an indication of inefficient adaptation to the intracellular lifestyle.

The intracellular phenotype of *B. abortus flbS1* was restored only by complementation with the full-length *flbS* expressed *in trans*. Although the N-truncated FlbS* protein bound haem and exhibited the predicted dimerization, phosphorylation, kinase, and ATPase domains, it did not complement the intracellular mutant phenotype. Thus, it seems that *Brucella* requires the N-terminus of FlbS to remain connected to the ATPase domain for efficient infection. Interestingly, a derivative of the *S. meliloti* FixL protein that does not contain the transmembrane domain was completely inactive in nodules [22].

B. abortus growth as free organism under microaerobic conditions was not affected by mutation in*flbS*. FlbS may function at very low oxygen levels as it does in *Caulobacter crescentus* FixL [23], as such levels were reached in the intracellular compartment. It is possible that haem-bound FlbS senses CO or NO intracellular levels. Nonetheless, gas limitation is only one of the stress conditions found by bacteria inside the host. Further characterization of this two-component system, will indicate how FlbSR contributes to the bacterial intracellular survival.

In summary, FlbS is a haemoprotein that mediates *B. abortus* infection of non-professional and professional phagocytes. This protein seems to be the sensor of a novel two-component system, present in other pathogens, with characteristics that resemble those of FixLJ and NodVW. Future studies to characterize this system will help to explain the complex interaction between bacterialpathogens and their hosts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.047.

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