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A quorum-sensing regulator controls expression of both the type IV secretion system and the flagellar apparatus of *Brucella melitensis*

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Summary

Both a type IV secretion system and a flagellum have been described in *Brucella melitensis*. These two multimolecular surface appendages share several features. Their expression in bacteriological medium is growth curve dependent, both are induced intracellularly and are required for full virulence in a mouse model of infection. Here we report the identification of VjbR, a quorum sensing-related transcriptional regulator. A *vjbR* mutant has a downregulated expression of both *virB* operon and flagellar genes either during vegetative growth or during intracellular infection. In a cellular model, the vacuoles containing the *vjbR* mutant or a *virB* mutant are decorated with the same markers at similar times post infection. The *vjbR* mutant is also strongly attenuated in a mouse model of infection. As C_{12}-homoserine lactone pheromone is known to be involved in *virB* repression, we postulated that VjbR is mediating this effect. In agreement with this hypothesis, we observed that, as *virB* operon, flagellar genes are controlled by the pheromone. All together these data support a model in which VjbR acts as a major regulator of virulence factors in *Brucella*.

Introduction

Brucella is an α2-Proteobacteria considered as facultative intracellular pathogens of mammals, including humans (Young, 1983). The pathogenesis of the resulting zoonosis, called brucellosis, is mostly linked to the ability of *Brucella* to survive and replicate intracellularly, in both professional and non-professional phagocytic host cells (Gorvel and Moreno, 2002). Among the virulence factors identified to date in *Brucella* spp. (Delrue *et al*., 2004) two deserve further attention because they are multimeric structures found on the bacterial surface: the type IV secretion system (T4SS) and the flagellum. The T4SS of *Brucella* plays a crucial role in the maturation of the *Brucella*-containing vacuole (BCV) into an organelle permissive for replication (Comerci *et al*., 2001; Delrue *et al*., 2001; Celli *et al*., 2003). This maturation involves the acquisition of endoplasmic reticulum (ER) membranes via an ER-Golgi COPI-dependent vesicular transport (Celli *et al*., 2003). The subversion of intracellular trafficking is probably mediated by a T4SS-dependent translocation of yet unidentified bacterial effectors inside the cells. Although *Brucella* are described as non-motile bacteria lacking genes for chemotaxis (DelVecchio *et al*., 2002), they have all the structural genes for building a flagellum (Letesson *et al*., 2002). More recently we demonstrated not only that *Brucella* is able to assemble a polar and sheathed flagellar apparatus under some precise *in vitro* conditions and that the MS-ring gene is induced intracellularly, but also that a complete flagellum is needed for a normal infectious process in mice (Fretin *et al*., 2005). Up to now, the function of this structure is still unresolved.

The expression and assembly of multimolecular surface structures such as type III and type IV secretion system, flagellum and pili are energy dispendious processes requiring an intricate regulatory control to allow their expression at the very precise steps of the infection where they are needed (Wu and Fives-Taylor, 2001; Aldridge and Hughes, 2002; Muir and Gober, 2002). Accordingly, the *Brucella* T4SS and flagellar apparatus are tightly regulated along growth curve in bacteriological medium and strongly induced inside cells (Sieira *et al*., 2000; Boschiroli *et al*., 2002; Fretin *et al*., 2005). Up to now, many environmental signals were demonstrated to up- or downregulate *virB* expression (Boschiroli *et al*., 2002; Taminiau *et al*., 2002). Among these signals, acidity which mimics at least one of the intracellular signals encountered by *Brucella* upregulates expression of *virB* (Boschiroli *et al*., 2002). On the other hand, the addition of N-dodecanoyl-homoserine lactone (C_{12}-HSL), the quorum-sensing (QS) pheromone of *Brucella melitensis* to the culture medium

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downregulates *Brucella virB* transcription (Taminiau *et al*., 2002). However, up to now, the transcriptional regulators of *Brucella* spp. involved in the control of *virB* and flagellar gene expression are unknown.

Here we report the identification of a QS-related regulator, named VjbR. A *vjbR* mutant of *B. melitensis* has a downregulated expression of both *virB* operon and flagellar genes either during culture in bacteriological medium or during intracellular infection. In a cellular infection model, the vacuoles containing the *vjbR* mutant or a *virB* mutant are decorated with the same markers at similar times post infection. Actually, the deletion of this putative QS transcriptional activator has the same effect on the expression of *virB* operon and flagellar genes as the exogenous addition of C_{12}-HSL to a culture of the wild-type (WT) strain. On these basis, we postulated that VjbR is the transcriptional regulator mediating this C_{12}-HSL effect. The *vjbR* mutant is also strongly attenuated in a mouse model of infection. All together these data support a model in which VjbR acts as a major regulator of virulence factors in *Brucella*.

Results

A mutation in a quorum sensing-related transcriptional regulator impairs intracellular replication of Brucella

We identified the 32D3 mutant in a screen of transpositional mutants of *B. melitensis* 16M defective for intracellular replication (Delrue *et al*., 2001). In this mutant, hereunder named *vjbR::mTn5*, the mini-transposon interrupts the 780 bp CDS BMEII1116 at nucleotide position 132. This gene, called *vjbR* (for vacuolar hijacking *Brucella* regulator), encodes a putative protein of 260 amino acids that exhibits significant homology to *N*-acylhomoserine lactone (AHL)-dependent transcriptional regulators of the LuxR family. Residues crucial for AHL binding (Vannini *et al*., 2002; Zhang *et al*., 2002) are conserved in the N-terminal part of VjbR (Fig. S1A in *Supplementary material*) suggesting that, despite the low level of similarity in this domain, the AHL-binding property may be retained. The DNA-binding domain of VjbR is also homologous to the corresponding domain in regulators of the LuxR family.

The *vjbR* putative coding sequence is located at the border of one of the three flagellar loci of *B. melitensis*. The genomic organization of this locus is completely conserved in the homologous locus of *Sinorhizobium meliloti* (Sourjik *et al*., 2000) (Fig. S1B in *Supplementary material*).

VjbR is involved in B. melitensis *16M virulence*

To confirm that the defective intracellular growth of the *vjbR::mTn5* mutant resulted from the disruption of *vjbR*, a new mutant in *B. melitensis* 16M was constructed by gene replacement (Δ*vjbR*). The behaviour of this strain and of the complemented strain (Δ*vjbR*c) were compared with WT *B. melitensis* 16M phenotype using infectious models described for the study of *Brucella* virulence.

In HeLa cells, the number of intracellular *vjbR* mutants, either Δ*vjbR* or *vjbR::mTn5*, was almost the same at 2 and 48 h post inoculation (p.i.) (Fig. 1A) suggesting a defective intracellular growth. This defect is also detected for Δ*vjbR* during infection of bovine macrophages (Fig. S2A in *Supplementary material*). In both cell lines, the expression *in trans* of *vjbR* in the Δ*vjbR* strain (Δ*vjbR*c) restores intracellular growth to WT level (Fig. 1A and Fig. S2A in *Supplementary material*). The complementation was also effective for the mutant *vjbR::mTn5* (data not shown).

A gentamicin assay demonstrated that the *vjbR::mTn5* and *virB2::mTn5* strains are not less invasive than the WT in HeLa cells in contrast to a *bvrR* mutant used as control for invasion defect (Sola-Landa *et al*., 1998) (Fig. S2B in *Supplementary material*).

The intracellular growth defect might result from either a lowered multiplication rate or an increased susceptibility to killing. To discriminate between these events, we evaluated the ability of Δ*vjbR*, Δ*vjbR*c and parental strains to replicate intracellularly by estimating the number of intact intracellular bacteria at 48 h p.i. in macrophages. The majority of cells infected with Δ*vjbR* mutants contains less than 10 intact intracellular bacteria. Cells infected with WT or Δ*vjbR*c strain contain a very large (uncountable) number of intracellular bacteria (Fig. 1B). In cell monolayers infected with the Δ*vjbR* strain, no bacterial debris were observable. Taken together, these results suggest that VjbR is involved in intracellular replication of *B. melitensis* 16M.

To determine whether the defective intracellular replication of *vjbR* mutants is correlated with *in vivo* attenuation, we infected groups of four BALB/c mice with the Δ*vjbR* and the WT parental 16M strains. After 1 week of infection, the Δ*vjbR* mutant was recovered from spleens at markedly lower levels (2 log) than the isogenic parental strain (Fig. 1C). By 4 and 10 weeks p.i., the number of Δ*vjbR* was three orders of magnitude lower than the WT. Moreover, two out of four mice and all mice have cleared the Δ*vjbR* strain from their spleen after 10 and 14 weeks of infection respectively. Thus, the *B. melitensis* VjbR protein is critically required for the infection in this mouse model, and the basis for the attenuation of the Δ*vjbR* mutant is likely to be its defect in intracellular survival.

The maturation of BCVs into ER-derived organelle depends on a functional VjbR

The impaired intracellular replication of the *vjbR* mutant

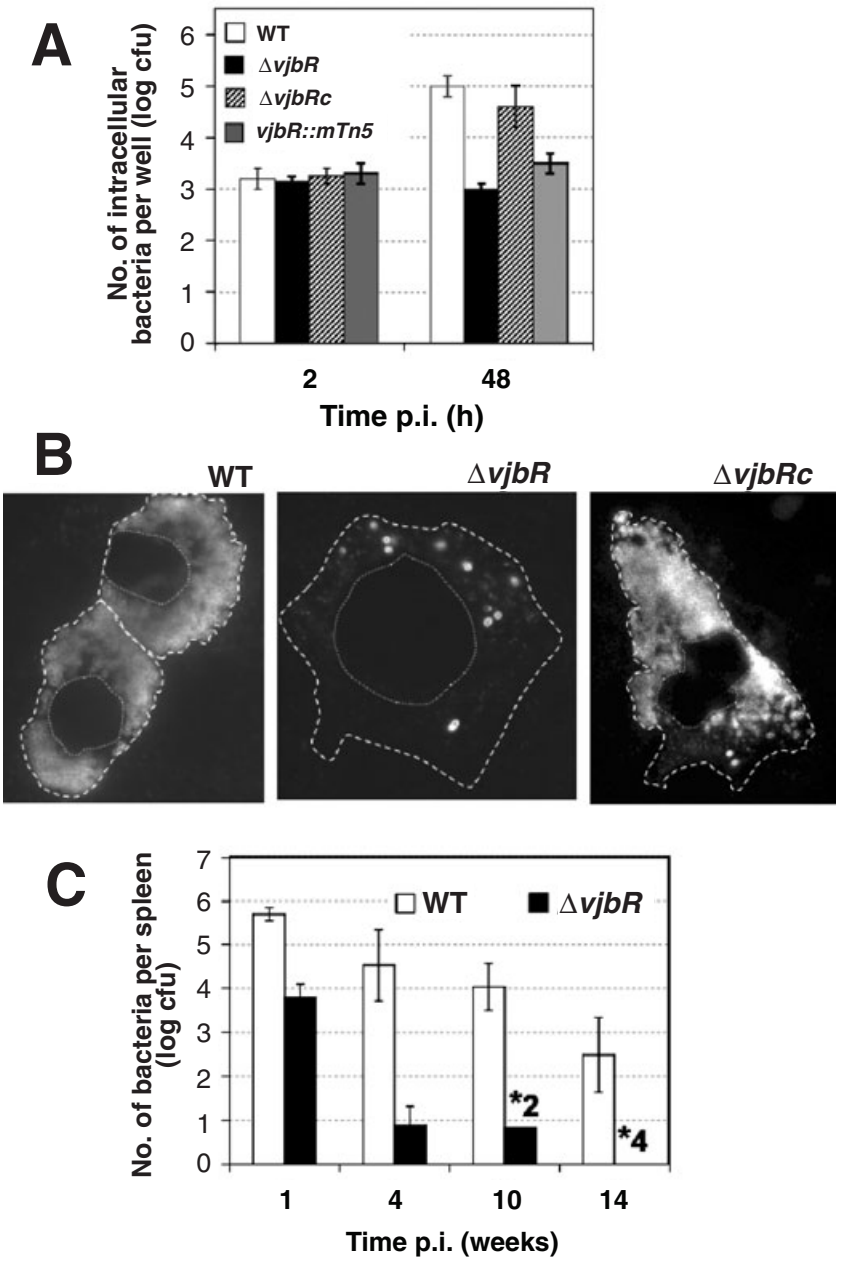

Fig. 1. Virulence of *B. melitensis* 16M *vjbR* mutant.
A. Intracellular replication of *B. melitensis* 16M Nal^R (WT), *vjbR* mutant (Δ*vjbR* or *vjbR::mTn5*) and complemented *vjbR* mutant (Δ*vjbR*c) in HeLa cells or bovine macrophages. Data are the averages of log number of colony-forming units (cfu) per well ± standard deviation (SD) ($n = 3$) from a representative experiment.
B. Intracellular growth phenotypes of WT, Δ*vjbR* and Δ*vjbR*c in bovine macrophages cells at 48 h p.i. The bacterial cells are detected using fluorescence microscopy (see *Experimental procedures*). The same experiments performed with HeLa cells instead of macrophages gave similar results (data not shown).
C. Accelerated clearance of the *vjbR* mutant from experimentally infected BALB/c mice. Mice were infected by intraperitoneal injection with 2×10^4 brucellae. Data are the averages (log number of cfu per spleen) ± SD ($n = 4$). The stars indicate the number of mice that cleared the splenic infection, at 10 and 14 weeks post infection.

(Fig. 1B) could result from an inability either to reach the replicative compartment or to multiply within it. We therefore focused on defining the nature of the *vjbR::mTn5*-containing vacuoles in HeLa cells at 24 h p.i. At this time, *Brucella* has normally reached its ER-derived replicative compartment (Pizarro-Cerda *et al.*, 1998; Delrue *et al.*, 2001; Celli *et al.*, 2003). The sec61β marker is used to identify ER compartments, and LAMP-1 is a marker for lysosomal vesicules. As WT strains replicate in sec61β+ and LAMP-1– compartments while *virB* mutants are blocked in sec61β+ and LAMP-1+ vacuoles (Pizarro-Cerda *et al.*, 1998; Celli *et al.*, 2003), the LAMP-1 marker was also used to characterize the *vjbR* mutant trafficking.

At 24 h p.i., the *vjbR::mTn5* strain was localized in compartments positive for sec61β (69 ± 9%) (Fig. 2A) and LAMP-1 markers (91 ± 1%) (Fig. 2B). In contrast, the majority of WT BCVs positive for sec61β (95 ± 1%) were negative for the LAMP-1 marker (only 7 ± 5% were positive) (Fig. 2B). No colocalization of Brucellae either with the EEA1 (an early endosomal marker), the Ci-M6PR (a late endosomal marker) or the cathepsin D (a lysosomal marker) was detected at 24 h p.i. whatever the strain tested (data not shown), suggesting that LAMP-1 found on *vjbR::mTn5* BCVs did not originate from interactions with endocytic pathway and could be the intermediate BCVs described by Celli *et al.* (2003).

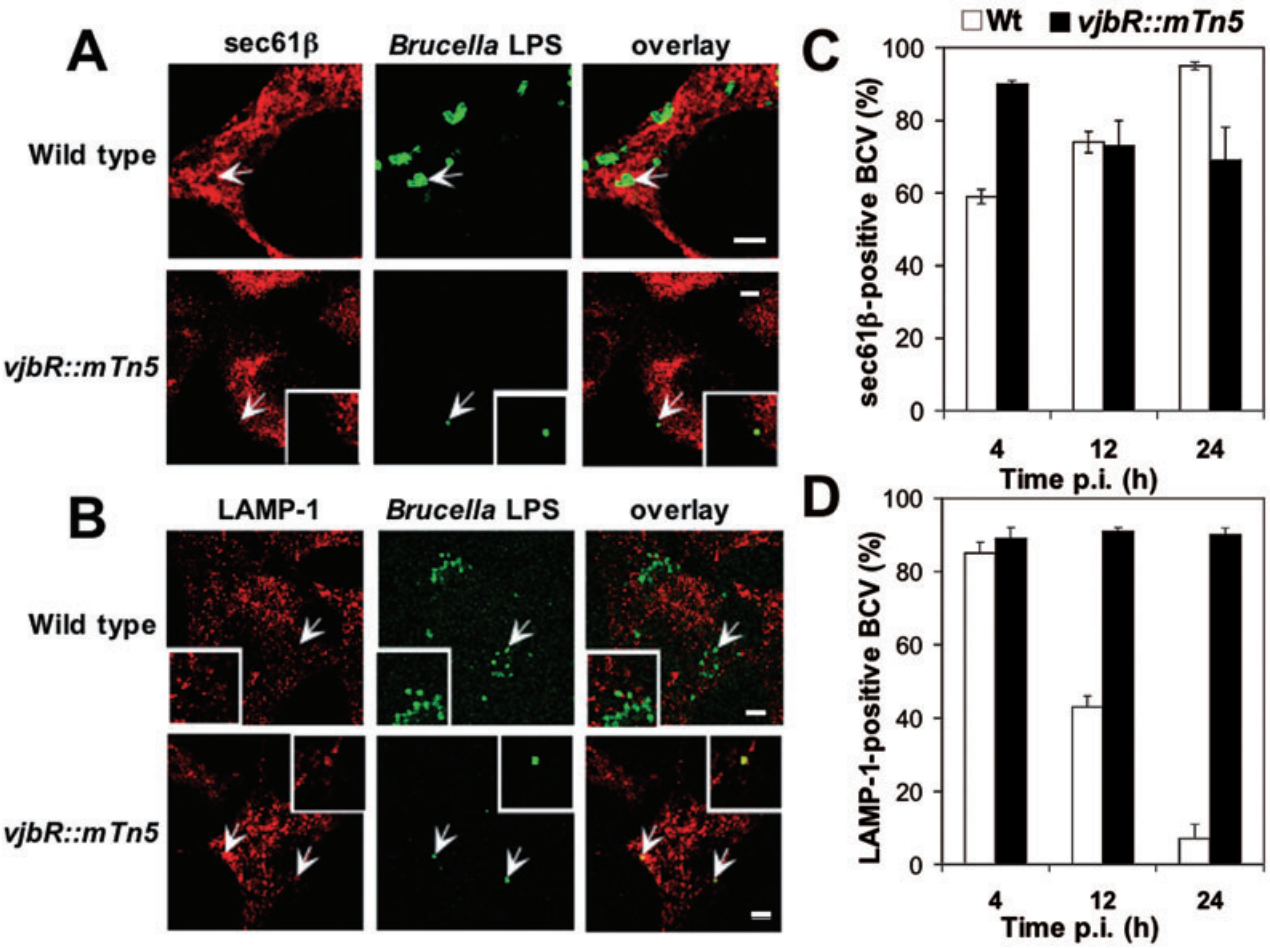

Fig. 2. The *vjbR::mTn5* containing vacuoles are decorated by sec61β marker and LAMP-1. HeLa cells were infected with the *Brucella* WT and *vjbR::mTn5* strains for various times. Samples were fixed and processed for immunofluorescence as described in *Experimental procedures*.
A. Representative confocal image of the BCVs interaction with sec61β marker at 24 h p.i. Arrowheads indicate BCVs.
B. Representative confocal image of the BCVs interaction with LAMP-1 marker at 24 h p.i. Arrowheads indicate BCVs.
In (A) and (B), sec61β and LAMP-1 markers are coloured in red.
C. Quantification of BCV acquisition of sec61β+ structures.
D. Quantification of BCV acquisition of LAMP-1+ structures.
For (A) and (B), bars correspond to 5 μm. For (C) and (D), data are means ± SD of three independent experiments.

In order to more precisely define the nature of the *vjbR::mTn5* BCVs, we evaluated their acquisition of the sec61β and LAMP-1 markers at various time points after infection. At 4 h p.i., we observed that the majority of vacuoles containing WT or *vjbR::mTn5* strain were sec61β+ and LAMP-1+ (Fig. 2C and D). However, although the percentage of LAMP-1+ WT BCVs decreased at later times of infection as expected (Pizarro-Cerda *et al*., 1998; Celli *et al*., 2003), vacuoles containing the *vjbR::mTn5* strain remained positive (Fig. 2D). This indicates that *vjbR::mTn5* is deficient in controlling vacuole maturation in HeLa cells after 4 h of infection as previously described for *virB2*, *virB4* and *virB9* mutants in epithelial cells (Delrue *et al*., 2001). This is in agreement with the replication deficiency of the *vjbR* mutants.

Inactivation of vjbR *downregulates the transcription of the T4SS and flagellar genes of* B. melitensis *16M*

The similar phenotypes of *vjbR* and *virB* mutants and the homology of VjbR with QS-related transcriptional regulators suggest that VjbR may be an activator of *virB* expression, required during a cellular infection. In addition, based on the synteny between *B. melitensis* and *S. meliloti* genomic flagellar loci (Fig. S1B in *Supplementary material*), it was tempting to speculate that in the same way as VisR, which is a global regulator of flagellar genes in *S. meliloti* (Sourjik *et al*., 2000), VjbR could be a transcriptional regulator of flagellar genes in *B. melitensis*. The *fliF* promoter is an interesting target in this context, as *fliF*, coding for the MS-ring, is a class II gene in the

flagellar cascade of gene expression (Aldridge and Hughes, 2002). The *flgE* gene product (flagellar hook monomer) was used for monitoring the production of class III proteins.

The activity of both *virB* and *fliF* promoters is known to be regulated in a density-dependent fashion during *Brucella* vegetative growth (Boschiroli *et al*., 2002; Rouot *et al*., 2003; Fretin *et al*., 2005). The contribution of VjbR to this regulation was tested by following, along a growth curve in 2YT medium, the expression of both *virB* and *fliF* promoters in a Δ*vjbR* and a WT strains.

When *B. melitensis* 16M harbouring a *pfliF–lacZ* translational fusion (pBBCmpfliF-lacZ) is grown in 2YT broth, the putative *fliF* promoter is transiently induced during the early exponential phase (Fig. 3A). The peak of β-galactosidase activity is detected around 10 h (OD_{600}: 0.3). When the WT strain harbouring a *pvirB–luxAB* transcriptional fusion on a plasmid is grown in 2YT broth, the putative *virB* promoter is transiently induced later in the exponential phase (Fig. 3B). The peak of luciferase activity is detected around 20 h (OD_{600}: 1.5). In the Δ*vjbR* strain, the activity of both promoters was very strongly downregulated (Fig. 3A and B). These results were confirmed by Western blotting analysis of the abundance of VirB8 and FlgE proteins in Δ*vjbR*, Δ*vjbR*c and WT cells harvested at a phase of the growth curve in 2YT medium where the expression is maximal (OD_{600}: 0.3 or 1.5 for the respective detection of FlgE and VirB8). We observed that proteins VirB8 and FlgE of *B. melitensis* 16M are detected in *B. melitensis* 16M and in Δ*vjbR*c, but not in the Δ*vjbR* strain (Fig. 4A and B).

The activity of *virB* and *fliF* promoters are also known to be induced intracellularly (Fretin *et al*., 2005). Their expression by a Δ*vjbR* and a WT strain were also evaluated in the course of a cellular infection, using a *gfp* reporter gene. HeLa cells were infected with bacteria grown overnight in 2YT medium. At 1 h and 24 h p.i., infected cells were fixed, bacteria were labelled with Texas-Red-labelled anti-lipopolysaccharide (LPS) antibodies and examined by fluorescence microscopy. At 24 h p.i. and whatever the promoter tested, green fluorescent protein (GFP) was detected in a large number of replicating WT *Brucella*, but GFP was never observed in intracellular Δ*vjbR* (Fig. S3 in *Supplementary material*) suggesting that, also intracellularly, VjbR is a transcriptional activator of both *virB* operon and *fliF* gene.

The C_{12}-HSL produced by B. melitensis *16M regulates expression of flagellar genes*

Sequence analysis suggested that VjbR contains both an AHL- and a DNA-binding domain as described for AHL-dependent QS regulators (Fig. S1A in *Supplementary material*), and data presented here suggest that VjbR directly or indirectly activates the transcription of *virB* operon and flagellar genes. Actually, the deletion of this putative QS transcriptional activator has the same effect on the *virB* operon as the exogenous addition of C_{12}-HSL to a culture of the WT strain (Taminiau *et al*., 2002). On these basis, we postulated that VjbR is the transcriptional regulator mediating the C_{12}-HSL effect on *virB* expression. Accordingly, the VirB8 protein was less abundant when WT bacteria were grown in a medium containing C_{12}-HSL (Fig. 4C), confirming data previously obtained at the transcriptional level (Taminiau *et al*., 2002).

If VjbR is the transcriptional regulator mediating the C_{12}-

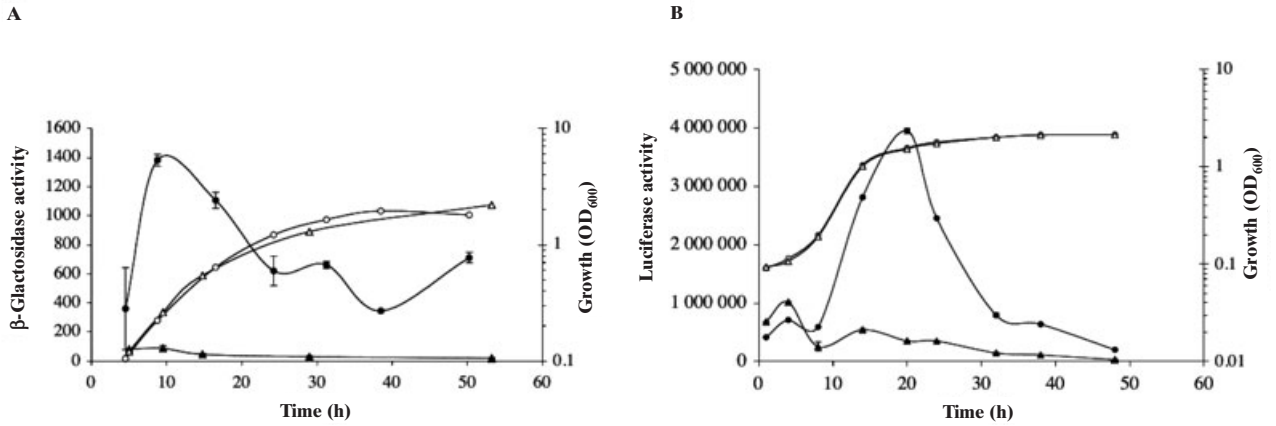

Fig. 3. Activities of *fliF* and *virB* promoters during vegetative growth of *B. melitensis* in rich medium.
A. The activity of *fliF* promoter was followed using β-galactosidase activity expressed as mean ± SD of three replicates (black forms). WT (circle) and Δ*vjbR* (triangle) bearing pBBCmpflif-lacZ were grown in 2YT and cell density was determined for each culture when β-galactosidase activity was evaluated (open forms).
B. The activity of *virB* promoter was followed using luciferase activity (black forms). WT (circle) and Δ*vjbR* (triangle) bearing pJD27-virB were grown in 2YT and cell density was determined for each culture when luciferase activity was evaluated (open forms).
These data are representative of three independent experiments.

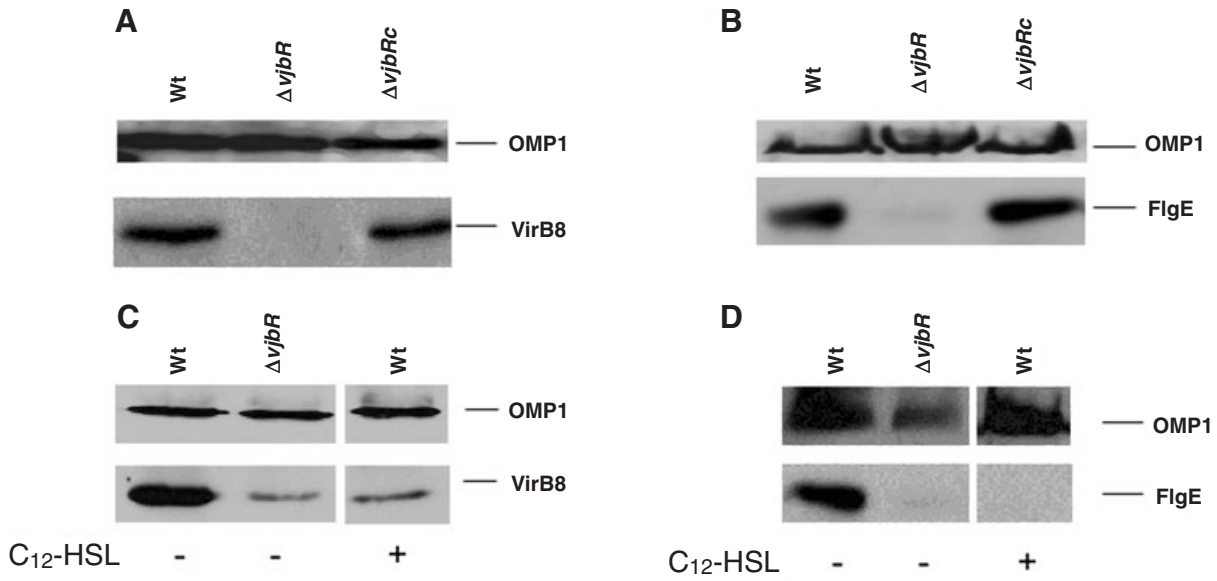

Fig. 4. Contribution of VjbR and C_{12}-HSL to the regulation of the abundance of T4SS and flagellar proteins. VirB8 (A) and FlgE (B) production in bacterial cells was evaluated by SDS-PAGE analysis of cell lysates, followed by Western blotting with VirB8-specific and with FlgE-specific antisera respectively. The VirB8 and FlgE proteins were detected at their expected size (31.5 kDa and 41 kDa respectively) (Rouot *et al*., 2003). OMP1 production was used to normalize total protein content using an anti-OMP1 monoclonal antibody. Western blotting was also used to monitor the effect of pheromone on VirB8 (C) and FlgE (D) abundance in the wild type (WT) and Δ*vjbR* strains. The strains were grown in rich medium in the presence (+) or in the absence (–) of 5 μM of synthetic C_{12}-HSL.

HSL effects, the flagellar genes expression should also be C_{12}-HSL dependent as this expression is also controlled by VjbR (Fig. 4B and Fig. S3 in *Supplementary material*). As expected, the abundance of flagellar proteins (FlgE in Fig. 4D) is strongly reduced when C_{12}-HSL are added to the culture medium, suggesting that the effect of this AHL is indeed mediated by VjbR.

Discussion

In this report, we describe the identification and the characterization of a LuxR-related transcriptional regulator (VjbR) essential for the *in vitro* and *in vivo* expression of two major virulence factors of *B. melitensis*: the T4SS and the flagellum. During vegetative growth in 2YT medium (referred here as rich medium) the transient expression of flagellar genes in early log phase (Fretin *et al*., 2005) and of *virB* operon in late log phase (Sieira *et al*., 2000; 2004; Rouot *et al*., 2003; den Hartigh *et al*., 2004) is almost abolished in a *VjbR* mutant background (Fig. 3A and B). The attenuation of the *vjbR* mutant in cellular model of infection (Fig. 1A and B and Fig. S2 in *Supplementary material*) is certainly not linked to its inability to express flagellar genes because neither the internalization nor the replication in cells was affected in flagellar mutants (Fretin *et al*., 2005). Actually, a *vjbR* mutant displays the same behaviour as *virB* mutants during a cellular infection, i.e. they avoid the phagolysosomal fusion and are blocked at a similar stage during the maturation of their respective BCVs being unable to exclude the LAMP-1 marker from the vesicle (Fig. 2B and D) (Delrue *et al*., 2001). The behaviour of *vjbR* mutant in BALB/c mice is also indicative for its role as global regulator. It is attenuated by more than 3 log at 4 weeks after infection as compared with the WT and almost cured from the spleen at later time points (10 and 14 weeks p.i.) (Fig. 1C). These data are coherent with the described attenuation of *virB* (den Hartigh *et al*., 2004) and flagellar mutants (Fretin *et al*., 2005) at similar times p.i. The nearly 2 log attenuation of *vjbR* mutant at 1 week p.i. (Fig. 1C) cannot be attributed to a downregulation of flagellar genes because flagellar mutant are not attenuated at this stage of infection (Fretin *et al*., 2005). The question whether this attenuation at 1 week could be linked to a defect in *virB* expression is questionable. Actually, *virB* mutants have never been isolated from STM screens in acute phase of infection in mice (Lestrate *et al*., 2003) but only by STM screen at later time point (Hong *et al*., 2000).

In the most widespread QS system of Gram-negative bacteria the activity of LuxR type regulators are modulated by binding to an AHL (Luo and Farrand, 1999; Egland and Greenberg, 2000; Fuqua *et al*., 2001). The expression of complex bacterial surface structures such as flagellum and bacterial conjugation system is regulated by QS in *S. meliloti* and *Agrobacterium tumefaciens* respectively (Piper *et al*., 1993; Sourjik *et al*., 2000; Anand

and Griffiths, 2003; Falcao *et al*., 2004). We have recently described a C_{12}-HSL produced by *B. melitensis* 16M (Taminiau *et al*., 2002). While direct binding of this C_{12}-HSL by the VjbR regulator described here remains to be confirmed, this is quite likely as: (i) the VjbR-deduced sequence contains the conserved residues critical for AHL binding in other QS regulators (Fig. S1A in *Supplementary material*); (ii) the complementation of the Δ*vjbR* strain by a *vjbR*D82A allele with a mutation in a conserved residue critical for AHL binding or a *VjbR* allele lacking the predicted AHL-binding domain results in a lack of repression by C_{12}-HSL of *virB* expression (S. Bonnot, unpublished results) and (iii) the C_{12}-HSL downregulates expression of *virB* and flagellar genes (Fig. 4C and D) (Taminiau *et al*., 2002). The above data suggest that C_{12}-HSL inhibits the function of VjbR probably through a change of its oligomerization state, as observed for many other LuxR homologues (Qin *et al*., 2000; Kiratisin *et al*., 2002; Ledgham *et al*., 2003). What makes this *Brucella* QS regulation peculiar among other described systems is the fact that VjbR appears to be an activator in the absence of AHL (meaning that AHL has a repressive effect). Actually, the LuxR-related regulators are described either as activator only in the presence of AHL (e.g. TraR in *A. tumefaciens*; Qin *et al*., 2000) or as repressor inactivated by AHL (i.e. EsaR in *Pantoea sterwatii*; Minogue *et al*., 2002; von Bodman *et al*., 2003).

While the nature of the environmental signals that lead to the regulated expression of these two supramolecular surface structures during vegetative growth in rich medium is only partially resolved, both the *virB* promoter and the *fliF* promoter have a common denominator: they are activated by VjbR and repressed by C_{12}-HSL (Fig. 4). Whether VjbR is acting directly by binding to these promoters or indirectly by modulating another regulator is presently unknown. But whatever the situation may be, the differential expression of these promoters should be explained by additional different regulatory inputs. How do these data fit with the available literature? The expression of the *virB* operon has been recently described as subjected to a complex regulatory network encompassing both activation and repression events. In rich medium the transcription of *virB* is strongly downregulated in the absence of integration host factor (IHF) binding (Sieira *et al*., 2004). Combined with our data, this fact suggests that the activity of VjbR is dependent on IHF which is a bending factor acting on the *virB* promoter topology to allow transcription to proceed. The timing of expression of *virB* in late exponential phase could then be related to the increasing expression of IHF-encoding genes preparing the starvation response of the cells in stationary phase as described in other bacteria (Ali Azam *et al*., 1999). This hypothesis is coherent with the observation that in minimal medium *virB* is expressed in early exponential phase (Boschiroli *et al*., 2002; Sieira *et al*., 2004) and that the alarmone of the stringent response ppGpp, known to control the expression and the activity of IHF (Aviv *et al*., 1994), is crucial for *virB* expression (M. Dozot and S. Köhler, pers. comm.). Moreover, recent evidence suggests that QS and starvation sensing pathways converge to regulate the entry of cell into the stationary phase in other bacteria (Lazazzera, 2000).

Also in agreement with this hypothesis, we know that a transcriptional fusion between *pvjbR* and a *luxAB* reporter gene is already expressed in early exponential phase (our unpublished data) and that VjbR is in fact present at this stage as attested by the expression of the promoter of *fliF*, the flagellar MS gene (Fig. 3A).

Accordingly, the activator role of VjbR on the *fliF* promoter in early exponential phase is predicted to be less dependent on the IHF activity. In fact, the flagellation cascade is regulated both by environmental factors and by growth phase in other bacteria (Soutourina and Bertin, 2003). Nevertheless in α-Proteobacteria the nature of the transcriptional regulator found at the very top of the hierarchy (class I gene) of the flagellar regulon differs from the systems found in β- or γ-Proteobacteria. In *Caulobacter crescentus*, CtrA, a response regulator regulating the asymmetric cell cycle, is also the class I gene controlling the flagellar cascade (Soutourina and Bertin, 2003). While the asymmetry and the CtrA phosphorelay are conserved in Rhizobiaceae (*Agrobacterium*, *Brucella*, *Mesorhizobium*, *Sinorhizobium*), CtrA-binding boxes have not been identified in the promoter of flagellar class II gene (i.e. *fliF*) in these genus (Hallez *et al*., 2004). Actually, a peculiar flagellar hierarchy was identified in *S. meliloti* (Sourjik *et al*., 2000). At the top of the hierarchy is the master operon *visRN*, encoding the LuxR-related regulators VisR and VisN that control class II genes (basal body and motor genes) in this organism. The *visNR* operon is located downstream and in reverse orientation of a set of flagellar genes and this organization is completely conserved in *Brucella* species (see Fig. S1B in *Supplementary material*). Nevertheless, in *Brucella*, among the two regulators found at this locus only VjbR belong to the LuxR family (but displays only 22% identity with VisR). The second regulator is of the TetR family and its function is presently unknown. This difference in regulator should be linked to the difference in lifestyle between the symbiot *S. meliloti* and the animal pathogen *Brucella* leading to a difference is environmental challenging signals these bacteria have to cope with.

Brucella could constitute a second example where the initial regulator of the flagellar cascade could be of the LuxR type, VjbR. Indeed, here we show that a Δ*vjbR* strain does not express the *fliF* gene and does not produce the FlgE protein, which are likely to be class II and class III components in the flagellar hierarchy.

In addition to VjbR, the QS-related regulator described here, the intricate regulatory network controlling the expression of both the flagellar and the T4SS apparatus will obviously involve other, yet unidentified, regulatory partners (i.e. other transcriptional regulators, sigma factors, histone-like proteins, etc.) as described in other bacteria (Ali Azam *et al*., 1999; Soutourina and Bertin, 2003). For example, the activity of the *pfliF* promoter is turned off almost at a stage in the growth curve where the T4SS is turned on, meaning that VjbR is still able to activate *virB* transcription. This could indicate that, in addition to VjbR, regulation of the flagellar cascade is depending on another growth phase-dependent regulator.

In the future and because other QS-related systems appears to be involved in global signalling (Withers *et al*., 2001), it will be interesting to get further insight into the VjbR regulon and to decipher how the QS system of *Brucella* is switched on and interplays with other global regulatory systems not only during vegetative growth but also intracellularly. In this later case, VjbR could be playing a role in 'diffusion sensing' (Redfield, 2002), when *Brucella* is confined in its ER-derived replicative compartment.

Experimental procedures

Bacterial *strains and media*

Brucella strains used in this study were derived from a spontaneous nalidixic acid-resistant mutant of the *B. melitensis* 16M strain obtained from A. Macmillan, Central Veterinary Laboratory, Weybridge, UK. *B. melitensis* 16M strains were grown in 2YT medium (10% yeast extract, 10 g l^{-1} tryptone, 5 g l^{-1} NaCl). When necessary, antibiotics were added to a final concentration of 50 μg ml^{-1} ampicilin and/or kanamycin, 20 μg ml^{-1} chloramphenicol and 25 μg ml^{-1} nalidixic acid. The *Escherichia coli* strains used in this study, DH10B (Gibco BRL) and S17-1 (Simon *et al*., 1983), were grown on Luria–Bertani (LB) medium with appropriate antibiotics. Synthetic C_{12}-HSL (N_Lauroyl-DL-homoserine, Sigma Aldrich) was prepared in acetonitrile (ACN) and added to bacterial growth media at 5 μM final concentration. The same volume of ACN was used as negative control.

Molecular techniques

DNA manipulations were performed according to standard techniques (Ausubel *et al*., 1991). The mini-Tn5 mutant strains (*vjbR::mTn5* and *virB2::mTn5*) were selected from the transpositional library described previously (Delrue *et al*., 2001).

A *vjbR* knockout mutant of *B. melitensis* 16M (Δ*vjbR*) was constructed by gene replacement. Briefly, upstream and downstream regions flanking *vjbR* were amplified from *B. melitensis* 16M NalR genomic DNA by the following primer pairs: (i) Fam (5′-ggATCCAATCCCTTCAggCgATgA-3′) and Ram (5′-CCTTggTgATgAAACCATg-3′); (ii) Fav (5′-AAgCTTTATCCgggATgTCgTTTCTg-3′) and Rav (5′-CCCgAggTACAgCATCTC-3′). The two polymerase chain reaction (PCR) products were first subcloned into pSKoriT plasmid (Tibor *et al*., 2002) in *Bam*HI–*Sma*I and *Eco*RV sites, respectively, to generate pSKoriT-vjbRamont and pSKoriT-vjbRaval. The *aph* cassette was cloned into the *Eco*RI site in pSKoriT-vjbRamont to generate pSKoriT-vjbRamont-*aph.* The vjbRamont-*aph* fragment was excised with *Spe*I and *Eco*RV restriction enzymes and inserted into *Spe*I–*Sma*I sites in the pSKoriT-vjbRaval to generate the final plasmid pSKoriT-Δ*vjbR*.

For the construction of the complementation plasmid, the *vjbR* CDS (BMEII1116 in *B. melitensis* 16M genome sequence, GenBank file: NC_003318, peptidic sequence Accession No. AAL54358) was amplified by PCR from *B. melitensis* 16M genomic DNA with primers VjbR1 (5′-TCATTgCTCgAgAgCCCg CATggTTTCATCA-3′) and VjbR2 (5′-ATgCTTggATCCTCCgCgC gACCATgTTgAT-3′) and cloned into the *Eco*RV site into pBBR4MCS plasmid (Kovach *et al*., 1994), downstream of the *lac* promoter generating the pBBR-vjbR plasmid.

Construction of plasmid p*virB–luxAB* transcriptional fusion (pJD27-*pvirB*). In order to amplify a 508 bp fragment upstream of *virB* operon, a PCR was carried out using primer pvir1 (5′-ATAAgCTTTCACCggCTAgCTGA-3′) and pvir2 (5′-CAAAgCT TAggATCgTCTCCTTCTCAgA-3′). The PCR product was cloned into pGEMT-easy (Promega), generating the plasmid pGEMT-easy *pvirB*. By using PGEMT-easy *pvirB* as template, a PCR was performed with the primers: M13R (5′-GGAAACAGCTATGAC CATG-3′) and M13F (5′-GTTTTCCCAGTCACGACG-3′) generating 0.762 kb PCR product containing the *virB* promoter. This PCR product has been digested with *Spe*I restriction enzyme yielding two fragments of 0.126 and 0.636 kb. The 0.636 kb fragment corresponding to the *virB* promoter has been ligated to the *Eco*RV and *Spe*I sites of pJD27 (Dr Essenberg, Department of Biochemistry, Oklahoma State University) generating pJD27-*pvirB*.

The suicide plasmid pFTvirB was constructed as follow: 0.5 kb of the *virB* promoter region was amplified by PCR with the pair of primers pVirBm (5′-AgTACTTCACCggCTAgCTgAAATC-3′) and VFTvirB (5′-TCATAggATCgTCTCCTTCT-3′). The PCR product was cloned into the *Eco*RV site of the pSKoriTcatgfp2 plasmid (J.-M. Delroisse and X. DeBolle, unpublished data). pFTvirB construction was introduced by conjugation into *B. melitensis* 16M.

The plasmids pBBpflif-gfp and pBBCmpflif-lacZ were constructed by Fretin *et al*. (2005).

All plasmids were conjugatively transferred into *B. melitensis* 16M NalR strains. Plasmid insertion and gene replacement were validated by Southern blot analysis (data not shown).

Measurement of luciferase and β-galactosidase activity

Luciferase assay. Bacterial strains were grown overnight in 2YT with aeration at 37°C. Cultures were centrifuged and bacterial pellets were resuspended and washed twice in 2YT. For each strain, 3×50 ml of cultures in 2YT at an initial optical density at 600 nm (OD_{600}) of 0.05 were incubated at 37°C with shaking.

At each sampling time, the OD_{600} was measured and 0.2 ml of samples were harvested. N-decanal substrate was added to a final concentration of 0.145 mM (stock concentration: 5.8 mM in Ethanol 50%). After 10 min, light production [Relative Luminescence Units (RLU)] was measured for 5 s using a luminometer

Microlumat LB96P (EG and Berthold). Luciferase activity is expressed as the RLU per OD_{600} at a given time point. Measures were performed in triplicate for each culture at various time p.i.

β-Galactosidase assay. β-Galactosidase assay was performed as described by Fretin *et al*. (2005).

Detection of VirB8 and FlgE by Western blotting

Strains were grown overnight in 2YT at 37°C with shaking. Bacterial cultures were diluted to OD_{600} 0.05 in 50 ml of 2YT and grown at 37°C. Samples (10 ml) from different times were inactivated for 2 h at 80°C. After standardizing for total protein content, samples were subjected to electrophoresis on 12% SDS-PAGE and transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech) by the semi-dry transfer technique. Immunodetection of proteins was performed with polyclonal anti-VirB8 (Rouot *et al*., 2003) or anti-FlgE (Fretin *et al*., 2005) antisera (diluted 2500 times) and with monoclonal anti-Omp1 antibody A53 10B2 (Cloeckaert *et al*., 1990) (diluted 1000 times) as a loading control. Bound antibodies were detected using chemiluminescence with peroxydase-conjugated secondary antibodies and the ECL Western blotting reagents RPN2209 as recommended by the manufacturer (Amersham Pharmacia Biotech).

Mice and cellular infections

Mice infection protocol is described by Fretin *et al*. (2005). To quantify the extent of bacterial survival in cellular model and to characterize intracellular BCVs, the infections of HeLa cells and bovine macrophages were performed as described previously (Delrue *et al*., 2001; Lestrate *et al*., 2003). A *BvrR* mutant was used as control for invasion defect (Sola-Landa *et al*., 1998) during the gentamicin assay (Delrue *et al*., 2001). *BvrR* encodes a regulator of a two-component system controlling outer membranes properties and virulence (Lopez-Goni *et al*., 2002).

Immunofluorescence techniques

Fluorescent antibodies. Rabbit polyclonal anti-human Lamp-1 (M. Fukada, The Burnham Institute, La Jolla, CA); rabbit polyclonal anti-cathepsinD (S. Kornfeld, Washington University School of Medecine, USA); rabbit polyclonal anti-sec61β (B. Dobberstein, Universität Heidelberg, Heidelberg, Germany) and anti-*Brucella* LPS O-chain monoclonal antibody 12G12 (Cloeckaert *et al*., 1993) were used. Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated anti-mouse immunoglobulin G (IgG), and Texas red (TxR)-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Immunotech, Marseille, France).

Intracellular trafficking. At different times after inoculation, coverslips were washed to remove non-adherent bacteria (five times with PBS) and fixed for 15 min in 3% paraformaldehyde pH 7.4 at room temperature. Immunostaining was performed as described in Delrue et al. (2001). Hela cells were observed on a Leyca TCS 4DA microscope using 100× oil immersion objective. The laser lines used were 488 nm (FITC) and 568 (TxR). To determine the percentage of bacteria which colocalized with the studied intracellular markers, a minimum of 100 intracellular bacteria was counted.

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Supplementary material

The following material is available for this article online:

Fig. S1. A. Multiple sequence alignment (CLUSTALW) between VjbR, CinR (*S. meliloti*), TraR (*A. tumefaciens*) and the consensus of the DNA-binding domain of LUXR type regulators (GerE). B. Synteny between *B. melitensis* 16M and *S. meliloti* 1021 in the genomic region containing the *vjbR* coding sequence (CDS).

Fig. S2. Virulence and internalization of *Brucella melitensis* 16M *vjbR* mutant.

Fig. S3. Contribution of VjbR to the regulation of *vibR* operon and flagellar genes.