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The stringent response mediator Rsh is required for *Brucella melitensis* and *Brucella suis* virulence, and for expression of the type IV secretion system *virB*

Marie Dozot,[1] Rose-Anne Boigegrain,[2,3] Rose-May Delrue,[1] Régis Hallez,[1] Safia Ouahrani-Bettache,[2,3] Isabelle Danese,[1] Jean-Jacques Letesson,[1] Xavier De Bolle[1] and Stephan Köhler[2,3]*

[1]*Research Unit in Molecular Biology (URBM), University of Namur, 61 rue de Bruxelles, B-5000 Namur, Belgium.*
[2]*Institut National de la Santé et de la Recherche Médicale, U-431, Montpellier F-34095, France.*
[3]*Université Montpellier II, Montpellier F-34095, France.*

Summary

Physiological adaptation of intracellular bacteria is critical for timely interaction with eukaryotic host cells. One mechanism of adaptation, the stringent response, is induced by nutrient stress via its effector molecule (p)ppGpp, synthesized by the action of RelA/SpoT homologues. The intracellular pathogen *Brucella* spp., causative agent of brucellosis, possesses a gene homologous to *relA*/*spoT*, named *rsh*, encoding a (p)ppGpp synthetase as confirmed by heterologous complementation of a *relA* mutant of *Sinorhizobium meliloti*. The Rsh deletion mutants in *Brucella suis* and *Brucella melitensis* were characterized by altered morphology, and by reduced survival under starvation conditions and in cellular and murine models of infection. Most interestingly, we evidenced that expression of *virB*, encoding the type IV secretion system, a major virulence factor of *Brucella*, was Rsh-dependent. All mutant phenotypes, including lack of VirB proteins, were complemented with the *rsh* gene of *Brucella*. In addition, RelA of *S. meliloti* functionally replaced *Brucella* Rsh, describing the capacity of a gene from a plant symbiont to restore virulence in a mammalian pathogen. We therefore concluded that in the intramacrophagic environment encountered by *Brucella*, Rsh might participate in the adaptation of the pathogen to low-nutrient environments, and indirectly in the VirB-mediated formation of the final replicative niche.

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*For correspondence. E-mail kohler@univ-montp2.fr; Tel. (+33) 4 67 14 42 38; Fax (+33) 4 67 14 33 38.

Introduction

In order to promptly react to variations in their environment, bacteria have developed complex mechanisms of regulation that allow rapid modulations of their cellular functions. Among these regulation systems is the so-called stringent response that enables bacteria to face starvation (Cashel *et al*., 1996; Chatterji and Ojha, 2001). This pleiotropic regulatory response is triggered by an alarmone called (p)ppGpp (guanosine tetra- or pentaphosphate), and results in rapid inhibition of stable RNA and ribosomal proteins synthesis, and in growth arrest. Stasis survival and stress defence are simultaneously stimulated, and several biosynthetic operons are activated. The way of action of (p)ppGpp is partially understood (Magnusson *et al*., 2005). The alarmone binds the RNA polymerase (RNAP) ββ′-subunits (Toulokhonov *et al*., 2001), resulting notably in alteration of competition between sigma factors for RNAP-binding, and therefore promoter selection (Jishage *et al*., 2002). The enzymes that synthesize (p)ppGpp can be either bifunctional, catalysing both (p)ppGpp synthesis and hydrolysis, as it is the case for SpoT protein of *Escherichia coli*, or in some cases monofunctional, acting as (p)ppGpp synthetase, like RelA in *E. coli* (Mittenhuber, 2001).

Recently, the stringent response was shown to be involved in the adaptation of several symbiotic or pathogenic bacteria to their intracellular lifestyle (Godfrey *et al*., 2002; Braeken *et al*., 2006). In *Sinorhizobium meliloti*, the stringent response is involved in the establishment of successful symbiosis, as ppGpp is required for nodule formation and regulation of succinoglycan production (Wells and Long, 2002; Wells and Long, 2003). In *Legionella pneumophila* (p)ppGpp synthesized by RelA triggers entry into stationary phase and expression of virulence-associated genes, promoting transmission to new host cells (Hammer and Swanson, 1999). It has also been shown that (p)ppGpp is required for *Salmonella typhimurium* invasiveness *in vitro* and virulence *in vivo*, and that it plays a critical role in the regulation of virulence genes expression (Pizarro-Cerda and Tedin, 2004).

Brucella is a Gram-negative intracellular pathogen belonging to the alpha subclass of *Proteobacteria* (Moreno *et al*., 1990). This bacterium is the etiologic agent of brucellosis, a worldwide zoonosis affecting a wide vari-

ety of mammals, and it is responsible for abortion in pregnant females and sterility of males. Brucellosis is endemic in the Mediterranean region, in Africa, Asia and South America where it causes important economic losses. Six recognized *Brucella* species were defined according to their host preferences (Corbel and Brinley-Morgan, 1984). The two *Brucella* species considered in this work are *Brucella melitensis* and *Brucella suis*; the first is frequently found in goats and sheep, and the second infects mainly pigs. Both species are also human pathogens causing Malta fever, a serious debilitating disease (Young, 1995).

Brucella invades and replicates in professional and non-professional phagocytes by inhibiting fusion between phagosome and lysosome, and by subsequently creating an intracellular niche inside which it replicates (Gorvel and Moreno, 2002; Celli *et al*., 2003). This deviation of classical intracellular trafficking depends on the presence of VirB, a type IV secretion system (T4SS) encoded in the genome of *Brucella* spp. (O'Callaghan *et al*., 1999; Comerci *et al*., 2001; Delrue *et al*., 2001; Celli *et al*., 2003). Although the nature of the effectors secreted by VirB is presently unknown, the regulation of *virB* expression has been studied. Several signals controlling *virB* expression have been identified, among which are a quorum-sensing system (Taminiau *et al*., 2002; Delrue *et al*., 2005), acid pH and nutrients availability (Boschiroli *et al*., 2002; Delrue *et al*., 2005). Moreover, recent studies showed that various metabolic pathways of *Brucella* are required for full virulence (Köhler *et al*., 2002; Köhler *et al*., 2003; Delrue *et al*., 2004). Together, these data led to the hypothesis that, during trafficking within the host cell, *Brucella* encounters a nutrient-poor environment which may trigger *virB* expression. The Rsh (for RelA/SpoT homologue) protein identified in *Brucella* appears to be a good candidate to sense intracellular nutrient availability and to regulate *virB* expression. Indeed, transposon mutants in *rsh* were isolated in two intracellular screens for attenuated mutants, suggesting that a functional *rsh* gene is essential for *Brucella* virulence (Köhler *et al*., 2002; Kim *et al*., 2003; Kim *et al*., 2005).

In this report, we analysed the phenotypes of *rsh* deletion mutants in *B. melitensis* 16M and in *B. suis* 1330, and of an overexpression mutant of *rsh* in *B. melitensis* 16M. Our results clearly demonstrated that Rsh played a critical role in *Brucella* replication in mammalian cells and in the BALB/c murine model. Moreover, *virB* expression was strongly modified either in an *rsh* deletion mutant background or in an *rsh* overexpression mutant. This suggested that Rsh, possibly by sensing nutrient-poor conditions, contributes to VirB production, hence allowing *Brucella* to establish its intracellular replicative niche. In addition, we observed reciprocal heterologous complementation between *Brucella* and the plant symbiont *S. meliloti*, restoring virulence of the first, and stringent response in the latter. Therefore, stringent response may be a common mechanism used by symbionts and pathogens to adapt to their respective intracellular niches.

Results

The rsh *gene in the genus* Brucella

The genomic sequencing of *Brucella* species – *B. melitensis* 16M (DelVecchio, 2002) and *B. suis* 1330 (Paulsen *et al*., 2002) – allowed us to identify a unique *relA/spoT* homologue called *rsh*. The *rsh* gene is predicted to encode a 751 amino acids protein with a molecular mass of 85 kDa. Homology analysis suggested that Rsh may be bifunctional, because critical residues for (p)ppGpp synthetase and (p)ppGpp hydrolase activities were conserved (Hogg *et al*., 2004) (see Fig. S1). *B. melitensis* 16M and *B. suis* 1330 Rsh proteins were nearly identical (99% identity), and slightly more similar to SpoT (36% identity) than to RelA (28% identity) of *E. coli*. Rsh protein of *Brucella* showed significant homology to the RelA/SpoT proteins of alphaproteobacteria, and particularly to the RelA/SpoT homologue of *S. meliloti* (70% identity) (see Fig. S1).

The Brucella rsh *mutants display phenotypes expected for (p)ppGpp° mutants*

To analyse the role of Rsh in mediating *Brucella*'s stringent response, two deletion mutants of the *rsh* gene, Δrsh_{Bm} and Δrsh_{Bs}, were created in *B. melitensis* 16M and in *B. suis* 1330 respectively. The mutant Δrsh_{Bm} was constructed by replacing an internal fragment of *rsh* by the non-polar *aphA4* allele (see *Experimental Procedures*). The Δrsh_{Bs} was generated by truncating the *rsh* gene using a *sacB* suicide vector (see *Experimental Procedures*). Non-polar deletion techniques were used, because *rsh* gene is encoded in a putative operon immediately upstream of the *pyrE* gene.

Using scanning electron microscopy, we observed that Δrsh_{Bm} mutant presented morphological abnormalities in comparison to the wild-type strain when cultured in bacteriological medium, as a high proportion of the bacteria was branched or unusually swollen (Fig. 1A). The same observations were made for the Δrsh_{Bs} mutant (data not shown). This phenotype, previously described as the result of cell cycle defects, was also observed for (p)ppGpp° mutants in *E. coli* (Cashel *et al*., 1996), in *Campylobacter jejuni* (Gaynor *et al*., 2005), and more recently, in *Brucella abortus* (Kim *et al*., 2005).

Given that in many bacteria stringent response is directly linked to nutrient availability, we then tested the ability of Δrsh mutants to survive under starvation conditions. As expected, Δrsh_{Bs} was unable to grow in Ger-

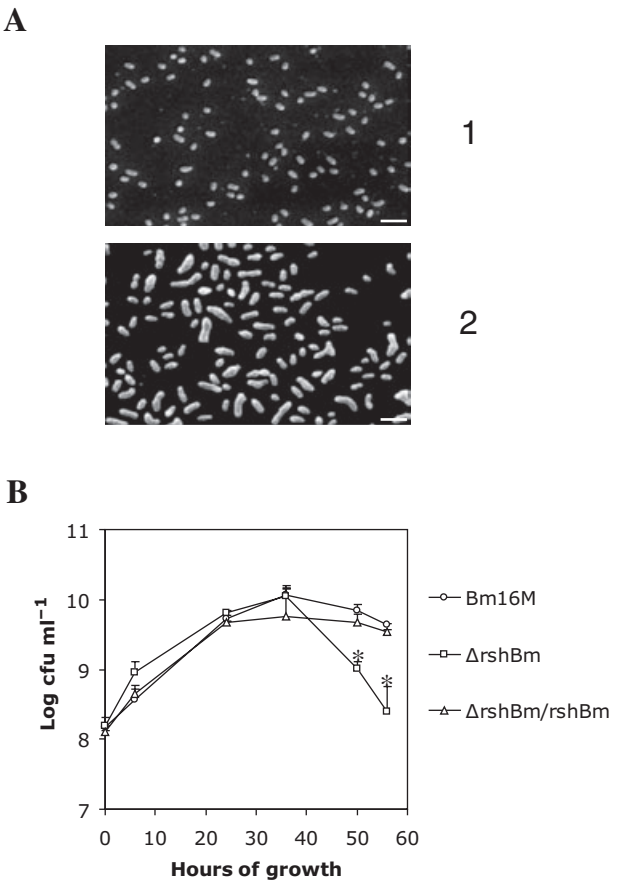

Fig. 1. A. Morphological aberrancies resulting from *rsh* deletion. *B. melitensis* 16M wild-type (1) and the *rsh* deletion mutant Δrsh_{Bm} (2) cultivated in 2YT complex medium were prepared for scanning electron microscopy. Bars: 2 μm.
B. Stationary phase survival of *B. melitensis* 16M (+pMR10*cat*), Δrsh_{Bm} (+pMR10*cat*) and Δrsh_{Bm} /rsh_{Bm} in 2YT complex medium. Strains were grown in liquid culture overnight, and diluted to 1×10^8 bacteria ml^{-1}. Concentrations of live bacteria ml^{-1} were determined at different times by plating serial dilutions. Values are the means [log number of colony-forming units (cfu) ml^{-1} of culture] ± SD ($n = 3$) of one representative experiment performed in triplicate. The asterisk means significant for $P < 0.001$. The *P*-value was calculated by one-way ANOVA analysis (Scheffé, 1959).

hardt's modified minimal medium, in contrast to the wild-type and the complemented mutant strains (data not shown). Moreover, Δ*rsh* mutants rapidly died upon entry in stationary phase, another phenotype shared with (p)ppGpp° mutants (Gaynor *et al*., 2005). Indeed, in contrast to the wild-type strain *B. melitensis* 16M and the complemented mutant $\Delta rsh_{Bm}/rsh_{Bm}$, Δrsh_{Bm} was unable to persist during stationary phase, presumably because of nutrient limitation occurring during this growth phase (Fig. 1B). Similar results were obtained for the Δrsh_{Bs} mutant (data not shown). These observations, all consistent with the absence of (p)ppGpp synthesis in the Δ*rsh* mutants, allowed us to consider Rsh as a good candidate for mediating stringent response in *Brucella*.

The product of rsh *restores the stringent response in the* relA *mutant of* S. meliloti

The functional role of the *Brucella rsh* gene product as a (p)ppGpp synthetase was demonstrated by heterologous complementation of the *relA* mutant DW186 of *S. meliloti* (Wells and Long, 2002). The (p)ppGpp production in *S. meliloti* by Rsh from *B. melitensis* was indirectly evidenced by using a plate assay which consists in reporting (p)ppGpp-dependent induction of histidine biosynthesis (Gropp *et al*., 2001; Wells and Long, 2002). In this assay, growth of the bacterium on a minimal medium depleted for histidine, and in the presence of aminotriazole (3-AT, an inhibitor of histidine biosynthesis), is possible only when enough (p)ppGpp is produced. In contrast to the wild-type strain, the *relA* mutant of *S. meliloti*, deficient in (p)ppGpp synthesis, cannot grow on this medium because it is not able to induce histidine biosynthesis (Fig. 2). The constitutive expression of rsh_{Bm} or $relA_{Sm}$ in the *S. meliloti relA* mutant restored growth of this strain on a medium lacking histidine in the presence of 3-AT (Fig. 2). This result indicated that the rsh_{Bm} gene was able to induce the stringent response in *S. meliloti*, most likely via (p)ppGpp synthesis, and to enhance histidine biosynthesis. Therefore, these data strongly suggested that the *Brucella rsh* gene encoded a functional (p)ppGpp synthetase.

Rsh is required for Brucella *replication in cultured cells and for survival in the murine BALB/c model of infection*

A previous study, aiming at the isolation of transposon mutants of *B. suis* defective for intracellular replication in macrophages, identified *rsh* as an important factor for

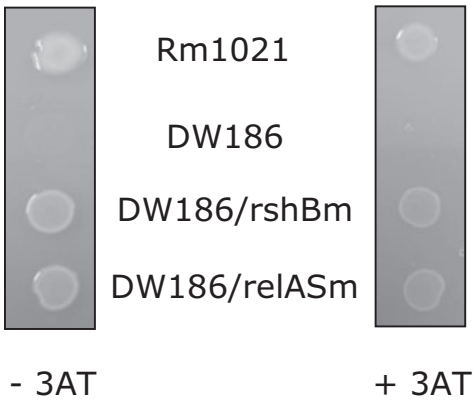

Fig. 2. Heterospecific complementation of an *S. meliloti relA* mutant with the *rsh* gene from *B. melitensis*. The *S. meliloti* wild-type strain Rm1021, the *relA* mutant DW186 (Wells and Long, 2002), and DW186 complemented with *rsh* from *B. melitensis* (rsh_{Bm}), or with *relA* from *S. meliloti* ($relA_{Sm}$) were tested for growth on M9 sucrose minimal medium without histidine, and without (left panel) or with (right panel) aminotriazole (3-AT).

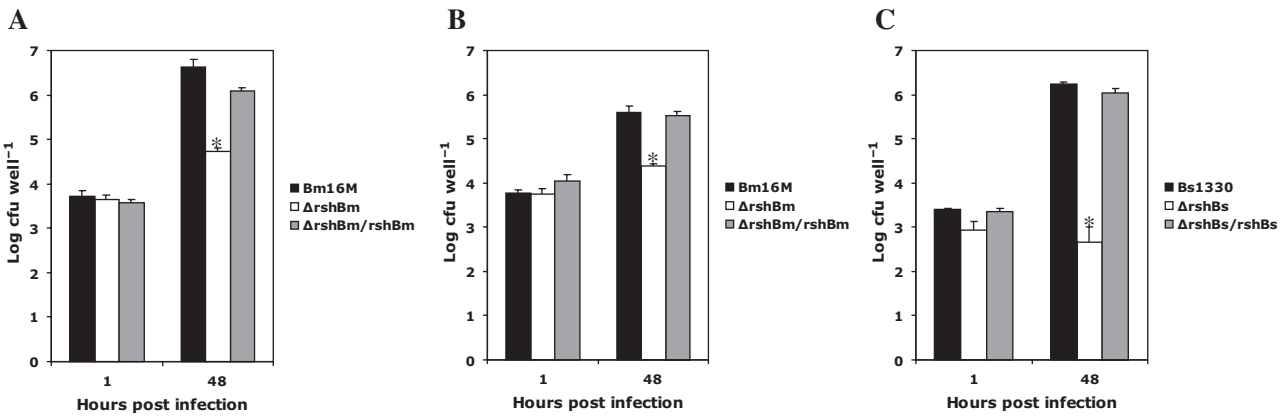

Fig. 3. Role of *rsh* in intracellular multiplication of *Brucella* in various cellular models of infection: replication of *B. melitensis* 16M (+pMR10*cat*), Δrsh_{Bm} (+pMR10*cat*), and of the mutant complemented with *rsh* from *B. melitensis* (Δrsh_{Bm}/rsh_{Bm}) in HeLa cells (A), and in ovine MOLC3 macrophages (B), and multiplication of *B. suis* 1330 (+pBBR1 MCS-1), Δrsh_{Bs} (+pBBR1 MCS-1), and Δrsh_{Bs}/rsh_{Bs} in human THP-1 macrophage-like cells (C). Values represent the means (log number of cfu well^{-1}) ± SD of one representative experiment performed in triplicate. The asterisk means significant for $P < 0.001$ (Scheffé, 1959).

multiplication (Köhler *et al*., 2002). The same gene, termed *spoT*, was also isolated in a screen for transposon mutants of *B. abortus* defective for infection of HeLa cells (Kim *et al*., 2003). As mentioned above, the *rsh* gene is encoded in a putative operon with the *pyrE* gene, the product of which may also be important for *Brucella*'s virulence, as other *pyr* genes have been identified to be virulence-related (Köhler *et al*., 2002). To check that attenuation of the *rsh* transposon mutant was not resulting from a polar effect on the neighbouring *pyrE* gene, capacity of the two non-polar mutants Δrsh_{Bs} and Δrsh_{Bm} to multiply within cultured cells was analysed. As shown in Fig. 3C, the Δrsh_{Bs} mutant was characterized by an important growth defect in human THP-1 macrophage-like cells. Similarly, the Δrsh_{Bm} mutant was shown to be defective for growth in human HeLa cells (Fig. 3A) and in ovine macrophages MOCL3 (Fig. 3B). Taken together, these results demonstrated that Rsh was essential for intracellular growth of the pathogen, suggesting an important role for this factor in mediating adaptation of *Brucella* to its intracellular host environment. Moreover, very effective complementation of Δ*rsh* mutants by expression *in trans* of *rsh* indicated that these mutants were indeed non-polar, having no effect on the downstream *pyrE* gene (Fig. 3). By contrast, the intramacrophagic survival of the original mutant of *B. suis* containing a mini-Tn5 transposon in *rsh* was even lower (data not shown), indicating a probable additional polar effect on *pyrE*.

To determine whether the stringent response mediated by Rsh was also involved in adaptation of *Brucella* to the host *in vivo*, the ability of Δrsh_{Bm} to infect BALB/c mice was assessed. *In vivo* assays were performed by infecting mice intraperitoneally with Δrsh_{Bm} and with the *B. melitensis* 16M parental strain. The number of viable bacteria in the spleen was evaluated at different times post infection. At 7 days post infection, Δrsh_{Bm} replication in the spleen was indistinguishable from that of the wild-type strain (Table 1). However, after 4 weeks, the Δrsh_{Bm} mutant was markedly attenuated as compared with the wild-type strain (2-logs difference). These results revealed that Rsh was necessary for persistence in mice, and confirmed the role of Rsh in adaptation of brucellae to their environment in the host.

rsh *deletion results in reduced production of VirB*

The expression of a major virulence factor of brucellae – the T4SS VirB – is induced under nutrient-poor conditions (i.e. stringent conditions). Indeed, in *B. suis* 1330, expression of the *virB* operon is induced in minimal medium at acid pH (pH 4.5) (Boschiroli *et al*., 2002). In *B. melitensis* 16M, the activity of the *virB* promoter is increased during late log phase, another nutrient-limiting condition (Delrue *et al*., 2005) and consistently, Western blot analysis using anti-VirB8 antisera (Rouot *et al*., 2003) revealed increase of VirB protein occurring during late exponential phase and stationary phase (Fig. 4A). To evaluate the role of Rsh

Table 1. Participation of Rsh in *Brucella* virulence in BALB/c mice. Values represent means (log cfu of bacteria per spleen) ± SD ($n = 4$).

	Number of bacteria/spleen (log cfu)	
	1 week post infection	4 weeks post infection
B. melitensis 16M	6.32 ± 0.15	4.66 ± 0.11
B. melitensis Δ*rsh*	6.51 ± 0.24	2.53 ± 0.86*

*Significant for $P < 0.01$. The *P*-value was calculated by one-way ANOVA analysis (Scheffé, 1959).

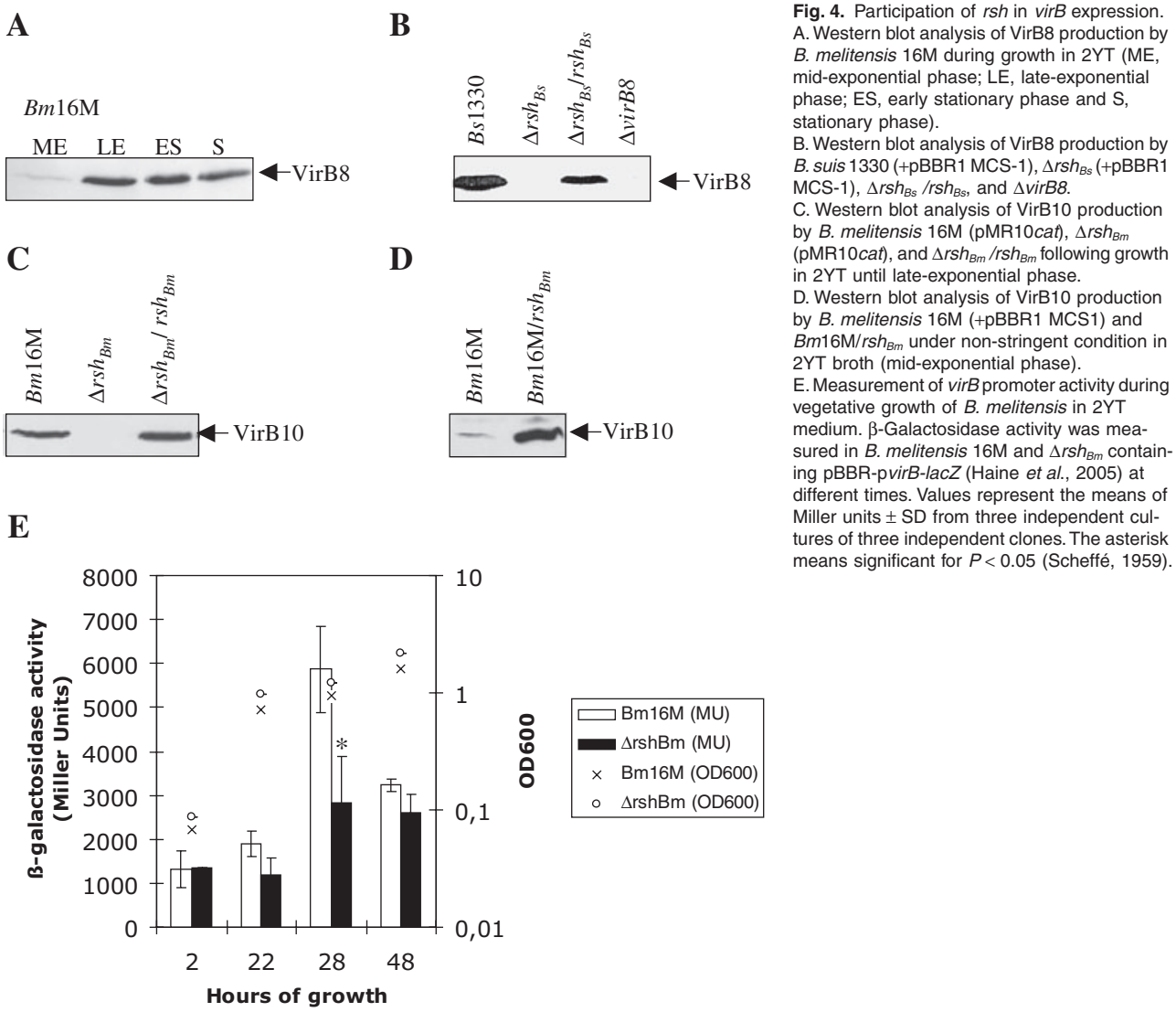

Fig. 4. Participation of *rsh* in *virB* expression.
A. Western blot analysis of VirB8 production by *B. melitensis* 16M during growth in 2YT (ME, mid-exponential phase; LE, late-exponential phase; ES, early stationary phase and S, stationary phase).
B. Western blot analysis of VirB8 production by *B. suis* 1330 (+pBBR1 MCS-1), Δ*rsh*$_{Bs}$ (+pBBR1 MCS-1), Δ*rsh*$_{Bs}$ /*rsh*$_{Bs}$, and Δ*virB8*.
C. Western blot analysis of VirB10 production by *B. melitensis* 16M (pMR10*cat*), Δ*rsh*$_{Bm}$ (pMR10*cat*), and Δ*rsh*$_{Bm}$ /*rsh*$_{Bm}$ following growth in 2YT until late-exponential phase.
D. Western blot analysis of VirB10 production by *B. melitensis* 16M (+pBBR1 MCS1) and *Bm*16M/*rsh*$_{Bm}$ under non-stringent condition in 2YT broth (mid-exponential phase).
E. Measurement of *virB* promoter activity during vegetative growth of *B. melitensis* in 2YT medium. β-Galactosidase activity was measured in *B. melitensis* 16M and Δ*rsh*$_{Bm}$ containing pBBR-p*virB-lacZ* (Haine *et al.*, 2005) at different times. Values represent the means of Miller units ± SD from three independent cultures of three independent clones. The asterisk means significant for $P < 0.05$ (Scheffé, 1959).

in controlling *virB* expression, the production of VirB proteins in the wild-type and the Δ*rsh* mutant was compared. Crude extracts of bacteria grown under *virB* induction conditions were analysed by Western blot using antisera directed against different VirB subunits. In the Δ*rsh*$_{Bs}$ mutant incubated for five hours in acidified minimal medium (pH 4.5), no VirB8 proteins were detected, in contrast to the parental strain *B. suis* 1330, and the complemented mutant Δ*rsh*$_{Bs}$/*rsh*$_{Bs}$ (Fig. 4B). Similarly, VirB10 protein was less abundant or not detected in Δ*rsh*$_{Bm}$ cultivated in rich medium until late exponential phase, in contrast to the wild-type strain and the complemented mutant Δ*rsh*$_{Bm}$/*rsh*$_{Bm}$ (Fig. 4C). Similar results were obtained with anti-VirB5 and anti-VirB9 antisera (data not shown). Moreover, analysis of VirB production at different times during growth in rich medium revealed that the Δ*rsh*$_{Bm}$ strain was characterized by very low levels or absence of VirB at all time points (data not shown). Together, these results suggested that Rsh was required for the production and stability of several VirB subunits in *B. melitensis* and *B. suis* under their respective conditions of T4SS induction.

To determine whether the potential regulatory role of Rsh on *virB* expression was operated at the transcriptional level, the activity of the *virB* operon promoter (p*virB*) was compared in *B. melitensis* 16M wild-type strain and in Δ*rsh*$_{Bm}$ strain during growth in rich medium using a *lacZ* reporter gene fusion (Haine *et al.*, 2005). The p*virB* activity was strongly downregulated in a Δ*rsh* background, as compared with the wild-type strain which displayed an increased p*virB* activity during late exponential phase (Fig. 4E). This correlated with levels of VirB proteins evidenced in total lysates of these two strains under these conditions. Our results therefore suggested that Rsh regulated *virB* expression at the transcriptional level.

Constitutive overexpression of rsh *in* B. melitensis *results in early production of VirB*

In his review, Cashel *et al*. (1996) prescribe the confrontation of two parallel approaches to investigate the involvement of (p)ppGpp in a process: the first is to analyse the phenotype of a (p)ppGpp° mutant, and the second is to study the effect of (p)ppGpp accumulation in the absence of nutritional stress. Accordingly, to further characterize the potential role of (p)ppGpp on *virB* expression, we studied the effect of the constitutive overexpression of rsh_{Bm} on VirB proteins production in *B. melitensis* 16M. An overexpression mutant of *rsh* (*Bm*16M/rsh_{Bm}) was constructed by transferring a pBBR1 vector containing the rsh_{Bm} gene cloned downstream of the *lac* promoter (pRH002-rsh_{Bm}) into *B. melitensis* wild-type strain. First, we observed using optical microscopy that *Bm*16M/rsh_{Bm} strain did not display severe morphological abnormalities, in contrast to the Δrsh_{Bm} strain (data not shown). This suggested that the effects of rsh_{Bm} overexpression in *B. melitensis* 16M were at least not as pleiotropic as those due to abolition of (p)ppGpp production. We then compared VirB proteins production in *B. melitensis* 16M wild-type strain and *Bm*16M/rsh_{Bm} strain under non-stringent conditions (i.e. during mid exponential phase) by Western blot analysis, using antisera directed against VirB5 and VirB10 proteins (Rouot *et al*., 2003). Three independent clones were tested in each experiment. Very low levels of VirB10 protein were detected in the *B. melitensis* wild-type strain during mid exponential phase (Fig. 4A and D). In contrast, *B. melitensis* overexpressing rsh_{Bm} produced higher levels of VirB10 protein during the same growth phase (Fig. 4D). Similar results were obtained with anti-VirB5 antisera (data not shown). These results suggest that excessive (p)ppGpp production induced early *virB* expression independently of the growth phase, and thus reinforced the hypothesis of a physiological role for (p)ppGpp as a positive effector of *virB* expression.

The relA *gene from* S. meliloti *complements* Δrsh *defects in* Brucella

RelA from *S. meliloti*, the homologue of Rsh in brucellae, synthesizes (p)ppGpp under low-nutrient conditions, and this protein is required for establishment of symbiosis with the host plant alfalfa (Wells and Long, 2002). In *Brucella*, we demonstrated that Rsh was required for growth in minimal medium, for successful infection of cellular models (Fig. 3), and that expression of the T4SS VirB was dependent on the presence of *rsh* (Fig. 4). To determine whether these phenotypes resulted from the absence of (p)ppGpp synthesis, heterologous complementation of Δrsh_{Bs} with the gene *relA* from *S. meliloti* ($relA_{Sm}$) was performed. Results demonstrated that expression of *relA* from *S. meliloti* in Δrsh_{Bs} completely restored growth capacity in Gerhardt's modified minimal medium (data not shown), and intracellular growth in THP-1 macrophage-like cells (Fig. 5A). Heterologous expression of *relA* from *S. meliloti* in Δrsh_{Bs} also complemented for *virB8* expression after induction in acid minimal medium (Fig. 5B). We concluded that the negative effect of *rsh* deletion on virulence, and on *virB* expression can be explained by the abolishment of stringent response, as it has been evidenced for *S. meliloti* that a disabled stringent response was due to *relA* inactivation, resulting in the incapacity to accomplish symbiosis. This is to our knowledge the first evidence of restoration of a virulent phenotype in a pathogen of mammals by heterologous complementation with a gene from a plant symbiont.

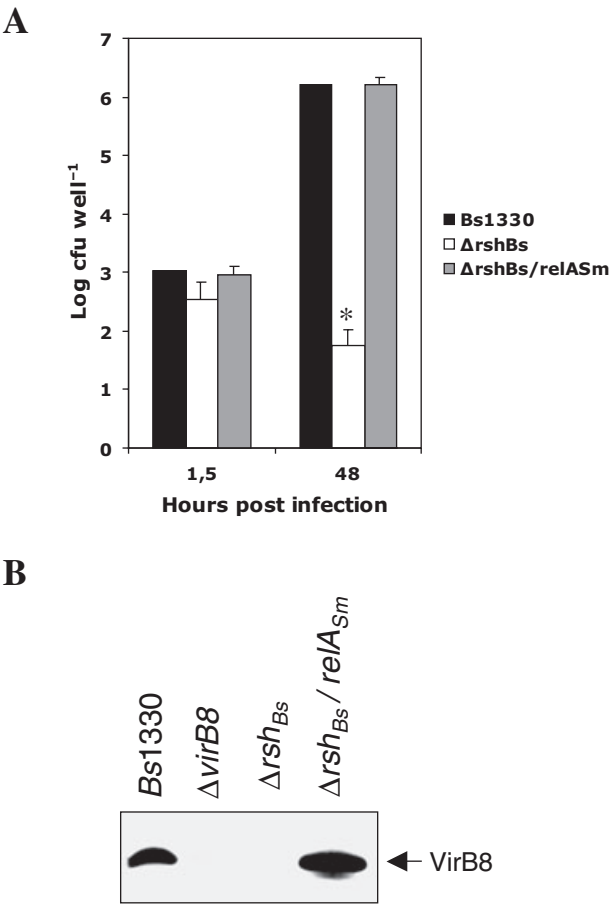

Fig. 5. Heterologous complementation of the *B. suis rsh* mutant with the *S. meliloti relA* gene.
A. Intracellular multiplication of *B. suis* 1330 (+pMR10), Δrsh_{Bs} (+pMR10), and Δrsh_{Bs}/$relA_{Sm}$ in human THP-1 macrophage-like cells. Values represent the means (log number of cfu well^{-1}) ± SD of one representative experiment performed in triplicate. The asterisk means significant for $P < 0.001$ (Scheffé, 1959).
B. Western blot analysis of VirB8 production by *B. suis* 1330 (+pMR10), Δ*virB8*, Δrsh_{Bs} (+pMR10), and Δrsh_{Bs}/$relA_{Sm}$.

Discussion

The role of stringent response in adaptation to intracellular environment has been recently evidenced for several pathogenic or symbiotic bacteria (Hammer and Swanson, 1999; Godfrey *et al*., 2002; Wells and Long, 2002). In this report, we describe the phenotypes of *rsh* mutants (for *relA spoT* homologue) of both *B. melitensis* 16M and *B. suis* 1330. Our analysis revealed that the *rsh* gene of *Brucella* encoded a functional (p)ppGpp synthetase, and that *rsh* mutants presented altered morphology, inability to face starvation and stationary phase defect. We also demonstrated the crucial role of Rsh in virulence, because *rsh* mutants were unable to grow in cellular models and were attenuated in mice. Finally, we analysed the involvement of Rsh in regulation of a major virulence factor in *Brucella* species: the T4SS VirB.

Genomes of the intracellular pathogens *B. melitensis* and *B. suis* contain a single *relA/spoT* homologue (DelVecchio, 2002; Paulsen *et al*., 2002). According to analysis of conserved residues in Rsh, this protein is predicted to be a functional (p)ppGpp synthetase/hydrolase (Hogg *et al*., 2004). Our experimental data strongly suggested that the *Brucella* Rsh proteins conserved at least a functional (p)ppGpp synthetase activity, because Rsh of *B. melitensis* restored (p)ppGpp-dependent growth of an *S. meliloti* (p)ppGpp° mutant (called *relA*) in the absence of histidine (Wells and Long, 2002). Conversely, heterospecific complementation of a *B. suis* Δ*rsh* mutant with the *S. meliloti relA* gene restored growth in minimal medium (data not shown), multiplication in human THP-1 macrophage-like cells, and expression of *virB*. Taken together, these data allowed us to consider Rsh of *Brucella* as a functional (p)ppGpp synthetase, efficient *in vivo*.

Moreover, *rsh* deletion in *Brucella* results in several phenotypes shared with other (p)ppGpp° mutants. First, Δ*rsh* presented altered morphology during vegetative growth similarly to other *relA spoT* mutants like *E. coli* (p)ppGpp° strains (Cashel *et al*., 1996), *spoT* mutant of *C. jejuni* (Gaynor *et al*., 2005), and, a recently described *spoT* transposon mutant of *B. abortus* (Kim *et al*., 2005). Next, consistent with the role of Rsh as a mediator for adaptation to nutrient limitation (Cashel *et al*., 1996), we observed that *B. suis rsh* mutants failed to grow in Gerhardt's minimal medium. Finally, our data clearly demonstrated that in *B. melitensis* and *B. suis*, the *rsh* gene was essential for survival during stationary phase in complex medium, as opposed to the observations described by Kim *et al*. (2005) for *B. abortus*. This last phenotype was also shared with the (p)ppGpp° mutant of *C. jejuni* (Gaynor *et al*., 2005) and it was in agreement with the involvement of (p)ppGpp in triggering entry into stationary phase (Cashel *et al*., 1996). These observations allowed us to conclude that Δ*rsh* mutants of *B. melitensis*, *B. suis*, and probably also *B. abortus* (Kim *et al*., 2005) are affected in multiple cellular processes as it is expected for (p)ppGpp° mutants (Cashel *et al*., 1996; Dahl *et al*., 2003; Gaynor *et al*., 2005).

At least both the inability of *rsh* mutants to grow in minimal medium, and to survive in stationary phase might be correlated with their inability to grow within eukaryotic cultured cells. Indeed, as mentioned above, several studies have proposed that the intracellular environment colonized by *Brucella* is nutrient-poor (Köhler *et al*., 2002; Köhler *et al*., 2003; Delrue *et al*., 2004) and evidence for a role of stationary phase physiology in successful adaptation of brucellae to the intracellular host environment has been demonstrated previously by Robertson and Roop (1999). Thus, we propose that the adaptation of *Brucella* to a harsh, nutrient-deprived intracellular environment was at least partially mediated by Rsh. Our data also demonstrated that Rsh is required for persistence in the mouse model of infection. In their work, Robertson and Roop identified an *hfq* homologue essential for stress resistance of *Brucella* during stationary phase. Long-term survival in late stationary phase, and persistence in BALB/c mice were affected in an *hfq* mutant (Robertson and Roop, 1999). These two phenotypes are shared with *rsh* mutants, suggesting that there may be a link between Rsh and Hfq regulatory networks.

Recently, Kim *et al*. (2005) confirmed with *B. abortus* the previously reported attenuation of a *B. suis rsh* mutant in a macrophage model of infection (Köhler *et al*., 2002), and described the reduced virulence of the mutant in mice. In contrast to the results obtained by Kim *et al*. (2005), however, we did not observe a reduced acid resistance for the *rsh* mutant of *B. suis* at pH 4.5 over an incubation period of 5 h (data not shown). Their hypothesis that (p)ppGpp may control resistance of brucellae to early phagosome acidification can therefore not be applied to all species.

The role of the (p)ppGpp alarmone in the control of virulence factors expression has recently been evidenced in several pathogenic bacteria (Hammer and Swanson, 1999; Godfrey *et al*., 2002; Haralalka *et al*., 2003; Pizarro-Cerda and Tedin, 2004; Gaynor *et al*., 2005). In this report, we studied the role of (p)pGpp in the regulation of the *Brucella* T4SS, VirB. VirB is a major virulence factor because it is required for the establishment and maintenance of the intracellular replicative niche of *Brucella* (O'Callaghan *et al*., 1999; Comerci *et al*., 2001; Delrue *et al*., 2001; Celli *et al*., 2003). Several studies on the regulation of *virB* expression revealed that it is induced under stringent conditions (i.e. nutrient deprivation conditions). Indeed, *virB* expression is enhanced in minimal medium (Boschiroli *et al*., 2002), and expression of the T4SS is induced in late log phase during vegetative growth in rich medium, coinciding with decrease of nutri-

ents availability (Sieira *et al*., 2000; Rouot *et al*., 2003; den Hartigh *et al*., 2004; Sieira *et al*., 2004; Delrue *et al*., 2005; this work). Our data clearly demonstrated that Rsh was required for *Brucella* T4SS expression because the *virB* promoter was downregulated in a Δ*rsh* background. This was correlated with the very low level of several VirB subunits evidenced by Western blot in total lysates of Δ*rsh* mutants. Although the involvement of (p)ppGpp in regulation of T4SS expression is consistent with the induction of VirB under stringent conditions, the pleiotropic nature of Δ*rsh* mutants imposed some caution. Indeed, the strongly reduced *virB* expression in Δ*rsh* mutants might be a secondary effect, far beyond the primary defect. For that reason, and as advised by Cashel *et al.* (1996), a second approach was used to evaluate the role of (p)ppGpp in *virB* regulation. A mutant constitutively overexpressing *rsh* was constructed in *B. melitensis* 16M, and *virB* expression under non-stringent conditions (i.e. during mid exponential growth phase in rich medium) was analysed in this strain. Early VirB production in strain 16M/rsh_{Bm} which overexpressed *rsh* was a strong additional argument suggesting that *virB* expression was directly correlated with (p)ppGpp level in the bacteria.

The mechanism by which Rsh controls *virB* expression is not yet understood. A current model of the mechanism of action of (p)ppGpp involves binding of (p)ppGpp to RNAP (Toulokhonov *et al*., 2001), resulting in alteration of competition between sigma-factors for RNAP-binding, and therefore promoter selection (Jishage *et al*., 2002). The sigma-factor RpoS, which regulates entry in stationary phase (Hengge-Aronis, 1996), would have been a good candidate for mediating (p)ppGpp-dependent regulation, because *virB* induction in rich medium is triggered during entry in stationary phase. However, *Brucella* spp. genomes do not contain a *rpoS* homologue (M. Delory, pers. comm.), but at least two sigma factors involved in the control of *virB* expression in *B. melitensis* 16M have been identified recently (Delory *et al*., submitted). We can hypothesize that one of these two sigma factor might play the role of RpoS in *Brucella* and therefore mediate (p)ppGpp-dependent regulation of *virB*. In their work, Sieira *et al*. (2004) identified a stationary phase regulator, the integration host factor IHF. This regulator binds to the *virB* promoter, and enhances *virB* expression in *B. abortus*. Interestingly, IHF was shown to be under the control of (p)ppGpp and RpoS in *E. coli* (Aviv *et al*., 1994). According to these data, it is possible that the starvation signals sensed by Rsh and mediated by one of the sigma factor mentioned above are transferred to the *virB* promoter by the action of IHF. Recently, the quorum-sensing-related regulator VjbR was shown to play a crucial role in the control of *virB* expression (Delrue *et al*., 2005). A previous study suggested that starvation-sensing and quorum-sensing pathways coregulate each other, and converge to regulate entry into stationary phase (Lazazzera, 2000). In addition (p)ppGpp participates in the regulation of quorum-sensing actors in several species (van Delden *et al*., 2001; Zhang *et al*., 2004; Moris *et al*., 2005). As previously suggested by Delrue *et al*. (2005), it would be interesting to investigate the presence of a potential link between the starvation-sensing pathway mediated by Rsh, and the quorum-sensing pathway mediated by VjbR in *Brucella*. We may then obtain further insight into the regulatory network controlled by (p)ppGpp, and the cascade by which starvation signals are transmitted to the *virB* promoter.

It has been suggested that certain bacterial virulence factors, notably T4SSs, may have been acquired by horizontal gene transfer from environmental bacteria, and adapted to the needs of pathogenic bacteria (Baron *et al*., 2002). In a second step, these virulence genes may then have passed under the control of conserved global regulators such as IHF or Rsh-dependent (p)ppGpp. Our data obtained with heterocomplementation assays between *Brucella* and *S. meliloti* supported this hypothesis, as the heterologous *rsh* genes restored stringent response in *S. meliloti*, and *virB* expression coupled to a virulent phenotype in *Brucella*. Restoration of the virulence of a mammalian pathogen by expression of a gene involved in global gene regulation in a plant symbiont emphasized the close relationship between these organisms, raising the question of the definition of common mechanisms of sensing and regulation, and distinct mechanisms of specific adaptation to the respective niches in the host organisms. Altogether, we conclude that Rsh might play a key role in the setup of two major strategies adopted by *Brucella* to face its intracellular environment, i.e. adaptation to low-nutrient environment, and, indirectly, escape to a safe replicative niche via VirB.

Experimental procedures

Bacterial strains and culture conditions

All *Brucella* strains used in this study were derived from *B. melitensis* 16M Nal^R (spontaneous nalidixic acid resistant mutant selected from *B. melitensis* 16M, received from A. Macmillan, Central Veterinary Laboratory, Weybridge, UK) or *B. suis* 1330 (American Type Culture Collection 23444), and were routinely cultivated in 2YT and tryptic soy (TS) broth respectively. *B. suis* growth curves in minimal medium were performed as follows: a stationary-phase overnight culture obtained in TS was washed once in phosphate-buffered saline (PBS) prior to a 1:100 dilution in Gerhardt's modified minimal medium (Lestrate *et al*., 2000), followed by incubation at 37°C with shaking. Growth was measured by reading the optical density of the cultures at 600 nm. *B. melitensis* 16M growth curves in 2YT broth were performed as followed: a late-exponential overnight culture obtained in 2YT was back-diluted to 1×10^8 bacteria ml^{-1} in 2YT for growth at 37°C with shaking. Concentrations of live bacteria ml^{-1} were determined at different times by plating serial dilutions. *E. coli*

strains DH10B (Invitrogen Life-Technologies), DB3.1 (Invitrogen Life-Technologies), S17-1 (Simon *et al*., 1983), DH5α (Bethesda Research Laboratories) and MT616 (Finan *et al*., 1986) were cultivated in Luria–Bertani broth. Antibiotics were used at the following concentrations when appropriate: nalidixic acid, 25 μg ml^{-1}; kanamycin, 50 μg ml^{-1}, chloramphenicol, 20 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; gentamicin, 50 μg ml^{-1}. *S. meliloti* strains used in this study were all derived from Rm1021 and DW186 (Wells and Long, 2002), and were cultivated as described by Wells and Long (2002). For stringent response assays on plates, we used M9 sucrose medium supplemented with 1 mM adenine, 1 mM thiamine and all amino acids except histidine and alanine, either with or without 15 mM 3-amino-1,2,4-triazole (Gropp *et al*., 2001; Wells and Long, 2002).

Molecular techniques and plasmids

DNA manipulations were performed according to standard techniques (Ausubel, 1989). *B. melitensis* 16M and *B. suis* 1330 knock out mutants were both obtained by gene replacement. Briefly, for *B. melitensis rsh* mutant strain (Δ*rsh*$_{Bm}$), upstream and downstream regions flanking *rsh* gene were amplified by polymerase chain reaction (PCR) from *B. melitensis* 16M Nal^R genomic DNA using the following primer pairs (Sigma): (i) FPrshB (5′-CCGGATGATCTGAAGGAA-3′) and RPrshB (5′-AATTCCCCCGGGGAGATCTCCGCGCATCATCTGCCGAAA-3′); (ii) FTrsh (5′-GGAGATCTCCCCGGGGGAATTGTCTGGGACCT CAAGCAT-3′) and RTrsh (5′-CCCGTGGTGACGATATCT-3′). A second PCR was used to ligate the two PCR products by cohesive ends. The PCR product (*rsh* upstream/downstream) was inserted into the NotI site of pSKoriT*cat* (I. Danese and P. Lestrate, unpublished) to generate pSKoriT*cat*-Δ*rsh*. A non-polar deletion cassette, called *aphA4*, was constructed based on the *aphA3* cassette described by Menard *et al*. (1993). The *aphA4* cassette was obtained by PCR amplification of the *aph* gene on plasmid pUC4K (Pharmacia) using the primers FaphA4B (5′-AACTGCAGGTGACTAATTAGCAAGGGGTGTTATGAGCCATA-3′) and RaphA4B (5′-TTCTGCAGTTCATTATTCCCTCCAGGGATT AGAAAAACTCATCGAGCAT-3′), and subsequently inserted into the PstI sites of pUC4K, replacing the classical *aph* cassette. Finally, the *aphA*4 cassette was excised from pUC4*aphA4* with BamHI, and subsequently cloned into the BglII site of pSKoriT-*Cat*-Δ*rsh* to generate plasmid pSKoriT*Cat*-Δ*rsh*::*aphA4*. This final construction was transformed into *E. coli* strain *S17-1*, and introduced into *B. melitensis* 16M Nal^R strain by conjugation. Clones for which a double recombination event occurred (Cm^s Kan^r) were selected, and their genotype was verified by Southern blot analysis using a specific probe hybridizing with the *rsh* downstream region. To construct a non-polar *rsh* mutant of *B. suis* (Δ*rsh*$_{Bs}$), a 3.2 kb HincII–KpnI genomic DNA fragment containing *rsh* and cloned into pUC18 (Invitrogen) (pUC18*rsh*$_{Bs}$) was identified by *E. coli* colony blot analysis using a probe obtained by PCR on *B. suis* total genomic DNA with the primers 5′-GGTTCGAGG-GAGTTTCGGC-3′ and 5′-GCGATTGAGATGCTTGAGGTCC-3′, and random primed-labelling with digoxigenin (Boehringer Mannheim). DNA digestion with NcoI deleted three contiguous fragments of a total size of 1.63 kb, internal to *rsh*. The plasmid was religated, and the remaining 1.6 kb chromosomal DNA fragment was then inserted as an SacI–SalI fragment into pCVD442. This vector carries the *sacB* gene conferring sucrose sensitivity to Gram-negative bacteria (Donnenberg and Kaper, 1991), hence allowing to apply selective pressure for allelic exchange which will then yield sucrose-resistant clones. To this purpose, following electroporation, brucellae were first plated in the presence of ampicillin to select for transformants. Positive clones were restreaked onto solid medium containing 5% sucrose, and ampicillin-sensitive but sucrose-resistant colonies were isolated. Gene replacement was confirmed by PCR using the *rsh* primers (not shown).

Construction of complementation plasmid pRH001-*rsh*$_{Bm}$ (pMR10 derived vector) and overexpression plasmid pRH002-*rsh*$_{Bm}$ (pBBR1 MCS-1 derived vector) was performed using the Gateway™ technique (Invitrogen). The *rsh* CDS (BMEI1296 in *B. melitensis* 16M genome sequence, GenBank file AE009567.1) was amplified by PCR from *B. melitensis* 16M Nal^R genomic DNA with Gateway™ primers rshB1 (5′-GGGGACAAGTTTGTAC AAAAAAGCAGGCTCGATGATGCGCCAATATGAGCTT-3′) and rshB2 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTATCCG TTCACACGCTTTGCGCT-3′) (Invitrogen Life-Technologies), and cloned in the entry vector pDONR201 (Invitrogen Life-Technologies) as previously described by Dricot *et al*. (2004). Destination vectors pRH001 and pRH002 (R. Hallez, unpublished) bear a chloramphenicol resistance marker, and the toxic cassette *ccdB* flanked by *att*R1 and *att*R2 recombination sites. LR recombination cloning procedure was performed as recommended by the manufacturer (Invitrogen Life-Technologies). Resulting vectors pRH001-*rsh*$_{Bm}$ and pRH002-*rsh*$_{Bm}$ were transferred, respectively, into Δ*rsh*$_{Bm}$ and *B. melitensis* 16M wild-type strains by conjugation to generate complemented strain Δ*rsh*$_{Bm}$/*rsh*$_{Bm}$ and overexpression strain 16M/*rsh*$_{Bm}$. Simultaneously, vectors pMR10*cat* (R. Roberts, unpublished) and pBBR1 MCS-1 (Kovach *et al*., 1994) were transferred into *B. melitensis* 16M wild-type and Δ*rsh*$_{Bm}$ strains by conjugation. The *B. suis* mutant Δ*rsh*$_{Bs}$ was complemented as follows: the *B. suis* genomic DNA fragment containing *rsh* was excised from pUC18*rsh*$_{Bs}$, and recloned as a HincII–KpnI fragment into vector pBBR1-MCS (Kovach *et al*., 1994) which replicates in brucellae. The plasmid pBBR1-*rsh*$_{Bs}$ was then transformed into the Δ*rsh*$_{Bs}$ strain by electroporation, resulting in complemented strain Δ*rsh*$_{Bs}$/*rsh*$_{Bs}$. Simultaneously, pBBR1 MCS-1 was transferred to *B. suis* 1330 wild-type and Δ*rsh*$_{Bs}$ strains by electroporation.

For heterologous complementation, the complete *rsh* gene from *S. meliloti* (termed *relA* in the publication by Wells and Long (2002)) was cloned following amplification by PCR on genomic DNA isolated from strain Rm1021. The following primers were used: 5′-CGAGATCGCCGATGAAACCC-3′ and 5′-CTTTCGG TATCGCGACCTGC-3′, yielding a PCR product of 2.6 kb which was then cloned into plasmid pGEM-T Easy (Promega). In a second step, this fragment was excised using *Eco*RI, and inserted into the *Eco*RI site of pMR10 (R. Roberts, unpublished). The obtained plasmid pMR10*rsh*$_{Sm}$ was transformed into the Δ*rsh*$_{Bs}$ strain by electroporation, resulting in complemented *B. suis* strain Δ*rsh*$_{Bs}$ /*rsh*$_{Sm}$ expressing the *rsh* gene from *S. meliloti*.

Plasmids pRH001*rsh*$_{Bm}$ and pMR10-*rsh*$_{Sm}$ were transferred to *S. meliloti* strain DW186 by triparental mating using MT616 *E. coli* as a helper strain (Finan *et al*., 1986), as described previously by Glazebrook and Walker (1991).

Infections of mice and of cultured cells

Infections of HeLa cells and ovine macrophages MOCL3 (Olivier *et al*., 2001) by *B. melitensis* were performed as described pre-

viously (Delrue *et al*., 2001), and human macrophage-like THP-1 cells were infected by *B. suis* strains as described by Ekaza *et al*. (2001). Experiments were performed twice in triplicate. Infections of BALB/c mice were performed as described recently (Fretin *et al*., 2005; Delrue *et al*., 2005). Briefly, four mice per strain and time point were infected by intraperitoneal injection with 5×10^5 brucellae, and the number of live bacteria in the spleens was determined at 1 and 4 weeks post infection. Spleens were aseptically removed and homogenized, prior to plating of the diluted homogenates on 2YT agar.

Detection of VirB proteins by Western blot analysis

For VirB analysis in *B. melitensis* 16M, strains were grown at 37°C in 2YT broth to stationary phase and then back-diluted for cultivation at 37°C until expected growth phase. Samples of culture were inactivated for 1 h at 80°C and standardized for same OD_{600}. Following sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot, immunodetection of VirB5, VirB8, VirB9 and VirB10 in total lysates was performed with a polyclonal rabbit anti-VirB5 (Rouot *et al*., 2003), -VirB8, -VirB9 and VirB10 antisera at respective dilutions of 1/5000, 1/2500, 1/2500 and 1/2000. For VirB analysis in *B. suis*, strains were grown at 37°C in TS broth to stationary phase. Four volumes of PBS were added before centrifugation at 2500 *g* for 25 min, and the pellets were resuspended in PBS at a final OD_{600} of 0.5. Two-millilitre aliquots of the bacterial suspensions were centrifuged for 2 min at 13 000 *g*, and the pellets were then resuspended in 0.5 ml of minimal medium at pH 4.5 (Alvarez-Martinez *et al*., 2001). After cultivation for 5 h at 37°C, bacteria were centrifuged, directly resuspended in sample buffer, and heated at 100°C for 5 min. Following SDS-PAGE and Western blot, immunodetection of VirB8 in total lysates was performed with a polyclonal rabbit anti-VirB8 antiserum at a dilution of 1/5000 (Rouot *et al*., 2003).

Measurement of β-galactosidase activity

Plasmid pBBR-*pvirB-lacZ* (Haine *et al*., 2005) was transferred into *B. melitensis* 16M Nal^R and Δrsh_{Bm} strains by conjugation, and samples were harvested at different times during growth at 37°C in 2YT broth supplemented with chloramphenicol. β-Galactosidase assays were performed according to the method described by Miller (1972).

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Multiple sequence alignment between Rsh_{Bm} (*Brucella melitensis*), Rsh_{Bs} (*Brucella suis*), $RelA_{Sm}$ (*Sinorhizobium meliloti*) and Rel*Seq* (*Streptococcus dysgalactiae* ssp. *equisimilis*). Synthetase and hydrolase domains are underlined, and boxed residues are amino acids that were shown to be involved in synthetase or hydrolase activities for Rel*Seq* (Hogg *et al.*, 2004). Red residues are identical for the four proteins, green residues are strongly similar and blue residues are weakly similar.

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