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Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the *Brucella* Type IV secretion system

Maarten F. de Jong^{1,2}, Yao-Hui Sun¹, Andreas B. den Hartigh¹, Jan Maarten van Dijk², and Renée M. Tsolis¹

¹Department of Medical Microbiology and Immunology, School of Medicine, University of California at Davis, One Shields Ave., Davis, CA 95616-8645, USA ²Department of Medical Microbiology, University Medical Center Groningen and University of Groningen, Hanzeplein 1, 9700 RB Groningen, the Netherlands

Abstract

Survival and replication inside host cells by *Brucella* spp. requires a type IV secretion system (T4SS), encoded by the *virB* locus. However, the identity of the molecules secreted by the T4SS has remained elusive. We hypothesized that proteins translocated by the T4SS would be co-regulated with the *virB* operon. The LuxR family regulator VjbR, known to regulate *virB*, bound a fragment of the *virB* promoter containing an 18-bp palindromic motif (*virB* promoter box), showing that VjbR regulated the *virB* operon directly. To identify *virB*-coregulated genes, we searched the *B. suis* 1330 and *B. abortus* 2308 genomes for genes with an upstream *virB* promoter box. 144 promoters in the two genomes contained the *virB* promoter box, including those of *fliC* encoding flagellin and *cgs* encoding cyclic β -glucan synthetase. Thirteen of these proteins were tested for VirB dependent translocation into macrophages using a β -lactamase reporter assay. This analysis resulted in the identification of the proteins encoded by BAB1_1652 (VceA) and BR1038/BAB1_1058 (VceC) as novel protein substrates of the *Brucella* T4SS. VceC could also be translocated by the *L. pneumophila* Dot/Icm T4SS into host cells. Our results suggest that VjbR coordinates expression of the T4SS and at least two of its secreted substrates.

Introduction

In order to cause persistent infection of the reticuloendothelial system, all human pathogenic *Brucella* species require a type IV secretion system (T4SS) known as VirB (Hong *et al.*, 2000, O'Callaghan *et al.*, 1999). T4SS are multi-component protein structures in the bacterial envelope used by many Gram-negative bacterial pathogens of animals and plants for the translocation of virulence factors into eukaryotic host cells (Backert & Meyer, 2006). Examples of well-studied T4SS are the VirB system of the plant pathogen *Agrobacterium tumefaciens*, which facilitates export of T-DNA together with at least 3 effector proteins into plant cells (Berger & Christie, 1994, Fullner, 1998, Vergunst *et al.*, 2000), and the Dot/Icm system of the accidental human pathogen *Legionella pneumophila*, which is used for secretion of more than 40 effector proteins into host cells (Nagai & Roy, 2001, Chen *et al.*, 2004, Conover *et al.*, 2003, Shohdy *et al.*, 2005, Luo & Isberg, 2004). Together with *Agrobacterium tumefaciens*, *Brucella* spp. are classified in the α -2 group of the α -proteobacteria (Moreno *et al.*, 1990), and correspondingly, their T4SS are closely related. However, the *Brucella* genome does not encode any homologs of *A. tumefaciens* effectors, probably because these two pathogens have different lifestyles. *Agrobacterium* is a soil

dwelling bacterium that infects root cells of plants, and while remaining extracellular, uses its T4SS to induce tumorigenesis (Christie, 2004).

Brucella utilizes a similar T4SS, VirB, for a different purpose. *Brucella* spp. are facultative intracellular pathogens of many wild and domestic animals and can cause zoonotic disease in humans. *Brucella* spp. can survive within the phagocytic cells of the host and are able to evade normal mechanisms of bacterial killing by altering the intracellular trafficking of their vacuole (Arenas *et al.*, 2000, Pizarro-Cerda *et al.*, 1998a, Pizarro-Cerda *et al.*, 1998b). The ability of phagocytosed *Brucella* to evade fusion of their endosomal vesicles with lysosomes requires the VirB T4SS (Comerci *et al.*, 2001, Delrue *et al.*, 2001, Sieira *et al.*, 2000, Celli *et al.*, 2003, Celli *et al.*, 2005). Secretion of effector proteins by the *Brucella* T4SS likely alters this pathway, allowing the bacteria to reside in vacuoles with properties of rough endoplasmic reticulum, thereby promoting survival and replication (Celli *et al.*, 2003, Celli *et al.*, 2005). Mutants of *Brucella* lacking a functional T4SS are highly attenuated in vitro in macrophages and in vivo in the mouse model of infection (O'Callaghan *et al.*, 1999, Delrue *et al.*, 2001, den Hartigh *et al.*, 2004, Sieira *et al.*, 2000, Hong *et al.*, 2000).

It has been shown that for both Type III secretion systems (T3SS) and T4SS, genes encoding the secretion apparatus are often co-regulated with the secreted substrates. For example, the same regulators control expression of genes encoding the structural components of *Salmonella enterica* serotype Typhimurium T3SS-1 and T3SS-2 and their effectors (Thijs *et al.*, 2007, Worley *et al.*, 2000). Further, in *L. pneumophila* the two-component system regulators PmrA and CpxR were found to regulate genes of the Dot/Icm T4SS as well as several Dot/Icm effector proteins (Zusman *et al.*, 2007, Altman & Segal, 2008). Based on these findings, we hypothesized that secreted substrates of the *B. abortus* T4SS would be co-regulated with the structural components of the secretion apparatus by the same transcriptional regulator.

The *Brucella* T4SS is induced during intracellular infection (Sieira *et al.*, 2004, Sieira *et al.*, 2000, Boschiroli *et al.*, 2002). The only direct regulator of the *virB* genes shown to date is integration host factor (IHF), which was shown to bind to the *virB* promoter (P_{virB}) of *B. abortus* (Sieira *et al.*, 2004). IHF was found to be necessary for activity of P_{virB} inside the host cell and during vegetative growth, likely by its well characterized DNA bending activity, which is thought to provide the correct promoter structure for the action of additional transcriptional regulators. Recently, a transcriptional activator (VjbR) of the *B. melitensis virB* genes was described (Delrue *et al.*, 2005, Delrue *et al.*, 2001). In a *B. melitensis vjbR* mutant expression of the T4SS is greatly reduced (Delrue *et al.*, 2005). Furthermore, the *B. melitensis vjbR* mutant is highly attenuated in both cellular and mouse models of infection. The VjbR protein belongs to the family of LuxR quorum-sensing (QS) regulators and contains a conserved motif required for binding to acylhomoserine lactone (AHL) pheromones. It was shown that the QS pheromone N-dodecanoylhomoserine lactone (C_{12} -HSL) inhibits the activation of *virB* genes via VjbR (Delrue *et al.*, 2005). The expression of VirB is downregulated by C_{12} -HSL in *B. melitensis* (Delrue *et al.*, 2005, Taminiau *et al.*, 2002, Uzureau *et al.*, 2007). However, it remains unknown whether VjbR binds directly at the *virB* promoter to activate its transcription, or whether intermediate regulators are involved.

In this report we present evidence for direct activation of P_{virB} by VjbR in *Brucella abortus*. Furthermore, by determining a conserved motif in P_{virB} required for activation by VjbR, we identified 143 additional promoter regions in the *B. abortus* 2308 and *B. suis* 1330 genomes containing this motif, including the promoters of *vceA* and *vceC*, encoding substrates of the VirB T4SS.

Results

1. VjbR of *B. abortus* activates $P_{virB}::lacZ$ and $P_{tetR}::lacZ$ in *E. coli*

We hypothesized that a common regulator would control expression of both the *virB* genes encoding the T4SS and its secreted effector proteins. Therefore, as a first step toward identification of genes that are co-regulated with the *virB* genes, we performed experiments to identify a direct regulator of the *virB* operon. In previous studies VjbR was found to be an activator of the *B. abortus* VirB system (Delrue *et al.*, 2005). Further, *Brucella melitensis* strains lacking *arsR6*, *araC8*, *deoR1* or *gntR4* have reduced expression levels of *virB* genes, suggesting that they may also function as activators of *virB* expression (Haine *et al.*, 2005). However it remained unknown whether the effect of all of these regulatory proteins on expression of *virB* genes is direct or is part of a regulatory cascade. In order to distinguish between these two possibilities, we reconstituted *virB* regulation in *E. coli*. A transcriptional fusion of the *virB1* upstream region to *lacZ* ($P_{virB}::lacZ$) was constructed and the resulting plasmid pSURS2, was introduced into *E. coli* BL21 *lacZ* (Supplementary table 1). In addition, we constructed plasmids encoding IPTG-inducible copies of *vjbR*, *arsR6*, *araC8*, *deoR1*, *gntR4* or a predicted two-component response regulator that did not affect expression of *virB* (BAB1_0345; unpublished results). Expression of each regulator together with the $P_{virB}::lacZ$ construct into *E. coli* strain BL21 *lacZ* allowed us to determine whether the regulator of interest could affect expression of the $P_{virB}::lacZ$ construct (pSURS2). To determine whether the corresponding regulatory proteins alter expression of a $P_{virB}::lacZ$ reporter construct, we measured the β -galactosidase activity of the $P_{virB}::lacZ$ fusion in *E. coli* strains expressing VjbR, ArsR6, AraC8, DeoR1, GntR4, or the control protein BAB1_0345 (Figure 1 and Figure 2). Data shown in Figure 1 demonstrate that induction of *vjbR* expression activated transcription of the $P_{virB}::lacZ$ reporter construct on pSURS2 (Fig. 1A). Furthermore, the induction of P_{virB} activity correlated well with the expression of VjbR as shown by Western blotting (Figure 1A, top panel). Induction of the control protein BAB1_0345 caused a slight reduction in expression of the $P_{virB}::lacZ$ reporter, which is likely due to T7 promoter-driven overexpression of the protein, as we have observed this effect with overexpression of other proteins as well (Fig. 1A). To correct for this effect of protein overproduction on expression of the *virB* reporter construct, we used our negative control to normalize data presented in subsequent figures.

Since C_{12} -homoserine lactone (C_{12} -HSL) inhibits transcriptional activation by VjbR in *Brucella* (Delrue *et al.*, 2005, Uzureau *et al.*, 2007), we determined whether addition of C_{12} -HSL would reduce VjbR-mediated activation of the $P_{virB}::lacZ$ reporter construct. Addition of 30 μ M and 60 μ M C_{12} -HSL to *E. coli* cultures reduced activity of P_{virB} in a dose-dependent manner only in the strains expressing VjbR, but not in strains expressing the negative control protein BAB1_0345 (Fig. 1A). The residual expression of the $P_{virB}::lacZ$ reporter in cultures treated with 60 μ M C_{12} -HSL is likely due to the high expression level of VjbR from the T7 promoter in *E. coli*. Notably, the β -galactosidase activity levels observed for cells carrying the $P_{virB}::lacZ$ fusion on pSURS2 were significantly higher than the background β -galactosidase levels observed in cells carrying the promoterless control vector pSURS1 (Fig. 1B).

Only expression of *vjbR*, but not of *arsR6*, *araC8*, *deoR1* or *gntR4*, activated expression of the $P_{virB}::lacZ$ reporter construct in *E. coli*, suggesting that whereas VjbR is able to activate *virB* expression in the absence of other *Brucella*-specific factors, ArsR6, AraC8, DeoR1, GntR4 may require additional *Brucella*-specific gene products to activate expression of the *virB* genes (Fig. 2B). We therefore focused on identifying additional members of the VjbR regulon to identify proteins secreted by the *B. abortus* T4SS.

Members of the LuxR regulator family in other bacteria (e.g. *Vibrio fischeri*) are known to regulate their own expression (Shadel & Baldwin, 1991, Shadel & Baldwin, 1992). We therefore examined the effect of VjbR on its own promoter. Since *vjbR* is predicted to be in an operon with an upstream gene encoding a *tetR* family regulator (Fig. 2A), we constructed *lacZ* fusions to the promoter directly upstream of *tetR* (BAB2_0117; P_{tetR}) and to the *tetR-vjbR* intergenic region (P_{vjbR}; 2C and 2D). *LacZ* promoter fusions were introduced into *E. coli* strains expressing *vjbR*, *arsR6*, *araC8*, *deoR1* or *gntR4* or BAB1_0345 genes and induction of *lacZ* expression was measured using β -galactosidase assays. The results from the assays showed that VjbR activated its own expression via P_{tetR} (Fig. 2C). Compared to the negative control BAB1_0345, induction of *tetR::lacZ* expression by VjbR was approximately 3 fold higher. We also examined the regulatory effect of VjbR on the intergenic region between the *tetR* and *vjbR* genes using the same system and, although this promoter showed background activity in *E. coli*, no activation by VjbR was observed (Fig. 2D). Since in *B. melitensis* it was recently shown that VjbR regulates its own expression (Rambow-Larsen *et al.*, 2008), these findings suggest that the promoter in the *tetR-vjbR* intergenic region may control VjbR expression in a VjbR-independent manner or that it may require *Brucella*-specific factors in addition to VjbR for transcriptional regulation.

2. VjbR-His₆ binds directly to both a fragment of P_{virB} and a fragment of the virB1-virB2 intergenic region

The results presented above suggested direct activation of P_{virB} by VjbR due to binding of this regulator to the *virB* promoter. To test this idea, we performed electrophoretic mobility shift assays (EMSA) using purified, His-tagged *B. abortus* VjbR protein and a 123 bp fragment containing the DNA region directly upstream of the *virB* promoter (Fig. 3). Addition of increasing amounts of VjbR-His₆ to the ³²P labeled P_{virB} fragment resulted in an increased intensity of a band with reduced electrophoretic mobility (Fig. 3B). Competition with the unlabeled P_{virB} probe reduced the intensity of the shifted band, and this effect was not observed when the same amount of an unlabeled and unrelated probe was added to the binding reaction. These results demonstrate a specific binding of VjbR to the upstream region of the *B. abortus virB* operon.

Since the *virB1-virB2* intergenic region is also predicted to contain a promoter, we tested binding of VjbR by EMSA to a ³²P labeled 172 bp DNA fragment containing the intergenic region (Fig. 3A). We found that purified VjbR-His₆ bound to this region similarly as to the P_{virB} fragment (Fig. 3C). Addition of increasing amounts of VjbR-His₆ to the fragment containing the *virB1-virB2* intergenic region resulted in an increased intensity of a band with reduced electrophoretic mobility (Fig. 3C). As a control for nonspecific binding of VjbR, we used a ³²P labeled 139 bp internal fragment of the housekeeping *gyrA* gene (Fig. 3D). No band shift was observed for the ³²P labeled *gyrA* (BAB1_1121) fragment at any of the VjbR concentrations examined, demonstrating a specific binding of VjbR to the *virB1-virB2* intergenic region. However, in *E. coli*, induction of *vjbR* expression did not increase expression of a *virB1-virB2::lacZ* transcriptional fusion (pSURS3; data not shown). It is possible that for proper VjbR mediated activation of the *virB1-virB2* region other *Brucella* specific factors are required that are not present in *E. coli*. Collectively, these data suggested that VjbR binds specifically to two promoter regions within the *virB* operon of *B. abortus*.

3. A conserved 18 bp sequence in P_{virB} is important for activation by VjbR

Since VjbR was found to activate P_{virB} and P_{tetR} in our *E. coli* expression system, we examined the DNA sequence of both promoters for the presence of conserved motifs. We identified a conserved palindromic sequence of 18 bp in P_{virB} and 19 bp in P_{tetR} (Fig. 3A). We also found a similar sequence in the intergenic region between *virB1* and *virB2* (Fig. 3A). In *B. abortus* P_{virB} the 18 bp box is positioned at -37 relative to the transcription start

site. Remarkably, this box is similar to the lux box consensus sequence that has been implicated in promoter activation by the regulator TraR of *A. tumefaciens* (White & Winans, 2007, Pappas & Winans, 2003). To investigate whether this sequence is required for activation by VjbR, a *lacZ* fusion was constructed to a 460 bp fragment of P_{virB} in which bp 2–7 in the upstream half of the 18 bp box was substituted with a 6 bp *HindIII* site (TGACCG to AAGCTT), thereby disrupting the palindrome, but not the putative –35 regulatory sequence. Control of the promoter activity of this construct by VjbR was monitored in *E. coli* using a β -galactosidase assay, as described above. Compared to wild type P_{virB} , the background activity of the mutated promoter remained the same (about 1300 Miller Units) but activation by VjbR was significantly reduced (Fig. 4). Replacement of bp 7–12 or bp 13–18 of the promoter box consensus sequence by AAGCTT also reduced activation of the resulting $P_{virB}::lacZ$ fusions by VjbR (data not shown). However, these changes to the *virB* promoter box also resulted in an overall decrease of background promoter function, possibly because the downstream half of the box overlaps the putative –35 regulatory sequence of the promoter (Fig. 3A).

4. Many promoter regions in the Brucella genome contain a conserved *virB* promoter box

Since the 18 bp lux box-like sequence was required for full P_{virB} induction, we performed a bioinformatic search for the *virB* promoter box in intergenic regions of the *B. suis* 1330 and *B. abortus* 2308 genomes using the motif alignment search tool MAST (<http://meme.ncbr.net/meme/>) (Halling *et al.*, 2005, Chain *et al.*, 2005, Paulsen *et al.*, 2002, Bailey & Gribskov, 1998). In the first search, a consensus of the three motifs in P_{virB} , P_{tetR} and the *virB1-virB2* intergenic region was used, resulting in the identification of 45 promoter regions containing similar motifs. A selection of 9 promoters was fused to *lacZ* for expression analysis in our *E. coli* strains containing *vjbR* or BAB1_0345 under control of a T7 promoter. Out of these 9 promoters we found 5 to be activated by VjbR, and the conserved motifs in these promoters were used to refine the consensus sequence for a new round of promoter searches. After repeating the promoter prediction process 3 times, a total of 144 promoters were predicted to contain the consensus 18 bp *virB* promoter box (Supplementary Table 2). Among genes directly downstream of these promoters, 13 are predicted to encode transcriptional regulators, including FtcR, whose expression was shown previously to be partially under control of VjbR (Leonard *et al.*, 2007). Other genes include proteins of hypothetical function (40), enzymes of unknown specificity (22) and transport and binding proteins (11). The list also includes 14 proteins predicted to be involved in adaptation of *Brucella* to atypical conditions such as the intracellular environment, including RelA/SpoT, BacA and cyclic β -glucan synthetase (Arellano-Reynoso *et al.*, 2005, Dozot *et al.*, 2006, LeVier *et al.*, 2000).

Based on known or predicted roles in host-pathogen interaction, 24 promoters were tested for VjbR-dependent transcriptional activation in *E. coli*. Transcriptional fusions of 15 of these promoters to *lacZ* were activated by induction of *vjbR* expression (Fig. 5 and Supplementary Table 3). These promoters included those upstream of two genes required for virulence in mice: *fliC* encoding flagellin and *cgs*, encoding cyclic β -glucan synthetase (Fretin *et al.*, 2005, Arellano-Reynoso *et al.*, 2005, Briones *et al.*, 2001). Expression of two regulators was shown to be induced by VjbR, LysR12 (BAB2_0329) which is required for virulence of *B. melitensis* during infection of mice (Haine *et al.*, 2005) and OmpR (BAB2_0762/3), predicted to encode the response regulator of the OmpR/EnvZ two-component system. In addition, *virB* promoter box-containing upstream regions of several uncharacterized genes were found to be activated by VjbR, including BAB2_0328, BAB1_1881, BAB1_1066, BAB1_1837, BAB1_1994, BR0951 (ORF BR0951 is not annotated in *B. abortus*), BAB1_1652, BRA1111, BAB1_1058, BAB2_0403 and BAB1_0604. BAB2_0880 was predicted in our first generation screen to contain an

upstream *virB* promoter box, however we found the BAB2_0880::*lacZ* transcriptional fusion not to be activated by VjbR in *E. coli* (Fig. 5). In the subsequent rounds of promoter searches the promoter of BAB2_0880 was no longer predicted to contain a *virB* promoter box, indicating that the consensus sequence predictions became more refined as we included more motifs from promoters that were activated by VjbR in *E. coli*.

5. TEM1 β -lactamase can be used to detect translocation of T4SS effector proteins into macrophages

TEM-1 β -lactamase has been used by our lab and others (Charpentier & Oswald, 2004, Raffatellu *et al.*, 2005, Sun *et al.*, 2007) to demonstrate cellular translocation of Type III secretion system (T3SS) substrates, including *Salmonella enterica* serovar Typhi SipA and serovar Typhimurium Phase I flagellin. Since the requirements for passage through the T3SS needle and the T4SS apparatus may differ, we tested the utility of this reporter using a well characterized T4SS effector, *Legionella pneumophila* RalF. For these experiments, we generated translational fusions to the C terminus of TEM-1 β -lactamase (see Materials and Methods). To this end, each protein was fused to the C terminus of a modified TEM-1, in which the N-terminal Sec-dependent signal sequence was replaced by a 3 \times FLAG tag (Raffatellu *et al.*, 2005). The resulting constructs were introduced into *L. pneumophila* Philadelphia 1 or a *dotA* mutant, in which the Dot/Icm T4SS is inactivated (Fig. 6). The strains expressing the TEM-1 fusion proteins were then used to infect J774 macrophages for detection of protein translocation, as has been reported previously (Raffatellu *et al.*, 2005, Sun *et al.*, 2007, Charpentier & Oswald, 2004). Translocation of a TEM1-RalF fusion by *L. pneumophila* into a host cell loaded with the fluorescent β -lactamase substrate (CCF2/AM) should lead to a shift in color of the cells from green to blue. At 4h after infection of J774 cells, we observed translocation of TEM1::RalF into macrophages (Fig. 6A). A RalF mutant protein in which a lysine residue in the C terminus was replaced by alanine (K368A) was translocated, as reported previously, but replacement of the essential leucine residue at the C terminus by alanine (L372) strongly reduced translocation. Finally, the C-terminal 20 amino acids of RalF mediated translocation of TEM1 into J774 cells, in agreement with a previous report (Nagai *et al.*, 2005). Figure 6B shows a blue cell infected with Lp01 expressing dsRed and TEM1::RalF and a green cell infected with the *dotA* mutant expressing the same fusion. A lack of translocation of all the fusion proteins by the *dotA* mutant was not the result of reduced intracellular bacteria or decreased expression of the fusion proteins, as both of these were similar between Lp01 and the *dotA* mutant (Fig. 6C and 6D). These results showed that TEM-1 β -lactamase is a useful reporter for detection of T4SS-mediated protein translocation.

6. Translocation of BAB1_1652 (VceA) and BAB1_1058/BR1038 (VceC) into macrophages by *B. abortus* is dependent on the VirB T4SS

We analyzed the 144 candidate genes with the 18 bp P_{virB} promoter motif for features typical of translocated bacterial effector proteins. Since no predicted function was found initially for some T4SS effectors identified in other bacterial pathogens (de Felipe *et al.*, 2005), we considered 13 hypothetical proteins from this group as candidate effectors. To determine whether these effector candidates were substrates of the T4SS, we generated translational fusions to the C terminus of TEM-1 β -lactamase, as described above for RalF (see Materials and Methods). The resulting constructs were introduced into the *B. abortus* wild type or a *virB2* mutant (ADH3; (den Hartigh *et al.*, 2004). The strains expressing the TEM-1 fusion proteins were then used to infect J774 macrophages for detection of protein translocation. For 10 of these constructs, we observed fewer than 0.5% blue cells, suggesting that these fusion protein are not translocated into host cells similar to what was observed for our negative control protein FLAGTEM1::GST (Fig. 7A and 7B). However, we did observe blue cells in macrophage cultures infected with *B. abortus* expressing TEM1::BAB1_1652,

B. suis TEM1::BR1038 or a TEM1 fusion to its *B. abortus* orthologue (TEM1::BAB1_1058), suggesting that these proteins are translocated into macrophages by *B. abortus* (Fig. 7A and 7B). No blue cells were seen in macrophage cultures infected with a *virB2* mutant (ADH3) expressing these fusion proteins, indicating that an intact T4SS is required for cellular translocation of TEM1::BAB1_1652, TEM1::BR1038 and TEM1::BAB1_1058 (Fig. 7A–B). Western blotting of wild type and *virB2* mutant *B. abortus* expressing TEM1::BAB1_1652, TEM1::BR1038 or TEM1::BAB1_1058 showed that both strains expressed the fusion protein at equivalent levels (Fig. 7C).

To ensure that the lack of detectable translocation from the *virB2* mutant compared to wild type *B. abortus* was not due to a lower number of T4SS mutant *Brucella* associated with the macrophages, we determined the kinetics of intracellular survival of each strain after a 90 minute treatment with gentamicin to kill extracellular bacteria (Fig. 8A). At time points up until 11h, we recovered equal numbers of *B. abortus* wild type and the *virB2* mutant, which demonstrated that failure of the *virB* mutant to translocate the TEM1 fusion proteins is not due to intracellular killing of the *virB* mutant. Based on these findings, we designated TEM1::BAB1_1652 as *vceA* (for *virB*-coregulated effector A), BAB1_1058 as *B. abortus vceC* and BR1038 as *B. suis vceC*.

Expression of the *B. abortus virB* genes has been shown to be induced upon phagosomal acidification and to reach its maximal expression level at 5h after infection (Sieira et al., 2004). Since we initially screened for translocation at 16h after infection, we determined the kinetics of translocation of VceA and VceC into macrophages (Fig. 8B). Translocation of VceA and VceC was first detectable at 7h after macrophage infection. These findings were consistent with a requirement for induction of the T4SS genes for translocation of the effectors into host cells.

7. VceA and VceC are conserved in all sequenced *Brucella* genomes

VceA is a protein of 105 amino acids that is conserved in all of the sequenced *Brucella* genomes. It is not well conserved in close phylogenetic relatives of *Brucella* spp., including *Ochrobactrum anthropi*, *O. intermedium*, *Bartonella* spp., or *A. tumefaciens*, however a conserved domain of this protein is found in sequenced genomes of some environmental strains of Alpha- and Betaproteobacteria, such as *Paracoccus denitrificans* and *Burkholderia* spp. This information did not allow us to infer a putative function for this protein.

The VceC protein of *B. abortus* contains 418 amino acids, with a proline rich central domain. BLASTP searches using the VceC amino acid sequence showed that an N-terminal region of approximately 100 amino acids was conserved in proteins of several α -proteobacteria including *Mesorhizobium loti*, *Methylobacterium radiotolerans* and *Bartonella* spp., among others. However, most of the full-length protein was specific to *Brucella* spp. and its close relatives, *Ochrobactrum* species *anthropi* and *intermedium* (unpublished genome sequence). Figure S1 shows a multiple alignment of different VceC proteins revealing a high level of conservation among VceC proteins from *O. anthropi*, *O. intermedium* and *Brucella* spp. (see also Table S3). A PSI-BLAST search using the conserved central proline rich region of VceC showed similarity to several eukaryotic proteins of hypothetical function containing proline rich regions. Interestingly, the C-termini of the *B. suis* and *B. abortus* VceC proteins differed, as a result of 1bp that is missing in *B. suis vceC*, leading to a frameshift in the C-termini of *B. suis* and *B. canis* (Figure S1). This difference was not the result of a sequencing error, as we re-sequenced these genes in our laboratory to confirm the different C termini (data not shown). The differences in the C terminus of *B. abortus* and *B. suis* variants of VceC suggest that the Type IV translocation signal may not need to be completely conserved. Based on this finding, as well as similarity

of the proline-rich region of VceC to eukaryotic proteins, we chose to characterize VceC further.

8. The C terminal 20 amino acids of VceC are required for its translocation into host cells

For several Type IV effectors, including *L. pneumophila* RalF (Nagai et al., 2005), *A. tumefaciens* VirF (Vergunst et al., 2005), *Bartonella henselae* BepA-D (Schulein et al., 2005), and *Helicobacter pylori* CagA (Hohlfeld et al., 2006), a C-terminal domain is important for translocation into host cells. To determine whether the C terminus of *B. abortus* VceC was required for its translocation into macrophages, we generated two sets of truncated TEM1::VceC fusions, using the *B. suis* and *B. abortus* variants (Fig. 9A): one in which the C-terminal 20 amino acids were deleted (*Ba* VceC_{303–398} and *Bs* VceC_{303–390}) and one in which the C-terminal 49 (*Bs* VceC) or 41 (*Ba* VceC) amino acids were deleted (TEM1::VceC_{303–369}). Each of these truncations abrogated translocation into macrophages, showing that despite their divergence in the C terminus of the protein, the C-terminal 20 amino acids of both *B. abortus* VceC and *B. suis* VceC are required for VirB-dependent translocation into macrophages. Truncation of the C terminus did not reduce stability of the proteins, as shown by Western blot (Fig. 7C). Alignment of the C termini of *B. abortus* VceC and *B. suis* VceC with that of VceA revealed no obvious motif that could be involved in translocation, although both VceA and VceC had a C-terminal K-X-K-X-K/H motif reminiscent of the C-terminus of *A. tumefaciens* effectors (Vergunst et al., 2005).

9. Translocation of TEM1::VceC results in a cytotoxic effect on J774 macrophages

Since we observed in some translocation assays a large number of blue cell “ghosts” suggestive of lysis of blue cells, we tested whether a cytotoxic phenotype may contribute to the low number of β -lactamase positive blue cells in our assays. To test this idea, we assayed for LDH release from J774 macrophages infected with *B. abortus* wild type or *virB2* mutant expressing TEM-1 β -lactamase fusions. At the multiplicity of infection of 500 used for these assays, at 11h post infection we observed 30% cytotoxicity in cells infected with *B. abortus*, while cells infected with the *virB2* mutant were indistinguishable from uninfected controls (Fig. 8C). This likely represents the T4SS-dependent macrophage cytotoxicity described by Pei et al using a similar multiplicity of infection (Pei et al., 2008). However strikingly we found that 60% of cells infected with *B. abortus* 2308 expressing the TEM1::VceC fusion were lysed by 11h (Fig. 9C). Truncation of the C terminus of VceC by 20 or 49 amino acids led to a reduction in cytotoxicity that corresponded with the reduced ability of these fusion proteins to be translocated into host cells (Fig. 9A–C). In a second experiment, we determined the time course of lysis in cells infected with *B. abortus* 2308 expressing the TEM1::VceC fusion (Fig. 10D). At 8h and 11h post infection, we observed increasing lysis of infected cells with kinetics paralleling those of blue cell appearance in the cultures (Fig. 8B). This finding suggested that under the conditions used for this assay, cells into which the TEM1::VceC fusion is translocated may ultimately lyse, thereby limiting our ability to detect translocation of this fusion protein.

10. The C terminus of *B. suis* VceC contains a translocation signal that is functional in *L. pneumophila*

T4S signals from some substrates, such as that of RSF1010 MobA, are able to function in heterologous systems, including *Bartonella henselae* (Schulein et al., 2005) and *Helicobacter pylori* (Hohlfeld et al., 2006), suggesting that some of these secretion signals share common features. To determine whether the C-terminal signal of VceC can also function in a heterologous system, we expressed the C-terminal 107 or 115 amino acids of *B. abortus* and *B. suis* VceC (CyaA-VceC) as a fusion to adenylate cyclase in both *B. abortus* and *L. pneumophila*. Translocation was detected by infecting CHO-FcR cells with either *B. abortus* or *L. pneumophila* expressing the fusion proteins and detection of cyclic AMP. While

translocation of the CyaA-VceC fusions by *B. abortus* could not be detected, *L. pneumophila* translocated the *B. suis* CyaA-VceC fusion protein into the CHO-FcR cells at a level similar to that of the Dot/Icm T4SS substrate RalF (Fig. 11 A and 11B). The *B. abortus* CyaA-VceC fusion protein was translocated, albeit at a lower level. Translocation of both VceC fusion proteins was dependent on the Dot/Icm T4SS, since a *dotA* mutant did not translocate either VceC variant. Examination of the C terminus of both proteins showed that similar to RalF, *B. suis* VceC had a Leu residue at the -3 position, as well as the motif K-X-K-X-K directly upstream of the Leu. *B. abortus* VceC lacked both of these features, suggesting that they may contribute to recognition of the *B. suis* VceC secretion signal by the Dot/Icm T4SS (Fig. 11A).

11. VjbR-His₆ binds directly to P_{vceC}

To determine whether VjbR would directly bind a promoter of a translocated effector identified in the bioinformatic screen, we performed EMSAs with P_{vceC} in a similar manner as with the P_{virB} fragments. In EMSAs with P_{vceC} we used a ³²P labeled 317 bp fragment containing a DNA region upstream of the *vceC* start codon (Fig. 12A). Fig. 12B shows that the intensity of a band with decreased electrophoretic mobility increased as increasing amounts of purified VjbR-His₆ were added to the binding reaction. These results demonstrated direct binding of VjbR to P_{vceC}, and together with the expression analysis in *E. coli* (Fig. 5) strongly supported VjbR mediated co-regulation of *vceC* and the *virB* genes in *B. abortus*.

12. VjbR activates expression of vceC in B. abortus

To assess the biological significance of the VjbR-dependent regulation of *vceC* in *E. coli* and of results from *in vitro* EMSAs, we constructed chromosomal *lacZ* fusions to the *virB1* and *vceC* promoters in the *B. abortus* wild type (2308) and in a *vjbR* mutant (ADH17) background. The β-galactosidase activity of these strains was measured at several time points during growth in modified minimal E-medium at pH 5, which has been shown to induce expression of the *virB* genes (Kulakov *et al.*, 1997, Patey *et al.*, 2006). As expected, P_{virB}::*lacZ* expression was reduced in the *vjbR* mutant (Fig. 13A) compared to wild type *B. abortus*. This was also shown by Western blot, in which the VirB8 protein levels were markedly lower in the *vjbR* mutant than the wild type (Fig. 13C). Similarly, our results showed that *vceC*::*lacZ* expression was down regulated in the *B. abortus vjbR* mutant (ADH17; Fig. 13B). These data suggested that VjbR is required for optimal expression of both *vceC* and the *virB* operon, indicating that this activator coordinates expression of the *B. abortus* T4SS and one of its secreted targets.

Discussion

Type IV secretion systems are used by many Gram-negative bacteria to manipulate their host's cells for optimal survival and multiplication in a hostile environment. In a wide variety of eukaryotic hosts such protein secretion systems therefore play a critical role in pathogenesis (Backert & Meyer, 2006). All *Brucella* species including the human pathogens *B. abortus*, *B. suis* and *B. melitensis* require a T4SS for intracellular survival and persistent infection, however to date no substrates of the T4SS have been identified (Delrue *et al.*, 2001, den Hartigh *et al.*, 2004, Hong *et al.*, 2000, O'Callaghan *et al.*, 1999, Sieira *et al.*, 2000). We hypothesized that in order to function optimally, the T4SS should be expressed together with its secreted effectors. To test this idea, we screened for genes co-regulated with the VirB system to identify an effector of the *B. abortus* T4SS. The LuxR-family quorum sensing regulator VjbR, is an important activator of *virB* gene expression (Delrue *et al.*, 2005), however, it is unclear from this work whether this regulation is direct or occurs as part of a regulatory cascade. Binding of VjbR to a 123 bp fragment of P_{virB} in an EMSA

provided evidence that this regulator directly activates *virB* transcription. Activation of a $P_{virB}::lacZ$ transcriptional fusion by VjbR in *E. coli* was reduced by the quorum sensing pheromone C₁₂-HSL, which was in accordance with recent work showing inhibition of transcriptional activation by VjbR in the presence of C₁₂-HSL (Uzureau *et al.*, 2007).

Other regulators of the LuxR family, such as TraR of *A. tumefaciens*, are known to bind a palindromic motif of 18 bp often located close to the -35 regulatory sequence of promoters (Pappas & Winans, 2003, White & Winans, 2007). We identified a structurally related palindromic motif that enabled us to identify candidate VjbR-regulated genes in *B. abortus* and *B. suis*.

The *virB* promoter box was found upstream of 144 *B. abortus* and *B. suis* genes. Since some of these genes are predicted to be part of operons, this finding suggested that the VjbR regulon may include more than 144 genes. VjbR regulated several genes required for virulence of *Brucella*, including *cgs* and *fliC* (Briones *et al.*, 2001, Arellano-Reynoso *et al.*, 2005, Fretin *et al.*, 2005). The gene *cgs* encodes the protein required for synthesis of cyclic β -1,2-glucan, which mediates evasion of lysosomal fusion with the *Brucella*-containing vacuole (Briones *et al.*, 2001, Arellano-Reynoso *et al.*, 2005, Fretin *et al.*, 2005) and *fliC* encodes flagellin, a component of the flagellum that has been shown to be required for persistent colonization of the reticuloendothelial system in mice (Briones *et al.*, 2001, Arellano-Reynoso *et al.*, 2005, Fretin *et al.*, 2005). A gene with potential function in host-pathogen interactions, the *bopA* gene (BRA1111), was also found to contain a *virB* promoter box. This gene, shown to be regulated in *B. melitensis* by VjbR, encodes a protein with similarity to T3SS effector protein HopAN1 from the plant pathogen *Pseudomonas syringae* (Boch *et al.*, 2002, Lindeberg *et al.*, 2005, Rambow-Larsen *et al.*, 2008). Thus, VjbR may be a global regulator of genes involved in interactions with host cells.

To determine whether the reporter TEM-1 would be useful for detection of T4SS, we showed translocation of a TEM1::RalF fusion protein by *L. pneumophila* (Fig. 6). Our results are in agreement with a recently published study showing translocation of several new Dot/Icm effectors designated Legs (de Felipe *et al.*, 2008). Interestingly, the same authors were unable to detect translocation of several of the Legs using translational fusions to *Bordetella pertussis* adenylate cyclase (CyaA), suggesting that TEM-1 may be a more sensitive translocation reporter for at least a subset of effectors (de Felipe *et al.*, 2005). Based on our success in demonstrating cytosolic translocation of *S. enterica* serovar Typhimurium flagellin into macrophages using this system (Sun *et al.*, 2007), we chose it as a sensitive reporter assay for translocation of candidate *B. abortus* effectors. We assayed for translocation into host cells of 13 hypothetical proteins containing upstream *virB* promoter boxes. Ten hypothetical proteins were not translocated into J774 cells by *B. abortus* in our experiments. For BAB2_0056 and BAB2_0095 this might be caused by the fact that we constructed a fusion with TEM fused to the N-terminus of these proteins, possibly disrupting an N-terminal Sec (BAB2_0056) or TAT (BAB2_0095) secretion signal. In this respect, it should be noted that the precise route of translocation across the cell envelope of most T4SS effectors is still unknown. Therefore, it is possible that some *Brucella* effectors are exported to the periplasm prior to translocation into the host cell by the T4SS, as is the case for subunits of the *B. pertussis* pertussis toxin (Covacci & Rappuoli, 1993, Weiss *et al.*, 1993).

Importantly, our screen identified the first effectors of the *Brucella* spp. T4SS, VceA and VceC. Both of these effectors were co-regulated with the *virB* genes via VjbR. Further, the timing of secretion (7–9h after infection of macrophages) coincided with the time at which the *B. abortus* *virB* genes encoding the T4SS apparatus are induced in intracellular bacteria (Sieira *et al.*, 2004). The percent of cells positive for translocated VceA and VceC ranged from 0.5% to 2.5%, which is similar to translocation levels reported for the *Bartonella*

henselae BepB, BepC and BepD secretion signals using the Cre reporter assay for translocation, a method that, similar to the TEM-1 reporter, allows quantification of the number of cells containing translocated proteins (Schulein et al., 2005). It is possible that we were able to detect translocation of TEM-1 fusions by *B. abortus*, but not CyaA fusions (data not shown) to these proteins, because with the low number of cells into which proteins are translocated, the signal of the few cells containing translocated protein would be diluted out by the majority of cells in a lysate that do not contain cytosolic protein. It has been reported previously, that in macrophage cultures infected with *Brucella*, only few cells contain replicating bacteria, while in the majority of cells, bacteria do not increase in numbers (Celli et al., 2003). Thus, it is possible that while many cells contain *Brucella*, only a few contain bacteria that successfully inject effector proteins and go on to replicate intracellularly.

The C terminus of VceC was required for optimal secretion, similar to what has been found for other Type IV effector proteins (Vergunst et al., 2005). VceC of *B. suis* had a positively charged C-terminal region, which could form a secretion signal similar to that of *A. tumefaciens* T4SS effector proteins (Vergunst et al., 2005). However, for VceC, a R/K-X-R/K-X-R/K motif found in the C terminus was not essential, since this motif was present in the C terminus of *B. suis* 1330 VceC but not *B. abortus* 2308 VceC, both of which were translocated with similar frequency into macrophages by *B. abortus*. There is evidence that domains of effector proteins other than the C terminus contribute to T4SS-dependent translocation in other organisms (Cambronne & Roy, 2007, Hohlfield et al., 2006, Schulein et al., 2005), which could explain why the C-terminal regions of VceC proteins were not conserved between *B. abortus* and *B. suis*, yet both could be translocated into macrophages. Interestingly, *B. suis* CyaA-VceC was translocated into CHO-FcR cells by *L. pneumophila*, dependent on the Dot/Icm T4SS, which suggests the T4S signal of VceC is recognized by the Dot/Icm T4SS. Additional studies will be required to define the precise residues of the VceC translocation signal that are required for its transfer to the host cell.

In summary, this report provided the first evidence that VjbR coordinates expression of the *virB* T4SS with its translocated effectors. This situation is reminiscent of other T4SS, including the Dot/Icm T4SS of *L. pneumophila*, in which the two-component system regulators PmrA and CpxR regulate genes of the Dot/Icm T4SS as well as several Dot/Icm effector proteins (Zusman et al., 2007, Altman & Segal, 2008), and the *Mesorhizobium loti* R7A VirB/D4 T4SS, in which the *virB* and *virD4* genes are coregulated with the effectors msi059 and msi061 via a VirA/VirG two-component regulatory system (Hubber et al., 2007). The discovery of new host cell targets for VceA and VceC should shed light on their role in facilitating intracellular *Brucella* infection.

Experimental Procedures

Bacterial strains and plasmids

The *Brucella abortus* and *Escherichia coli* strains used in this study are listed in Table S1. *B. abortus* 2308 was used as a wild type strain. *B. abortus* strains were cultured on tryptic soy agar (TSA; Difco/Becton-Dickinson, Sparks, Md.), in tryptic soy broth (TSB) with appropriate antibiotics, or in modified E-medium (Kulakov et al., 1997). *E. coli* strains were grown on Luria Bertani (LB) agar. Antibiotics were used at the following concentrations for *E. coli* and *B. abortus*: carbenicillin (Carb), 100 µg/ml; kanamycin (Kan), 100 µg/ml; chloramphenicol (Cm), 30 µg/ml. *E. coli* and *B. abortus* were grown at 37°C. Work with *B. abortus* was performed at biosafety level 3. DNA techniques were performed according to standard protocols. Restriction enzymes were purchased from New England Biolabs and primers from Operon Technologies. Wild type *L. pneumophila* strain Lp01 (Berger and Isberg, 1993) and *dotA* mutant were grown on CYE plates or in AYE broth as described

previously (Feeley et al., 1979). Antibiotics were used at the following concentrations: chloramphenicol (10 µg/ml) or kanamycin (Kan; 50 µg/ml).

Construction of plasmids for LacZ fusions

To obtain a low copy number plasmid for study of *B. abortus* promoters in *E. coli*, pSURS was constructed. To this end, the P15A origin of replication together with a Cm resistance gene were amplified by PCR from pSU19 (a derivative of pACYC184) using primers pACYC184-F and pACYC184-R (Table S4). The resulting 1900 bp product was digested with PstI and SalI and ligated to a 7500 bp fragment of pRS528, containing *lacZYA*, which was also digested with PstI and SalI, to yield pSURS1. Promoters of interest were introduced in the 5' region of *lacZ* between the BamHI and EcoRI sites of pSURS. A 463 bp fragment of P_{virB} was PCR amplified using primers PVirBBamH1-F and PVirBEcoR1-R, digested with BamHI and EcoRI, and ligated into pSURS1. The resulting plasmid was named pSURS2 (see Table S1 for all the pSURS plasmids constructed).

For construction of *lacZ* fusions in *B. abortus*, regions of *virB1* and *vceC* were PCR amplified using primers virB-1F and virB874R for *virB1* and VceC-Pst1-F and VceCXba1-R for *vceC*. These amplicons were digested with PstI and XbaI and inserted between PstI and XbaI sites in the 5' region of *lacZ* of plasmid pUJ10. The resulting plasmids were introduced into *B. abortus* 2308 and ADH17 strains by electroporation. Recombinants carrying the plasmid integrated into the chromosome were selected on ampicillin.

β-galactosidase assays

For β-galactosidase expression assays in *E. coli*, BL21 *lacZ*⁻ (Stratagene) was transformed with a pET103 plasmid containing the *vjbR* gene or ORF BAB1_0345 (a randomly selected putative transcriptional regulator of *Brucella*, which was used as negative control). Subsequently a pSURS plasmid containing a promoter of interest fused to *lacZ* was introduced into this strain. For the expression assay strains were grown overnight in LB containing 20 mM glucose. The overnight cultures were diluted 1:20 in LB with 20 mM glucose and grown for 2 h to an OD_{600nm} of 0.4, after which expression of regulators was induced by adding IPTG to a final concentration of 1 mM to the cultures. For some experiments, N-Dodecanoyl-DL-homoserine lactone (C₁₂-HSL from Sigma-Aldrich, Switzerland; dissolved in acetonitrile- to a final concentration of 30 µM or 60 µM) was added to inhibit *virB* promoter activity. Cultures were then grown for an additional 1.5 h until the OD_{600nm} reached about 1.0.

For β-galactosidase assays in *B. abortus*, wild type and *vjbR* mutant strains containing single-copy transcriptional fusions of *lacZ* to the *virB1* or *vceC* genes were grown in TSB overnight and then switched to modified minimal E-medium, pH 5.0 (Kulakov *et al.*, 1997). Samples were then taken at different time points for β-galactosidase activity measurement and Western blotting. β-galactosidase assays in *E. coli* and *B. abortus* were performed according to the protocol developed by Miller (1972). Assays were performed at least 3 times independently for each strain. Western blotting was performed to measure VirB8 protein expression after switching to modified minimal E-medium (pH 5.0) at the same time points β-galactosidase activity was measured. Bacteria were pelleted, resuspended in 2X Laemmli sample buffer and heated at 100°C for 15 min. The total protein equivalent of 1 × 10⁸ CFU was loaded and run on a 12% polyacrylamide gel and then transferred to a nitrocellulose membrane. The membranes were blocked in 2% non-fat skim milk powder in PBS for 1 h and probed with rabbit anti-VirB8 polyclonal antibody (1:5000), kindly provided by C. Baron (Rouot *et al.*, 2003). Goat-anti-rabbit IgG antibody (Biorad) conjugated with horseradish peroxidase (HRP) was used (1:5000) as a secondary antibody and HRP activity was detected with a chemiluminescent substrate (Perkin-Elmer).

Purification of VjbR-His₆

To generate a C-terminally His-tagged VjbR protein, the *vjbR* gene was PCR amplified from *B. abortus* 2308 DNA using the primers VjbR-F and VjbR-R. The PCR product was digested with *Nde*I and *Sal*I and cloned in pET103 digested with the same enzymes. The plasmid was introduced into *E. coli* BL21 by electroporation. The C-terminal His-tagged fusion protein was purified as follows. A culture of BL21 pET103-vjbR was grown overnight in LB containing 10 mM glucose and carb. The culture was diluted 1:50 in LB with carb and grown for an additional 24 hours at room temperature without the addition of Isopropyl β -D-thiogalactopyranoside (IPTG), in order to obtain more correctly folded recombinant VjbR-His₆ protein. Purification of VjbR-His₆ was performed according to standard protocols (Qiagen).

Electrophoretic mobility shift assays (EMSA)

A 123 bp fragment upstream of the *virB1* start codon was amplified from *B. abortus* 2308 DNA by PCR using the primers PvirB100-F and PvirB463-R and cloned into pCR2.1 to give rise to pWIL1 (Table S1). The fragment was isolated from pWIL1 by digestion with *Eco*RI. For EMSAs with the intergenic region *virB1-virB2*, a 172 bp fragment containing the putative P_{virB} box was PCR amplified from *B. abortus* 2308 DNA using primers PvirB1ig2-F and virB1369R. A 317 bp fragment of P_{vceC} (BAB1_1058) was obtained from pSURS31 by digestion with *Bam*HI and *Hind*III (P_{vceC} in *Brucella* contains a *Hind*III site). A 139 bp fragment of the *gyrA* gene served as a negative control. This fragment was PCR amplified from *B. abortus* 2308 DNA using primers RTgyrA-F and RTgyrA-R. All fragments were purified on a 5% polyacrylamide gel. The purified fragments were 5' labeled using T4 polynucleotide kinase (New England Biolabs) and γ -³²P-dATP (GE-healthcare). Binding reactions contained 20,000 cpm (0.4 ng) of radio labeled DNA and varying concentrations of VjbR protein in a final volume of 20 μ l. Furthermore, each reaction contained 10 mM Tris HCl (pH 7.4), 50 mM KCl, 1mM DTT, 6% glycerol, 0.5 mM EDTA, 50 μ g/ml BSA and 50 μ g/ml Poly(dI-dC) (Pierce Nucleic Acid). Samples were incubated at room temperature for 30 min and electrophoresed on a 5% non-denaturing polyacrylamide gel in 0.5x TBE. Gels were dried and exposed to an X-ray film for several hours. EMSAs were repeated at least 3 times with similar results.

Prediction of promoter regions containing a P_{virB} consensus box

A consensus prediction of the P_{virB} box was made using the online program MEME (<http://meme.nbcrl.net/meme/>) using motifs found in the promoter regions upstream of *virB1* and *tetR* (BAB2_0117) and in the intergenic region *virB1-virB2*. The resulting consensus was used to find related boxes in other promoters by searching the *B. abortus* intergenic nucleotide sequences (TIGR) with the online motif alignment search tool MAST (<http://meme.nbcrl.net/meme/>). Several promoter regions identified in this first generation search were PCR amplified from *B. abortus* 2308 genomic DNA, cloned into pSURS and analysed as described earlier for the *virB* promoter for activation by VjbR. Promoters with P_{virB} boxes, which were found to be activated by VjbR in the *E. coli* system were further used to refine the box consensus sequence and the search for new promoters containing this motif. In total this refining process was performed three times leading to a fourth generation promoter prediction.

Effector translocation assays

For fusions to the C terminus of TEM-1 β -lactamase or adenylate cyclase (CyaA), genes encoding candidate effectors were PCR amplified without their start codons (see Supplementary table 5 for primers), digested with *Xba*I and *Pst*I and cloned into pFlagTEM1 (Raffatellu *et al.*, 2005) or pFlagCya, which were also digested with *Xba*I and

PstI. pFlagTEM1 encodes a copy of TEM1 β -lactamase, in which the Sec-dependent signal sequence has been deleted and replaced with a 3xFLAG tag at the N terminus (Raffatellu *et al.*, 2005). All plasmid constructs were checked by DNA sequencing. Flag-TEM1-effector or CyaA-effector fusion constructs were introduced into *Brucella abortus* 2308 and ADH3 (*AvirB2*) strains by electroporation. Expression of the fusion proteins in *Brucella* was confirmed by Western blot using anti-Flag antibodies (Sigma). For the translocation assay 6×10^4 J774.A1 mouse macrophages were seeded in 96-well plates and infected with *B. abortus* 2308 or ADH3 expressing TEM-1 fusion proteins at a multiplicity of infection of 500:1. Plates were centrifuged for 5 min at $250 \times g$ at room temperature. Cells were incubated for 20 min at 37°C in 5% CO_2 and washed 2 times with phosphate-buffered saline (PBS) to remove free bacteria. Then 0.2 ml new DMEMsup plus 1 mM IPTG were added to each well and plates incubated at 37°C in 5% CO_2 . After different time points after infection (3, 5, 7, 9 and 16 hours), cells were washed once with Hank's balanced salt solution (Invitrogen) and loaded with a solution containing the fluorescent substrate CCF2/AM (Zlokarnik *et al.*, 1998) at a final concentration of 1mM, for 1.5h at room temperature using the standard loading protocol recommended by the manufacturer (Invitrogen). Fluorescence microscopy analysis was performed inside a BSL3 facility using an Axiovert M200 (Carl Zeiss, Germany), equipped with a CCF2 filter set (Chroma Technology, Brattleboro, VT, USA). Fluorescence micrographs were captured using a Zeiss Axiocam MRC5 and Zeiss AxioVision 4.5 software.

To detect translocation of CyaA fusion proteins, CHO-FcR cells, seeded 2×10^4 in 96 well plates, were infected by opsonized *B. abortus* or *L. pneumophila* strains expressing CyaA-effector fusions. Plates were centrifuged for 5 min at $250 \times g$ at room temperature and the cells were incubated for 60 min at 37°C in 5% CO_2 . After 3 washes with PBS, cells were incubated for another 5 h at 37°C in 5% CO_2 . Then cells were lysed in 0.1 M HCL/0.5 % Triton X-100 for 10 min at room temperature followed by heating for 10 min at 95°C . cAMP levels were determined by using the Direct cAMP Correlate-EIA Kit (Assay Designs).

To detect translocation of TEM-1 fusion proteins by *L. pneumophila*, pFLAGTEM1 plasmids expressing GST and RalF (full length, mutated or truncated) were introduced into *L. pneumophila* Lp01 wild type or *dotA* mutant by electroporation. To label *Legionella* with DsRed a constitutive promoter was amplified from pJC43 (Celli *et al.*, 2005) and then was assembled with the dimer Tomato (dTomato) gene, which was obtained from Dr. R. Tsien (Shaner *et al.*, 2004). The gene encoding dTomato, driven by the *aphA* promoter, was then cloned into pFT/RalFc (see above) to give rise to pTFT/RalFc, which was confirmed by DNA sequencing to have all components in the correct reading frame, and introduced into *L. pneumophila* Lp01 wild type and *dotA*. For the translocation assay 1×10^5 J774.A1 mouse macrophages were seeded in 24-well plates and infected with *L. pneumophila* expressing TEM1 fusion proteins at a multiplicity of infection of 100:1. Plates were centrifuged for 5 min at $250 \times g$ at room temperature. Cells were incubated for 60 min at 37°C in 5% CO_2 and washed 3 times with phosphate-buffered saline (PBS) to remove free bacteria. Then 0.5 ml fresh medium containing 2 mM IPTG and 50 $\mu\text{g}/\text{ml}$ gentamicin, was added to each well and plates were incubated at 37°C in 5% CO_2 . After 3 hours, cells were treated and processed as described above for *B. abortus*. To determine the bacterial loads, cells were lysed in 0.5% Tween 20 and scraped from each well. Expression of the fusion proteins to TEM-1 in *L. pneumophila* was confirmed by Western blot using anti-Flag antibodies (Sigma), as described above for *B. abortus*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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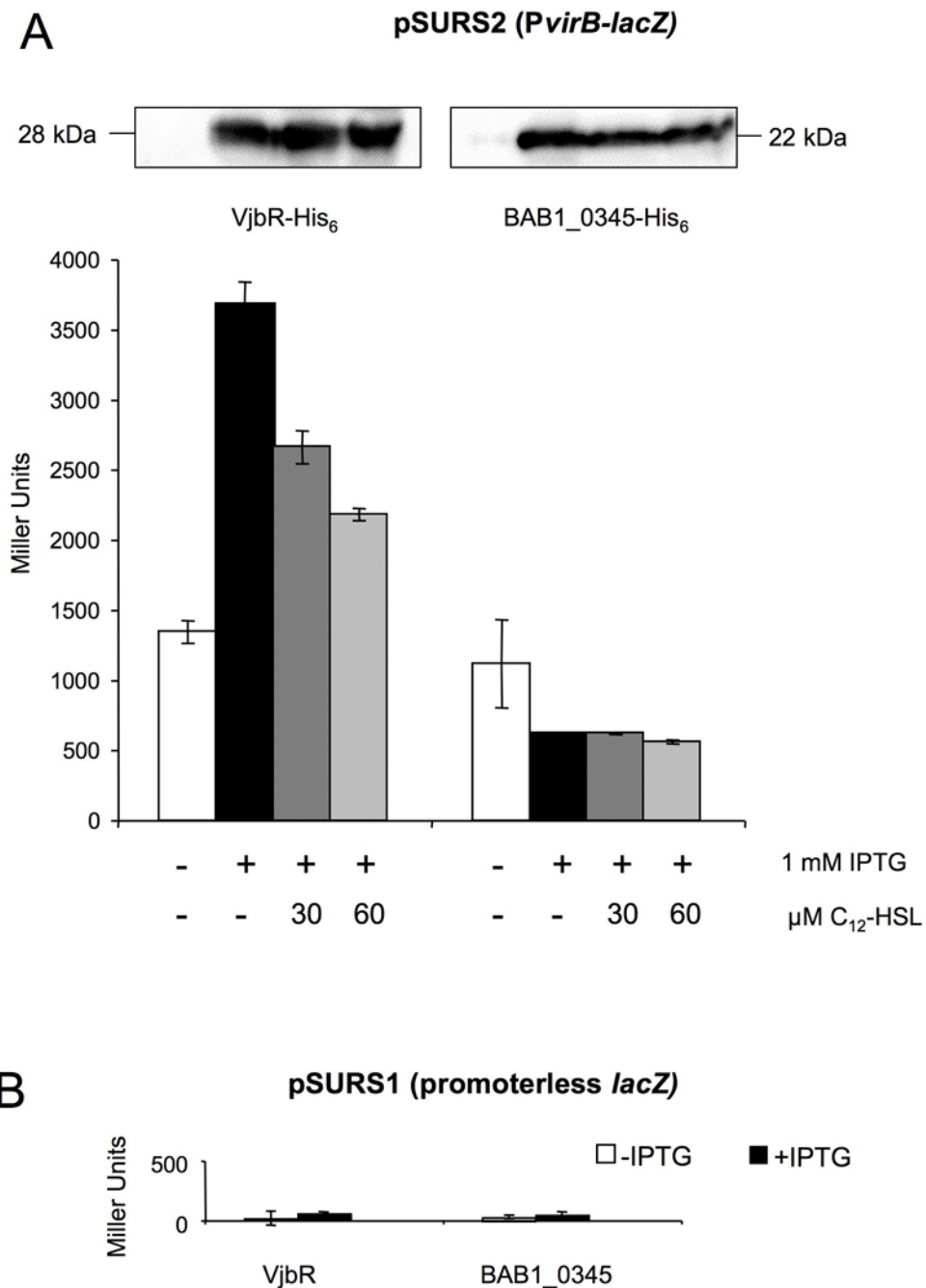
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**Figure 1.**

VjbR activates the promoter upstream of *virB1* in *E. coli*. **(A) Top:** Western blot using anti-His antibodies showing expression of VjbR-His₆ or BAB1_0345-His₆ after induction with IPTG and showing that expression of VjbR-His₆ is not reduced after addition of C₁₂-HSL. **Bottom:** β-galactosidase activity of *E. coli* with pSURS2 was measured after no induction (white bars) or induction with IPTG (black bars) of VjbR-His₆ and a negative control BAB1_0345-His₆. Hatched bars indicate induction with IPTG and the addition of 30 μM or 60 μM C₁₂-HSL to the culture. Values are the averages ± standard deviations of duplicate samples from a representative experiment that was repeated at least three times

independently. **(B)** β -galactosidase activity of *E. coli* with empty pSURS1 vector and expressing VjbR-His₆ or BAB1_0345-His₆.

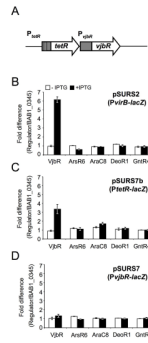


Figure 2.

(A) Map of the *tetR-vjbR* operon. (B–D) Fold difference of β -galactosidase activity of P_{virB} (B), P_{tetR} (C) and P_{vjbR} (D) in *E. coli* VjbR, ArsR6, AraC8, DeoR1 or GntR4 strain versus the negative control strain. White bars represent the ratio of measured Miller Units in uninduced *E. coli* expressing one of the regulators versus uninduced *E. coli* BAB1_0345. Black bars represent the ratio of measured Miller Units in IPTG induced *E. coli* expressing one of the regulators versus induced *E. coli* expressing BAB1_0345.

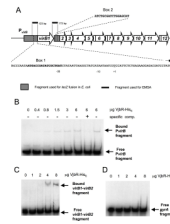


Figure 3.

(A) Schematic representation of the *virB* operon in the *B. abortus* chromosome II including *P_{virB}* and intergenic regions. The putative *P_{virB}* box 1 and box 2 that were used in the first prediction round are shown and the fragments that were used in EMSAs are indicated in black. (B–D) EMSA experiments showing specific binding of VjbR-His₆ to regions directly upstream and downstream of *virB1* (B) VjbR-His₆ binding to a 123 bp fragment of *P_{virB}* upstream of *virB1*. In the first lane no VjbR-His₆ was added to the binding reaction, and in the subsequent five lanes VjbR-His₆ was added in increasing amounts. In lanes 7 and 8 VjbR-His₆ protein concentration was the same as in lane 6, but here a 100-fold excess of unlabeled specific (*P_{virB}*) or non-specific (*vjbR* gene) DNA fragment was added to the reaction. (C–D) VjbR-His₆ binding to a 172 bp fragment of the *virB1-virB2* intergenic region (C), and not to a 139 bp fragment of the gene *gyrA* (D) when VjbR-His₆ was added in the same, increasing amounts to the binding reactions.

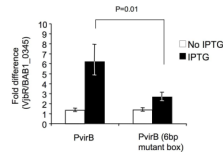


Figure 4. VjbR-dependent expression of $P_{virB}::lacZ$ constructs containing the native wild type (pSURS2) or mutated P_{virB} box (pSURS2b) in *E. coli* expressing *vjbR*. Values are presented as fold difference above *E. coli* expressing the negative control gene BAB1_0345. White bars represent expression levels in the absence of *vjbR* induction (no IPTG), and black bars represent expression after induction of *vjbR* expression with IPTG.

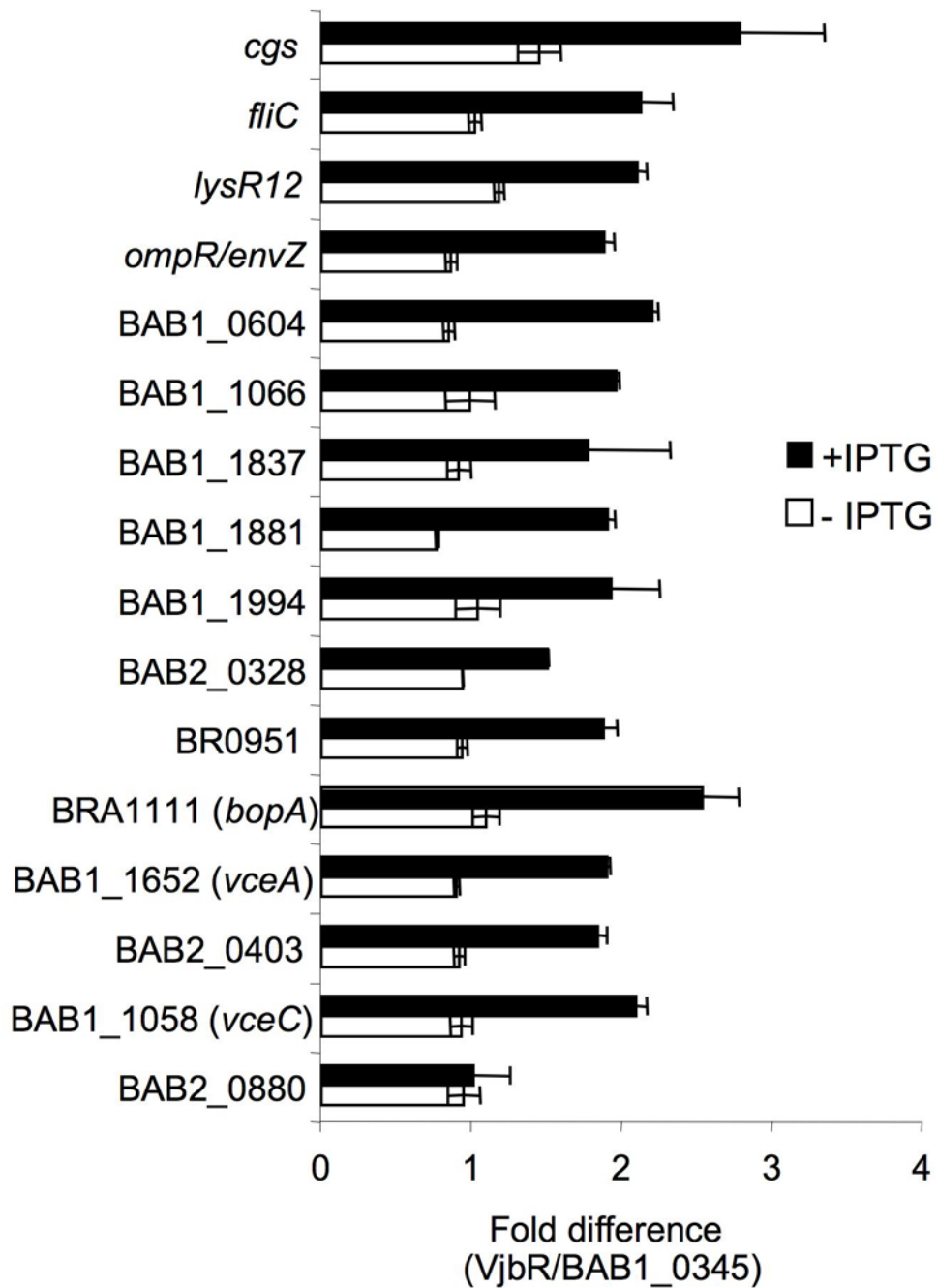


Figure 5. VjbR-dependent expression in *E. coli* of *lacZ* transcriptional fusions to 16 promoter regions containing a putative P_{virB} box. Values are presented as fold difference above *E. coli* expressing the negative control gene BAB1_0345. White bars represent expression levels in the absence of *vjbR* induction (no IPTG), and black bars represent expression after induction of *vjbR* expression with IPTG.

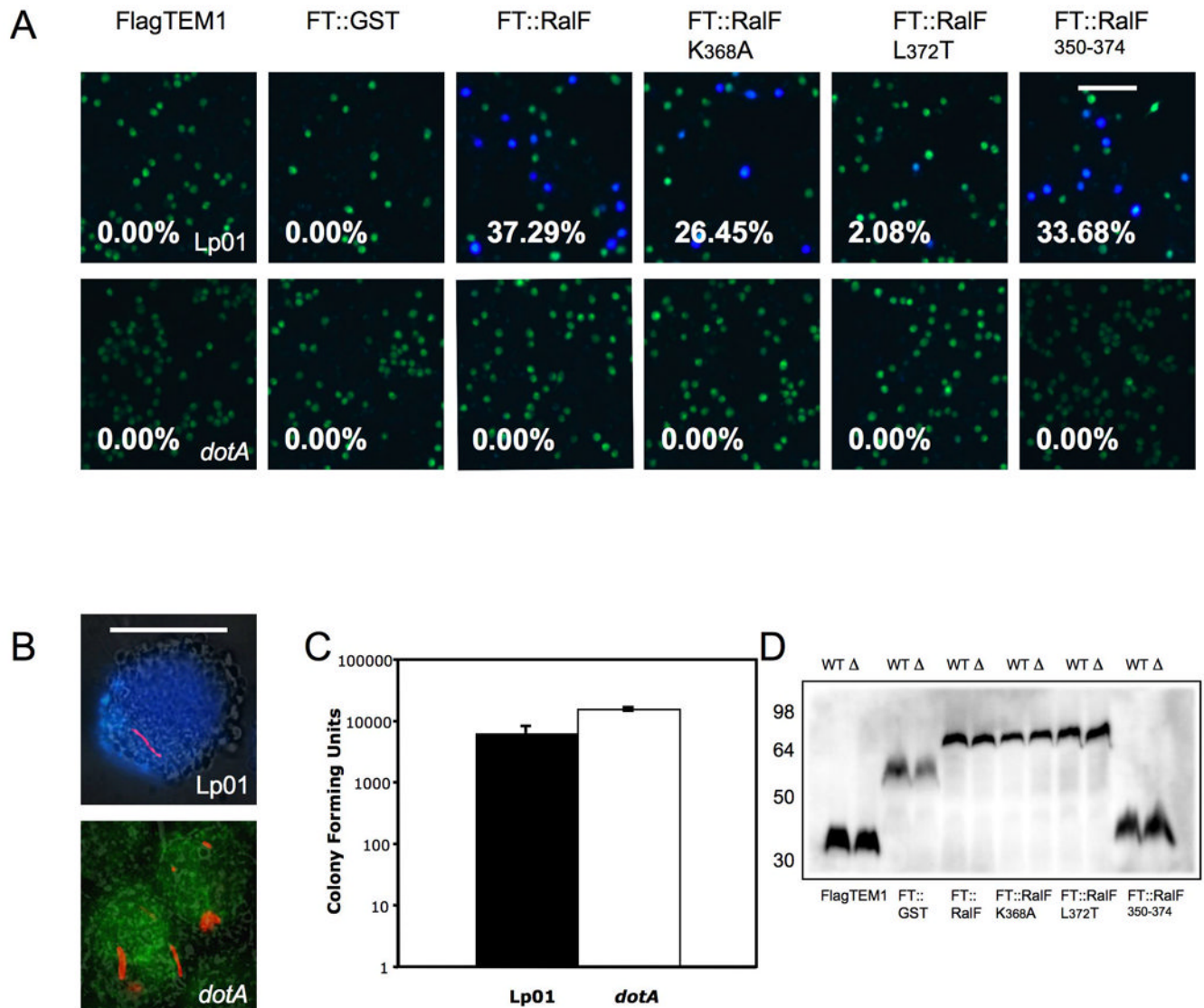
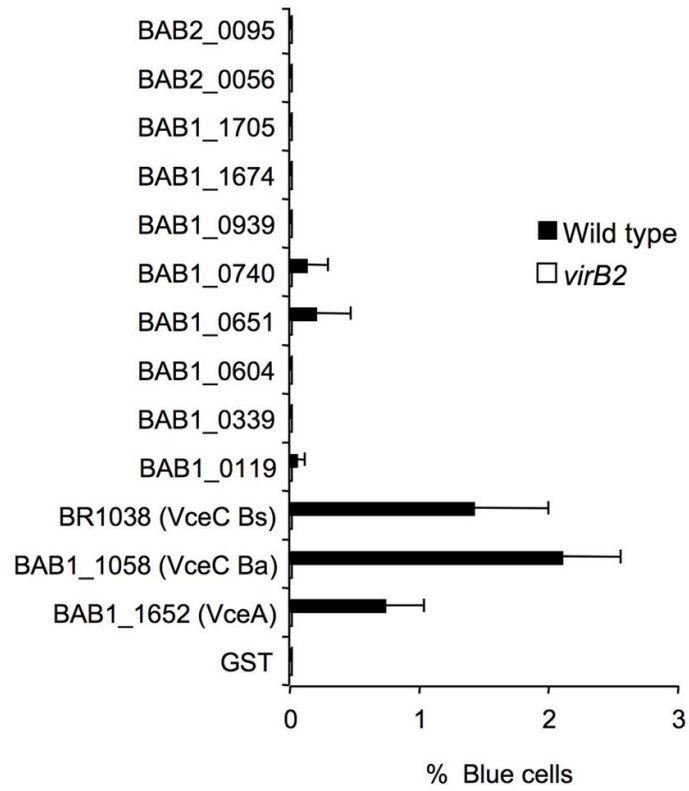
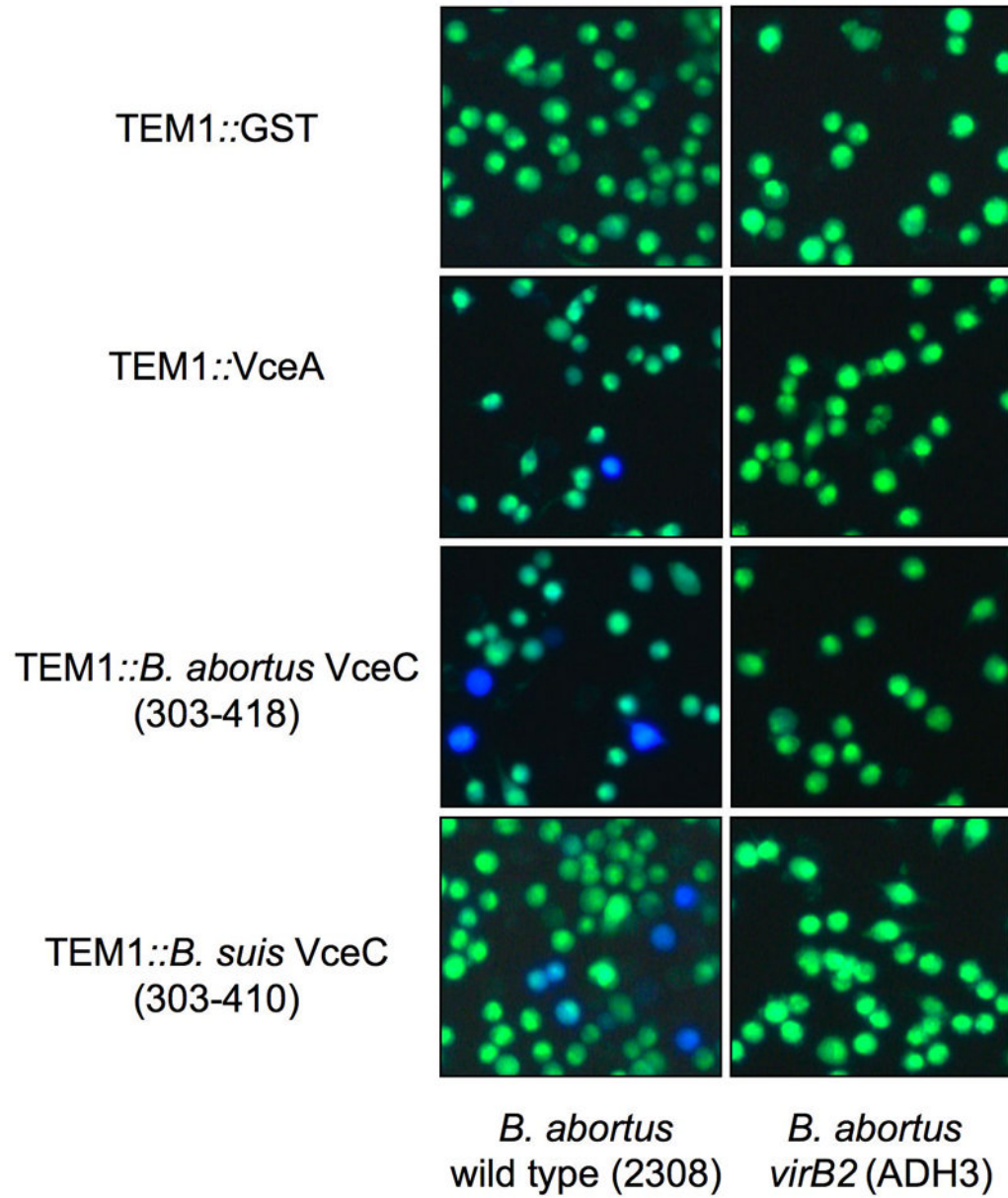


Figure 6. Translocation of TEM1::RalF into J774 macrophages by *L. pneumophila*. (A) J774 macrophages were infected with Lp01 (top row) or *dotA* (bottom row) mutant transformed with plasmids expressing FLAG-TEM-1 fusions for a total of four hours. Translocation efficiency is given as percentage of blue cells. Data is representative of three experiments that produced similar results. Bar, 100 μ m. (B) Same experiment as above by using DsRed labeled Lp01 and *dotA* mutant bearing a plasmid expressing FT::RalF₃₅₀₋₃₇₄. These high magnification images were merged from the β -lactamase color channel (Blue and Green), DsRed mono channel (Red) and phase contrast control. The upper blue cell contains Lp01 expressing FT::RalF₃₅₀₋₃₇₄ and the bottom green cell contains the *dotA* mutant expressing the same fusion protein. Bar, 5 μ m. (C) Number of bacteria per well. At the end of experiment cells were lysed in 0.5% Tween 20 after incubation for 90 min with 50 μ g/ml gentamicin. Colony forming units were determined by serial dilution and plating on BYCE plates. (D) Western blot using anti Flag, showing all β -lactamase fusions were expressed and their expression were similar between Lp01 (WT) and DotA mutant (Δ). Numbers on right indicate protein standard in kDa.

A



B

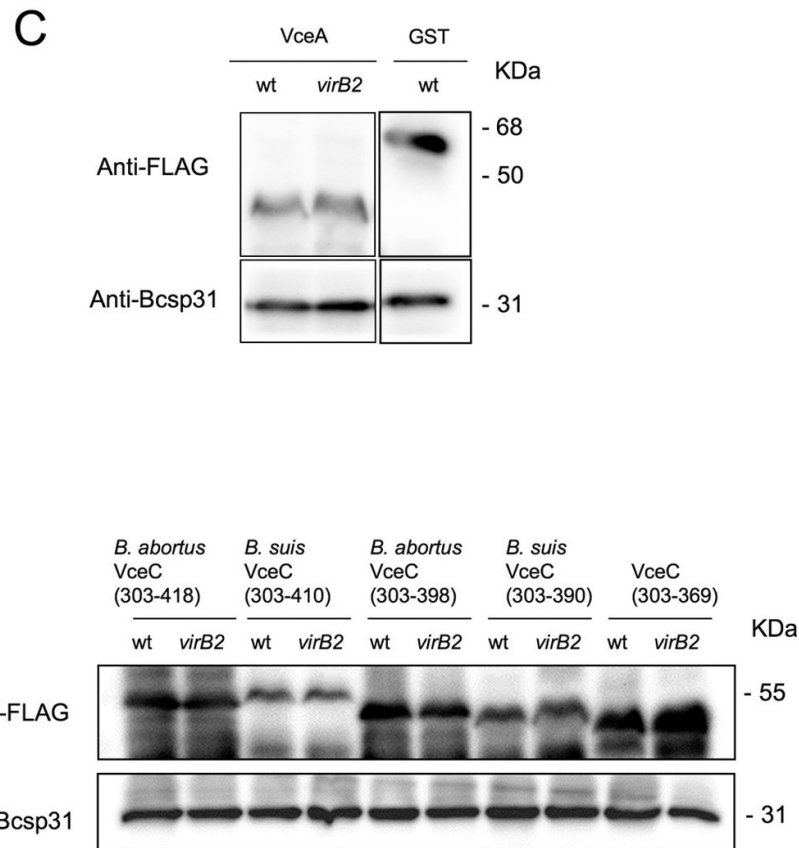
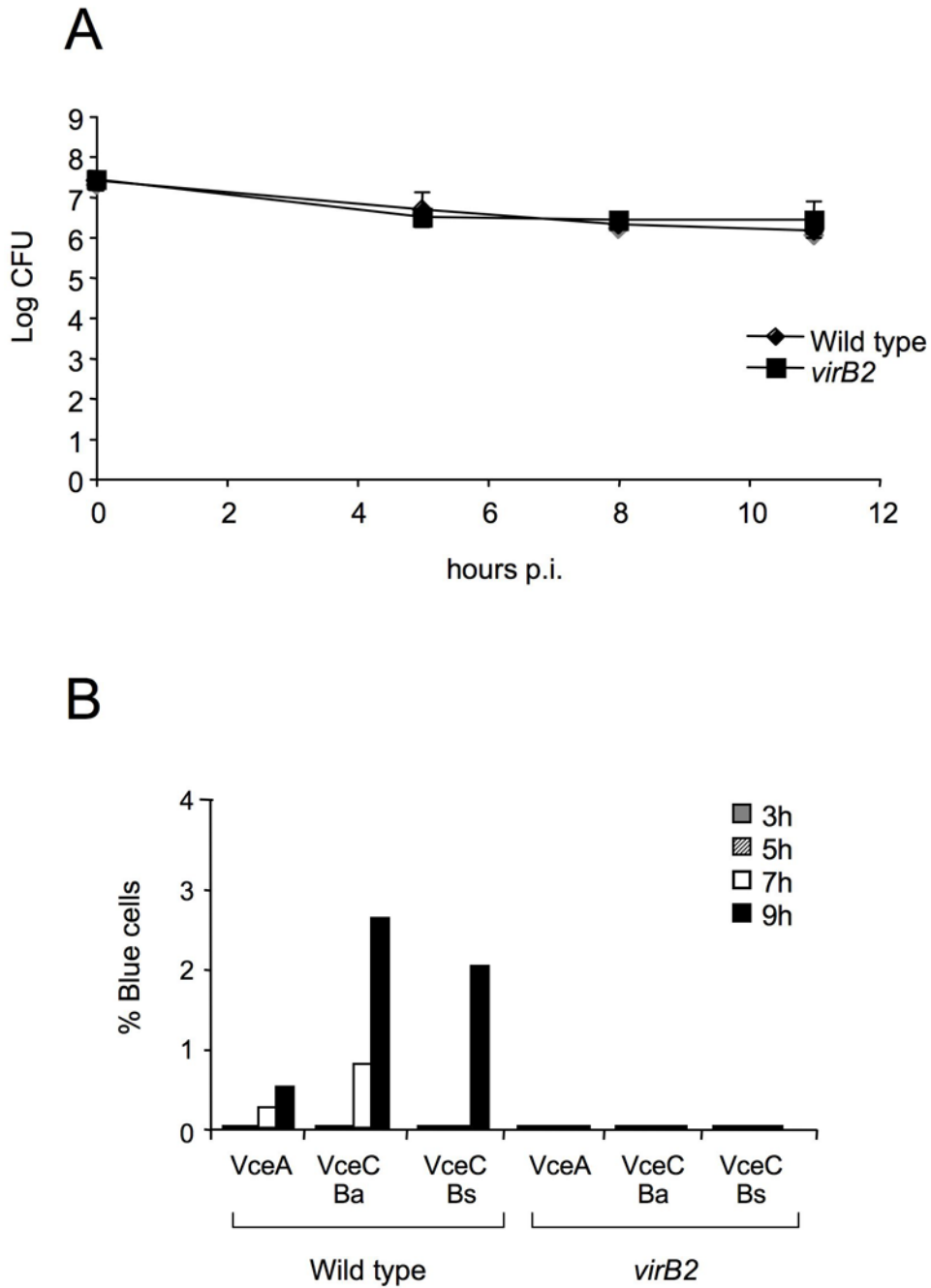
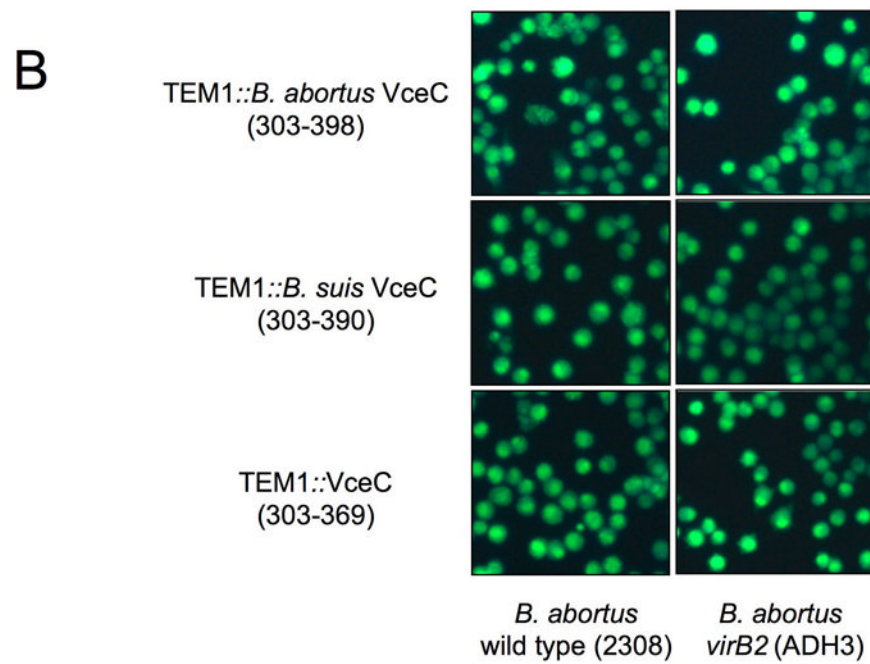
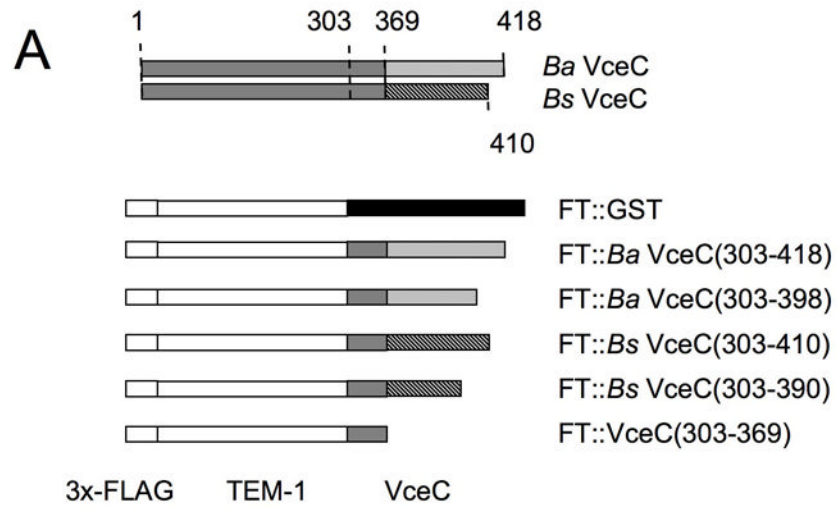


Figure 7.

Translocation of TEM1::VceA and TEM1::VceC into J774 macrophages. Cytosolic translocation of β -lactamase by wild type (2308) or $\Delta virB2$ mutant strain (ADH3) of *B. abortus* was assessed by fluorescence microscopy. Cells in which translocation of the fusion protein has occurred appear blue. (A) Quantification of effector translocation. Cells from ten independent random microscope fields were counted and the percent blue cells calculated. Results shown are the mean \pm SD of three independent experiments. (B) **Top** *B. abortus* 2308 containing the control fusion protein pFlagTEM1-GST, showing lack of translocation of FLAG-TEM1::GST. **Second from top to bottom** *B. abortus* 2308 (left) or *virB2* mutant (right) expressing FLAG-TEM-1 fused to *B. abortus* VceA or the C-terminal domain of VceC from *B. abortus* or *B. suis*. Results are from a representative individual experiment that was repeated three times independently. (C) Western blots showing equal expression levels of FLAG-TEM1 fusion proteins in *B. abortus* wild type and *virB2* mutant. Proteins were detected using anti-FLAG antiserum (upper row). As a loading control, the blot was probed with anti-Bcsp31 (lower row).

**Figure 8.**

Translocation of TEM1::VceA and TEM1::VceC into J774 macrophages at different time points after infection. (A) Survival of wild type (2308) and *virB2* mutant (ADH3) *B. abortus* in J774 macrophages after infection at an MOI of 500 bacteria per macrophage. (B) Cytosolic translocation of TEM1::VceA and TEM1::VceC by wild type (2308) or $\Delta virB2$ mutant strain (ADH3) *B. abortus* was assessed by fluorescence microscopy at 3, 5, 7 and 9 hours post infection (MOI 1:500). Cells in which translocation of the fusion protein has occurred appear blue and were quantified from independent random microscope fields from a single experiment and the percent blue cells was calculated.



C

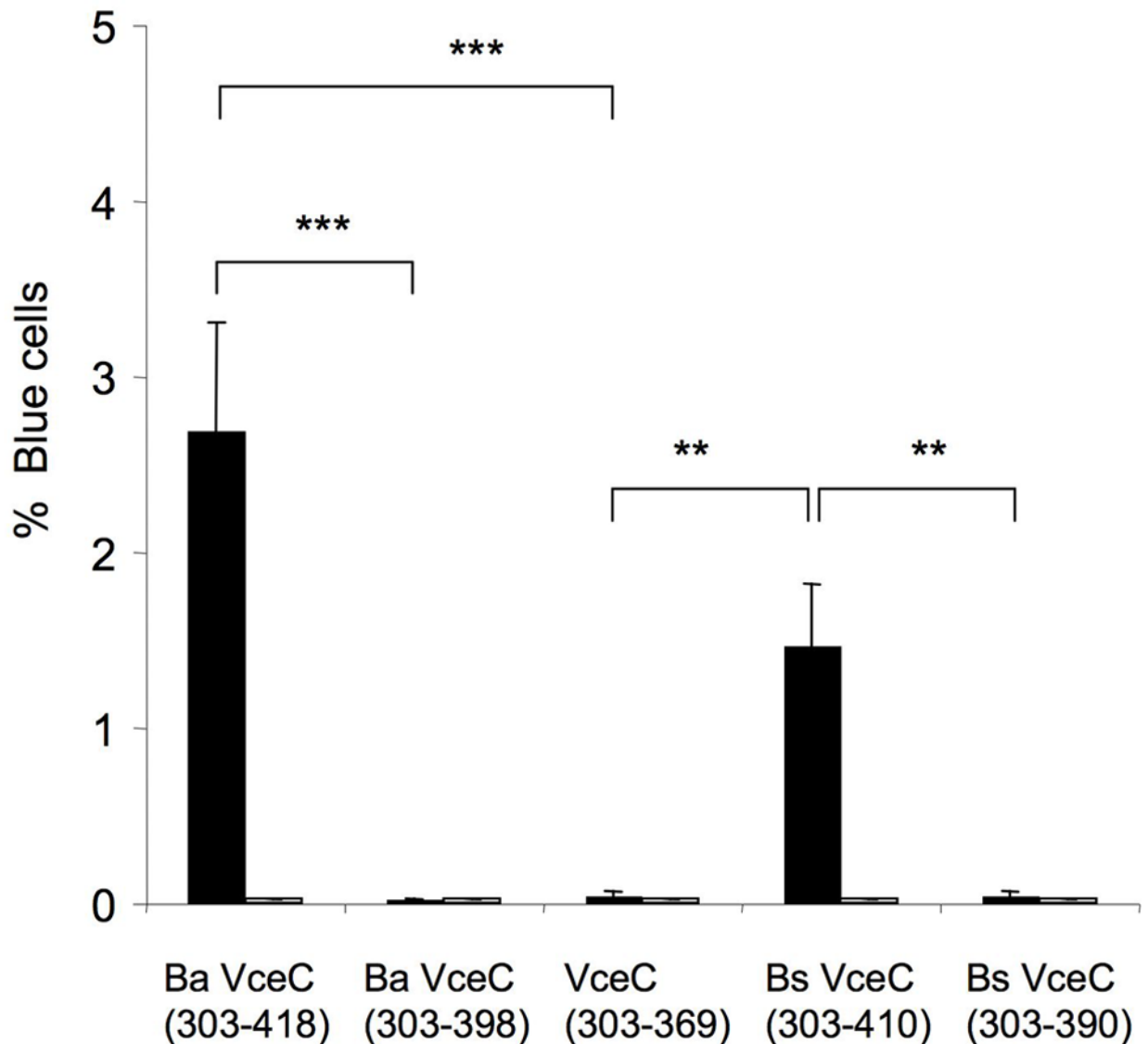


Figure 9.

The C-terminal 20 amino acids of both *B. abortus* VceC and *B. suis* VceC are required for translocation into infected macrophages. (A). Schematic representation of VceC in *B. abortus* and *B. suis* which differ in their last 49 or 41 amino acids respectively. For translocation experiments the C-terminal 115 (*B. abortus*) or 107 (*B. suis*) amino acids of VceC and truncations lacking the last 20 amino acids were fused to TEM1. Also a truncation of VceC (303–369) lacking the entire C-terminal region that differs between VceC *B. abortus* and *B. suis* was fused to TEM1 and tested for translocation in macrophages. (B). Translocation of TEM1-VceC fusions into J774 macrophages. Cytosolic translocation of β -lactamase by wild type (2308) or $\Delta virB2$ mutant strain (ADH3) of *B. abortus* was assessed by fluorescence microscopy. (C) Quantification of effector translocation. Cells from ten independent random microscope fields were counted and the percent blue cells calculated. Results shown are the mean \pm SD of three independent experiments. For statistical analysis

and calculation of SD, data were logarithmically transformed and significance of differences between cultures infected with wild type and *virB2* mutant *B. abortus* was analysed using a Student's t test. ***, $p < 0.001$; **, $p < 0.01$.

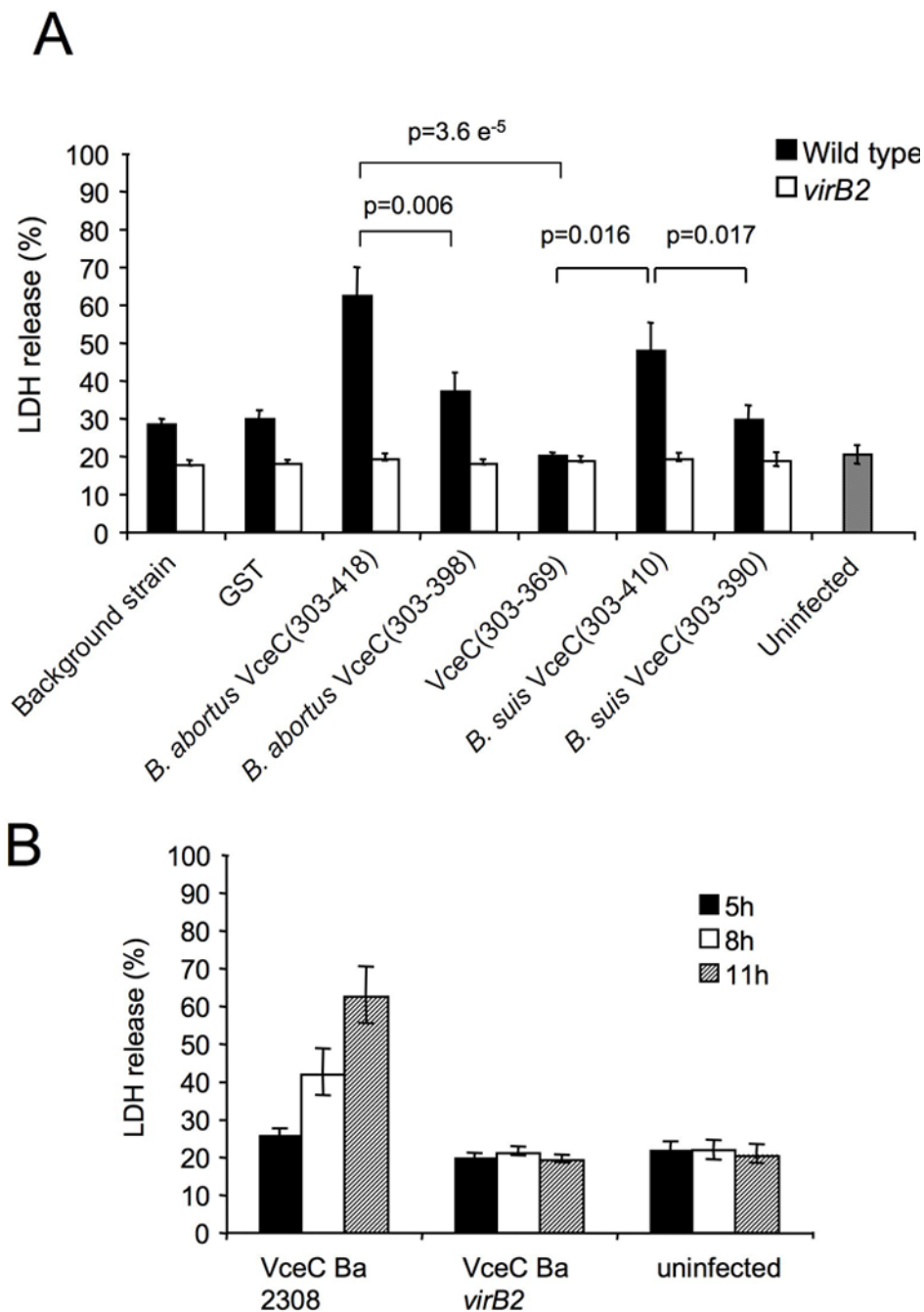


Figure 10.

(A) Cytotoxicity assay showing % LDH release at 11 hours post infection from J774 macrophages infected at MOI 1:500 with either wild type or *virB2* mutant *Brucella abortus* expressing TEM1-GST or TEM1-effector fusions. (B) Cytotoxicity assay showing % LDH release at 5, 8 and 11 hours post infection from J774 macrophages infected at MOI 1:500 with either wild type or *virB2* mutant *Brucella abortus* expressing TEM1::VceC. Results shown in A and B are the mean \pm SD of three independent experiments. For statistical analysis and calculation of SD, data were analysed using a Student's t test. ***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$.

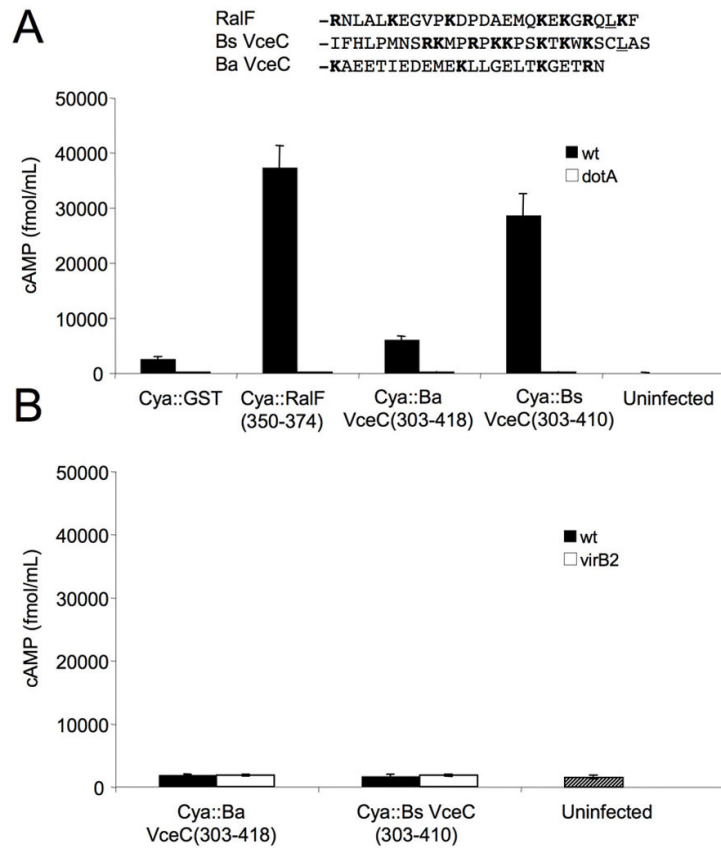


Figure 11.

The C-terminus of *B. suis* VceC contains a signal that is recognized by the *L. pneumophila* Dot/Icm T4SS (A) Translocation of CyaA::VceC into CHO-FcR cells by *L. pneumophila* wild type or *dotA* mutant. (B) Translocation of the same fusions by *B. abortus* cannot be detected. Results shown are representative of two independent experiments.

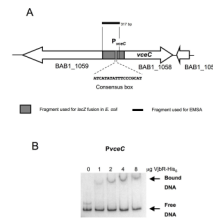
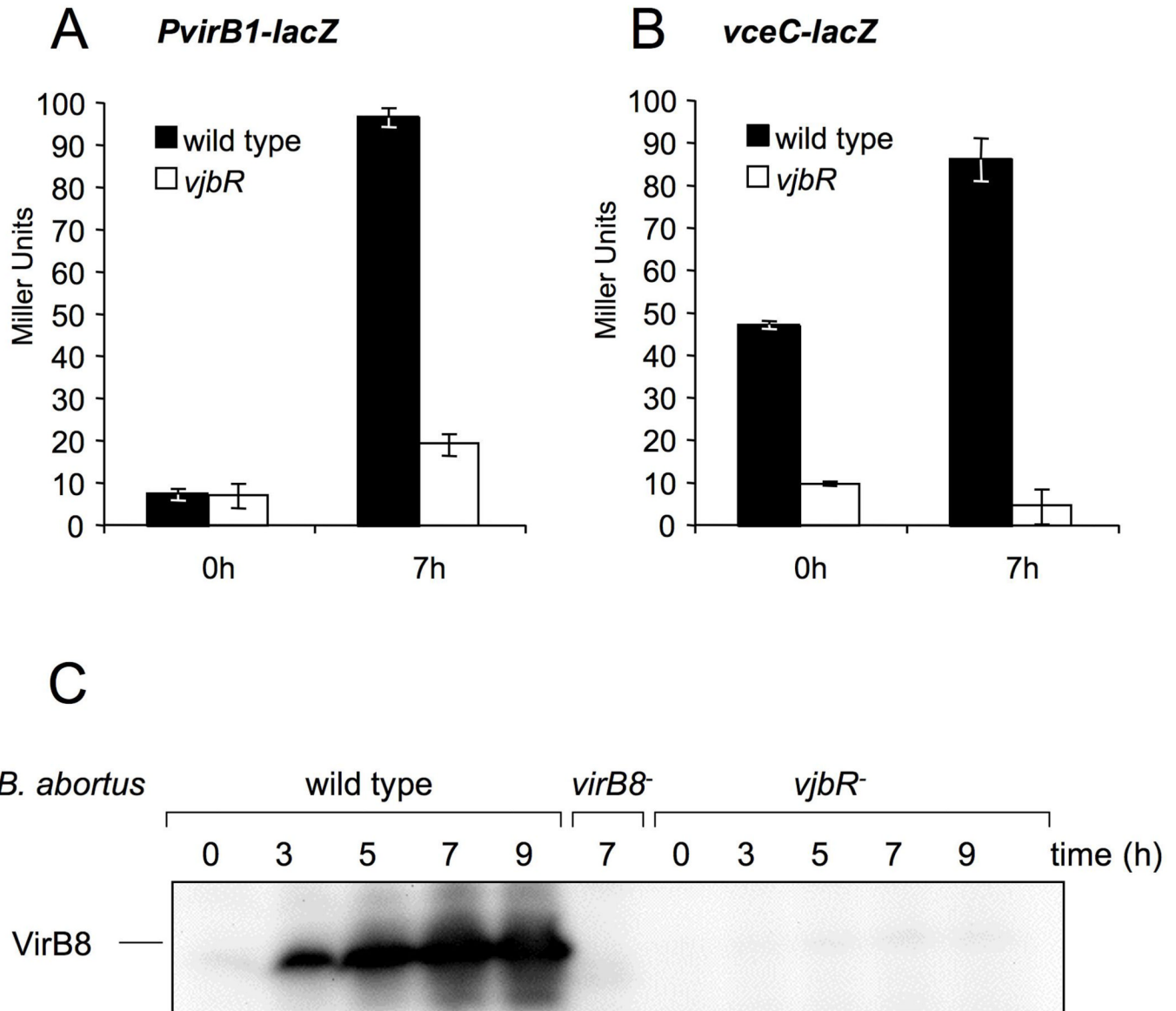


Figure 12.

VjbR binds to P_{vceC} (A) Schematic representation of *VceC* in chromosome I of *B. abortus* showing the putative P_{virB} box and promoter fragments used for EMSA and *lacZ* fusions. (B) EMSA showing binding of VjbR-His₆ to a ³²P labeled DNA fragment containing 317 bp upstream of the *vceC* start codon.

**Figure 13.**

VjbR-dependent activation of *virB* and *vceC* expression in *B. abortus* at different time points after switching from TSB to modified minimal E-medium, pH 5. At time points 0 h and 7 h after switching media, β -galactosidase activity was measured in samples of *B. abortus* wild type and *vjbR* mutant strains containing a chromosomal *virB1::lacZ* fusion (A) or *vceC::lacZ* fusion (B). C. Western blot using anti-VirB8 antiserum shows protein levels of VirB8 in wild type and *vjbR* mutant *B. abortus* in samples taken at several time points after switching to modified minimal E-medium at pH 5. In each lane, the total protein equivalent to 1×10^8 CFU was loaded, as determined by measurement of OD₆₀₀ for each culture.

Table 1

VjbR regulated proteins translocated into macrophages

ORF	Name	Aa	C-terminus ^a
BAB1_1652	VceA	105	TMKVVAG KVKRY GDGTPAKDKGHAPKN
BR1038	<i>B. suis</i> VceC	410	IFHLPMN SRKMPRPKPSKTKWKS CLAS
BAB1_1058	<i>B. abortus</i> VceC	418	DEQPEDAK KA EETIEDEME KLLGELTKGETRN

^a*Brucella abortus/suis* candidate T4SS substrates. Positively charged amino acids in the C-termini are shown in bold.