Identification and characterization of *Brucella* effector proteins

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The cover shows a convocal microscopic image of HeLa cells infected for 24 hours with *Brucella abortus* (expressing DsRed). Cells were stained for ER marker Calreticulin (blue) and lysosome marker LAMP-1 (green). The image was made together with Dr. Tregei Starr in the laboratory of Dr. Jean Celli.

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Contents

List of abbreviations

T4SS: Type IV secretion system

LPS: lipopolysaccharide

TLR: Toll-like receptor

BCV: Brucella containing vacuole

ER: endoplasmic reticulum

C_{12}-HSL: N-dodecanoyl homoserine lactone

NF-κB: **N**uclear **f**actor **kappa** light chain enhancer of activated **B** cells

IHF: integration host factor

Sec: general secretion system

TAT: twin arginine translocation

EMSA: electrophoretic mobility shift assay

GFP: green fluorescent protein

MM: minimal medium

CFU: colony forming unit

p.i.: post infection

TM: transmembrane

SP: signal peptide

PR: proline-rich

HA tag: hemagglutinin tag

IP: immunoprecipitation

LAMP1: Lysosomal-associated membrane protein 1

SNAP23: Synaptosomal-associated protein, 23 kDa

kDa: kilodalton

Vce: **v**irB **c**o-regulated **e**ffector

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Chapter 1 Introduction - Brucellosis and Type IV Secretion

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Abstract

Brucellosis is a global disease of domestic and wild mammals that is caused by intracellular bacteria of the genus *Brucella*. Although humans are not a natural reservoir for *Brucella*, infection in the human population is common in many countries, and brucellosis is one of the most common zoonotic infections. *Brucella* species have evolved to avoid the host's immune system and infection is usually characterized by long-term persistence of the bacteria. One important *Brucella* virulence factor for intracellular survival and persistence in the host is the type IV secretion system (T4SS). This review will discuss the *Brucella* T4SS in detail, including current knowledge of architecture and regulation as well as the newly identified effector substrates that this system transports into host cells.

Brucellosis

Brucella *species*

The genus *Brucella* is named after David Bruce, who was the first to isolate the bacterium from the spleen of a fatal case of brucellosis in Malta in 1886, which was then known as Malta fever (Bruce 1888). The bacteria Bruce found were Gram-negative, coccobacilli now known as *Brucella melitensis*. Later, goats were found to be the natural hosts of *B. melitensis*. Since then, more *Brucella* species were discovered including *B. abortus* (natural host cattle), *B. suis* (swine), *B. ovis* (sheep), *B. canis* (dogs), *B. neotomae* (rats) and recently *B. microti* (voles), *B. ceti* (dolphins and whales) and *B. pinnipedialis* (seals). These species are classified on their preference for specific animal hosts. Based on 16S rRNA sequence homology, *Brucella* is classified in the α-2 group of the α-proteobacteria together with the plant pathogen *Agrobacterium,* and intracellular mammalian pathogens of the genera *Bartonella, Anaplasma and Rickettsia* (Moreno, Stackebrandt et al. 1990).

Transmission

Brucellosis is a disease of domestic and wild animals that is transmissible to humans, which defines it as a zoonosis (Godfroid, Cloeckaert et al. 2005). Brucellosis in humans is mainly caused by *B. melitensis* and *B. abortus,* and the majority of human infections occur via the consumption of unpasteurized milk and milk products from goats and cattle. Contact with infected goats, sheep, cattle, pigs or dogs, is also an important route of transmission for human pathogenic *Brucella* species. As a result, farmers, slaughterhouse workers, hunters or veterinarians that handle infected animals or aborted fetuses, are at risk of infection. Exposure to *Brucella* cultures in clinical laboratories is also an important source of infection.

Box 1. *Brucella*

Brucella bacteria

- Gram-negative
- Size: 0.5-1.5 μm
- Coccobacilli
- Aerobic
- Non-motile
- No pili
- No toxins
- Urease positive (most sp)

Important virulence factors

- LPS
- Cyclic 1,2 β-glucan
- T4SS

Symptoms

After an exposure to *Brucella*, the bacteria invade the human body via mucosal surfaces of the digestive or respiratory tracts. This invasion does not elicit an inflammatory response, which likely reflects both the low number of infecting bacteria and the ability of *Brucella* to evade innate immune detection, and as a result, patients most frequently present with symptoms between 2 and 8 weeks after exposure (Barquero-Calvo, Chaves-Olarte et al. 2007). In tissues, *Brucella* is then ingested by phagocytes, which transport the bacteria to systemic sites (Archambaud, Salcedo et al. 2010). This explains why different routes of infection such as inhalation of aerosols, oral ingestion or a breach of the skin all lead to similar symptoms and clinical signs of *Brucella*.

Box 2. Brucellosis

- *Brucella* is a pathogen of mammals. Infection of humans occurs mostly through contact with wild or domesticated animals or consumption of unpasteurized milk or milk products.
- There are >500,000 reported new cases of human Brucellosis each year.
- Human Brucellosis is mainly caused by *B. melitensis, B. abortus* and *B. suis*. Most cases are in Mediterranean and Middle Eastern countries, Mexico and South America.
- *Brucella* are able to survive inside phagocytic cells of their hosts, avoid host innate immune system and cause a persistent infection.
- Main target organs of *Brucella* are spleen, lymph nodes and liver. Enlargement of these organs during *Brucella* infection is common.
- Common symptoms include intermittent fevers, malaise, anorexia, night sweats and joint pain.
- Diagnosis is performed by detection of antibodies against *Brucella* with serum agglutination tests and ELISA or by blood or bone marrow culture.
- Treatment occurs usually with a combination of the antibiotics rifampin, doxycycline and streptomycin for at least 6 weeks.
- Other names for Brucellosis in humans are Malta fever, Mediterranean fever or Undulant fever.

Brucellae are intracellular pathogens that have the ability to survive and multiply inside professional and nonprofessional phagocytic cells of the host. In these cells *Brucella* proliferates in vacuoles with properties of the endoplasmic reticulum (ER) (Pizarro-Cerda, Meresse et al. 1998; Pizarro-Cerda, Moreno et al. 1998; Arenas, Staskevich et al. 2000; Celli, de Chastellier et al. 2003). Preferred host cells include macrophages and dendritic cells (Billard, Cazevieille et al. 2005; Copin, De Baetselier et al. 2007; Salcedo, Marchesini et al. 2008; Archambaud, Salcedo et al. 2010), which explains the tropism of these bacteria for lymph nodes, spleen, bone marrow and liver. However, focal complications of brucellosis can occur in almost any tissue, especially the large joints (osteoarticular brucellosis) and spine (*Brucella* spondylitis). Rare but serious focal complications include *Brucella* endocarditis, which can occur in patients with pre-existing rheumatic or congenital heart disease, and neurobrucellosis, which can lead to permanent neurological deficits (Madkour 2001).

The clinical presentation includes a wide variety of nonspecific symptoms, which often makes diagnosis difficult (Franco, Mulder et al. 2007). Reports published before the discovery of antibiotics, provide details of the clinical presentation and course of untreated *B. melitensis* infection in humans (Bruce 1889; Hardy, Jordan et al. 1936). Characteristic symptoms of infection in humans in-

clude chills, night sweats and fever of varying intensity throughout long periods of time, loss of weight and strength, anorexia, arthritis, sometimes orchitis in men, and in most cases enlarged spleen. Relapses occurred frequently and periods of fever commonly alternated with intermissions. During these periods the fever exhibited a tendency to be undulant. Also, a common effect of *Brucella* infection was involvement of the nervous system with headaches during periods of fever. Although the disease was reported to persist for many years in some patients, most cases of untreated brucellosis were reported to result in complete recovery (Bruce 1889). *B. canis*, which was discovered later, was also found to be infectious for humans (Swenson, Carmichael et al. 1972). The characteristic symptoms of brucellosis caused by *B. canis* are similar to those caused by *B. melitensis*, *B. abortus* or *B suis*, however *B. canis* is less infectious for humans and symptoms are less severe. Currently, once brucellosis is diagnosed, treatment is effective with antibiotics and symptoms in most cases disappear within several days (Pappas, Akritidis et al. 2005). However, the initial infection can go unnoticed or untreated, leading some patients to first seek medical treatment for focal complications such as brucellar arthritis or spondylitis. A panel of clinical experts on brucellosis has recommended, as the most effective treatment regimen for primary brucellosis infection, a six-week regimen of doxycycline combined either with streptomycin for 2–3 weeks, or rifampicin for six weeks. Therapy with a single antibiotic is not recommended, as it has been associated with a high rate of relapse (Ariza, Bosilkovski et al. 2007).

Virulence factors

In general, *Brucella* species do not express toxins or virulence factors that cause direct damage to the host. Instead this pathogen's strategy is to persist long enough in the infected host until transmission can occur, which in the natural hosts is usually through abortion or sexual contact. Since in nature, the hosts of some *Brucella* species, can breed only once (cattle and goats) or twice (swine and sheep) per year, persistent infection may be an adaptation that *Brucella* needs to remain hidden from the host immune system for a considera-

ble time. Upon initial infection with *Brucella*, innate immune responses are evaded. For example surface exposed *lipopolysaccharide* (LPS) from *B. abortus* is poorly endotoxic to hosts as it does not bind complement and is poorly recognized by the innate immune sensor Toll-like receptor (TLR) 4 (Hoffmann and Houle 1983; Ferguson, Datta et al. 2004; Lapaque, Forquet et al. 2006; Barquero-Calvo, Chaves-Olarte et al. 2007; Parent, Goenka et al. 2007). Also, *Brucella* smooth LPS, which contains full length O-antigen, is required for entry of the bacteria in host cells via lipid rafts and subsequent trafficking inside the cells (Porte, Naroeni et al. 2003). During infection *Brucella* mainly has an intracellular lifestyle, which hides the organism from antibodies. Cells infected with *Brucella* remain viable, as *Brucella* is able to inhibit apoptosis using yet unknown virulence factors (Gross, Terraza et al. 2000). Important virulence factors for intracellular survival are *cyclic β 1,2-glucan* and the *type IV secretion system (T4SS)*. Cyclic β 1,2-glucan is a molecule secreted into the periplasm of *Brucella* and is required for intracellular *Brucella* to avoid fusion of the phagosome with lysosomes (Arellano-Reynoso, Lapaque et al. 2005). The T4SS translocates *Brucella* proteins, also called effectors, into host cells and is critical for both *Brucella* survival and replication in infected host cells. The T4SS will be discussed in more detail below.

The Brucella T4SS

Architecture of the Brucella T4SS

The *Brucella* T4SS is a complex of 12 proteins that assembles in the envelope of the bacterium. Once assembled, this system can transport effector proteins, from *Brucella* into infected host cells. The *Brucella* T4SS is encoded by the *virB* operon, which consists of genes *virB1* to *virB12* and controlled by a main promoter upstream of *virB1* (Figure 1). Most encoded VirB proteins show similarity to components of T4SS in other bacteria, including those of *Agrobacterium tumefaciens* and *Bordetella pertussis* (O'Callaghan, Cazevieille et al. 1999). *Brucella* strains lacking a functional T4SS are highly attenuated in macrophages and mice and in the natural host, the goat (O'Callaghan, Cazevieille et al. 1999;

Sieira, Comerci et al. 2000; den Hartigh, Sun et al. 2004; Zygmunt, Hagius et al. 2006; den Hartigh, Rolan et al. 2008). In transposon mutagenesis screens designed to find *Brucella* virulence factors, insertions in *virB* genes or genes affecting *virB* expression have consistently been found (Foulongne, Bourg et al. 2000; Hong, Tsolis et al. 2000; Delrue, Martinez-Lorenzo et al. 2001; Kim, Watarai et al. 2003; Lestrate, Dricot et al. 2003; Wu, Pei et al. 2006). This underscores the importance of the T4SS for *Brucella* virulence. However, not all VirB proteins are equally important for a functioning T4SS. By deleting each *virB* gene individually in *B. abortus* it was shown that *virB1*, *virB7* and *virB12* are dispensable for persistence of *B. abortus* in the mouse model (den Hartigh, Sun et al. 2004; Sun, Rolan et al. 2005; den Hartigh, Rolan et al. 2008). VirB1, VirB7 and VirB12 are not known to be part of the T4SS core translocation apparatus or the pilus in *Brucella* or other bacteria containing a T4SS (figure 1). Therefore it is possible that these proteins aid or enhance T4SS function but are not essential. For example, VirB1 is a lytic transglycosylase and degrades peptidoglycan to provide space for the T4SS to be assembled (Höppner, Liu et al. 2004). However this function could be redundant as the *Brucella* genome encodes other similar enzymes (den Hartigh, Sun et al. 2004). The core structure of a T4SS was shown to be composed of the proteins VirB8, VirB9 and VirB10 (Chandran, Fronzes et al. 2009; Fronzes, Schafer et al. 2009). This appears to be also the case in *Brucella* as these three proteins interact with each other (Sivanesan, Hancock et al. 2010). Other proteins such as VirB6 and VirB7 could have a stabilizing role in the formation of the core complex (Baron, Thorstenson et al. 1997; Jakubowski, Krishnamoorthy et al. 2003). Furthermore, the ATPases VirB4 and VirB11 are essential for T4SS function by providing energy for T4SS assembly and transport of the effector proteins (Watarai, Makino et al. 2002; Alvarez-Martinez and Christie 2009). The pilus is composed of the major component VirB2 and the minor component VirB5 (Schmidt-Eisenlohr, Domke et al. 1999) and assembly of the pilus is mediated by VirB3 (Shirasu and Kado 1993). The pilus is thought to attach to the host cell surface and to create a pore, through which effectors are translocated (Hwang and Gelvin 2004).

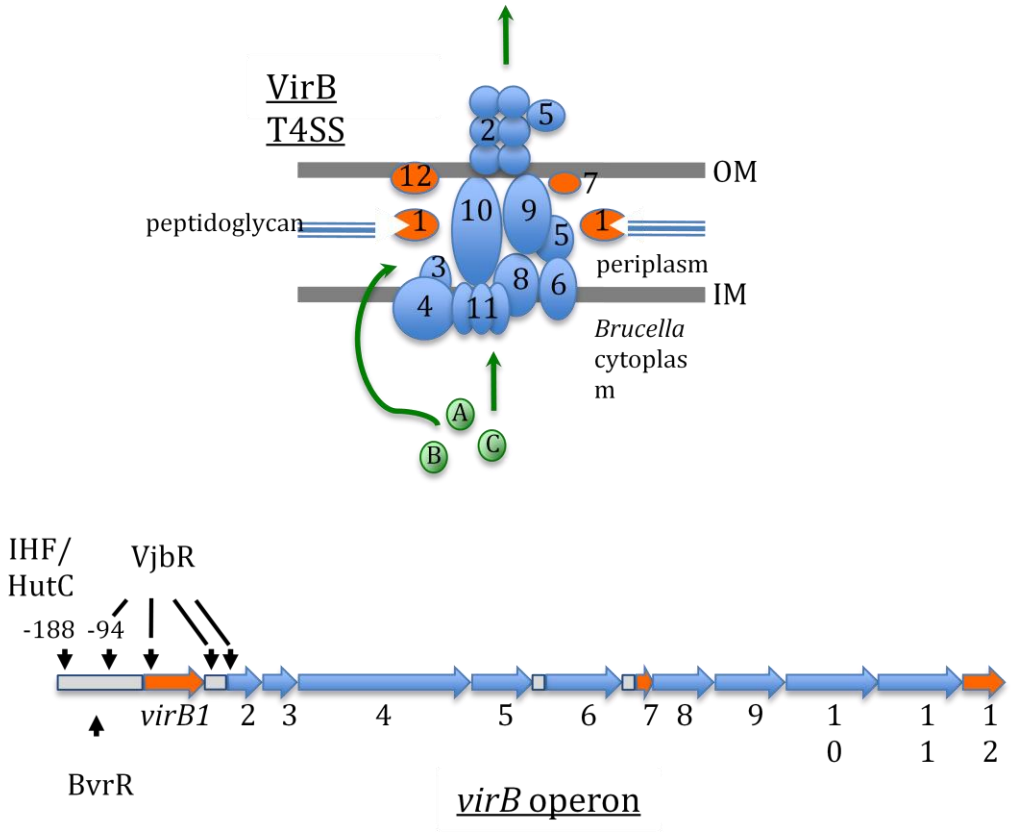


Figure 1. Schematic representation of the *virB* operon and the T4SS it encodes. Genes shown in blue are absolutely required for virulence of *Brucella in vitro* in macrophages or *in vivo* in mice. Genes shown in orange were found to be not essential for virulence in mice. Also direct regulators of the *virB* operon and their (approximate) binding regions are shown. Promoters and intergenic regions containing putative promoters are shown in grey. Substrate proteins are shown in green and green arrows show putative translocation pathways. OM, outer membrane, IM, inner membrane.

Function of Brucella T4SS during infection

All *virB* genes encoding the T4SS are conserved in all sequenced *Brucella* species (table 1), indicating this system is important for *Brucella*. The importance of the T4SS for *Brucella* virulence has been shown experimentally mostly *in vitro* in macrophages and *in vivo* in the mouse model of infection (O'Callaghan, Cazevieille et al. 1999; Hong, Tsolis et al. 2000; Sieira, Comerci et al. 2000; Delrue, Martinez-Lorenzo et al. 2001; Zygmunt, Hagius et al. 2006; Paixao, Roux et al. 2009). In both models the *Brucella* wild-type bacteria are able to persist and replicate, whereas T4SS mutants are slowly cleared. *In vivo* it has been shown that at later stages of infection, *B. melitensis* and *B. abortus* wild-

type bacteria elicit innate immune responses in mice, however T4SS mutants do not (Roux, Rolan et al. 2007). This on one hand confirmed the existing data on the stealthy nature of *Brucella* during infection of their hosts, but on the other hand it showed that later during infection the T4SS directly or indirectly serves as a signature that is recognized by the innate immune system (for review see (de Jong, Rolan et al. 2010)). *Brucella* may actively translocate a molecule through the T4SS into host cells that activates the innate immune system with the goal, for example, to polarize the immune response to Th1 by increasing interferon gamma production or to generate formation of granulomas (Rolan and Tsolis 2008; Rolan, Xavier et al. 2009). It is also possible that the difference in immune activation between *Brucella* wild-type and T4SS lacking strains is caused indirectly by a difference in intracellular trafficking and growth. In experiments using cultured cells, it was found that the *Brucella* T4SS is required for maturation of the *Brucella* phagosome into an ER-derived compartment (Celli, de Chastellier et al. 2003; Celli, Salcedo et al. 2005; Starr, Ng et al. 2008). Although *Brucella* phagosomes do transiently fuse with early and late endosomes and lysosomes, a fraction of intracellular *Brucella* containing a T4SS are eventually able to exclude endosomal and lysosomal markers from their phagosomes and avoid degradation in phagolysosomes (Starr, Ng et al. 2008). Instead, *Brucella* phagosomes acquire ER markers such as calreticulin. This process of excluding endosomal and lysosomal markers and acquiring ER markers is completed approximately 12 to 24 hours after *Brucella* infection of a host cell and requires the T4SS and presumably its translocated effectors. *Brucella* then starts to multiply to high numbers inside host cells, while *Brucella virB* mutants never reach the ER derived vacuole and are killed in phagolysosomes (Celli, de Chastellier et al. 2003; Celli, Salcedo et al. 2005; Starr, Ng et al. 2008).

Regulation of the Brucella T4SS

Once inside the host cell, *Brucella* encounters new environmental conditions that stimulate increased synthesis of VirB proteins. *Brucella* will need the T4SS only during defined and relatively short periods during intracellular infection, and tight regulation of its expression is therefore important. This period is several

hours after uptake of the bacterium into a host cell until destruction in phagolysosomes has been avoided and a vacuole in the ER suitable for replication has been established. After uptake by host cells expression of the T4SS is induced after acidification of the *Brucella* containing vacuole (Boschiroli, Ouahrani-Bettache et al. 2002). This occurs after the phagosome transiently fuses with early and late endosomes and lysosomes (Starr, Ng et al. 2008). Nutrient starvation could also be a signal to elicit expression of *virB* genes. In culture, expression of *virB* genes can be strongly induced after switching the bacteria from rich medium to minimal medium at low pH (Rouot, Alvarez-Martinez et al. 2003). Maximal expression of *virB* genes is reached around 5 hours after infection of host cells (Sieira, Comerci et al. 2004).

The transcriptional regulators involved

The first protein found to bind the main promoter of *virB* was integration host factor (IHF). In other bacteria, such as *E. coli*, IHF is known to act as a global regulator of transcription through its ability to bend target DNA, thereby providing the right structure for other transcription factors to bind (Browning, Grainger et al. 2010). It was shown that in *B. abortus* IHF was required for correct activation of the *virB* promoter in medium at neutral pH and inside host cells (Sieira, Comerci et al. 2004). In a later study the same group found a second regulator HutC that competed with IHF for binding to the same binding site centered at -188 relative to the transcription start site of the *virB* promoter. HutC is a repressor of the histidine utilization genes and was found to be required for *virB* activation under nutrient starvation and low pH conditions. Since *virB* gene expression is dependent on IHF at neutral pH and dependent on HutC at acidic pH, these regulators could be acting sequentially during acidification of the *Brucella* phagosome to activate *virB* genes (Sieira, Arocena et al. 2010). Both IHF and HutC could be involved in recruitment of, or providing the right promoter structure for activating transcription factors that bind the promoter closer to the transcription start site. One such activating transcription factor is VjbR, a regulator belonging to the LuxR family, and shown to be required for *virB* activation (Delrue, Deschamps et al. 2005). VjbR contains a DNA binding domain and a

homoserine lactone (HSL) binding domain. VjbR was found to be able to bind a 123 bp region of the *virB* promoter directly upstream of the *virB1* gene (de Jong, Sun et al. 2008). Later the binding site was determined to be a region of the *virB* promoter that is centered at -94 relative to the transcription start site (Arocena, Sieira et al. 2010). This is upstream of an 18 bp (*virB*) box that was shown to be important for activation of the *virB* promoter by VjbR in the heterologous host *E. coli* (de Jong, Sun et al. 2008). Many *Brucella* promoters were found to be activated by VjbR in the *E. coli* model, and these promoters contained a similar 18 bp *virB* box. It is possible that this box acts as a recognition site for VjbR prior to binding to the promoter at a different site. Alternatively, the 18 bp box could serve as binding site of a co-regulator of VjbR. Besides a DNA binding domain, VjbR contains a domain, which is able to bind C_{12}-HSL (Uzureau, Godefroid et al. 2007). Activation of *virB* genes by VjbR was inhibited by C_{12}-HSL and this was shown to be the result of decreased binding of VjbR to the *virB* promoter (Delrue, Deschamps et al. 2005; Uzureau, Godefroid et al. 2007; Arocena, Sieira et al. 2010). Another regulator that is able to bind the main promoter of *virB* is BvrR, the response regulator of the two-component system BvrR/S (Martinez-Nunez, Altamirano-Silva et al. 2010). This system was found to be involved in regulation of many genes involved in *Brucella* virulence including genes encoding outer membrane proteins and proteins required for LPS modifications (Viadas, Rodriguez et al. 2010). Both *Brucella* VjbR and BvrR/S mutants are highly attenuated for survival inside host cells and regulate an overlapping set of genes (Sola-Landa, Pizarro-Cerda et al. 1998; Viadas, Rodriguez et al. 2010). This suggests a connection between the two regulatory systems and indeed recently it was found that VjbR is under control of the BvrR/S system (Martinez-Nunez, Altamirano-Silva et al. 2010; Viadas, Rodriguez et al. 2010). It is possible that BvrR/S system senses the decrease in pH of the *Brucella* phagosome after *Brucella* entry of host cells and subsequently activates the T4SS both directly and through VjbR (Viadas, Rodriguez et al. 2010).

Recently a second LuxR regulator, designated BabR or BlxR, was shown regulate *virB* genes (Rambow-Larsen, Rajashekara et al. 2008; Uzureau, Lemaire et

al. 2010). When the set of genes regulated by VjbR and BabR/BlxR were compared it was found that there is an overlap between VjbR and BabR/BlxR regulated genes, however both regulators appear to regulate their target genes in an opposite way (Uzureau, Lemaire et al. 2010). Target genes include genes involved in virulence, stress response, metabolism, and bacterial replication (Rambow-Larsen, Rajashekara et al. 2008; Uzureau, Lemaire et al. 2010; Weeks, Galindo et al. 2010). Based on these results it could be hypothesized that BvrR/S and VjbR are required for adaptation of *Brucella* in nutrient limiting conditions directly after infection of the host cell until a replication permissive niche has been reached. Then BvrR/S and VjbR are turned off and BlxR/BabR is activated (Martinez-Nunez, Altamirano-Silva et al. 2010; Uzureau, Lemaire et al. 2010). This regulator acts in an opposite way to VjbR, and enables *Brucella* to replicate in the ER and down regulate expression of the T4SS, which is now no longer required.

Although *Brucella* does not contain any known genes that could synthesize C_{12}-HSL, this molecule was isolated in small amounts from *B. melitensis* culture supernatant (Taminiau, Daykin et al. 2002). It was proposed that during infection of a host cell by *Brucella* low production of C_{12}-HSL could lead to slow accumulation of this molecule in the *Brucella* phagosome (Delrue, Deschamps et al. 2005). When *Brucella* reaches the ER and the T4SS is no longer needed, C_{12}-HSL concentrations could be high enough to inhibit activity of VjbR and expression of the *virB* genes. Alternatively, it is possible that during *Brucella* infection VjbR or BabR/BlxR do not sense self produced C_{12}-HSL, but a molecule already present in the host cell. Perhaps this is a molecule only present in the ER, which provides a signal to *Brucella* that it reached its replication niche.

Effectors

The genes encoding *Brucella* effectors identified to date are scattered across the two *Brucella* chromosomes (Figure 2). In *Bartonella* species, the *virB* genes are located together with the genes encoding the effector substrates (Schulein, Guye et al. 2005). Since a similar situation does not exist in *Brucella*, identification of *Brucella* effectors has proven to be a challenging task.

The first substrates of the *Brucella* T4SS identified were VceA and VceC (de Jong, Sun et al. 2008). These effectors were found in a screen for *Brucella* promoters that were activated by VjbR in the heterologous host *E. coli*.

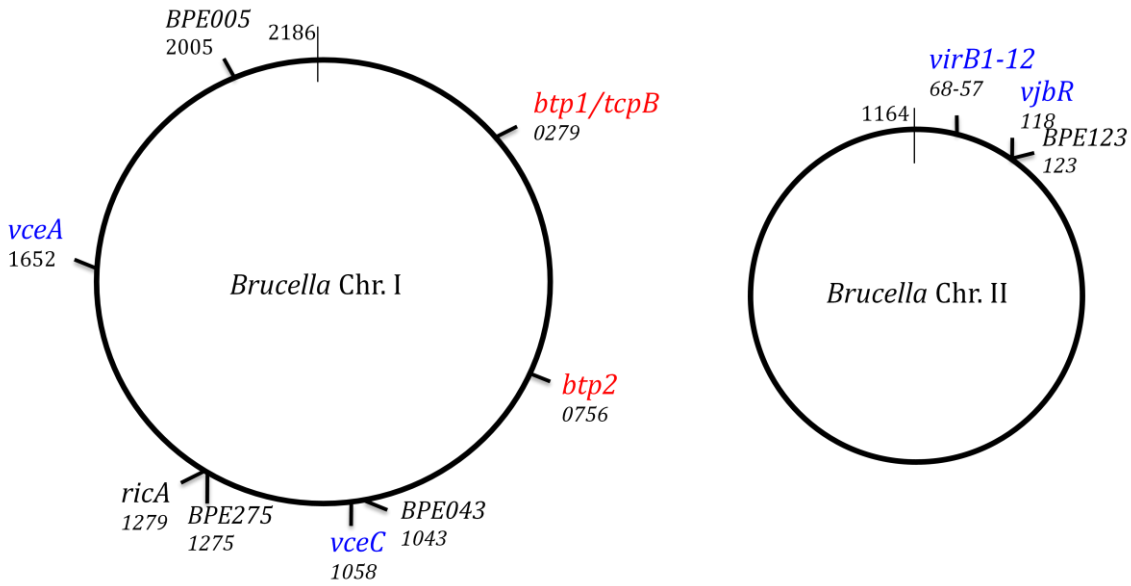


Figure 2. Schematic representation of the two chromosomes of *Brucella* species showing the position of effector genes, such as the *virB* genes and *vjbR*, in the chromosomes. Effectors shown in black and blue have been shown to be translocated by the T4SS into host cells. Those in red have not been shown to be translocated yet. Genes shown in blue have been shown to be regulated by VjbR.

Although screening for effector genes among *virB* co-regulated genes is a great method of narrowing down potential effector candidates, many candidates could also have been missed. For example regulation of effectors may be under the control of a different regulator (such as directly by BvrR) or regulators down-stream of VjbR. Also it could be hypothesized that activation of some effectors is not connected to activation of the *virB* genes. *Brucella* may already contain a 'ready for translocation' pool of effectors before entry into host cells, as has been shown for *Legionella pneumophila* (Kubori, Shinzawa et al. 2010). These effectors could be required early during infection of the host cell and expressed constitutively. Since nutrients in the early *Brucella* phagosome are limited, having a ready pool of effectors would save resources for *Brucella* for other functions. Recently, a different strategy to identify *Brucella* effectors was utilized by

screening all proteins of unknown function for eukaryotic-like domains or domains known to be involved in protein-protein interactions (Marchesini, Herrmann et al. 2011). This strategy has proven to be successful in identifying T4SS effectors of other intracellular pathogens, such as *L. pneumophila* and *Coxiella burnetii* (Pan, Luhrmann et al. 2008; Chen, Banga et al. 2010). Using this strategy, 6 proteins were identified that were translocated into mouse macrophages by *B. abortus*. Translocation into cells of 4 of these *Brucella* putative effector proteins (BPE123, BPE005, BPE275 and BPE043) was dependent on the VirB T4SS (table 1). Recently another protein was found that was translocated in a T4SS-dependent manner into macrophages during infection with *B. abortus*. This protein, named RicA, was found in a screen for *Brucella* proteins interacting with human proteins predicted to be associated with phagosomes. RicA was demonstrated to interact with Rab2, a GTPase involved in trafficking (de Barsy, Jamet et al. 2011). Rab2 has been shown before to localize to the *Brucella* phagosome. Furthermore, it was determined that Rab2 is important for intracellular replication of *B. abortus* (Fugier, Salcedo et al. 2009). In line with this, RicA, which preferentially binds to GDP bound Rab2, is involved in recruiting this GTPase to the *Brucella* phagosome (de Barsy, Jamet et al. 2011).

Potential effectors

There are several *Brucella* proteins that have eukaryotic domains or have been shown to interact with eukaryotic proteins. One of these proteins is Btp1/TcpB (Cirl, Wieser et al. 2008; Salcedo, Marchesini et al. 2008), which contains a TIR domain and interacts with the adapter MAL/TIRAP, thereby interfering with host TLR-2 and TLR-4 signaling and inhibiting NF-κB activation (Radhakrishnan, Yu et al. 2009; Sengupta, Koblansky et al. 2010). It is still unknown whether Btp1/TcpB is translocated by *Brucella* into host cells during infection and whether this depends on the T4SS, however its predicted site of activity in the host cell cytosol suggests a regulated release from *Brucella* during infection. Most *Brucella* species encode a second TIR domain-containing protein (table

1), however no experimental data showing a similar function of this protein to Btp1/TcpB are available.

Route of translocation

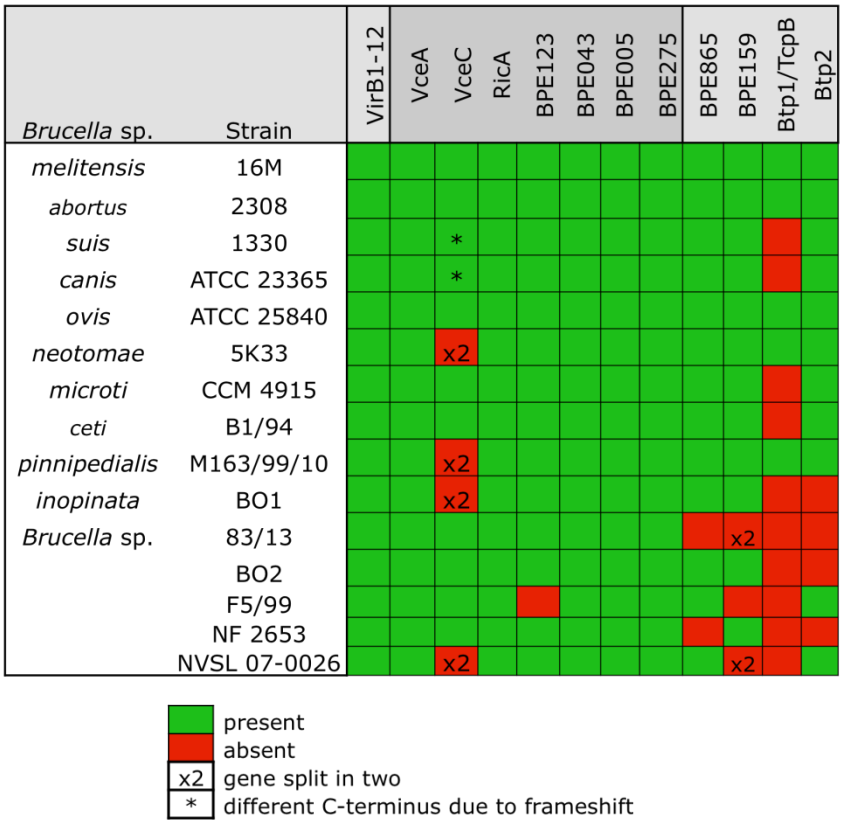
In most Gram-negative bacteria containing a T4SS, the route of protein substrate translocation across the two membranes is thought to be a one step process (Alvarez-Martinez and Christie 2009). A coupling protein (T4CP), such as VirD4, binds the effector in the bacterial cytoplasm and directs it to the ATPases VirB11 and VirB4. Then, the effector is transported through the channel across the inner membrane, periplasm and outer membrane (Atmakuri, Cascales et al. 2004; Cascales and Christie 2004; Jakubowski, Cascales et al. 2005). *B. pertussis*, which lacks a VirD4 T4CP, is known to secrete subunits of the pertussis toxin into the periplasm through the general secretion pathway (Sec) prior to translocation across the outer membrane (Covacci and Rappuoli 1993; Weiss, Johnson et al. 1993). *Brucella* also lacks a gene encoding a VirD4 homolog, suggesting a similar situation could exist in *Brucella* (Figure 1). This idea is supported with the recent finding that the effector BPE123 contains a predicted N-terminal Sec signal peptide. Also, the N-terminus of BPE123 is required for its translocation into host cells (Marchesini, Herrmann et al. 2011). Another *Brucella* effector with a predicted Sec signal peptide (SignalP (Emanuelsson, Brunak et al. 2007)) is VceA, however translocation into host cells was detected with a fusion of TEM-1 β-lactamase to the N-terminus of this protein (de Jong, Sun et al. 2008). This indicates that the C-terminus of VceA is sufficient for translocation, but does not exclude an additional translocation pathway involving a periplasmic intermediate for VceA. VceC contains a C-terminal secretion signal, as VceC constructs with truncations of the C-terminus were not translocated into host cells (de Jong, Sun et al. 2008). The C-terminal 115 amino acids of VceC are sufficient for translocation of TEM-1 into host cells by the *Brucella* T4SS. Furthermore, *Legionella pneumophila*, which encodes a quite distinct T4SS (dot/icm system), was able to translocate the VceC C-terminus into host cells (de Jong, Sun et al. 2008). Combined with the fact that

VceC does not contain a predicted Sec- or TAT-specific signal peptide, translocation of VceC across the bacterial envelope via the periplasm seems unlikely, and is probably a one step process. However, it is possible that some *Brucella* effectors are translocated into the periplasm, prior to translocation into host cells. Future experiments are required to address this question.

Conservation of effectors among Brucella species

The VirB T4SS is conserved in all *Brucella* species, however effectors are not (table 1). Therefore, it could be hypothesized that the set of effectors translocated by the T4SS system contributes to differences in virulence or host specificity between *Brucella* species. All currently known T4SS effectors are conserved in the human pathogenic *Brucella* species *B. abortus, B. melitensis, B. suis* and *B. canis* (table 1).

Table 1. Presence of T4SS effectors and possible T4SS effectors (BPE865, BPE159, Btp1, Btp2) in different *Brucella* species.



In *B. suis* and *B canis* VceC contains a different C-terminus due to a frameshift. However we have shown this does not affect the ability of *B. suis* VceC to be translocated into host cells (de Jong, Sun et al. 2008). All effectors are also conserved in *B. ovis* and *B. ceti*. Of these two species only *B. ceti* has zoonotic potential (Whatmore, Dawson et al. 2008). The lack of *B. ovis* pathogenicity in humans could be explained with other virulence factors that are missing in *B. ovis*. For example *B. ovis* is a naturally rough LPS strain and does not have urease activity, which is necessary for passage through the stomach (Tsolis, Seshadri et al. 2009). Other species lack one or more effectors in at least one sequenced strain. Future experiments are required to determine the expression level and contribution to virulence of identified effectors in different *Brucella* species.

Mutant strains of *B. abortus* and *B. melitensis* lacking a single effector have been constructed and tested for virulence compared to the wild-type strain in tissue culture and mouse models. All these *Brucella* mutants, including *ricA*, *BPE123* and *tcpB* were not attenuated in these models, suggesting a redundancy among effectors (Sengupta, Koblansky et al. 2010; de Barsy, Jamet et al. 2011; Marchesini, Herrmann et al. 2011). It is also possible that cultured cells and mice are not the correct model to observe the role of these effectors during infection.

T4SS inhibiting molecule as anti-Brucella medicine

Since the T4SS is an essential virulence factor for *Brucella*, targeting this system with a small inhibitory molecule may be an efficient way to treat brucellosis. A screen to find inhibitors of VirB8-VirB8 interactions in *Brucella*, has yielded promising candidates that were found not only to reduce *B. abortus* intracellular survival, but were also non-toxic to host cells (Paschos, den Hartigh et al. 2011). However, the effect of these molecules was not limited to inhibition of VirB8 dimerization but also reduced expression of all *virB* genes. This suggests that destabilizing the T4SS structure may generate a negative feedback loop, which reduces *virB* gene expression (Paschos, den Hartigh et al. 2011). If the

effect is limited to VirB T4SS stability and transcription, and no toxic side effect for animals and humans is determined, these or related molecules could potentially serve as useful anti-*Brucella* drugs.

Summary and future perspective

In order to cause the persistent disease brucellosis in humans and animals, *Brucella* relies on several virulence factors, including the VirB T4SS. The T4SS was identified as an important virulence factor for *Brucella* more than 10 years ago. Since other important bacterial pathogens of humans, such as *H. pylori* and *L. pneumophila*, were found to use a similar T4SS to translocate effector proteins into host cells, it was hypothesized that *Brucella* would also use its T4SS for this purpose. Although a direct involvement of the T4SS in intracellular phagosome trafficking cannot be ruled out, the *Brucella* T4SS has indeed been shown to translocate multiple effectors in infected host cells. Now that several effector substrates of the *Brucella* T4SS have been identified, the next step will be to determine the role of these effectors in intracellular survival of *Brucella*. Effector function could hypothetically be classified in several categories based on host cell pathways *Brucella* is known to interfere with, such as intracellular trafficking of the *Brucella* phagosome, manipulation of the host immune response and inhibition of apoptosis. Some *Brucella* effectors could be specific to one category, and others could have multiple functions in the host cell. Of the identified effectors so far, RicA probably interferes with trafficking of the *Brucella* phagosome, as it recruits the trafficking GTPase Rab2 to *Brucella* phagosomes. Although Btp1/TcpB has not yet been shown to be a T4SS substrate, the known function of this putative effector is inhibition of inflammation. Future research will reveal interesting host cell pathways that are manipulated by *Brucella* effectors and their contribution to *Brucella* infection.

Scope of this thesis

This thesis describes the identification and subsequent characterization of proteins that *Brucella* translocates into infected host cells through its VirB type IV secretion system (T4SS).

Chapter 2 describes a screen to identify *Brucella* effectors by searching the *Brucella* genome for genes that are co-regulated with the *virB* genes. It was hypothesized that proteins translocated by the T4SS would be co-regulated with the *virB* operon. The LuxR family regulator VjbR, known to regulate *virB*, was shown to bind a fragment of the *virB* promoter containing an 18-bp palindromic motif (*virB* promoter box). This finding demonstrated that VjbR regulates the *virB* operon directly. To identify *virB*-coregulated genes, the *B. suis* 1330 and *B. abortus* 2308 genomes were searched 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 the corresponding proteins were tested for VirB-dependent translocation into macrophages using a ß-lactamase reporter assay. This analysis resulted in the identification of the proteins VceA and VceC as novel protein substrates of the *Brucella* T4SS.

In **chapter 3** the role of VceC inside host cells is described. Snapin was identified by yeast two-hybrid and immuno precipitation assays as an interaction partner of VceC. Furthermore, ectopically expressed VceC was shown to be targeted to the ER of HeLa cells and this targeting required an N-terminal TM domain. In HeLa cells expressing VceC, co-localization of Snapin and VceC was demonstrated. A *B. abortus vceC* mutant was attenuated compared to wild-type bacteria in the mouse model of infection, but not in cultured macrophages or HeLa cells. The results suggest that VceC may have a role in immune evasion by *Brucella*.

Chapter 4 reports on the identification and characterization of VceB as a novel *Brucella* effector protein. VceB was shown to be translocated by the VirB system into mouse J774A.1 macrophages and both N- and C-termini of VceB were required for translocation. Using pulldown assays it was determined that VceB interacts with the host protein Lyric. VceB was also found to co-localize with

Lyric in the ER of HeLa cells. Lyric is a protein involved in NF-κB activation and, therefore, the effect of VceB on NF-κB activity was investigated. Experiments with transfected HeLa cells showed that VceB is able to inhibit activation of NF-κB when cells are stimulated with TLR4 or TLR5 ligands FliC or LPS, respectively.

Lastly, in **Chapter 5** the results described in this thesis are discussed and ideas for future research are presented.

2

Chapter 2 Identification of VceA and VceC, two members of the VjbR regulon that are translocated into macrophages by the *Brucella* Type IV secretion system

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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 ß-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 (O'Callaghan, Cazevieille et al. 1999; Hong, Tsolis et al. 2000). 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 and 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 and Christie 1994; Fullner 1998; Vergunst, Schrammeijer 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 and Roy 2001; Conover, Derre et al. 2003; Chen, de Felipe et al. 2004; Luo and Isberg 2004; Shohdy, Efe et al. 2005). Together with *Agrobacterium tumefaciens, Brucella* spp. are classified in the α-2 group of the α-proteobacteria (Moreno, Stackebrandt 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 (Pizarro-Cerda, Meresse et al. 1998; Pizarro-Cerda, Moreno et al. 1998; Arenas, Staskevich et al. 2000). The ability of phagocytosed *Brucella* to evade fusion of their endosomal vesicles with lysosomes requires the VirB T4SS (Sieira, Comerci et al. 2000; Comerci, Martinez-Lorenzo et al. 2001; Delrue, Martinez-Lorenzo et al. 2001; Celli, de Chastellier et al. 2003; Celli, Salcedo 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, de Chastellier et al. 2003; Celli, Salcedo 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, Cazevieille et al. 1999; Hong, Tsolis et al. 2000; Sieira, Comerci et al. 2000; Delrue, Martinez-Lorenzo et al. 2001; den Hartigh, Sun et al. 2004).

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 (Worley, Ching et al. 2000; Thijs, De Keersmaecker et al. 2007). 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, Aloni et al. 2007; Altman and 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, Comerci et al. 2000; Boschiroli, Ouahrani-Bettache et al. 2002; Sieira, Comerci et al. 2004). 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, Comerci 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, Martinez-Lorenzo et al. 2001; Delrue, Deschamps et al. 2005). In a *B. melitensis vjbR* mutant expression of the T4SS is greatly reduced (Delrue, Deschamps et al. 2005). Furthermore, the *B. melitensis vjbR* mutant is highly attenuated in both cellular and mouse models of infection. The VjbR protein be-

longs 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, Deschamps et al. 2005). The expression of VirB is downregulated by C_{12}-HSL in *B. melitensis* (Taminiau, Daykin et al. 2002; Delrue, Deschamps et al. 2005; Uzureau, Godefroid 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.

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, Guigue-Talet 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 37ºC. 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 (C_{12}-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, Guigue-Talet 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 x 10^8 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, Alvarez-Martinez 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-His_6

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-His_6 protein. Purification of VjbR-His_6 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 *Hin*dIII (P_{vceC} in *Brucella* contains a *Hin*dIII 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-^{32}P-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 μl. Furthermore, each reaction contained 10 mM Tris HCl (pH 7.4), 50 mM KCl, 1mM DTT, 6% glycerol, 0.5 mM EDTA, 50 μg/ml BSA and 50 μg/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.nbcr.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.nbcr.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 b-lactamase or adenylate cyclase (CyaA), genes encoding candidate effectors were PCR amplified without their start codons (see Supplementary table 5 for primers), digested with XbaI and PstI and cloned into pFlagTEM1 (Raffatellu, Sun et al. 2005) or pFlagCya, which were also digested with XbaI and PstI. pFlagTEM1 encodes a copy of TEM1 b-lactamase, in which the Sec-dependent signal sequence has been deleted and replaced with a 3xFLAG tag at the N terminus (Raffatellu, Sun 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 (*ΔvirB2*) 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 x 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 x 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, Negulescu 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 x 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 x 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, Campbell et al. 2004). The gene encoding dTomato, driven by the *aphA* promotor, was then cloned into pFT/RalFc (see above) to give rise to pdTFT/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 x 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 x g at room temperature. Cells were incubated for 60 min at 37°C in 5% CO2 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 μg/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*.

Results

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, Deschamps 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, Sinon 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 re-

sults). 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 (Figures 1 and 2). Data shown in Figure 1 demonstrate that induction of *vjbR* expression activated transcription of the P_{virB}*::lacZ* reporter construct on pSURS2 (Figure 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 (Figure 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, Deschamps et al. 2005; Uzureau, Godefroid 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 μM and 60μM 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 (Figure 1A). The residual expression of the P_{virB}*::lacZ* reporter in cultures treated with 60μM 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 (Figure 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 (Figure 2B). We therefore focused on identifying additional members of the VjbR regulon to identify proteins secreted by the *B. abortus* T4SS.

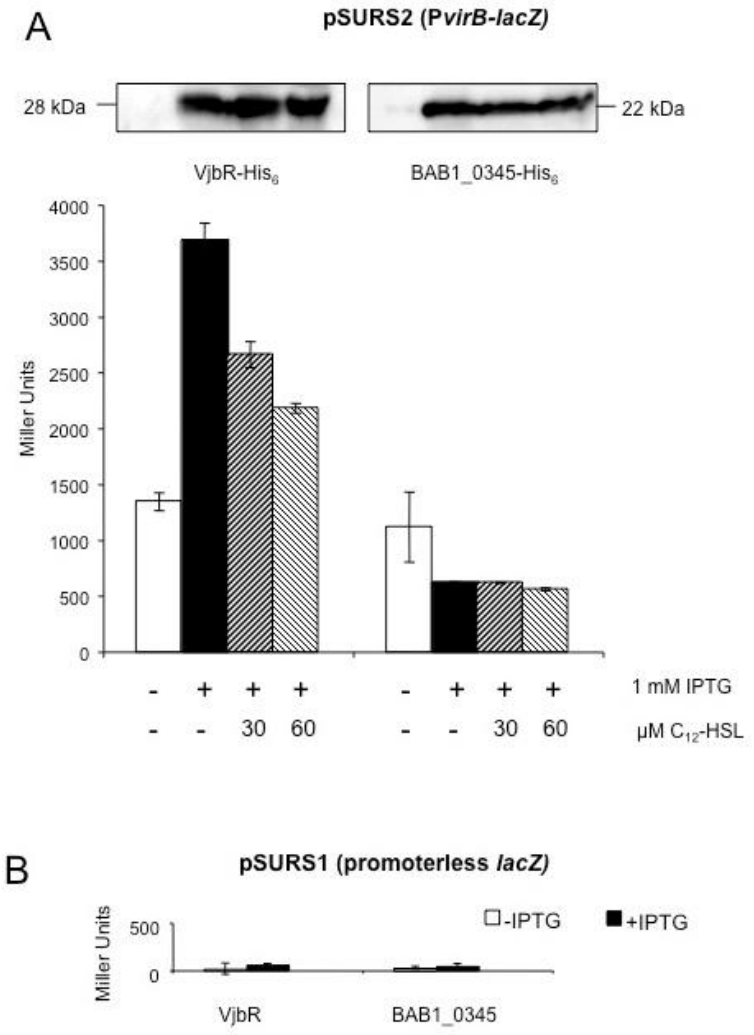


Figure 1. VjbR activates the promoter upstream of *virB1* in *E. coli*. **(A)** *Top:* Western blot using anti-His antibodies showing expression of VjbR-His_6 or BAB1_0345-His_6 after induction with IPTG and showing that expression of VjbR-His_6 is not reduced after addition of C_{12}-HSL. *Bottom:* β-galactosidase activity of *E. coli* with pSURS2 was measured after no induction (white bars) or induction with IPTG (black bars) of VjbR-His_6 and a negative control BAB1_0345-His_6. Hatched bars indicate induction with IPTG and the addition of 30 µM or 60 µM C_{12}-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-His_6 or BAB1_0345-His_6.

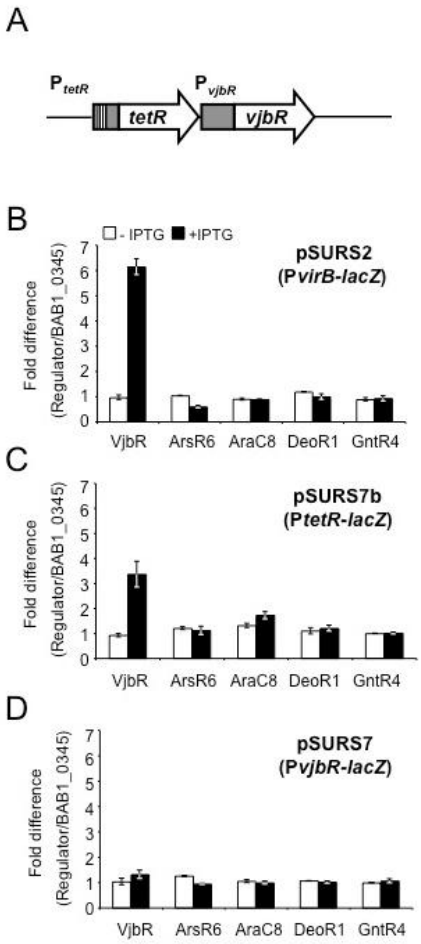


Figure 2. (A) Map of the *tetR-vjbR* operon. **(B-D)** Fold difference of β-galactosidase activity of P_{virB} (**B**), P_{tetR} (**C**) and P_{vjbR} (**D**) in *E. coli* VjbR, ArsR6, AraC8, DeoR1 or GntR4 strain versus the negative control strain. White bars represent the ratio of measured Miller Units 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.

Members of the LuxR regulator family in other bacteria (e.g. *Vibrio fischeri*) are known to regulate their own expression (Shadel and Baldwin 1991; Shadel and 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 (Figure 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} (Figure 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 (Figure 2D). Since in *B. melitensis* it was recently shown that VjbR regulates its own expression (Rambow-Larsen, Rajashekara 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.

VjbR-His$_6$ 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 (Figure 3). Addition of increasing amounts of VjbR-His$_6$ to the ^{32}P labeled P$_{virB}$ fragment resulted in an increased intensity of a band with reduced electrophoretic mobility (Figure 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.

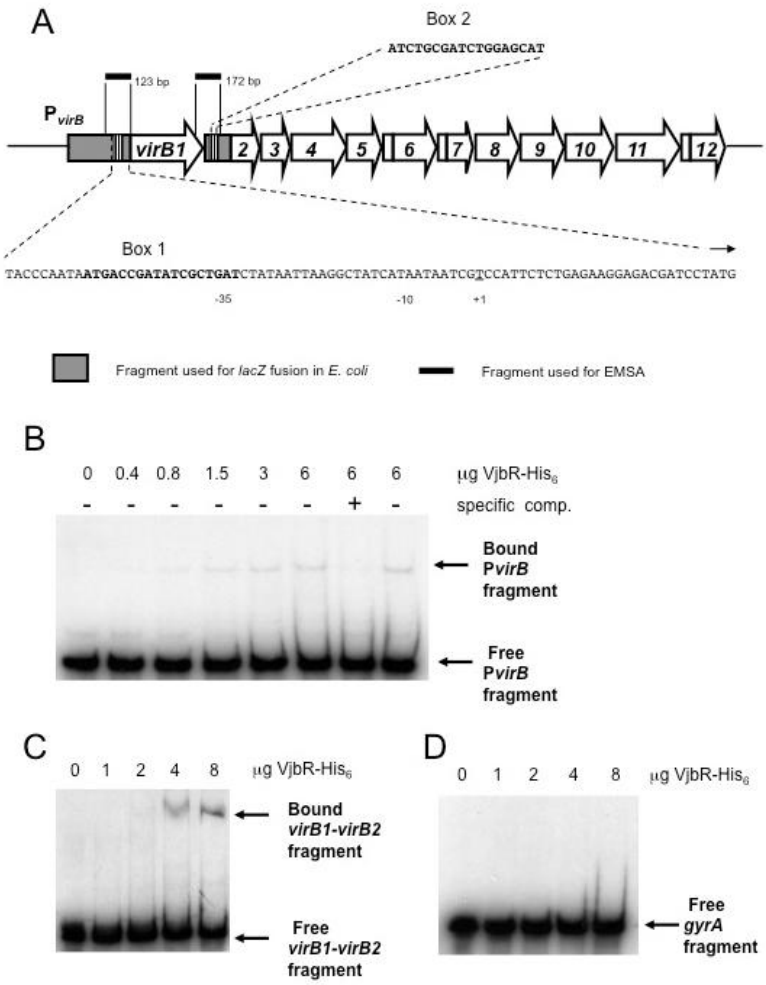


Figure 3. (A) Schematic representation of the *virB* operon in the *B. abortus* chromosome II including P_{virB} and intergenic regions. The putative P_{virB} box 1 and box 2 that were used in the first prediction round are shown and the fragments that were used in EMSAs are indicated in black. **(B-D)** EMSA experiments showing specific binding of VjbR-His_6 to regions directly upstream and downstream of *virB1*. **(B)** VjbR-His_6 binding to a 123 bp fragment of P_{virB} upstream of *virB1*. In the first lane no VjbR-His_6 was added to the binding reaction, and in the subsequent five lanes VjbR-His_6 was added in increasing amounts. In lanes 7 and 8 VjbR-His_6 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-His_6 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-His_6 was added in the same, increasing amounts to the binding reactions.

Since the *virB1-virB2* intergenic region is also predicted to contain a promoter, we tested binding of VjbR by EMSA to a ^{32}P labeled 172 bp DNA fragment containing the intergenic region (Figure 3A). We found that purified VjbR-His$_6$ bound to this region similarly as to the P_{virB} fragment (Figure 3C). Addition of increasing amounts of VjbR-His$_6$ to the fragment containing the *virB1-virB2* intergenic region resulted in an increased intensity of a band with reduced electrophoretic mobility (Figure 3C). As a control for nonspecific binding of VjbR, we used a ^{32}P labeled 139 bp internal fragment of the housekeeping *gyrA* gene (Figure 3D). No band shift was observed for the ^{32}P 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*.

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} (Figure 3A). We also found a similar sequence in the intergenic region between *virB1* and *virB2* (Figure 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* (Pappas and Winans 2003; White and Winans 2007). 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 substi-

tuted with a 6 bp *Hin*dIII site (TGACCG to AAGCTT), thereby disrupting the palindrome, but not the putative -35 regulatory sequence.

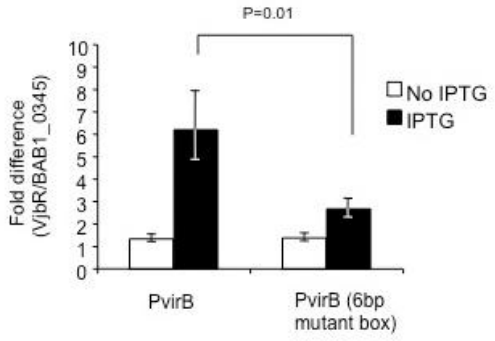


Figure 4. VjbR-dependent expression of of P_{virB}*::lacZ* constructs containing the native wild-type (pSURS2) or mutated P_{virB} box (pSURS2b) in *E. coli* expressing *vjbR*. Values are presented as fold difference above *E. coli* expressing the negative control gene BAB1_0345. White bars represent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

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 (Figure 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 (Figure 3A).

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.nbcr.net/meme/)(Bailey and Gribskov

1998; Paulsen, Seshadri et al. 2002; Chain, Comerci et al. 2005; Halling, Peterson-Burch et al. 2005). 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, Ferooz 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 b-glucan synthetase (LeVier, Phillips et al. 2000; Arellano-Reynoso, Lapaque et al. 2005; Dozot, Boigegrain et al. 2006).

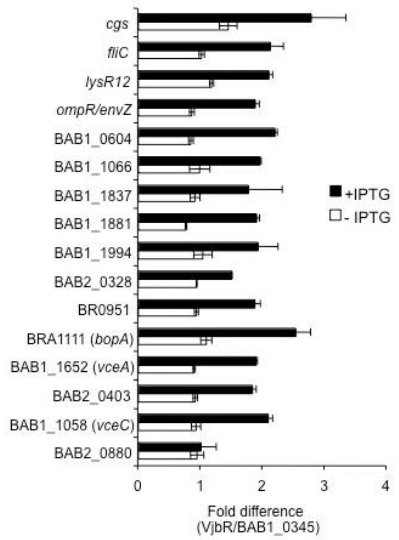


Figure 5. VjbR-dependent expression in *E. coli* of *lacZ* transcriptional fusions to 16 promoter regions containing a putative P_{virB} box. Values are presented as fold difference above *E. coli* expressing the negative control gene BAB1_0345. White bars represent expression levels in the absence of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

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 (Figure 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 b-glucan synthetase (Briones, Inon de Iannino et al. 2001; Arellano-Reynoso, Lapaque et al. 2005; Fretin, Fauconnier et al. 2005). 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, Sinon 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* (Figure 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*.

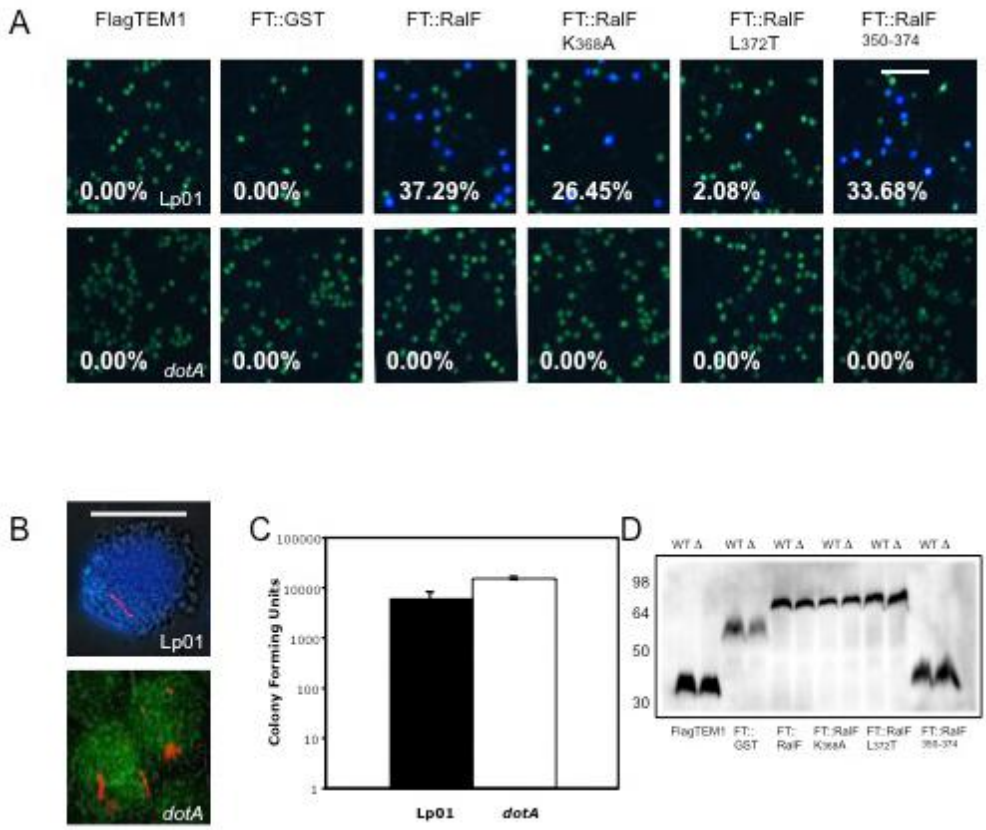


Figure 6. Translocation of TEM1::RalF into J774 macrophages by *L. pneumophila*. (A) J774 macrophages were infected with Lp01 (top row) or *dotA* (bottom row) mutant transformed with plasmids expressing FLAG-TEM-1 fusions for a total of four hours. Translocation efficiency is given as percentage of blue cells. Data is representative of three experiments that produced similar results. Bar, 100 μm. (B) Same experiment as above by using DsRed labeled Lp01 and *dotA* mutant bearing a plasmid expressing FT::RalF$_{350-374}$. These high magnification images were merged from the b-lactamase color channel (Blue and Green), DsRed mono channel (Red) and phase contrast control. The upper blue cell contains Lp01 expressing FT::RalF$_{350-374}$ and the bottom green cell contains the *dotA* mutant expressing the same fusion protein. Bar, 5 μm. (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 50μg/ml gentamicin. Colony forming units were determined by serial dilution and plating on BYCE plates. (D) Western blot using anti Flag, showing all b-lactamase fusions were expressed and their expression were similar between Lp01 (WT) and DotA mutant (Δ). Numbers on right indicate protein standard in kDa.

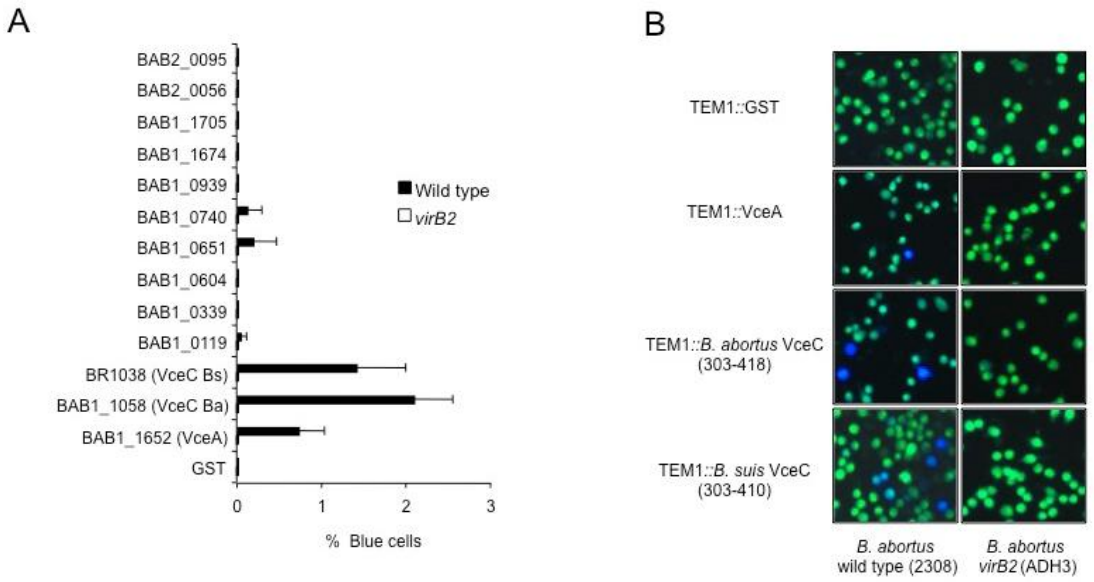
TEM1 b-lactamase can be used to detect translocation of T4SS effector proteins into macrophages

TEM-1 b-lactamase has been used by our lab and others (Charpentier and Oswald 2004; Raffatellu, Sun et al. 2005; Sun, Rolan et al. 2007) to dem-

onstrate 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 b-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 3xFLAG tag (Raffatellu, Sun 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 (Figure 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 (Charpentier and Oswald 2004; Raffatellu, Sun et al. 2005; Sun, Rolan et al. 2007). Translocation of a TEM1-RalF fusion by *L. pneumophila* into a host cell loaded with the fluorescent b-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 (Figure 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, Cambronne 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 (Figure 6C and 6D). These results showed that TEM-1 b-lactamase is a useful reporter for detection of T4SS-mediated protein translocation.

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, Pampou 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 b-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, Sun et al. 2004). The strains expressing the TEM-1 fusion proteins were then used to infect J774 macrophages for detection of protein translocation.



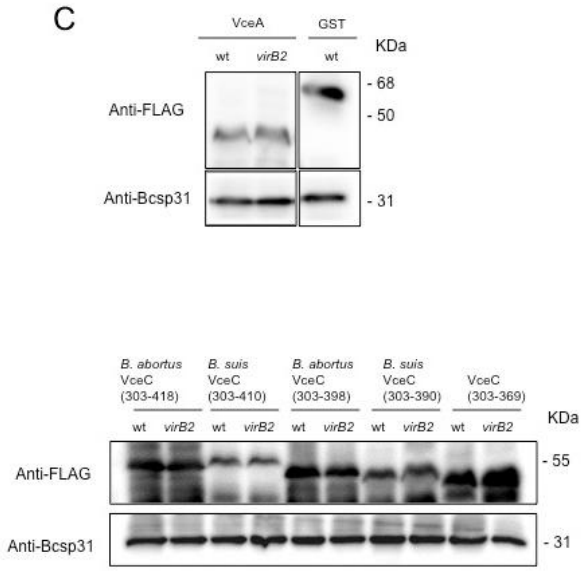


Figure 7. Translocation of TEM1::VceA and TEM1::VceC into J774 macrophages. Cytosolic translocation of b-lactamase by wild-type (2308) or Δ*virB2* mutant strain (ADH3) of *B. abortus* was assessed by fluorescence microscopy. Cells in which translocation of the fusion protein has occurred appear blue. **(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 ± 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).

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 (Figs. 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* (Figure 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 (Figure 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 (Figure 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 (Figure 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*.

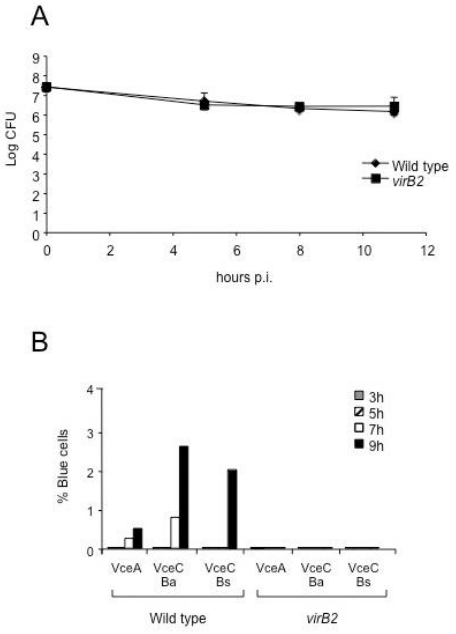


Figure 8. Translocation of TEM1::VceA and TEM1::VceC into J774 macrophages at different time points after infection. (**A**) Survival of wild-type (2308) and *virB2* mutant (ADH3) *B. abortus* in J774 macrophages after infection at an MOI of 500 bacteria per macrophage. (**B**) Cytosolic translocation of TEM1::VceA and TEM1::VceC by wild-type (2308) or Δ*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.

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, Comerci 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 (Figure 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.

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.

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, Cambronne et al. 2005), *A. tumefaciens* VirF (Vergunst, van Lier et al. 2005), *Bartonella henselae* BepA-D (Schulein, Guye et al. 2005), and *Helicobacter* pylori CagA (Hohlfeld, Pattis 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 (Figure 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 (Figure 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, van Lier et al. 2005).

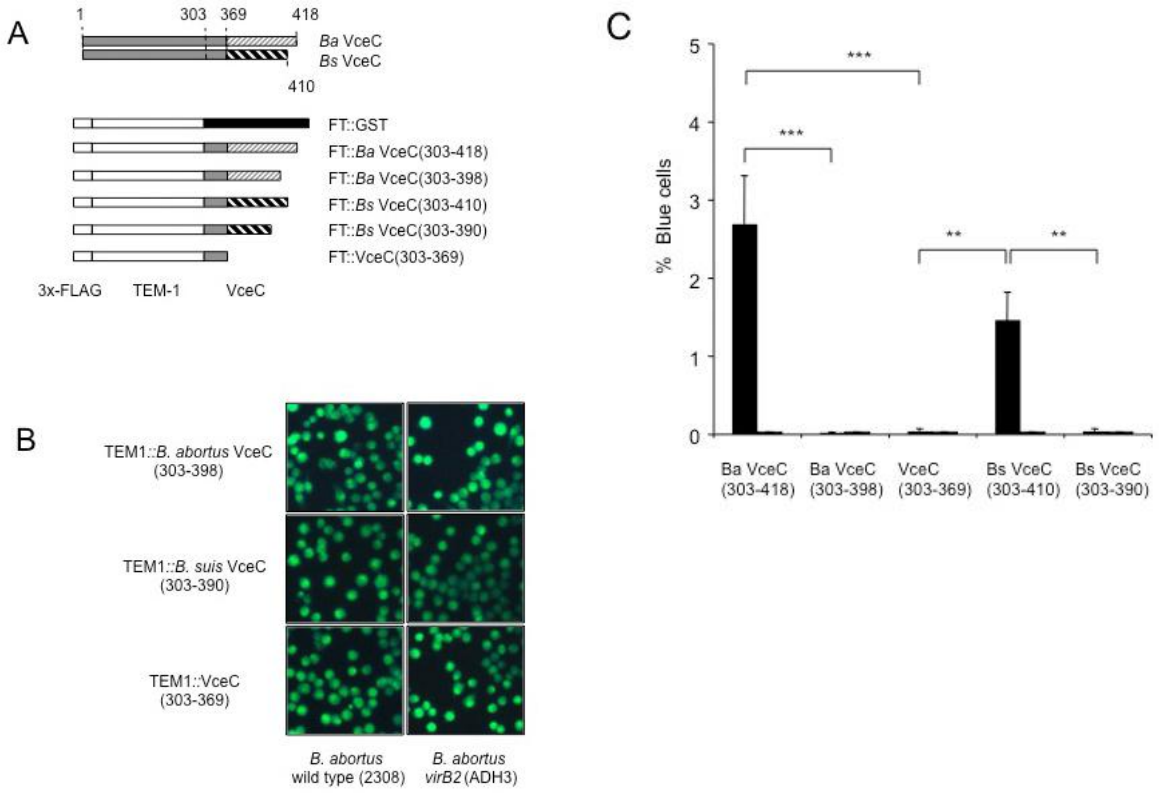


Figure 9. The C-terminal 20 amino acids of both *B. abortus* VceC and *B. suis* VceC are required for translocation into infected macrophages. **(A).** Schematic representation of VceC in *B. abortus* and *B. suis* which differ in their last 49 or 41 amino acids respectively. For translocation experiments 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 b-lactamase by wild-type (2308) or Δ*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 ± 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. ***, $p<0.001$; **, $p<0.01$.

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 b-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 b-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 (Figure 8C).

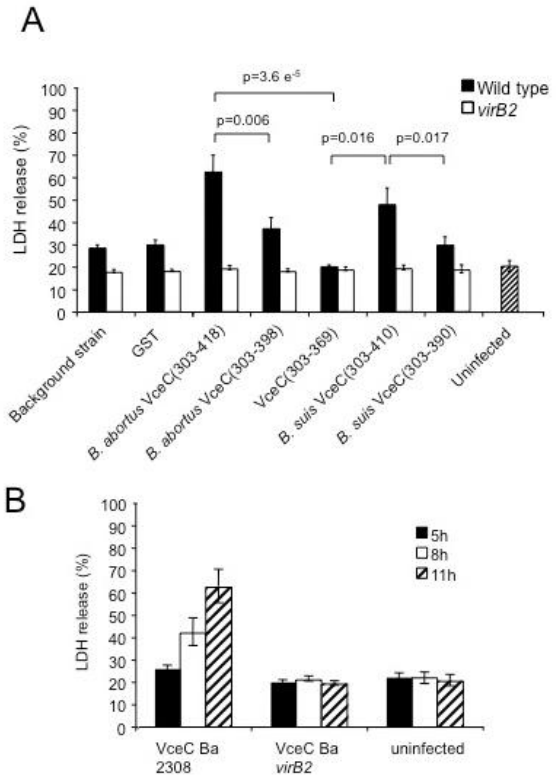


Figure 10. (**A**) Cytotoxicity assay showing % LDH release at 11 hours post infection from J774 macrophages infected at MOI 1:500 with either wild-type or *virB2* mutant *Brucella abortus* expressing TEM1-GST or TEM1-effector fusions. (**B**) Cytotoxicity assay showing % LDH release at 5, 8 and 11 hours 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 ± 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.

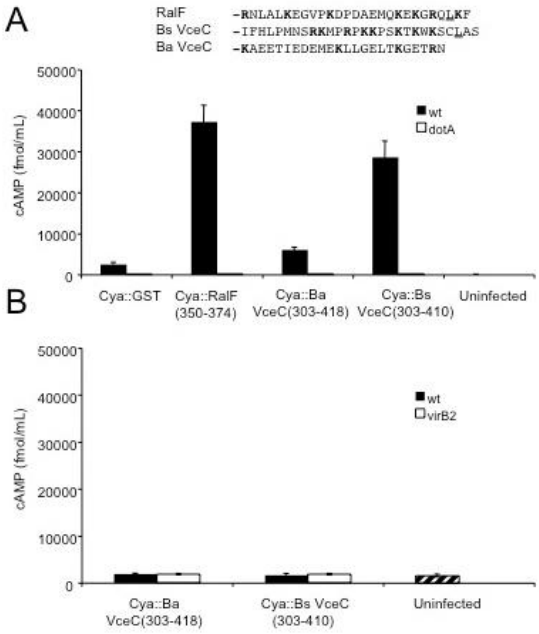


Figure 11. The C-terminus of *B. suis* VceC contains a signal that is recognized by the *L. pneumophila* Dot/Icm T4SS **(A)** Translocation of CyaA::VceC into CHO-FcR cells by *L. pneumophila* wild-type or *dotA* mutant. **(B)** Translocation of the same fusions by *B. abortus* cannot be detected. Results shown are representative of two independent experiments.

This likely represents the T4SS-dependent macrophage cytotoxicity described by Pei et al using a similar multiplicity of infection (Pei, Wu 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 (Figure 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 (Figure 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 (Figure 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 (Figure 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.

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, Guye et al. 2005)and *Helicobacter pylori* (Hohlfeld, Pattis 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 (Figs. 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 (Figure 11A).

Table 1. VjbR regulated proteins translocated into macrophages

ORF	Name	Aa	C-terminus[a]
BAB1_1652	VceA	105	TM**K**VVAG**K**V**KR**YGDGTPA**K**D**K**G**H**AP**K**N
BR1038	*B. suis* VceC	410	IFHLPMNS**RK**MP**R**P**KK**PS**K**T**K**W**K**SCLAS
BAB1_1058	*B. abortus* VceC	418	DEQPEDA**K**AEETIEDEME**K**LLGELT**K**GET**R**N

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

VjbR-His$_6$ 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 ^{32}P labeled 317 bp fragment containing a DNA region upstream of the *vceC* start codon (Figure 12A). Figure 12B shows that the intensity of a band with decreased electrophoretic mobility increased as increasing amounts of purified VjbR-His$_6$ 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* (Figure 5) strongly supported VjbR mediated co-regulation of *vceC* and the *virB* genes in *B. abortus*.

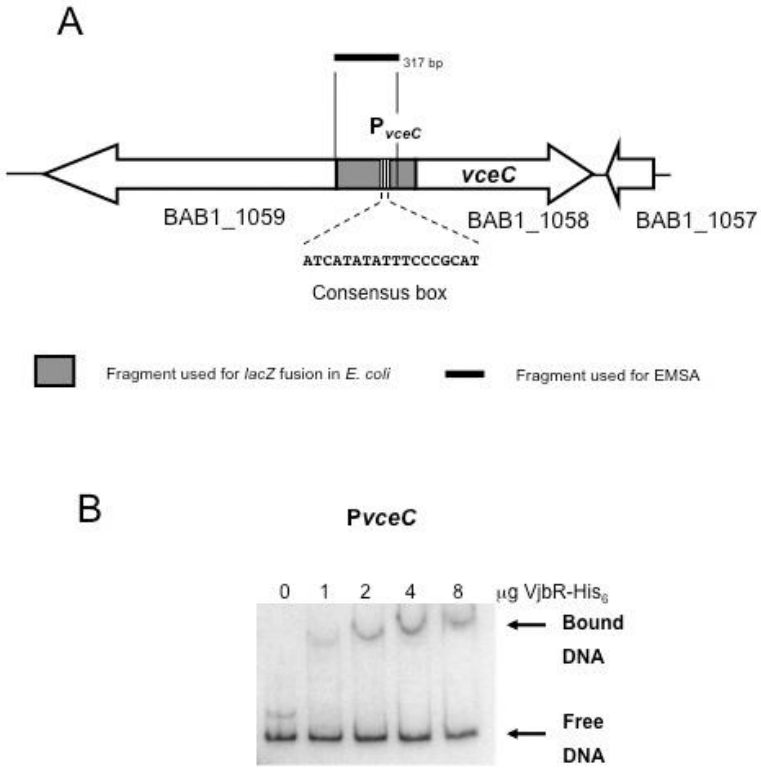


Figure 12. VjbR binds to P_{vceC}. **(A)** Schematic representation of VceC in chromosome I of *B. abortus* showing the putative P_{virB} box and promoter fragments used for EMSA and *lacZ* fusions. **(B)** EMSA showing binding of VjbR-His_6 to a ^{32}P labeled DNA fragment containing 317 bp upstream of the *vceC* start codon.

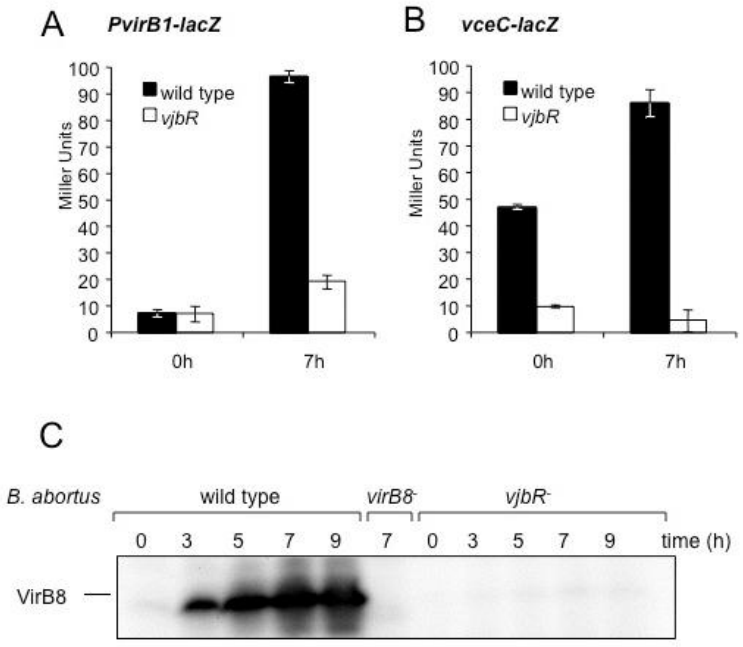


Figure 13. VjbR-dependent activation of *virB* and *vceC* expression in *B. abortus* at different time points after switching from TSB to modified minimal E-medium, pH 5. At time points 0 h and 7 h after switching media, β-galactosidase activity was measured in samples of *B. abortus* wild-type and *vjbR* 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 x 10^8 CFU was loaded, as determined by measurement of OD_{600} for each culture.

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, Guigue-Talet et al. 1997; Patey, Qi et al. 2006). As expected, P_{virB}::*lacZ* expression was reduced in the *vjbR* mutant (Figure 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 (Figure 13C). Similarly, our results showed that *vceC*::*lacZ* expression was down regulated in the *B. abortus vjbR* mutant (ADH17; Figure 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 and 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 (O'Callaghan, Cazevieille et al. 1999; Hong, Tsolis et al. 2000; Sieira, Comerci et al. 2000; Delrue, Martinez-Lorenzo et al. 2001; den Hartigh, Sun et al. 2004). 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, Deschamps 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 C_{12}-HSL, which was in accordance with recent work showing inhibition of transcriptional activation by VjbR in the presence of C_{12}-HSL (Uzureau, Godefroid 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 and Winans 2003; White and 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, Inon de Iannino et al. 2001; Arellano-Reynoso, Lapaque et al. 2005; Fretin, Fauconnier et al. 2005). The gene *cgs* encodes the protein required for synthesis of cyclic ß-1,2-glucan, which mediates evasion of lysosomal fusion with the *Brucella*-containing vacuole (Briones, Inon de Iannino et al. 2001; Arellano-Reynoso, Lapaque et al. 2005; Fretin, Fauconnier et al. 2005) and *fliC* encodes flagellin, a component of the flagellum that has been shown to be re-

quired for persistent colonization of the reticuloendothelial system in mice (Briones, Inon de Iannino et al. 2001; Arellano-Reynoso, Lapaque et al. 2005; Fretin, Fauconnier 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, Joardar et al. 2002; Lindeberg, Stavrinides et al. 2005; Rambow-Larsen, Rajashekara 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 T4S, we showed translocation of a TEM1::RalF fusion protein by *L. pneumophila* (Figure 6). Our results are in agreement with a recently published study showing translocation of several new Dot/Icm effectors designated Legs (de Felipe, Glover 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, Pampou et al. 2005). Based on our success in demonstrating cytosolic translocation of *S. enterica* serovar Typhimurium flagellin into macrophages using this system (Sun, Rolan 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 and Rappuoli 1993; Weiss, Johnson 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, Comerci 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, Guye 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, de Chastellier 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, van Lier 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, van Lier 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 (Schulein, Guye et al. 2005; Hohlfeld, Pattis et al. 2006; Cambronne and Roy 2007), 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, Aloni et al. 2007; Altman and 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, Sullivan 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.

Acknowledgements

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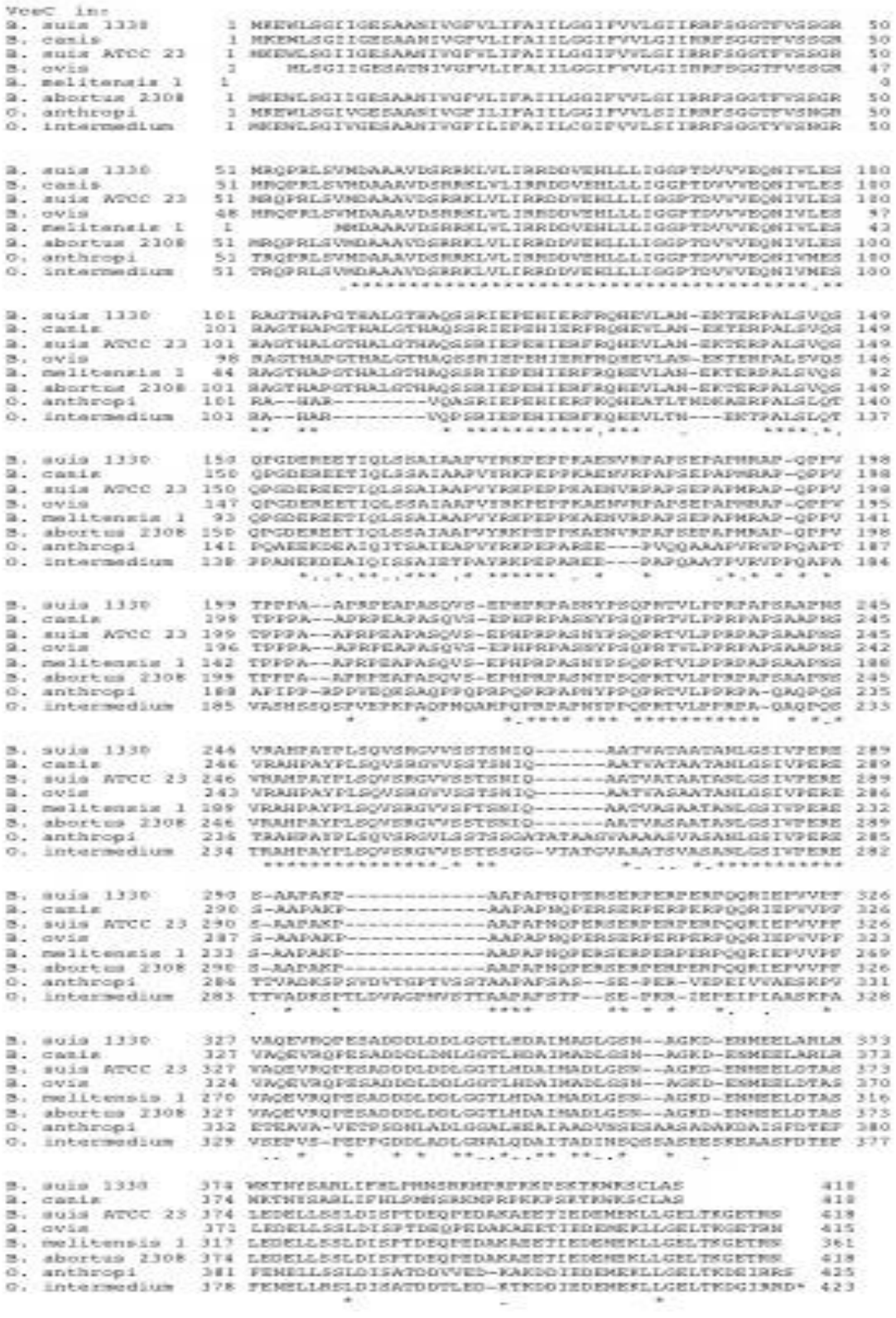
Supplementary data



Supplementary Figure 1. Multiple alignment of VceC amino acid sequences of *Brucella* species and *Ochrobactrum* species *anthropi* and *intermedium* using ClustalW (MacVector 7.2). Proteins aligned are BR1038, BCAN_A1051, BSUIS_A1081, BOV_1003, BMEI0948, BAB1_1058 and Oant_2123, and a predicted ORF from the *O. intermedium* genome (Brettin, Tsolis et al, unpublished results).

Supplementary table 1: Strains and plasmids used in this study

Strain or plasmid	Genotype and antibiotic resistance phenotype	Reference or source
E. coli strains		
Top10	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80*lacZ* ΔM15	Invitrogen
	*ΔlacX74 recA1 ara Δ139 Δ(ara-leu)*7697 *galU galK rpsL* (StrR) *endA1 nupG*	
BL21 (DE3)	F- *ompT gal [dcm] [lon] hsdSB (rB- mB-)* DE3	Studier et al., 1990
BL21 gold (DE3)	F- *ompT gal* [dcm] [lon] *hsdSB* (rB- mB-) Tetr	Stratagene
	DE3 *endA lacZ Hte.*	
E. coli plasmids		
pET101/103	CarbR	Invitrogen
pET-vjbR	VjbR fused to a 6x Histidine tag at C-terminus	This Study
pET-gntR4	GntR4 fused to a 6x Histidine tag at C-terminus	This Study
pET-araC8	AraC8 fused to a 6x Histidine tag at C-terminus	This Study
pET-arsR6	ArsR6 fused to a 6x Histidine tag at C-terminus	This Study
pET-deoR1	DeoR1 fused to a 6x Histidine tag at C-terminus	This Study
pSURS1	A fusion between pSU19 and pRS528 plasmids containing *lacZYA* and P15A ori for promoter expression analysis. CmR	This Study
pSURS2	463 bp fragment upstream of *virB1* cloned in BamHI/EcoRI sites of pSURS1	This Study
pSURS2b	463 bp fragment upstream of virB1 cloned in BamHI/EcoRI sites of pSURS1 containing HindIII site at *virB* promoter box	This Study
pSURS7	P*vjbR* 333 bp upstream of *vjbR* (BAB2_0118)	This Study
pSURS7b	P*tetR-vjbR* 473 bp upstream of *tetR* (BAB2_0117)	This Study
pSURS13r	323 bp fragment upstream of BAB2_0762	This Study
pSURS14	317 bp fragment upstream of BAB2_1069 (BRA1111)	This Study
pSURS17f	183 bp fragment upstream of BAB1_1881	This Study

pSURS18f	147 bp fragment upstream of BAB2_0328	This Study
pSURS18r	147 bp fragment upstream of BAB2_0329	This Study
pSURS19	175 bp fragment upstream of BAB1_1651	This Study
pSURS21	790 bp fragment upstream of BAB1_0108	This Study
pSURS22	320 bp fragment upstream of BR0951 (*B. suis*)	This Study
pSURS23	449 bp fragment upstream of BAB1_1066	This Study
pSURS25	375 bp fragment upstream of BAB1_1837	This Study
pSURS29	290 bp fragment upstream of BAB1_1994	This Study
pSURS30	637 bp fragment upstream of BAB2_1106	This Study
pSURS31	399 bp fragment upstream of BAB1_1058	This Study
pSURS32	432 bp fragment upstream of BAB1_0604	This Study
pSURS35	704 bp fragment upstream of BAB2_0403	This Study
pWIL1	pCR2.1 containing 123 bp fragment of P*virB*	This Study
B. abortus strains		
2308	Wild-type	Deyoe
ADH3	*ΔvirB2* (non polar) in 2308	Den Hartigh et al., 2004
ADH17	*ΔvjbR::kan* in 2308	This Study
MDJ15	pFT/BAB1_1652 in 2308	This Study
MDJ16	pFT/BAB1_1652 in ADH3	This Study
MDJ11	pFT/BR1038(303-410) in 2308	This Study
MDJ12	pFT/BR1038(303-410) in ADH3	This Study
MDJ42	pFT/BR1038(303-390) in 2308	This Study
MDJ43	pFT/BR1038(303-390) in ADH3	This Study
MDJ24	pFT/BAB1_1058(303-418) in 2308	This Study
MDJ26	pFT/BAB1_1058(303-418) in ADH3	This Study
MDJ40	pFT/BAB1_1058(303-398) in 2308	This Study
MDJ41	pFT/BAB1_1058(303-398) in ADH3	This Study
MDJ44	pFT/BAB1_1058(303-369) in 2308	This Study
MDJ45	pFT/BAB1_1058(303-369) in ADH3	This Study
MDJ50	pFT/BAB2_0403 in 2308	This Study
MDJ51	pFT/BAB2_0403 in ADH3	This Study
ADH58	P*virB1::lacZ* in 2308	This Study
ADH60	P*virB1::lacZ* in ADH17	This Study
MDJ30	P*vceC::lacZ* in 2308	This Study
MDJ31	P*vceC::lacZ* in ADH17	This Study
L. pneumophila strains		
Lp01	Wild-type	Berger and Isberg, 1993
	DotA mutant	
B. abortus or L. pneumophila plasmids		
pFLAGTEM1	β-lactamase reporter cloning vector	Raffatellu et al., 2005

pFT/GST	pFLAGTEM1 expressing FT::GST	This Study
pFT/BAB1_1652	pFLAGTEM1 expressing TEM1:: BAB1_1652	This Study
pFT/BAB2_0056	pFLAGTEM1 expressing TEM1::BAB2_0056	This Study
pFT/BAB2_0095	pFLAGTEM1 expressing TEM1::BAB2_0095	This Study
pFT/BAB1_0604	pFLAGTEM1 expressing TEM1::BAB1_0604	This Study
pFT/BR1038wt	pFLAGTEM1 expressing TEM1::BR1038(303-410)	This Study
pFT/BR1038-20	pFLAGTEM1 expressing TEM1::BR1038(303-390)	This Study
pFT/BAB1_1058wt	pFLAGTEM1 expressing TEM1::BAB1_1058(303-418)	This Study
pFT/BAB1_1058-20	pFLAGTEM1 expressing TEM1::BAB1_1058(303-398)	This Study
pFT/BAB1_1058-49	pFLAGTEM1 expressing TEM1::BAB1_1058(303-369)	This Study
pFT/BAB2_0403	pFLAGTEM1 expressing TEM1::BAB2_0403	This study
pFT/BAB1_1705	pFLAGTEM1 expressing TEM1::BAB1_1705	This study
pFT/BAB1_0119	pFLAGTEM1 expressing TEM1::BAB1_0119	This study
pFT/BAB1_1674	pFLAGTEM1 expressing TEM1::BAB1_1674	This study
pFT/BAB1_0939	pFLAGTEM1 expressing TEM1::BAB1_0939	This study
pFT/BAB1_0339	pFLAGTEM1 expressing TEM1::BAB1_0339	This study
pFT/BAB1_0651	pFLAGTEM1 expressing TEM1::BAB1_0651	This study
pFT/BAB1_0740	pFLAGTEM1 expressing TEM1::BAB1_0740	This study
pVjbR-F	up- and downstream fragments of *vjbR* separated by KIXX cassette	This study
pFT/RalF	pFlagTEM1 expressing FT::RalF	This Study
pFT/RalF K368A	pFlagTEM1 expressing FT::RalF(K368A)	This Study
pFT/RalF L372T	pFlagTEM1 expressing FT::RalF(L372T)	This Study
pFT/RalFc	pFlagTEM1 expressing FT::RalF(350-374)	This Study
pdTFT/RalFc	In pFT/RalFc expressing FT::RalF(350-374) and DsRed	This Study

Supplementary Table 2. *Brucella spp.* containing predicted consensus sequence in promoter

B. abortus 2308 ORF	*B. suis* 1330 ORF	*B. melitensis* 16M ORF	*virB* promoter box	Gene name/Predicted function	Downstream genes possibly in operon
Amino acid biosynthesis					
BAB1_0926	BR0908	BMEI1061	ATTAGAGGGTTTCGGCTT	aroQ	BAB1_0925-BAB1_0923
BAB1_1031	BR1013	BMEI0971	ATCAGCGCGACCACGCAT	Aminoacyl-tRNA synthetase	BAB1_1030-BAB1_1034
BAB1_0335	BR0305	BMEI1617	AACAGGCATAAAGAAAAC	metZ	BAB1_0334
BAB1_0813	BR0793	BMEI1166	ATCAGTCAATAAGCACTT	O-acetylhomoserine/O-acetylserine sulfhydrylase family protein	-
BAB2_0751	BRA0486	BMEII0781	CTCCCCGAAAGGGAGGAT	homoserine O-succinyltransferase	-
BAB1_2087	BR2086	BMEI2040	ATGCTCTAAAAAGATACC	hisE	BAB1_2088
Biosynthesis of cofactors, prosthetic groups, and carriers					
BAB1_1161	BR1138	BMEI0846	ATGAGCCAGTCAGGGGAT	triosephosphate isomerase, tpiA-1	BAB1_1161-BAB1_1148
BAB1_0972	BR0957	BMEI1020	ATGATCTATCTCGCAACC	mobA	BAB1_0971-BAB1_0968
BAB1_1719	BR1707	BMEI0329	ATGCCCGAATTCGATCAT	thiamine-phosphate pyrophosphorylase (putative)	BAB1_1720-BAB1_1726
Cell envelope: Biosynthesis and degradation of murein sacculus and peptidoglycan					
BAB1_0607	BR0583	BMEI1351	ATGAGGCAAATCTCGCCT	penicillin binding protein	BAB1_0606-BAB1_0604
BAB2_0312	BRA0923	BMEII0374	AAGAGCGCGGGCGCTGAT	alanine racemase	BAB2_0311

BAB2_0781	BRA0455	BMEII0811	AACCTCGATCCAGAAGCT	membrane protein	BAB2_0782
Cellular processes: Adaptations to atypical conditions/Pathogenisis/Detoxification					
BAB1_0672	BR0652	BMEI1296	ATGGCCGATATCTCTGTA	RelA/SpoT protein	BAB1_0673-BAB1_0675
BAB1_2150	BR2149	BMEI1980	GATCGCCATTTGCCGGAT	Dps family protein	-
BAB2_1106	BRA1147	BMEII0150	ATGCTTAAGGTGGAAATT	flagellin family protein	-
BAB1_1030	BR1012	BMEI0972	AACCTCTCGATAACGCTT	glutathione reductase	BAB1_1031-BAB1_1034
BAB1_1441	BR1422	BMEI0587	ATTCGATGGATGGAGCAT	competence protein ComL, putative	BAB1_1440-BAB1_1438
BAB1_1880	BR1878	BMEI0183	GTCTTCCAGATAAAGGTT	competence protein F	BAB1_1879-BAB1_1876
-	BR0971	BMEI1007	GGCCGCTTTTTTACTGTT	omp	
-	BRA0173	BMEII1069	ATCCGGCATATCTCTCAC	omp	
BAB1_0108	BR0111	BMEI1837	ATCCGTGATCTCGCAGCT	cyclic beta 1-2 glucan synthetase	BAB1_0109
BAB2_0067	BRA0068	BMEII0026	ATGCTCCAGATCGCAGAT	virB2	BAB2_0066-BAB2_0057
BAB2_0068	BRA0069	BMEII0025	ATGACCGATATCGCTGAT	virB1	BAB2_0067-BAB2_0057
BAB1_0402	BR0372	BMEI1553	ATCCTGCATATCTATGCC	bacA	-
BAB1_0801	BR0780	-	AACCTCCATGCCCCTGAT	sugE protein	-
BAB2_0205	BRA0211	BMEII1033	TGCAGCCATATCTTGTTT	pmbA	BAB2_0206-BAB2_0210

Central intermediary metabolism					
BAB1_1837	BR1829	BMEI0222	ATGCGCCATGCCGAACAT	carbonic anhydrase, putative	-
BAB2_0253	BRA0981	BMEII0316	ATGGTTCAAATGGCAAAT	2-deoxy-d-gluconate 3-dehydrogenase, putative	BAB2_0253-BAB2_0263
DNA metabolism: DNA replication, recombination, and repair					
BAB1_1224	BR1202	BMEI0787	ATTTTCGGAATTGCTGAT	recA	BAB1_1223
BAB2_0625	BRA0615	BMEII0656	AGGGCCGCTATAGCCGAT	UMUC-like DNA-repair protein	BAB2_0624
Energy metabolism: Amino acids and amines					
BAB2_0513	BRA0727	BMEII0559	ATGCGTTAAAGCGCGCCT	glycine cleavage system protein	BAB2_0514-BAB2_0516
BAB1_1806	BR1798	BMEI0252	GGTATCCATTTTCGGGAT	ATP synthase	BAB1_1807 and BAB1_1804
-	BR0202	BMEI1747	ATGTGCGGGATGACTCTT	aldehyde dehydrogenase family protein	-
BAB2_0835	BRA0386	BMEII0880	ATGCTTCAAATAGAAGAG	acetate kinase	BAB2_0836
BAB2_0459	BRA0779	BMEII0512	ATGCTGCAACTGGCAGAC	6-phosphogluconolactonase	BAB2_0458
BAB2_0460	BRA0778	BMEII0513	ATGTGTGCATTTGCGGTT	glucose-6-phosphate 1-dehydrogenase	BAB2_0459-BAB2_0458
BAB2_0935	BRA0268	BMEII0980	AGGAGTAAAACCGAAGAT	ribitol dehydrogenase	BAB2_0934

Fatty acid and phospholipid metabolism: Biosynthesis/Degradation

BAB1_0484	BR0459	BMEI1475	ATCCTGCATGCCGCTAAC	acpP	BAB1_0485-BAB1_0489
BAB1_1994	BR1994	BMEI0075	AACCGCCGTCTTCCTGAT	1-acyl-sn-glycerol-3-phosphate acyltransfe-rase, putative	BAB1_1993
BAB1_2173	BR2172	BMEI1957	AACCGCGAAGACGAGGCT	3-oxoacyl-(acyl-carrier-protein) synthase I	BAB1_2172
BAB1_2174	BR2173	BMEI1956	AAGATTGAACGGGCTGTT	3-hydroxyl decanoyl dehydratase fabA	BAB1_2173-BAB1_2172
BAB2_0668	BRA0572	BMEII0695	ATTCGCTGCCTGACGGTT	phosphatidylcholine synthase	BAB2_0669-BAB2_0673
BAB1_1528	BR1510	BMEI0503	ATGATTAATGAATATCAT	long-chain acyl-CoA thioester hydrolase, putative	-
BAB2_0439	BRA0799	BMEII0492	TTGCGCGATGTAGCTGTT	acyl-CoA dehydrogenase family protein	-

Hypothetical proteins: Conserved and not conserved

BAB1_0119	BR0122	BMEI1826	ACGAGTCATAAAACTGAT	hypothetical protein	BAB1_0120-BAB1_0121
BAB1_0294	BR0263	BMEI1658	AGCTTTCATAATGCTGAT	hypothetical protein	-
BAB1_0608	BR0584	-	AGGCGAGATTTGCCTCAT	conserved hypothetical protein	BAB1_0609
BAB1_0651	BR0628	BMEI1314	ATCTGGTTTAACGTGCAT	conserved hypothetical protein	BAB1_0650
BAB1_0939	BR0922	BMEI1051	ATCAGCCATTTTGCGCTG	conserved hypothetical protein	BAB1_0938
BAB1_1058	BR1038	BMEI0948	ATCATATATTTCCCGCAT	hypothetical protein	-
BAB1_1295	BR1276	BMEI0723	ATTTGCCCATGCGAGCAT	hypothetical protein	BAB1_1294-BAB1_1287
BAB1_1651	BR1633	-	ATTTGATATATGAAGGAT	hypothetical protein	BAB1_1652

BAB1_1678	BR1664	-	AACCCTGATGAAGCAGAT	hypothetical protein	BAB1_1677-BAB1_1674
BAB1_1708	BR1696	-	AGCTTCGGTTTCGCGCCT	hypothetical protein	BAB1_1707-BAB1_1693
BAB2_0038	BRA0039	BMEII0054	AGGTTCGCACTGCCGGAT	hypothetical protein	BAB2_0039-BAB2_0041
BAB2_0801	BRA0441	-	ATGAGCTATCTACCGCTT	conserved hypothetical protein	-
BAB2_0892	BRA0313	-	AAATATGAGATAGCCGAT	conserved hypothetical protein	-
-	BR0023	BMEI1919	AGTTGCGATTTAGCTCTT	acetoacetyl-COA synthase	
BAB1_0087	BR0090	BMEI1857	ATCATTAAACACGCACTT	hypothetical protein	-
BAB1_0101	BR0104	BMEI1844	ATCTGCGCATTGGCAGAT	conserved hypothetical protein	-
BAB1_0155	BR0156	BMEI1791	AACTGCGATATGGGGCGT	hypothetical protein	BAB1_0156-BAB1_0157
BAB1_0329	BR0299	BMEI1623	ATCTTCGTTATCCGGAAT	Pollen allergen Poa pIX/Phl pVI, C-terminal	-
BAB1_0336	BR0306	BMEI1616	GTTTTCTTTATGCCTGTT	2'-deoxycytidine 5'-triphosphate deaminase	BAB1_0337-BAB1_0340
BAB1_0339	BR0309	BMEI1613	TTCAGCGATACATCAGAT	conserved hypothetical protein	BAB1_0340
BAB1_0342	BR0312	BMEI1610	ATTTTCCATTTAGAGCAT	conserved hypothetical protein	BAB1_0341
BAB1_0343	BR0313	BMEI1609	ATGCTCTAAATGGAAAAT	conserved hypothetical protein	-
BAB1_0452	BR0426	BMEI1508	ATTTGCCGCATGGTGGTT	conserved hypothetical protein	-
BAB1_0587	BR0562	BMEI1371	ATGCTCTAAAGGTATAAC	conserved hypothetical protein	-
BAB1_0587	BR0562	BMEI1371	ATCTTCTATTTGAAGCAT	conserved hypothetical protein	-

BAB1_0597	BR0572	BMEI1361	GGTTGCGAGCTTCAGGAT	conserved hypothetical protein	BAB1_0598
BAB1_0598	BR0573	BMEI1360	CTGAGCCATATGGCGGTT	Amidase	-
BAB1_0604	BR0580	BMEI1354	ATCCGCTATGCCGACCAT	conserved hypothetical protein (effector cand.)	-
BAB1_0812	BR0792	BMEI1167	AAGTGCTTATTGACTGAT	conserved hypothetical protein	BAB1_0811-BAB1_0808
BAB1_0852	BR0831	BMEI1132	ATCATCGATCTGTCGGTC	hypothetical protein	BAB1_0853
BAB1_1529	BR1511	BMEI0502	ATGATATTCATTAATCAT	conserved hypothetical protein	BAB1_1530-BAB1_1533
BAB1_1881	BR1879	BMEI0182	ATGCTCGAAAGAGAAGAT	hypothetical protein	BAB1_1882
BAB2_0056	BRA0057	BMEII0037	AAGACCTATAGAGCGGTT	hypothetical protein	BAB2_0055
BAB2_0163	BRA0167	BMEII1075	TTTAGCAATATCGAGGAT	conserved hypothetical protein	BAB2_0164-BAB2_0165
BAB2_0328	BRA0907	-	AACCGCAAAACCGCTGAT	hypothetical protein	BAB2_0327
BAB2_0403	BRA0833	BMEII0457	ATCGGCCTCATCCAACAT	conserved hypothetical protein	-
BAB2_0616	BRA0627	BMEII0649	GTCAGCGATGTGGCGTAT	conserved hypothetical protein	-
BAB2_0947	BRA0256	BMEII0991	ATCAGCCAAAGCTGTTTT	lipase	BAB2_0946-BAB2_0934
BAB2_1069	BRA1111	BMEII0188	ATCAGCCATATCGATAAC	conserved hypothetical protein	BAB2_1071 (BRA1112)
BAB2_1131	BRA1172	BMEII0123	AGCCGCCTTTTTCATCAT	conserved hypothetical protein	BAB2_1132-BAB2_1135

Protein fate: Degradation/Secretion/Folding

BAB1_0118	BR0121	BMEI1827	ATCAGTTTTATGACTCGT	hypothetical protein	BAB1_0117-BAB1_0110
BAB1_2151	BR2150	BMEI1979	ATCCGGCAAATGGCGATC	protease	BAB1_2152-BAB1_2171
BAB2_0586	BRA0654	BMEII0626	ATTTGCCTTTTGCGGGTT	renal dipeptidase family protein	BAB2_0585-BAB2_0580
BAB1_0901	BR0882	BMEI1084	ATCGGAGATAGCAGGCAT	tatB	BAB1_0902-BAB1_0907
BAB1_1162	BR1139	BMEI0845	ATCCCCTGACTGGCTCAT	rotamase family protein	BAB1_1163-BAB1_1167
BAB2_0782	BRA0454	BMEII0812	TTCTTCTGTTTGAAGCAT	polypeptide deformylase	-

Protein synthesis

BAB1_1728	BR1716	BMEI0322	ATCCGCAGGAAAGCGCAT	ribosomal protein L31	-
BAB2_0269	BRA0966	BMEII0332	ATCCGCCAGTTGGCGGCC	ribosomal protein S21	-
BAB1_1183	BR1161	BMEI0824	ATCCGTCATATCCATGAT	translation elongation factor Ts	BAB1_1182-BAB1_1171
BAB1_1815	BR1807	BMEI0242	GTTCGCTTATTGCCGCAT	leuS	BAB1_1814
BAB1_1815	BR1807	BMEI0242	GATTTCCTCCTTCAGCAT	leuS	BAB1_1814

Purines, pyrimidines, nucleosides, and nucleotides

BAB2_0326	BRA0909	BMEII0387	AAAAGCGTTTTGGCTGAT	formyltetrahydrofolate deformylase purU	-

BAB2_0587	BRA0653	BMEII0627	AACCCGCAAAAGGCAAAT	adenine deaminase	-
Regulatory functions: DNA interactions					
BAB1_1201	BR1179	BMEI0808	ATCAGGAAAATAGCGATT	transcriptional regulator, MerR family	-
BAB1_2175	BR2174	BMEI1955	AACAGCCCGTTCAATCTT	regulator ferric uptake	BAB1_2176
BAB2_0117	BRA0118	BMEII1117	ATCAGCTTTATCAACGGAT	transcriptional regulator, TetR family	BAB2_0118 (vjbR)
BAB2_0250	BRA0984	BMEII0312	GGCTTCCAATTGAATGAT	transcriptional regulator	BAB2_0251
BAB2_0313	BRA0922	BMEII0375	ATCAGCGCCCGCGCTCTT	alanine catabolic operon transcriptional regulator	-
BAB2_0329	BRA0906	BMEII0390	ATCAGCGGTTTTGCGGTT	transcriptional regulator, LysR family	BAB2_0330
BAB2_0762	BRA0474	BMEII0791	ATCAGCCAGTAGGCTGAT	ompR response regulator	BAB2_0763 (envZ)
BAB2_0780	BRA0456	BMEII0810	AGCTTCTGGATCGAGGTT	bacterial regulatory protein, ArsR family	BAB2_0779-BAB2_0778
BAB2_1041	BRA1082	BMEII0219	AACCGTGAAAAAGAGAAT	transcriptional regulator, IclR family	BAB2_1042
BAB2_1099	BRA1140	BMEII0158	ATCATTTAGCCCGCTATT	transcriptional regulator (ftcR)	-
BAB2_1128	BRA1169	BMEII0127	ATGCTTCAAATATAGACT	transcriptional regulator IclR family protein	BAB2_1127-BAB2_1122
BAB1_1059	BR1039	BMEI0947	ATGCGGGAAATATATGAT	sensory box histidine kinase/response regulator	BAB1_1060-BAB1_1061
BAB1_1539	BR1522	BMEI0492	AACCGCAATGTGGTTCAT	osmolarity sensor protein EnvZ, putative	-

Tat (Sec-independent) protein export: has twin-arginine signal sequence					
BAB1_0047	BR0050	BMEI1893	AACCATAAGATCGCAAAT	lipoprotein, putative	-
Transcription: Transcription factors					
BAB1_0157	BR0158	BMEI1789	ATCTTTTATTTTAGGCAT	RNA polymerase sigma-54 factor	-
BAB1_1498	BR1479	BMEI0532	AACAGGAAATCGGCTACT	RNA polymerase sigma-70	BAB1_1497-BAB1_1495
BAB1_1498	BR1479	BMEI0532	ATGATGAAGTCCGATGCC	RNA polymerase sigma-70	BAB1_1497-BAB1_1495
BAB1_1665	BR1650	BMEI0378	ATGATGCATATATATGTC	RNA polymerase sigma-70 family protein	-
Transport and binding proteins					
?	BR1954	?	ATCATCCAACGATCACAT	ABC transporter	
BAB1_1627	BR1611	BMEI0412	AGGTTCCGGCTAAAGCAT	ABC transporter	BAB1_1626-BAB1_1624
BAB2_0611	BRA0632	-	ATGCCCCCAACCGCTGGT	bacterial extracellular solute-binding protein	BAB2_0612-BAB2_0615
BAB2_1040	BRA1081	BMEII0220	ATTCTCTTTTTCACGGTT	peptide ABC transporter, permease protein	BAB2_1040-BAB2_1035
BAB2_1129	BRA1170	BMEII0126	AGTCTATATTTGAAGCAT	Amino acid permease protein	BAB2_1130-BAB2_1135
-	BRA0304	BMEII0945	ATCTGATTTTTACATGTT	sugar ABC transporter	BRA0305-BRA0312
BAB1_0738	BR0720	BMEI1233	ATTTGCCCGTTGACGCTT	L-lactate permease, lldP	BAB1_0737-BAB1_0735
BAB2_0738	BRA0500	BMEII0770	AACATTGAAACATATGAT	monovalent cation/proton antiporter, MnhA/PhaA subunit	BAB2_0737-BAB2_0726

-	BRA0311	BMEII0935	GATTTCGCCATCGAGGTT	transporter	BRA0312
BAB1_1727	BR1715	BMEI0323	ATGCGCTTTCCTGCGGAT	ABC transporter, ATP binding/permease protein	-
BAB2_0113	BRA0115	BMEII1120	ATGCGCGTTATCGTTGAA	ABC transporter	BAB2_0112-BAB2_0110
Unknown function: General/Enzymes of unknown specificity					
BAB1_0739	BR0721	BMEI1231	AAGCGTCAACGGGCAAAT	oxidoreductase, putative	BAB1_0740
BAB1_1066	BR1046	BMEI0940	AACCGCCTTTTTGCGGTT	phosphoribosyltransferase family protein	BAB1_1065
BAB1_1664	BR1649	BMEI0379	AACCGCTCTTTTGGGAAT	acetyltransferase, GNAT family	-
BAB2_0097	BRA0099	BMEII1134	GTCCTCGATCTGGCGGTT	amidase	BAB2_0096-BAB2_0092
BAB2_0098	BRA0100	BMEII1133	AACCGCCAGATCGAGGAC	ornithine/DAP/arginine decarboxylase family	-
BAB2_0252	BRA0982	BMEII0314	ATTTGCCATTTGAACCAT	glutamine amidotransferase, class I	-
BAB2_0461	BRA0777	BMEII0514	AACCGCAAATGCACACAT	oxidoreductase, short-chain dehydroge-nase/reductase family	-
BAB2_0555	BRA0685	BMEII0598	ATCCGCGAAAGCCCTCTT	phosphatase, Ppx/GppA family	BAB2_0554
BAB2_0644	BRA0596	BMEII0673	GACGCCGATAAAGTTGAA	aminoacyl-tRNA synthetase	BAB2_0645-BAB2_0648
BAB2_1015	BRA1054	BMEII0246	AACCCCCATACAGAAGCC	nitroreductase family protein	BAB2_1016
-	BR0951	BMEI1023	ATGCTCTAAATAGAAAAT	glutathione S-transferase domain protein	-
-	BRA0301	BMEII0947	AAGATCAATATCTCGAAT	cyclic nucleotide-binding protein	-
BAB1_0882	BR0863	BMEI1103	AAGTTCAATTAAGCAGTT	TIM-barrel protein, yjbN family	-

BAB1_1106	BR1083	BMEI0899	ATGACCAAGAGCGCGGAT	BRO family protein: COG3617	BAB1_1105-BAB1_1103
BAB1_1133	BR1110	BMEI0873	ATGCCCGATAGCGATGCG	ATP/GTP-binding site motif A (P-loop)	BAB1_1132-BAB1_1129
BAB1_1147	BR1124	BMEI0859	TTCAGCCCCATCGGGGAT	lipoic acid synthetase, lipA	BAB1_1146
BAB1_1718	BR1706	BMEI0330	ATGCCCGAATTCGATCAT	hypothetical protein	BAB1_1717-BAB1_1709
BAB2_0307	BRA0928	BMEII0369	AAGCGAGAAAACGCTCAT	amidohydrolase	BAB2_0308
BAB2_0361	BRA0875	BMEII0419	AGTTTCGCGCTGCCTCTT	GTP binding protein	BAB2_0362-BAB2_0365
BAB2_0859	BRA0344	BMEII0904	ATGCTTCAAATAGAAGGT	EAL domain protein	-
BAB2_1072	BRA1113	BMEII0186	AGCATCATTATGGAGCAT	pemK family protein (transcriptional regulator, growth inhibitor)	-
BAB2_1072	BRA1113	BMEII0186	AGGAGTCATGCCGCTGAT	pemK family protein (transcriptional regulator, growth inhibitor)	-

Supplementary Table 3. Promoters with a putative P*virB* box activated by VjbR in *E. coli*.

ORF	P*virB* box	Gene name/Predicted function	Downstream genes
BAB2_0068	ATGACCGATATCGCTGAT	*virB1*	BAB2_0067-BAB2_0057 *virB2-virB12*
BAB2_0067	ATCTGCGATCTGGAGCAT	*virB2*	BAB2_0066-BAB2_0057 *virB3-virB12*
BAB2_0117	ATCAGCTTTATCAACGGAT	Transcriptional regulator (*tetR)*	BAB2_0118 *vjbR*
BAB2_0762	ATCAGCCAGTAGGCTGAT	*ompR* response regulator	BAB2_0763 *envZ* osmolarity sensor
BAB1_1881	ATGCTCGAAAGAGAAGAT	Hypothetical protein	BAB1_1882 transporter
BAB2_0328	AACCGCAAAACCGCTGAT	Hypothetical protein	BAB2_0327 aldehyde dehydrogenase
BAB2_0329	ATCAGCGGTTTTGCGGTT	Transcriptional regulator (*lysR12)*	BAB2_0330 oxidoreductase
BAB1_1651	ATTTGATATATGAAGGAT	Hypothetical protein	BAB1_1652 (*vceA*)
BAB1_1066	AACCGCCTTTTTGCGGTT	Phosphoribosyltransferase family	BAB1_1065 *mutT*
BAB1_1837	ATGCGCCATGCCGAACAT	Carbonic anhydrase, putative	-
BRA1111	ATCAGCCATATCGATAAC	Conserved hypothetical protein (*bopA*)	BRA1112 (BAB2_1071) hypothetical
BAB1_0108	ATCCGTGATCTCGCAGCT	Cyclic beta 1-2 glucan synthetase	BAB1_0109 *pncB*
BR0951	ATGCTCTAAATAGAAAAT	Glutathione S-transferase domain	BR0950 oxidoreductase (putative)
BAB2_1106	ATGCTTAAGGTGGAAATT	Flagellin family protein (*fliC*)	-
BAB1_0604	ATCCGCTATGCCGACCAT	hypothetical protein	-

BAB1_1058	ATCATATATTTCCCGCAT	hypothetical protein (*vceC*)	-
BAB1_1994	AACCGCCGTCTTCCTGAT	1-acyl-sn-glycerol-3-phosphate acyltransferase	BAB1_1993 inorganic pyrophosphatase
BAB2_0403	ATCGGCCTCATCCAACAT	hypothetical protein	-
consensus	ATCCGCGATATCGCGGAT		

Supplementary table 4. Similarity (%) between VceC amino acid sequences of *Brucella* spp. and *O. anthopi*. Amino acid sequences of BR1038, BCAN_A1051, BSUIS_A1081, BOV_1003, BAB1_1058, BruAb1_1043, BMEI0948, Oant_2123, and a predicted ORF from *O. intermedium* were aligned pairwise using ClustalW (MacVector 7.2).

VceC Identity (%)	*B. suis* 1330	*B. canis*	*B. suis* 23445	*B. ovis*	*B. abortus* 2308	*B. abortus* 9-941	*B. melitensis* 16M	*O. anthropi*
B. suis 1330	100							
B. canis ATCC 23365	99	100						
B. suis ATTC 23445	88	88	100					
B. ovis	87	87	98	100				
B. abortus 2308	88	88	99	98	100			
B. abortus 9-941	88	88	99	98	100	100		
B. melitensis 16M	75	75	85	86	85	85	100	
O. anthropi	56	55	63	61	62	62	50	100
O. intermedium	56	56	62	61	62	62	50	81

Supplementary table 5. Primers used in this study

Purpose[a]	Name	Sequence[b]
pET-vjbR	VjbR-F	AAACATATGAGTCTTGATCTCGTTCATTT
	VjbR-R	TCCGTCGACGAGATGCTGTACCTCGGG
pET-gntR4	GntR4-F	AAACATATGCGGCTGGTCGCGGAAGGTATC
	GntR4-R	TCCGTCGACGGATCTTGCGGCCTTCATGGC
pET-araC8	AraC8-F	AAACATATGTCCGTATTGCTGACAACG
	AraC8-R	TCCGTCGACGCATTCGTAATTTAAGCAATTTG
pET-deoR1	DeoR1-F	AAACATATGATACCGGCTGAACGGCAG
	DeoR1-R	TCCGTCGACGATTTTCCCTTCGACTTTGCC
pET-arsR6	ArsR6-F	AAACATATGACTAACAAAGTTACTTTTTATG
	ArsR6Xho-R	TCCCTCGAGTTGGTGCGCCACCGCCATCAT
pSURS1	pACYC184-R	TCCTTCCTGCAGCTGATGTCCGGCGGTGCTTTTG
	pACYC184-F	AATTAAGTCGACGCTAGCGGAGTGTATACTGGCT
pSURS2	PVirBBamH1-F	AAAGGATCCATCGCCATGACAGGCATATTTC
	PVirBEcoR1-R	TCCGAATTCTAGGATCGTCTCCTTCTCAGAG
pSURS7	PvjbRBamH1-F	AAAGGATCCATTCGGGACAATGTGGAGTCC
	PvjbREcoR1-R	TCCGAATTCTGGAAATATCCTTGGTGATGAA
pSURS7b	PvjbR2BamH1-F	AAAGGATCCGGGGATGTTTTCAATATAGCC
	PvjbR2EcoR1-R	TCCGAATTCAAAGCAGGACTCTTAACTTTTTTC
pSURS13R	13REcoR1-F	TCCGAATTCTTTGCTGTTCCATTGCCGT
	13RBamH1-R	AAAGGATCCGCGAAGTCTGCGCTATTGG
pSURS14	14BamH1-F	AAAGGATCCTGCGATGGGGCACCATCG
	14EcoR1-R	TCCGAATTCTGGTTCCTCTGGGGTCGCA
pSURS17F	17FBamH1-F	AAAGGATCCTGCATTCGCCCCCGTTC
	17FEcoR1-R	TCCGAATTCGGGATTCTATCTTCTCTTTCGAGC

pSURS18F	18FBamH1-F	AAAGGATCCTCCATTCTCGCATTAACATAGTT
	18FEcoR1-R	TCCGAATTCTGCGAAAATTTCCGTTGAAA
pSURS18R	18REcoR1-F	TCCGAATTCTCCATTCTCGCATTAACATAGTT
	18RBamH1-R	AAAGGATCCTGCGAAAATTTCCGTTGAAA
pSURS19	19BamH1-F	AAAGGATCCAGCAGAAATTCCAATGTATCTCC
	19EcoR1-R	TCCGAATTCCGGATTAATCGGTGTCGCAG
pSURS21	21BamH1-F	AAAGGATCCTATGCCCGGTGATTGAAGG
	21EcoR1-R	TCCGAATTCTCCTTCATATTGTTCGAGCGG
pSURS22	22BamH1-F	AAAGGATCCGAAACACGCCGCGCCTA
	22EcoR1-R	TCCGAATTCGGTTATACCTGTCGAATTGAGGA
pSURS23	23BamH1-F	AAAGGATCCAAGCGGTTTTGCGTCGGA
	23EcoR1-R	TCCGAATTCCGAAACGCTCCATGGATC
pSURS25	25BamH1-F	AAAGGATCCTCTGGCGCGCTACCTTTC
	25EcoR1-R	TCCGAATTCAGCGTTTGAATATCCCTATCG
pSURS29	29BamH1-F	AAAGGATCCGCTGCGTGTGCTCTTCACC
	29EcoR1-R	TCCGAATTCCGTATTGGCGAGGGCCC
pSURS30	30BamH1-F	AAAGGATCCATCTCAACCCCGCCTCTGTC
	30EcoR1-R	TCCGAATTCAGTTTTTTGCCCCTTGGGAA
pSURS31	31BamH1-F	AAAGGATCCGTGCTTCTCCGTTCAGCCG
	31EcoR1-R	TCCGAATTCCACGGATGGGCGGATATTAC
pSURS32	32BamH1-F	AAAGGATCCCCTGAACGACAATGTCTCCAA
	32EcoR1-R	TCCGAATTCTTTACATCCTGGGCGTTACG
pSURS35	35BamH1-F	AAAGGATCCGATGCGGGAGATCAGTGAGTT
	35EcoR1-R	TCCGAATTCCCGGTTCTCCCATGACAGG
pFT/GST	GST-F1	TCCATATGTCTAGATCCCCTATACTAGGTTATTG

	GST-R1	AACTGCAGTCAACGCGGAACCAGATCCGATTTTG
pFT/RalF	RalF-BspE1-F	ATCCGGACTCTCTAGACATCCAGAAATT-GAAAAAGCCCAAAGAGAG
	RalF-Bcl1-R	ATGATCACTGCAGGCCATTTCACCCAGATTTGTG
pFT/RalF(K368A)	RalF-K368A-R	ACTGCAGATTAAAATTTTAATTGTCTACCTGCTTC
pFT/RalF(L372T)	RalF-L372T-R	ACTGCAGATTAAAATTTTGTTTGTCTACCTTTTTC
pFT/RalF(350-374)	RalF-F	CGTCTAGACTGGCACTTAAGGAGGGCGTTC
	RalF-R	GCGCTGCAGGCCATTTCACCCAGATTTGTGGAG
pFT/BAB1_1652	BR1634-F	TCCTCTAGAAAAATCATCATCACGGCAGCA
	BR1634-R	AAACTGCAGCTAGTTCTTGGGCGCGTGGCC
pFT/BAB1_1058	BAB1_1058-F	TCCTCTAGAGAACGTTCAGAGCGTCCAGAA
	BAB1_1058-R	AAACTGCAGCTAATTGCGGGTTTCTCCCTTG
pFT/BR1038	BR1038-F2	TCCTCTAGAGAACGTTCAGAGCGTCCAGAA
	BR1038-R	AAACTGCAGTCAACTCGCCAAGCAGCTTTT
pFT/BAB1_1058-20	BAB1058-20-R	AAACTGCAGCTAGGTTTCTTCGGCCTTGGC
pFT/BR1038-20	BR1038-20-R	AAACTGCAGCTACCGGCTGTTCATCGGTAGG
pFT/BAB1_1058-49	BAB1058-49-R	AAACTGCAGCTACAACTCCTCCATATTTTCATCTTTAC
pFT/BAB2_0056	BRA0057-F	TCCTCTAGACGTGAAGCTCTGACAAGCGGA
	BRA0057-R	AAACTGCAGCTATTTTCTGGGGGCTTTTCC
pFT/BAB2_0095	BRA0097-F	TCCTCTAGACTGGTTTCGCTCTGGCCCAGG
	BRA0097-R	AAACTGCAGTCAGCCAGTCTTTCCTTTCGC
pFT/BAB1_0604	BAB0604-F	TCCTCTAGAGCGGTGAGTGTATGCCTACC
	BAB0604-R	AAACTGCAGCTATTTGACCGACCTGATGGG
BAB1-0119	BAB1-0119Xba1-F	TCCTCTAGAGCGACCAATGTTTTTCATGC
	BAB1-0119PstI-R	AAACTGCAGCTACAATAAAGGAAACCGGCC
BAB1-0651	BAB1-0651Xba1-F	TCCTCTAGAGAAAATTTTGGATTTGGACGC

	BAB1-0651PstI-R	AAACTGCAGTCATTCGTTCCTCCCAAAATG
BAB1-0740	BAB1-0740Xba1-F	TCCTCTAGATCGGTGATCGGTGATGTGTT
	BAB1-0740PstI-R	AAACTGCAGTCAGAATTTGTCTAGCAGGTCCTG
BAB1-1674	BAB1-1674Xba1-F	TCCTCTAGACTCGCCGCGATTTCGCT
	BAB1-1674PstI-R	AAACTGCAGTTAGCGGTCTTTTTTCTTGTCCG
BAB1-1705	BAB1-1705Xba1-F	TCCTCTAGAGCATCTGTTTCTTCGCAGGAA
	BAB1-1705PstI-R	AAACTGCAGTCAGCACTTGGCGCGACT
BAB2-0403	BAB2-0403Xba1-F	TCCTCTAGAAAGATGCGCCGCTTACCTG
	BAB2-0403PstI-R	AAACTGCAGTCAGTCGAACAGCGGTCCG
ADH17	VjbR-up-F	TATCCGCCTCCTGCCTGCCTG
	VjbR-up-R	CCCGGGAAGTATCGCTTTGAAAGGAAG
	VjbR-dn-F	CCCGGGTCAACATGGTCGCGCGGAAAC
	VjbR-dn-R	CTGCAGGCAGGAATTGCGCATGACCCG
ADH58, ADH60	virB-1F	CTGCAGGTGGGGCTATGAGGTGAT
	virB874R	TCTAGAAGGATCGTCTCCTTCTCA
MDJ30, MDJ31	vceC-Pst1-F	AAACTGCAGCTTTCGGTGATGGATGCCG
	vceCXba1-R	TCCTCTAGAGGCTGCCTGGATATTTGATGTG
EMSA	PvirB100-F	CCCTCACAAGCATATTTGTCC
	PvirB463-R	TAGGATCGTCTCCTTCTCAGAG
EMSA	PvirB1ig2-F	GACGGCGTAGTTGTTTTCTAA
	virB1369R	GCTCAATAAAAGGGAAATGCTCC
EMSA	RTgyrA-F	TGATGCCCTCGTGCGTATG
	RTgyrA-R	TTCCGTGACCTTTTCCAGACG

[a] plasmid constructs correspond to those listed in Table 1

[b] underlined nucleotides indicate restriction sites used for cloning the resulting amplicons

3

Chapter 3 The *Brucella* effector VceC is targeted to the Endoplasmic Reticulum of host cells and interacts with Snapin

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Abstract

Brucella spp. are Gram-negative intracellular bacteria that can chronically infect many mammalian species including humans. An important virulence factor of *Brucella* is the VirB Type IV secretion system. The conserved *Brucella* protein VceC is translocated by this secretion system into macrophages during infection with *Brucella abortus*. In the present study, we have characterized the role of VceC inside host cells. Importantly, a *B. abortus vceC* mutant was attenuated in a mouse infection model, but not in cultured macrophages or HeLa cells. Snapin was identified by yeast two-hybrid and immuno precipitation assays as an interaction partner of VceC. Ectopically expressed VceC was targeted to the ER of HeLa cells, which required the N-terminal transmembrane domain of VceC. Furthermore, the expression of VceC resulted in a disruption of ER structure. In HeLa cells expressing VceC, colocalization of Snapin and VceC was observed. These findings suggest that *Brucella* employs VceC to: (i) inhibit host cell protein secretion by inhibiting exocytosis, (ii) inhibit fusion of the *Brucella*-containing vacuole (BCV) with lysosomes, (iii) enhance BCV trafficking to the ER, or (iv) induce ER stress to provide more membrane for *Brucella* replication.

Introduction

Brucellosis is a chronic disease, characterized by long periods of general malaise, intermittent fever, anorexia and joint pain that can be caused by several different *Brucella* species. These species, which are intracellular Gram-negative bacteria, are classified by the animal host they naturally infect. All known *Brucella* species encode a Type IV secretion system (T4SS). This system, which is a complex of 12 proteins spanning the inner and outer membranes of the bacterium, is essential for virulence of *B. melitensis*, *B. abortus* and *B. suis* in cultured macrophages, mice and goats (O'Callaghan, Cazevieille et al. 1999; Hong, Tsolis et al. 2000; Sieira, Comerci et al. 2000; den Hartigh, Sun et al. 2004; Zygmunt, Hagius et al. 2006; den Hartigh, Rolan et al. 2008). The 12 proteins of the *Brucella* T4SS are encoded by the *virB* operon, which contains the genes *virB1* to *virB12. Brucella* mutant strains lacking genes in the *virB* operon are highly attenuated in macrophages and mice (den Hartigh, Sun et al. 2004; den Hartigh, Rolan et al. 2008). In host cells, expression of the *virB* operon is induced after acidification of the *Brucella*-containing vacuole (Boschiroli, Ouahrani-Bettache et al. 2002). The *Brucella* T4SS is required for intracellular survival of *Brucella*, by maturing the *Brucella* phagosome into an endoplasmic reticulum (ER)-derived compartment (Celli, de Chastellier et al. 2003; Celli, Salcedo et al. 2005; Starr, Ng et al. 2008). Intracellular *Brucella abortus*, containing a T4SS, are able to avoid degradation in phagolysomes and instead multiply within vacuoles containing ER markers such as calreticulin (Starr, Ng et al. 2008). As phagosomes containing *virB* mutant *Brucella* do not acquire ER markers it is possible that this process is mediated by effectors translocated by the *Brucella* T4SS into the host cytosol.

To date, several *Brucella* proteins that are translocated into the host cytosol have been identified, including VceA, VceC, RicA, BPE123, BPE005, BPE275 and BPE043 (de Jong, Sun et al. 2008; de Barsy, Jamet et al. 2011; Marchesini, Herrmann et al. 2011). Translocation of these effectors into host cells was demonstrated to be dependent on the *Brucella* T4SS.

Previously we demonstrated that VceC was translocated into J774A.1 mouse macrophages during infection with *B. abortus*. Also, heterologously expressed

VceC was translocated by *L. pneumophila* into CHO cells and similarly to what was observed with *B. abortus,* this depended on a functional T4SS (de Jong, Sun et al. 2008). In the present study, we characterized the function of *B. abortus* VceC inside host cells by determining the intracellular localization of VceC and also by identifying an interacting partner protein from the human host.

Experimental Procedures

Bacterial strains and plasmids

The *B. abortus* and *Escherichia coli* strains used in this study are listed in table 1. *B. abortus* 2308 was used as a wild-type strain. *Brucella* 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, Guigue-Talet 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 37ºC. 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.

Table 1. Plasmids and strains used in this study.

Strain or Plasmid	Genotype and antibiotic resistance phenotype	Reference or source
E. coli strains		
Top10	F- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) *Φ80lacZ ΔM15 ΔlacX74 recA1 ara Δ139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*	Invitrogen
B. abortus strains		
2308	Wild-type	Deyoe
ADH3	Δ*virB2* (non polar) in 2308	Den Hartigh et al. 2004
MDJ32	*vceC*::*kan* in 2308	This study

Strain or Plasmid	Genotype and antibiotic resistance phenotype	Reference or source
Plasmids		
pUKDVceC	*vceC*-upstream and downstream regions flanking a kanamycin resistance cassette (CarbR/KanR; for knockout in *B. abortus*)	This study
pEGFP-C1	Empty vector for EGFP-protein fusions	Clontech
pEGFP-N1	Empty vector for EGFP-protein fusions	Clontech
pEGFP-C1-VceC	VceC fused to C-terminus of GFP (KanR)	This study
pEGFP-N1-VceC	VceC fused to N-terminus of GFP (KanR)	This study
pEGFP-C1-VceC167	First 167 aa of VceC fused to C-terminus of GFP (KanR)	This study
pEGFP-C1-VceCdTM	VceC37-418 fused to C-terminus of EGFP (KanR)	This study
pCMV-Myc	Empty vector for Myc-protein fusions	Clontech
pCMV-HA	Empty vector for HA-protein fusions	Clontech
pCMV-Myc-VceC	VceC fused to C-terminus of Myc (CarbR)	This study
pCMV-HA-VceC	VceC fused to C-terminus of HA (CarbR)	This study
pCMV-Myc-VceCdTM	VceC37-418 fused to C-terminus of Myc (CarbR)	This study
pCMV-HA-VceCdTM	VceC37-418 fused to C-terminus of HA (CarbR)	This study
pCMV-HA-VceC100	First 100 aa of VceC fused to C-terminus of HA (CarbR)	This study

Construction of plasmids

All plasmids that were constructed are listed in Table 1. Genes and inserts were PCR-amplified from *B. abortus* 2308 genomic DNA, using forward and reverse primers listed in Table 2. Using restriction sites in the primers listed in Table 2 PCR products were then digested with the appropriate enzymes and cloned in destination vectors.

Table 2. Primers used in this study.

Purpose	Name	Sequence	Insert size	Restriction site
pUKDvceC	vceC-up-F	CCGCACGCGTCTCCTATAT	596	-
	vceC-up-R	TCC**CCCGGG**AGCGGTTTCTGTTTTAACAGAATC		SmaI
	vceC-dn-F	TCC**CCCGGG**GCGGATACCCTCTTACACTATAAA	625	SmaI
	vceC-dn-R	AAA**CTGCAG**GATCGCGAAACAGGAAATAGAT		PstI
pEGFP-C1-VceC	gfpC-vceC-F	T**TCCGGA**CTCAGCGGCATCATCGGTGAAAGCGCG	1239	BspE1
	gfpC-N-vceC-R	A**CTGCAG**GCGGGTTTCTCCCTTGGTCAACTCACC		Pst1
pEGFP-N1-VceC	gfpN-vceC-F2	T**AAGCTT**ATGCTCAGCGGCATCATCGGTGAAAGCGCG	1239	HindIII
	gfpC-N-vceC-R	A**CTGCAG**GCGGGTTTCTCCCTTGGTCAACTCACC		Pst1
pEGFP-C1-VceCdTM	gfpC-dTMvceC-F	T**TCCGGA**CGCCGTTTCAGCGGCGGCACATTTGTA	1140	BspE1
	gfpC-vceC167-R	A**CTGCAG**CGCCGCGATGGCCGAGCTTAGCTG		Pst1
pEGFP-C1-VceC167	vceC167EcoR1-F	T**GAATTC**CTCAGCGGCATCATCGGTGAAAGCGCG	489	EcoRI
	gfpC-N-vceC-R	A**CTGCAG**GCGGGTTTCTCCCTTGGTCAACTCACC		Pst1
pCMV-Myc or HA-VceC	vceCEcoR1-F	T**GAATTC**CTCAGCGGCATCATCGGTGAAAGCGCG	1239	EcoR1
	vceCXho1-R2	A**CTCGAG**CTAGCGGGTTTCTCCCTTGGTCAACTCACC		Xho1
pCMV-Myc or HA-VceCdTM	dTMvceCEcoR1-F	T**GAATTC**CGCCGTTTCAGCGGCGGCACATTTGTA	1140	EcoR1
	vceCXho1-R2	A**CTCGAG**CTAGCGGGTTTCTCCCTTGGTCAACTCACC		Xho1
pCMV-HA-VceC100	vceCEcoR1-F	T**GAATTC**CTCAGCGGCATCATCGGTGAAAGCGCG	291	EcoR1
	vceC100Xho1-R2	A**CTCGAG**CTACGATTCCAGAACGATATTCTGTTCGACA ACCAC		Xho1

Confocal microscopy

HeLa cells were seeded on 12 mm coverslips in 24 well plates at 5 x 10^4 cells per well. After 24 hours cells were transfected with the different constructs containing *vceC* fusions (table 1) using 0.5 μg of plasmid DNA at a ratio of 2μg of DNA to 4 μl of Fugene HD (Roche). After another 24 hours cells were washed three times with PBS and fixed with 3% paraformaldehyde for 10 minutes at 37°C. Fixed cells were washed again twice and NH_4Cl was added for 10 minutes. Then coverslips were incubated for 30 minutes in blocking buffer (PBS with 10 % horse serum, 0.1% Saponin (Sigma)), 40 minutes in blocking buffer containing primary antibody (at 1:1000 dilution), washed 3 times in PBS and then incubated again for 40 minutes in blocking buffer containing secondary antibody. Then the coverslips were washed 3 times in PBS and once in water and mounted on glass slides using Mowiol (Calbiochem). Primary antibodies used were mouse anti-HA or anti-Myc (Covance), rabbit anti-calreticulin (Thermo Scientific), rabbit anti-giantin (Covance), mouse anti-Snapin (Antibodies inc, Davis, CA), rat anti-VceC (Genovac). Secondary antibodies used were donkey anti-mouse (Alexa 488 or Alexa 647 conjugated) or donkey anti-rabbit (Alexa 488 or Alexa 568 conjugated) (From Invitrogen) or Cy5 conjugated anti-rat (Jackson ImmunoResearch).

Yeast two-hybrid assay

For the yeast-two hybrid screen *vceC* was cloned into plasmid pGBKT7 (Gal4-BD) as bait and a HeLa cell cDNA library (Clontech) in pGADT7 (Gal4-AD) was used as prey. The Yeast two-hybrid assay was performed according to manufacturers instructions (Clontech). pGADT7 prey plasmids were isolated from positive colonies, inserts were sequenced, and reintroduced into yeast together with pGBKT7 (empty) or pGBKT7-vceC to eliminate false positives.

Immunoprecipitation

For IP experiments 15 dishes containing 8 x 10^5 HeLa cells each were transfected with *vceA*- or *vceC*-containing vectors. For each dish 6 μg of plasmid DNA was added to 600 μl DMEM and 18 μl of Fugene HD transfection reagent was added. After 15 minutes this mixture was added to each dish and 40 hours later cells were scraped from the dishes and washed twice with cold PBS. The cells were lysed by adding lysis buffer (PBS, 50 mM Tris-HCl pH 7.6, 150 mM NaCl 0.1% (v/v) NP-40, 1 mM EDTA), containing phosphatase inhibitor (1:100) and protease inhibitor III (1:500) (Calbiochem). After 15 minutes the lysate was centrifuged at 3000 xg, for 15 minutes. The supernatant was then precleared by incubation with 70 μl 50% Protein A agarose (Amersham) slurry for 1 h at 4°C on a rotator. To pellet the agarose the lysate was centrifuged at 1000 xg for 2 minutes, and the supernatant was collected. To the supernatant 7.5 μl of anti-Snapin antibody (Antibodies inc., Davis, CA) was added (0.5 μg antibody per plate) and incubated while rotating for 1 h at 4°C. Then 70 μl protein A agarose slurry was added for 1-2 h. The agarose was pelleted by low speed centrifugation. Another 5 μl of anti-Snapin antibody was added to the supernatant for a second round of IP similar as described above. The agarose was washed 4 times with lysis buffer and then once with lysis buffer without NP-40. 30 μl hot 2x SDS sample buffer was added and eluates of both rounds of IP were pooled together and boiled for 5 minutes.

Infection of mice

Female BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and used at an age of 4-6 weeks. For infection experiments, groups of four or five mice were inoculated intraperitoneally (i.p.) with 0.2 ml of PBS containing a 1:1 mixture of 2 x 10^5 CFU of wild-type *B. abortus* and 2 x 10^5 CFU of MDJ32 (*vceC*). Infected mice were held in microisolator cages in a Biosafety Level 3 facility. At the appropriate time points, mice were euthanized by CO_2 asphyxiation and the spleens and livers collected aseptically at necropsy. Spleens and livers were homogenized in 3 ml of PBS and serial dilutions of the homogenate

plated on TSA containing antibiotics, as appropriate, for enumeration of CFU. All animal experiments were approved by the University of California, Davis, Institutional Laboratory Animal Care and Use Committee and conducted in accordance with institutional guidelines.

Macrophage killing assay

J774A.1 mouse macrophages were seeded in 24-well plates and infected 24 hours later with *B. abortus* 2308 or *vceC* mutant (MDJ32) at a multiplicity of infection of 100:1. Plates were centrifuged for 5 min at 250 x 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 0.5 ml fresh DMEM containing 50 µg/ml gentamicin was added to each well and plates were incubated at 37°C in 5% CO_2. At appropriate time points cells were washed 2 times with PBS and lysed in 0.5% Tween 20. Viable bacteria were quantified by serial dilution in sterile PBS and plating on TSA.

Western Blotting

Western blotting was performed to detect VceC protein expression in *Brucella* strains after switching to modified minimal E-medium (pH 5.0) similarly. Bacteria were pelleted, resuspended in 2x SDS sample buffer and heated at 100°C for 15 min. The total protein equivalent of 1 x 10^8 CFU was loaded and run on a 12% SDS-PAGE 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 rat anti-VceC antibody (Produced by Genovac) (1:5000). Anti-rat IgG antibody (Cell Signalling) conjugated with horseradish peroxidase (HRP) was used (1:5000) as a secondary antibody and HRP activity was detected with a chemiluminescent substrate (Perkin-Elmer). For Western blot detection of Snapin, GAPDH and VceA, primary antibodies used were mouse anti-Snapin (Antibodies inc., Davis, CA), rabbit anti-GAPDH (Cell Signaling) and anti-VceA (produced in rabbits), and secondary antibodies used were HRP conjugated

goat anti-rabbit (Biorad) or HRP conjugated goat anti-mouse (Jackson ImmunoResearch).

Results

VceC is expressed in B. abortus and B. melitensis

In our previous study we showed that the *vceC* gene is co-regulated with the *virB* operon (de Jong, Sun et al. 2008). Both *virB* and *vceC* expression increase after switching the *B. abortus* culture from rich TSB medium to minimal medium (MM) at an acidic pH of 5, which is a condition that likely mimics the intracellular environment. The gene encoding *vceC* is present but not identical in most sequenced *Brucella* species. In several *Brucella* species including *B. neotomae, B. pinnipedialis, B. inopinata* and a *Brucella* isolate from a baboon, *vceC* is probably a pseudogene as the gene in these species is predicted to contain an early stop codon. In *B. suis* 1330 and *B. canis vceC* genes contain an extra base pair compared to *vceC* of other *Brucella* species leading to a frameshift and a different C-terminus of the encoded VceC protein. To determine whether any of the genetic differences lead to different expression levels of VceC in *Brucella* species, we examined VceC expression by Western blot. Wild-type strains of *B. abortus* 2308, *B. melitensis* 16M, *B. suis* 1330, *B. canis*, *B. ovis*, *B. neotomae* and the baboon *Brucella* isolate (Schlabritz-Loutsevitch, Whatmore et al. 2009) were grown in rich medium for 24 hours and were then switched to MM at pH 5. Samples were taken for Western blotting at 0 and 7 hours after the switch. VceC proteins were detected on Western blot using anti-VceC serum.

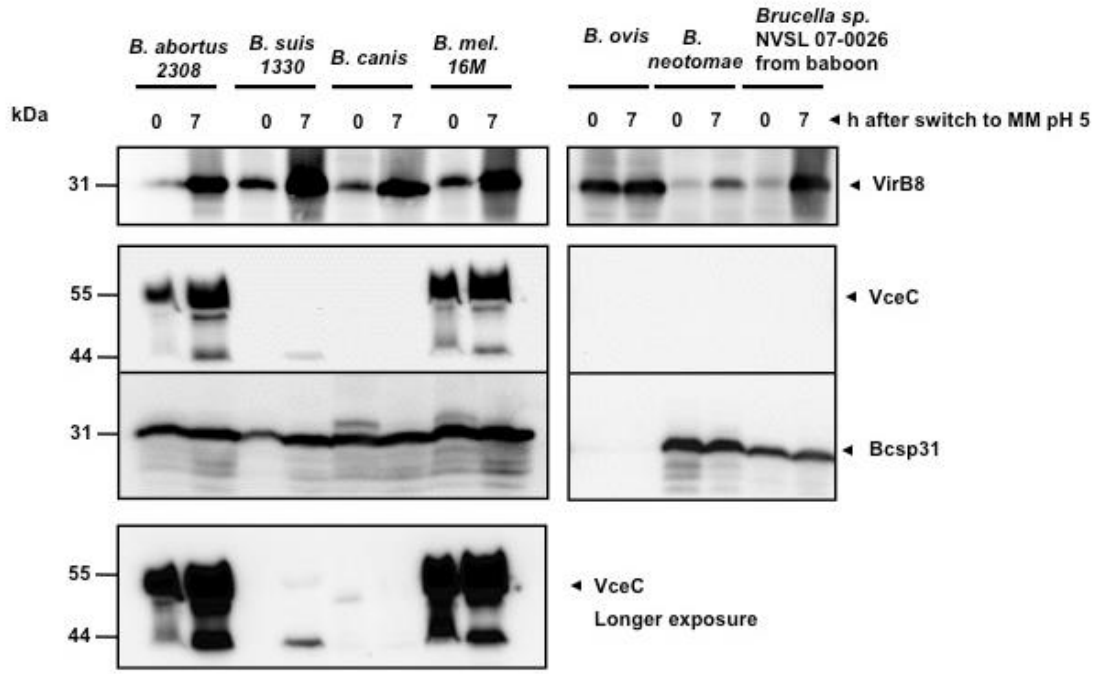


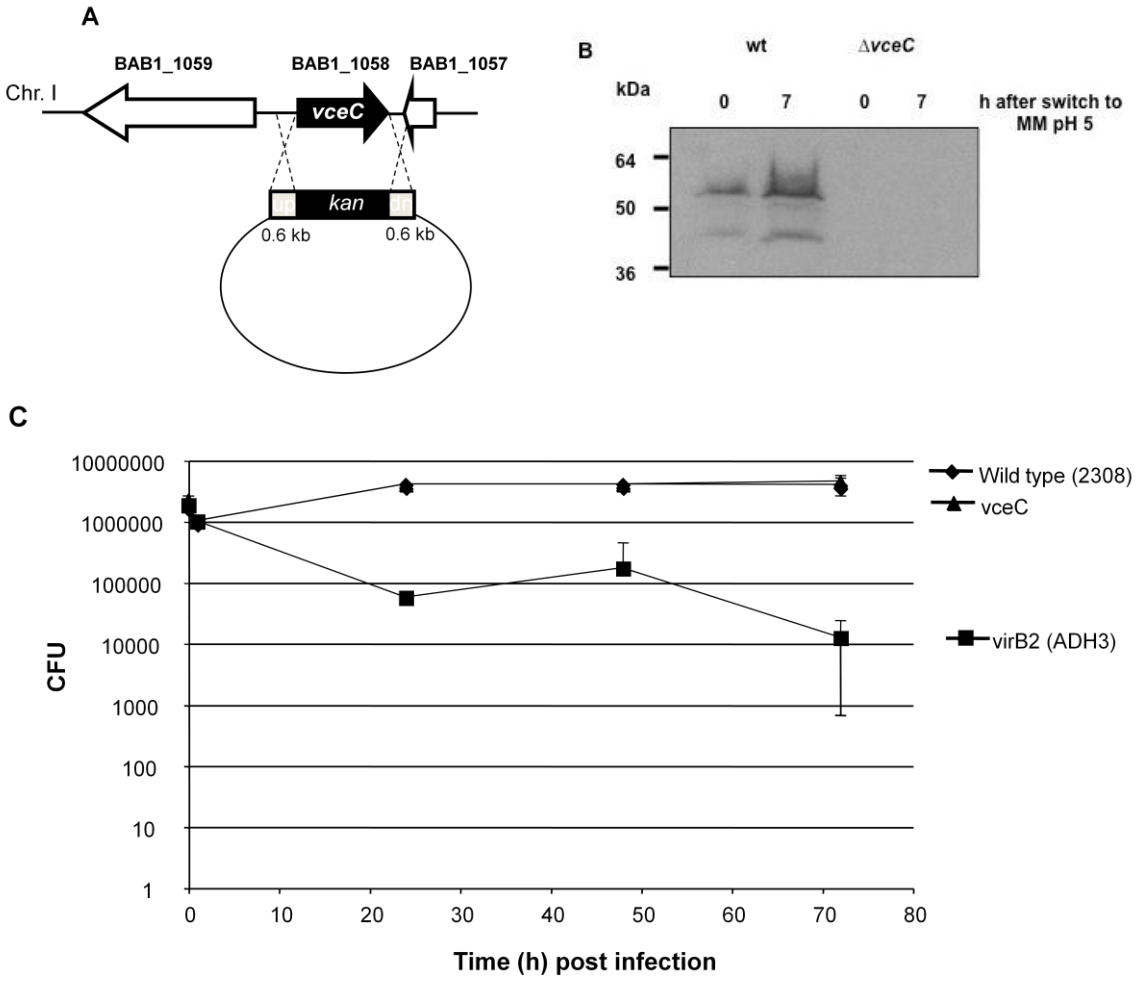
Figure 1. Differential expression of VirB8 and VceC in different *Brucella* species. All strains were grown for 24 h in TSB and then switched to MM at pH 5 and grown for an additional 0 or 7 h. Western blotting shows that under the tested conditions VirB8 is expressed in all *Brucella* species examined, whereas VceC is only expressed in *B. abortus*, *B. suis*, and *B. melitensis.* Bscp31 was used as a loading control. VceC expression in *B. suis is* visible in a longer exposure of the blot that was stained with anti-VceC antibodies.

As shown in Figure 1, we detected significant levels of VceC expression in *B. abortus* 2308 and *B. melitensis* 16M, and relatively low levels of VceC expression *B. suis* 1330. In contrast, no expression of VceC was detectable in other *Brucella* species under these growth conditions. VceC in *B. abortus, B. melitensis* and *B. suis* run at approximately 55 kDa (highest band) in the SDS-PAGE gel, which is higher than the predicted molecular weight of *B. abortus* VceC of 44 kDa. A band running at 44 kDa was also detectable, and this band was specific for VceC, since it was absent from samples of a *B. abortus vceC* mutant (Figure 1). Most likely, the 44 kDa band represents a breakdown or cleavage product of VceC, because a *B. abortus* VceC-6xHis recombinant protein that was expressed in *E. coli* was also found to run at 55 kDa upon SDS-PAGE (data not shown). VceC was expressed at a much lower level in *B. suis* 1330 and did not appear to be expressed by cells grown in rich medium (0 hours after

switch to MM). Only after 7 hours of growth of *B. suis* in MM at pH 5 some VceC was detectable (Figure 1). As expected, *B. neotomae* and the *Brucella* baboon isolate did not express VceC as the respective genes in these species are not complete. No expression of VceC was observed in *B. canis* and *B. ovis*, two species that do contain the complete *vceC* gene and promoter region. Together with the results showing low expression of VceC in *B. suis,* these observations indicate that the *vceC* gene regulatory networks may be different in different *Brucella* species.

Characterization of a B. abortus vceC mutant

Brucella strains mutated in their *virB* genes and lacking a functional T4SS are highly attenuated in both J774A.1 macrophages and in mice (den Hartigh, Sun et al. 2004; den Hartigh, Rolan et al. 2008). To study the contribution of VceC to *Brucella* survival in both these models, a *vceC* mutant was constructed in *B. abortus.* In the *B. abortus* Δ*vceC* strain the *vceC* gene was replaced with a kanamycin resistance cassette (Figure 2A). Deletion of *vceC* from the genome was confirmed by PCR and Southern blotting (not shown). Also a Western blot probed with anti-VceC antiserum showed the lack of expression of VceC in the *B. abortus vceC* mutant (Figure 2B). J774A.1 macrophages were infected with *B. abortus* wild-type (2308), Δ*vceC* or Δ*virB2* (ADH3) at an MOI of 1:100 and CFU counts were determined after several time points post infection. Unlike the Δ*virB2* strain, which was highly attenuated in the macrophages the Δ*vceC* strain was able to survive and replicate inside the macrophages, to a comparable level as the wild-type *B. abortus* strain (Figure 2C). To study the *in vivo* role of VceC during infection, BALB/c mice were infected with a 1:1 mixture of *B. abortus* wild-type and Δ*vceC* strains and the CFUs of both strains recovered from spleen and liver of the mice at 1, 4 and 8 weeks post infection were determined.


A
BAB1_1059
BAB1_1058
BAB1_1057
Chr. I
vceC
kan
0.6 kb
0.6 kb
B
wt
ΔvceC
kDa
0
7
0
7
h after switch to MM pH 5
64
50
36
C
10000000
1000000
100000
10000
1000
100
10
1
CFU
0
10
20
30
40
50
60
70
80
Time (h) post infection
Wild type (2308)
vceC
virB2 (ADH3)

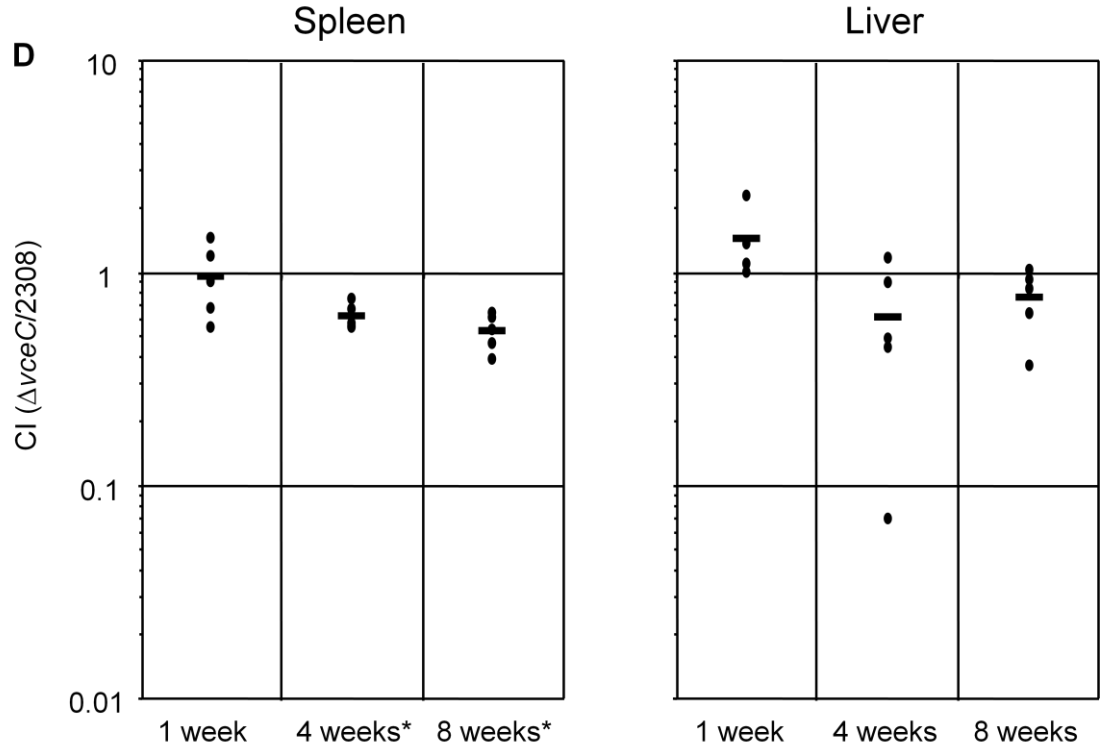


Figure 2. Construction and characterization of a *Brucella* Δ*vceC* mutant. (A) Schematic representation of the strategy to create a *B. abortus vceC* mutant. (B**)** Western blot showing VceC expression in *B. abortus 2308* or Δ*vceC* that were grown for 24 h in TSB and then switched to MM at pH 5 and grown for an additional 0 or 7 h. (C) Survival of *B. abortus* Δ*vceC* in J774A.1 cells compared to wild-type (2308) and a *virB2* mutant (ADH3). (D) Competitive infection of balb/c mice with a 1:1 mixture of *B. abortus* Δ*vceC* and wild-type (2308). After 1, 4 and 8 weeks p.i. CFU in spleen and liver were counted and the competitive index (CI) was calculated as Δ*vceC*/2308.

The ratio of recovered Δ*vceC*/wild-type showed that at one week post infection, both *B. abortus* Δ*vceC* and wild-type were surviving at comparable levels in the spleen and liver of the infected mice. However, at 4 and 8 weeks post infection approximately 50% less of the *B. abortus* Δ*vceC* mutant was recovered from spleens of infected mice compared to wild-type *B. abortus* (Figure 2D). Thus, VceC is required for optimal survival of *B. abortus in vivo* in the mouse model, but not *in vitro* in J774A.1 mouse macrophages.

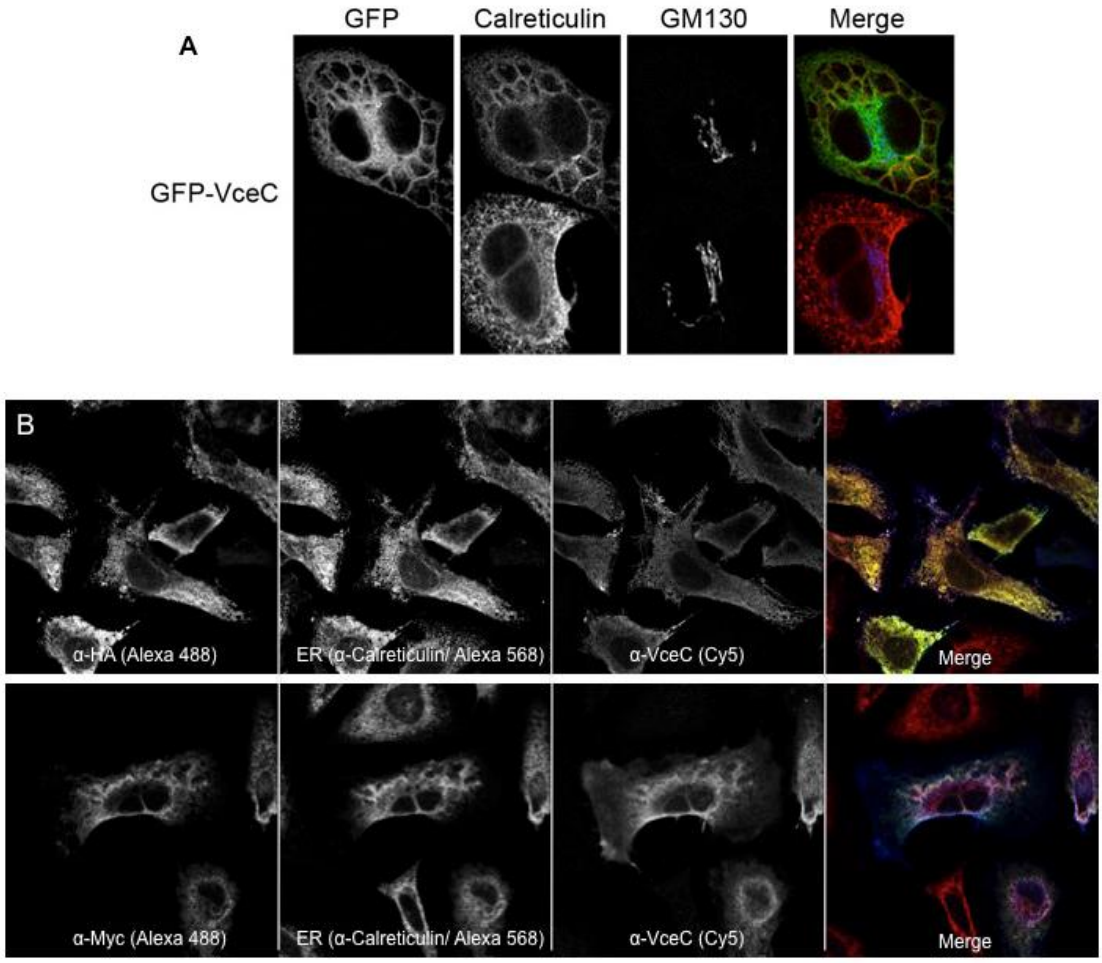


Figure 3. Localization of GFP-VceC (A), HA-VceC or Myc-VceC (B) fusions (green) in Hela cells. Transfected Hela cells grown on coverslips were stained with anti-calreticulin to visualize ER (red), anti-GM130 to visualize Golgi (blue) and anti-HA, Myc or VceC antibodies for VceC (blue in panel B). Secondary antibodies conjugated to Alexa 488, 568 or cy5 were used.

Ectopically expressed VceC localizes to the Endoplasmic Reticulum in HeLa cells and alters its structure

The finding that *vceC* is not in required for survival of *B. abortus* in cultured host cells suggests that the intracellular function of VceC is either redundant, or that VceC is not involved in trafficking of the *Brucella* phagosome and intracellular survival of *Brucella* but has another function instead.

To gain further insights into the intracellular function of VceC, we determined the localization of VceC inside host cells. To this end, fusion proteins were constructed in which *B. abortus* VceC was fused either to the N- or C-termini of

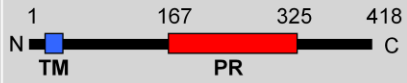
EGFP. Next, the VceC-GFP and GFP-VceC were ectopically expressed in HeLa cells grown on coverslips and fluorescence was examined using confocal microscopy. The VceC-GFP fusion protein was evenly distributed in the cytoplasm of the cells (not shown). In contrast, the GFP-VceC fusion protein was found to co-localize with the endoplasmic reticulum (ER) marker calreticulin (Figure 3A). Interestingly, the ER in cells expressing the GFP-VceC construct appeared to be highly vacuolated compared to normal ER in HeLa cells. To ensure the effect of GFP-VceC on the ER was not caused by the GFP-tag, experiments were repeated with HA-VceC or Myc-VceC fusions that were also ectopically expressed in HeLa cells. Similar to GFP-VceC, HA-VceC and Myc-VceC both localized to the ER of the HeLa cells (Figure 3B). Although not to the same extent as in GFP-VceC expressing cells, the ER structure was also disrupted in HA-VceC and Myc-VceC expressing cells.

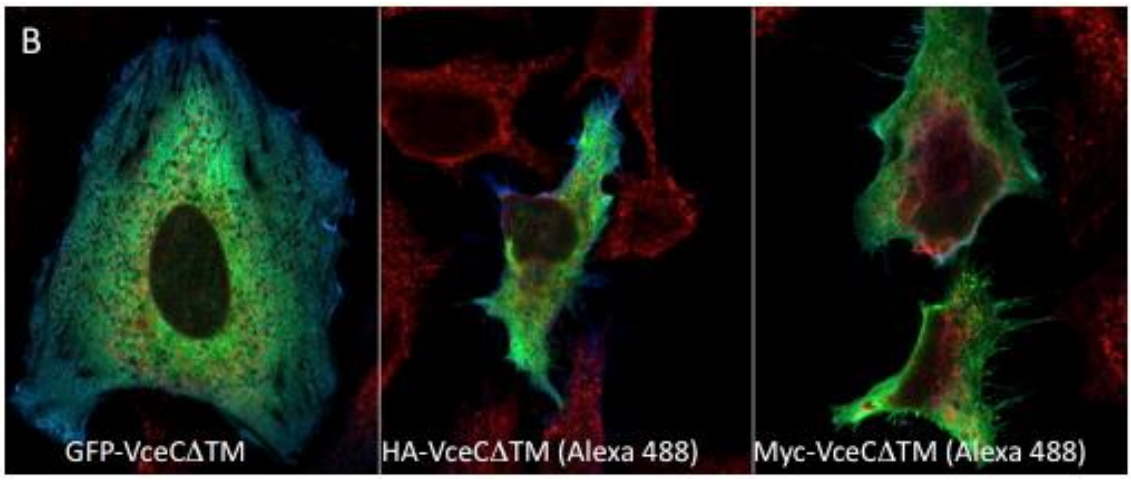
VceC requires a hydrophobic domain for its localization to the ER in HeLa cells

The *B. abortus* VceC protein is predicted to contain a hydrophobic transmembrane (TM) domain at its N-terminus (Figure 4A). Also, VceC contains a central proline-rich domain in which proline accounts for 25% of amino acids. To determine which domain is required for VceC localization in HeLa cells, we constructed GFP, HA or Myc fusions to truncated VceC proteins lacking either the N-terminal TM domain (VceCΔTM) or the central proline-rich domain (VceC167 or VceC100; Figure 4A). In contrast to full length VceC, the VceCΔTM constuct, N-terminally fused to GFP, HA or Myc, was not targeted to the ER, but was instead located in the cytoplasm of the HeLa cells (Figure 4B). This indicated that the N-terminal 37 amino acids containing the predicted TM domain are required for targeting of VceC to the ER. GFP-VceC167, which is missing the proline-rich domain, but contains the TM domain, was targeted to the ER. This confirmed that the N-terminal region is important for VceC localization in host cells. Also, the ER structure of HeLa cells expressing GFP-VceC167 appeared to be disrupted similar to the ER of HeLa cells expressing full-length VceC. These re-

sults suggested that the proline-rich domain is not required for the observed changes in the ER structure triggered by VceC (Figure 4C).

To determine whether the TM region of VceC is sufficient for targeting to the ER HeLa cells were transfected with constructs containing the first 100 amino acids of VceC fused to HA. A clumping of HA-VceC100 in an area around the nucleus was visible, and these clumps partially co-localized with the ER marker calreticulin, but not with the Golgi marker giantin (Figure 4D). However targeting of HA-VceC100 to the entire ER appeared to be disrupted, despite the presence of the TM domain. This suggests that the region between amino acid 100 and 167 of *B. abortus* VceC is also important for proper localization of this protein in the host cell.

A		LOCALIZATION	ER DISRUPTION
VceC full		ER	++
ΔTM		-	-
167		ER	+
100		-	-



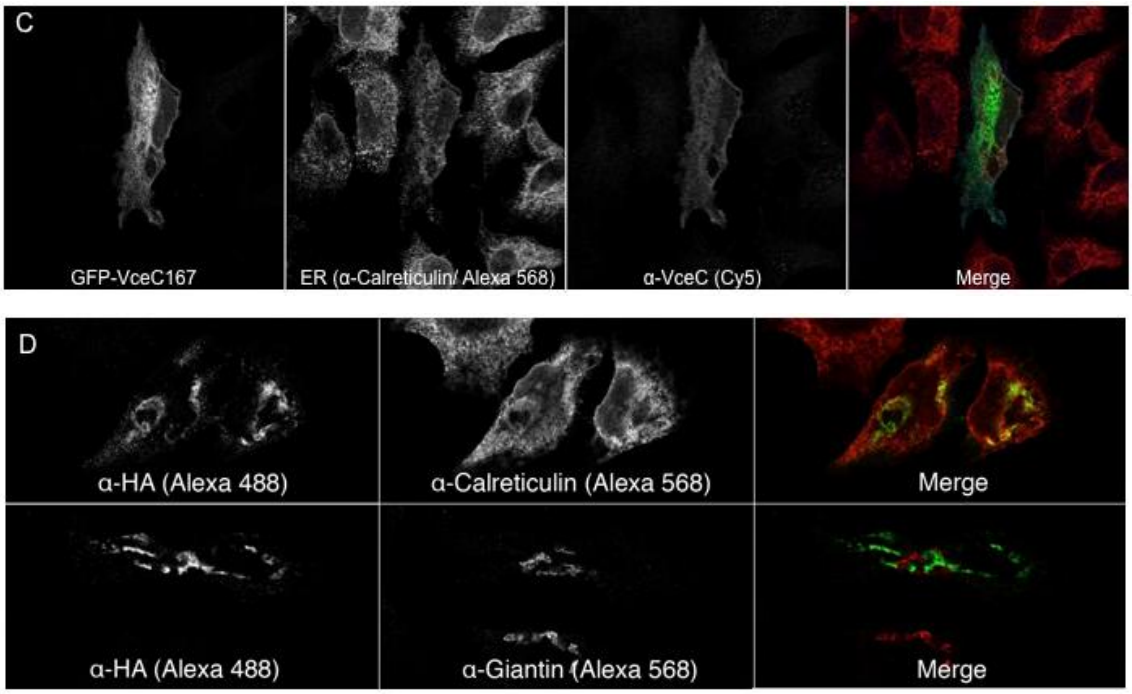


Figure 4. Localization of VceC truncations in HeLa cells. (A) Schematic representation of *B. abortus* VceC showing putative transmembrane (TM) and proline rich (PR) domains and the observed localization and effect on ER structure of the fusion proteins. (B) Localization of GFP-VceCΔTM, HA-VceCΔTM and Myc-VceCΔTM fusion proteins (green) in the cytoplasm of Hela cells. Cells were also stained with the ER marker α-calreticulin (Alexa 568, red) and α-VceC (Cy5, blue). (C and D) Localization of the GFP-VceC167 or HA-VceC100 fusion proteins (green) in HeLa cells stained with the ER marker α-calreticulin (red) and the Golgi marker α-giantin (red).

VceC interacts with the ER chaperone Bip

To identify potential interaction partners of VceC, we performed immunoprecipitation (IP) on HeLa cells expressing GFP-VceC or Myc-VceC fusions. In a first series of experiments, we used anti-GFP antibodies to pull down GFP-VceC from transfected HeLa cells. The IP fraction was loaded and run on a SDS-PAGE gel, which was then silver stained. Only one clearly identifiable band was visible at about 78 kDa, which was excised from the gel and subjected to LC/MS analysis. This resulted in the identification of the protein Bip/GRP78 (Data not shown). Bip is a chaperone found in the lumen of the ER where it is involved in the ER stress response (Dudek, Benedix et al. 2009). We confirmed the interaction of VceC with Bip by performing IP using anti-Myc antibodies on

HeLa cells transfected with the Myc-VceC construct (Figure 5). Western blotting showed the presence of Bip in the IP fraction of Myc-VceC transfected HeLa cells, but not in the IP fraction of untransfected cells. These results confirm the localization of ectopically expressed VceC to the ER as observed by microscopy. However, the interaction of VceC with Bip might result from GFP- or Myc-VceC overexpression and targeting to the ER in HeLa cells. Thus, Bip may not be a physiologically relevant interaction partner for VceC.

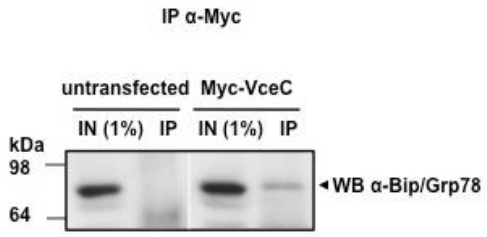


Figure 5. Interaction of VceC with the host ER chaperone Bip. A Western blot (WB) probed with anti-Bip, shows the presence of Bip in the immunoprecipitated (IP) fraction of Myc-VceC transfected cells, but not in the IP fraction of untransfected cells. The IP was performed using antibodies against the Myc tag. IN, 1% of input fraction (lysate).

Identification of Snapin as an interaction partner of VceC

In order to identify additional potential host interaction partners of VceC, we performed a yeast two-hybrid screen using VceC as bait and a HeLa cell cDNA library as prey. After sequencing the constructs from three positive colonies, one was found to encode the bait protein in the correct reading frame. This protein was identified as Snapin (Figure 6A), also known as SNAP-25 associated protein.

To confirm the direct interaction of VceC with Snapin and to determine whether the interaction also occurs in mammalian cells, we ectopically expressed either Myc-VceA (as negative control for overexpression of a Myc tagged protein) or Myc-VceC and performed IP using anti-Snapin antibodies. Snapin was present in both IP fractions, indicating the pulldown using the anti-Snapin antibodies was successful. Furthermore, VceC but not VceA was pulled down together with Snapin as Myc-VceC was present in the IP fraction of Myc-VceC cell lysates, whereas no Myc-VceA was present in the IP of Myc-VceA cell lysates (Figure 6B). As a second control, the abundant protein GAPDH was only present in lysates but not in IP fractions. Thus, Snapin specifically pulled down

VceC from the lysates.

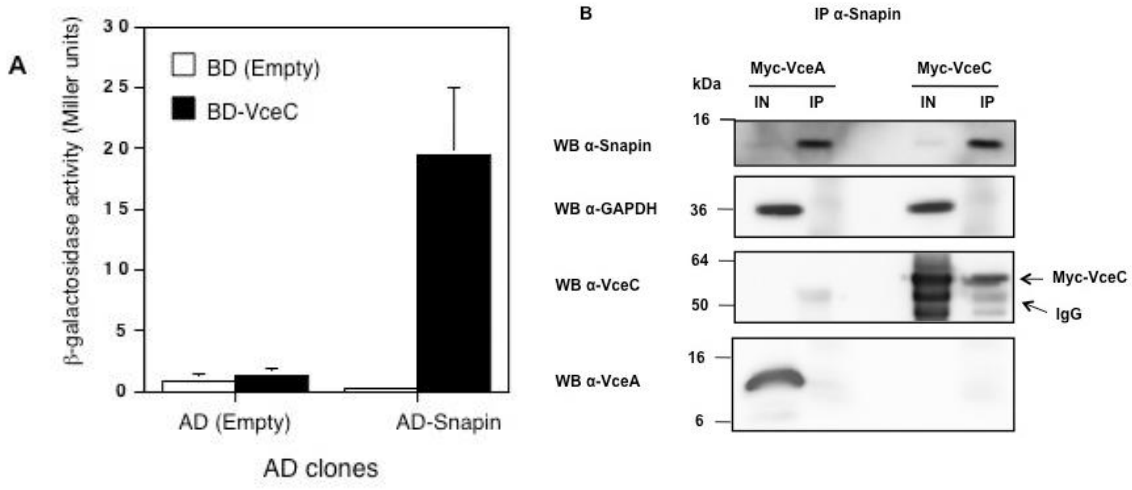


Figure 6. VceC interacts with Snapin. (A) Yeast 2-Hybrid screen with VceC as bait and a HeLa cell cDNA library as prey identifies Snapin as an interaction partner of VceC (Gal4-AD was used as prey domain and Gal4-BD as bait domain). (B) IP of Snapin from HeLa cells expressing Myc-VceC or Myc-VceA results in the co-IP of Myc-VceC but not Myc-VceA as shown with Western blotting. IN, 1% of input fraction (lysate). Also Western blots (WB) show GAPDH presence in only the input fractions.

VceC partly colocalizes with Snapin in HeLa cells

The results shown above demonstrate that Snapin directly interacts with VceC in HeLa cells, suggesting Snapin and VceC co-localize in these cells. Snapin has been described to localize to vesicles, the plasma membrane and cytosol of cells (Buxton, Zhang et al. 2003). To determine any co-localization of Snapin with VceC, HeLa cells expressing GFP-VceC were stained using anti-Snapin antibodies and examined by confocal microscopy. In HeLa cells, Snapin was observed to be distributed mostly as small blobs, which are probably small vesicles. In cells expressing the GFP-VceC fusion protein a partial co-localization of Snapin with VceC could be observed (Figure 7A). The co-localization of VceC with Snapin only occured in what appeared to be larger blobs or vesicles at the cell periphery. These results were similar in Myc-VceC expressing HeLa cells (Figure 7B).

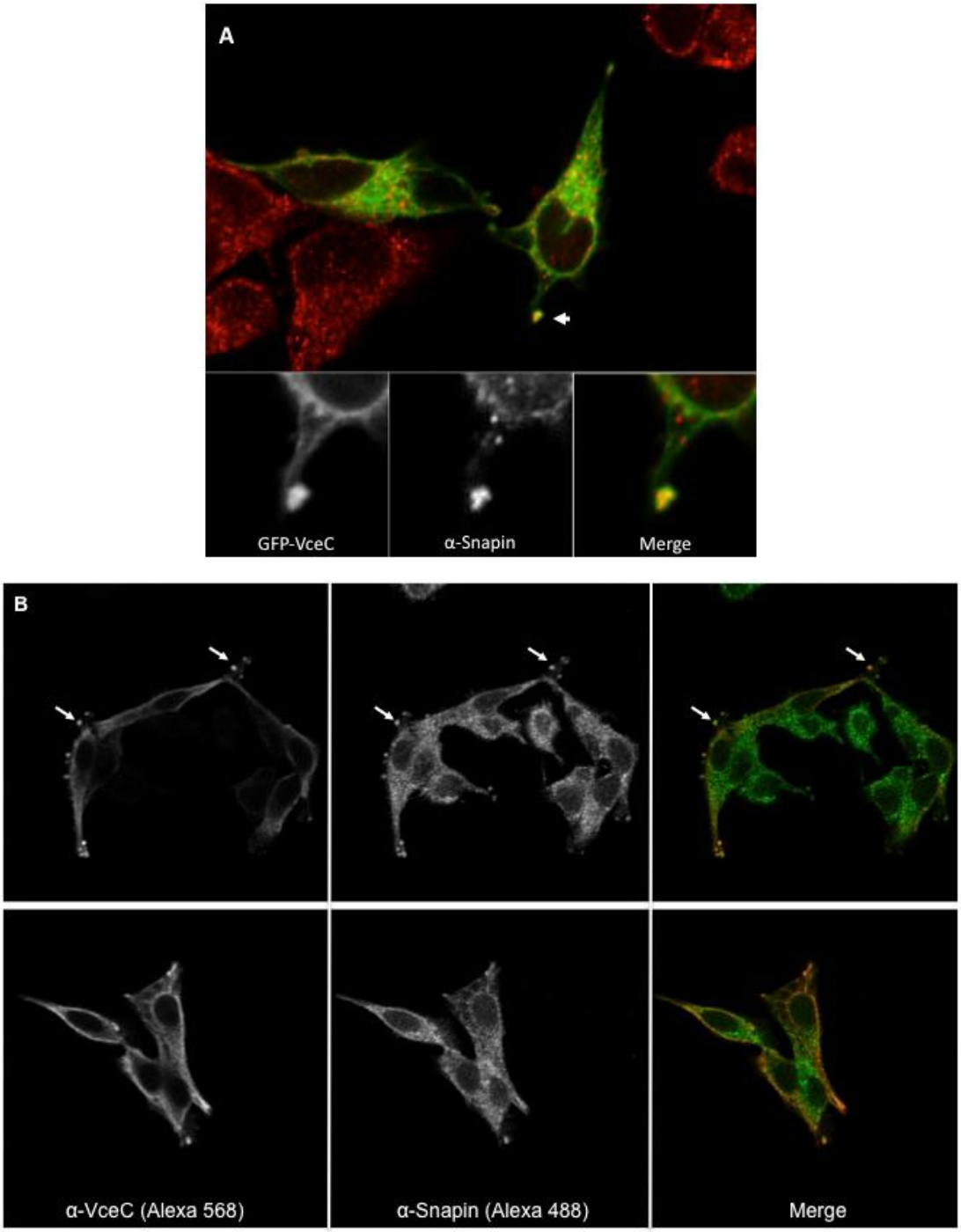


Figure 7. VceC partly co-localizes with Snapin in HeLa cells (A) Confocal micrograph showing colocalization of GFP-VceC (green) with Snapin (red) in vesicles at the cell periphery. Snapin was stained with anti-Snapin antibodies and secondary antibodies conjugated to Alexa 647. (B) Confocal micrographs showing colocalization of Myc-VceC (red) with Snapin (green). Snapin was stained with anti-Snapin antibodies and secondary antibodies conjugated to Alexa 488 and Myc-VceC was stained with anti-VceC antibodies and secondary antibodies conjugated to Alexa 568.

Discussion

The *Brucella* VirB T4SS is an important virulence factor for survival and proliferation of *Brucella* inside host cells. In this work we have characterized VceC, which is one of the substrates of the T4SS, and which was shown to be translocated into macrophages during *Brucella* infection (de Jong 2008). VceC was found to be expressed in *B. abortus*, *B. melitensis* and at low levels in *B. suis*, but not in other *Brucella* species, including *B. neotomae*, *B. canis*, *B. ovis* and a *Brucella* species isolated from baboons (strain NVSL 07-0026). The gene encoding VceC is a pseudogene in *B. neotomae* and the *Brucella* Baboon isolate, but is present in *B. suis*, *B. canis* and *B. ovis*, suggesting that *vceC* gene regulation is different in these *Brucella* species. Since VceC was expressed in *B. abortus*, a *B. abortus vceC* mutant was constructed and characterized. The *B. abortus vceC* mutant was not attenuated for survival in cultured cells, such as J774A.1 macrophages and HeLa cells, but persisted at lower levels in Balb/c mice comparted to wild-type *B. abortus*.

Furthermore, we found that VceC is targeted to the ER and causes disruption of ER structure. Also, VceC was shown to interact with Snapin in a Yeast 2-Hybrid screen. This interaction was confirmed by co-IP of VceC together with Snapin from HeLa cells and by fluorescence microscopy showing colocalization of VceC and Snapin in vesicles at the periphery of cells. In addition to the interaction with Snapin, VceC was found to interact with the ER chaperone Bip. Based on these results and available literature on the role of the interaction partners of VceC, several models for the function of VceC can be entertained.

Model 1. VceC inhibits host cell protein secretion by inhibiting exocytosis

In this study VceC was found to interact with Snapin. Snapin was initially found as a regulator of exocytosis in neuronal cells by binding to SNAP-25 (Ilardi, Mochida et al. 1999). Later it was found that Snapin also binds the SNAP-25 homologue SNAP-23 in non-neuronal cells, and also regulates exocytosis of proteins in these cells (Buxton, Zhang et al. 2003; Bao, Lopez et al. 2008). SNAP-25 and SNAP-23 are (target) Q-SNAREs located in the cell membrane, which interact with (vesicle) R-SNAREs (such as VAMP-2) during exocytosis of

vesicles (Sorensen, Nagy et al. 2003; Lang and Jahn 2008). Snapin acts as a positive regulator of plasma membrane to vesicle fusion and protein secretion (Bao, Lopez et al. 2008; Pan, Tian et al. 2009). Thus, it is possible that VceC inhibits host cell protein secretion by interfering with Snapin function or localization (Figure 8). For example, SNAP-23 is known to be involved in secretion of the proinflammatory TNFα from macrophages (Pagan, Wylie et al. 2003). VceC could function by inhibiting exocytosis of cytokines such as TNFα and lowering the inflammatory response of host macrophages to *Brucella* infection. Such a function of a *Brucella* effector would not be surprising, as *Brucella* is known to be a stealthy pathogen by both evading and actively inhibiting recognition of the bacteria by the host innate immune system (Barquero-Calvo, Chaves-Olarte et al. 2007; Parent, Goenka et al. 2007; Cirl, Wieser et al. 2008; Salcedo, Marchesini et al. 2008). Some of our data support the hypothesis that VceC could be involved in inhibiting host cell protein secretion. In HeLa cells expressing GFP-VceC or Myc-VceC, colocalization of VceC with Snapin was observed only in larger vesicles at the cell periphery. In untransfected cells, Snapin was associated mostly with small vesicles. The large Snapin and VceC positive vesicles at the cell periphery of VceC-expressing cells could be due to an accumulation of smaller vesicles that failed to fuse with the plasma membrane. Another line of evidence supporting a role of VceC in inhibition of host cell cytokine secretion is the finding that, compared to wild-type *B. abortus*, the *vceC* mutant is not attenuated for survival in HeLa cells or macrophages. This suggests that VceC is not required for trafficking of Brucella-containing vacuoles (BCVs). In contrast to cultured cells, the *vceC* mutant did not persist at similar levels as the wild-type *B. abortus* in the mouse model. Reduced persistence *in vivo* but not *in vitro* could be explained with the reduced ability of the *vceC* mutant to evade the host's immune system.

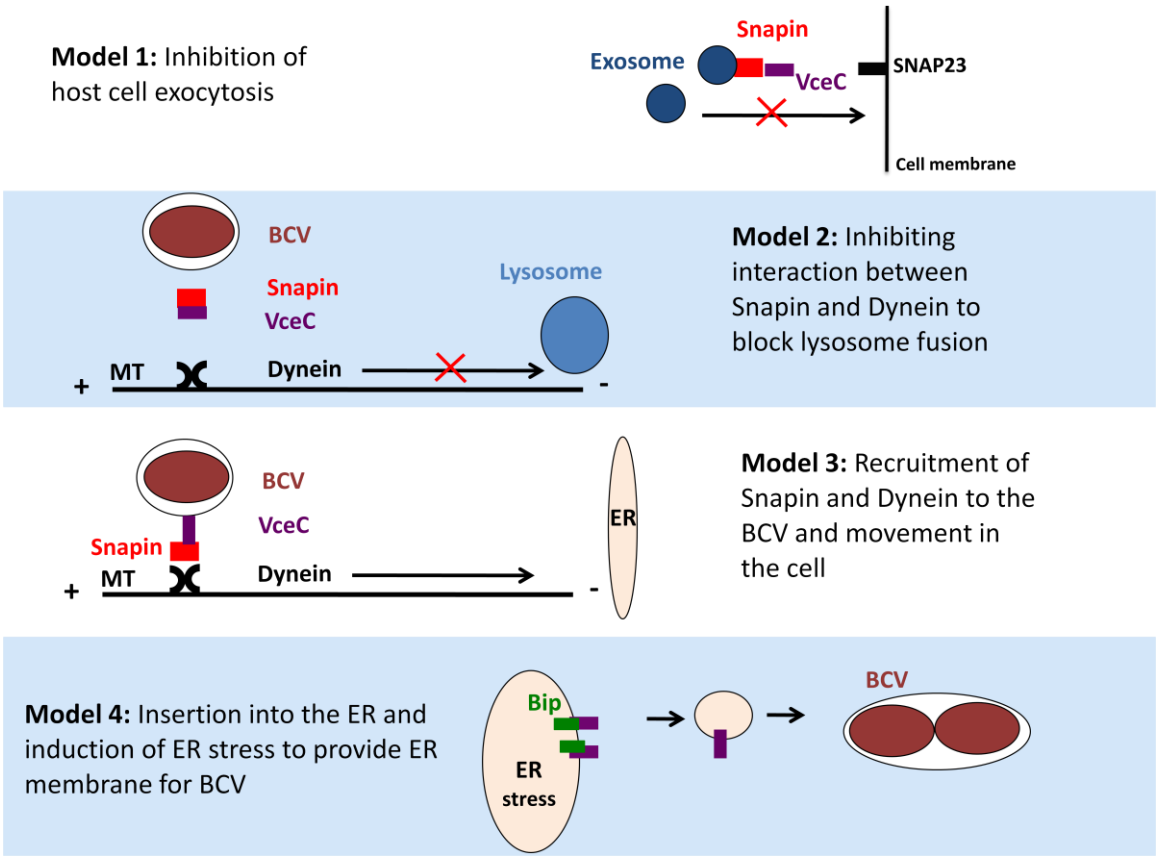


Figure 8. Schematic representation of four proposed models for VceC function in host cells. MT, microtubules, BCV, *Brucella*-containing vacuole.

Model 2. VceC Inhibits fusion of the BCV with lysosomes

In a more recent study it was found that in addition to its role in exocytosis, Snapin is involved in trafficking of late endosomes to lysosomes by connecting late endosomes to dynein for transport along microtubules towards lysosomes (Lu, Cai et al. 2009; Cai, Lu et al. 2010). In Snapin deficient cells, it was found that fewer late endosomes were transported from the cell periphery towards the soma and that the efficacy of late endocytic membrane trafficking was altered causing a reduced delivery of internalized materials to lysosomes for efficient degradation. Trafficking of hydrolase precursors or intermediates through late endosomes to acidic lysosomes for maturation was impaired in Snapin deficient cells, resulting in proliferation of immature lysosomes (Cai, Lu et al. 2010). After uptake by cells, the BCV is thought to first fuse with early and late endosomes, after which the BCV proceeds to fuse with lysosomes in a limited fashion (Starr, Ng et al. 2008). Vacuoles containing heat-killed or *virB* mutant *B. abortus* never

reach the ER and are killed in phagolysosomes. Wild-type *B. abortus* that successfully traffic to the ER also appear to be able to limit the amount of lysosomes that fuse to their vacuole, suggesting that VceC could manipulate Snapin function to alter late endosome to lysosome trafficking along microtubles (Figure 8).

One argument against the role of the T4SS and its effectors in the prevention of full fusion of BCVs to lysosomes is that the transient fusion of BCVs with lysosomes and the resulting acidification is required for *virB* expression and therefore occurs before *virB* expression (Boschiroli, Ouahrani-Bettache et al. 2002; Starr, Ng et al. 2008). However, it is possible that once the T4SS is expressed, translocated effectors, such as VceC, could prevent further fusion of lysosomes with BCVs and allow *Brucella* to redirect the trafficking to the ER.

Compared to wild-type *B. abortus*, the *vceC* mutant is not killed more by macrophages or HeLa cells and also does not have a defect in replication once the niche of *B. abortus* in the ER has been reached, which would argue against a role of VceC in BCV trafficking. However, the lack of phenotype of the *Brucella vceC* mutant in macrophages could be because the intracellular function of VceC is redundant and other *Brucella* effectors can have similar functions.

Model 3. VceC recruits Snapin and Dynein to the BCV to enhance trafficking to ER

The localization of VceC in the ER suggests that VceC is involved in trafficking of the *Brucella* phagosome to the ER, which is the target organelle of *Brucella*. Snapin was shown to interact with dynein, thereby connecting late endosomes to transport along microtubules towards lysosomes (Lu, Cai et al. 2009; Cai, Lu et al. 2010). As an alternative model to inhibition of BCV fusion with lysosomes (model 2), VceC could recruit Snapin to BCVs, thereby enhancing the fusion of these vacuoles with ER membranes. Recruitment of Snapin to BCVs by VceC could also connect BCVs with dynein for transport along microtubules toward the ER (Figure 8).

Model 4. VceC induces ER stress to provide more membrane for Brucella replication

In addition to an interaction with Snapin, we showed with pulldown experiments from HeLa cells, that VceC interacts with Grp78/BiP. Bip is an ER chaperone that plays a role in the assembly and folding of newly synthesized proteins, translocation of proteins across the ER membrane, regulation of calcium homeostasis and ER stress (Dudek, Benedix et al. 2009). Knockdown of Bip expression with siRNA leads to ER stress and vacuolization of the ER (Li, Ni et al. 2008). The observation of ER vacuolization and stress caused by VceC expression in HeLa cells may be caused by an interference of normal Bip functions by VceC. *Brucella* replicates in an ER derived vacuole, which requires the acquisition of additional ER membrane. The ER stress signaling protein IRE1α, which is activated by the unfolded protein response, was shown to be required for *Brucella* replication in the ER (Qin, Pei et al. 2008). Thus, binding of VceC to Bip could lead to an expansion of the ER to provide replicating *Brucella* with additional membrane (Figure 8). Since our data are obtained with HeLa cells that overexpress VceC it cannot be concluded that binding of VceC to Bip and disruption of normal ER structure also occurs during *Brucella* infection. While our data do not rule out Bip as a genuine interaction partner of VceC, the chaperone function of Bip suggests that it may bind VceC as a result of its overexpression and targeting to the ER.

The observed localization of VceC in the ER, the disruption of ER structure by VceC overexpression, and the interaction of VceC with both Snapin and Bip are hard to accommodate in a model in which VceC has only one intracellular function. Thus, it is conceivable that VceC could have multiple functions in host cells. Such a situation would be similar to that of CagA of *Helicobacter pylori* (Backert, Tegtmeyer et al. 2010). Future research is required to define the role or roles of VceC during *B. abortus* infection.

Acknowledgements

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4

Chapter 4 The *Brucella* effector VceB interacts with Lyric in the Endoplasmic Reticulum of host cells and inhibits NF-kappa B activation

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Abstract

Brucella species are Gram-negative intracellular pathogens of mammals that can also infect humans. The VirB Type IV secretion system is required for survival and growth of *Brucella* during host cell infection. This secretion system is known to translocate effector proteins into the infected host cells. In the present study we have identified a *Brucella* protein, VceB, that is translocated by the VirB system into mouse J774A.1 macrophages. Both the N- and C-termini of VceB were required for translocation. Pulldown assays revealed that VceB interacts with the host protein Lyric (also known as AEG-1 or MTDH). We also found that VceB colocalizes with Lyric in the ER of HeLa cells. The Lyric protein is involved in NF-κB activation, and we therefore investigated the effect of VceB on NF-κB activity. Experiments with transfected HeLa cells showed that VceB is able to inhibit activation of NF-κB when cells were stimulated with the TLR4 or TLR5 ligands LPS or FliC, respectively. Taken together, our results suggest that VceB is employed by *Brucella* to interfere with an important host cell response that counteracts the intracellular growth and survival of this pathogen.

Introduction

Brucella species are intracellular Gram-negative bacterial pathogens that cause the disease Brucellosis in animals and humans. *Brucella* requires a VirB Type IV secretion system (T4SS) to survive inside host cells and cause a persistent infection in the host. The *Brucella* T4SS is a complex of 12 VirB proteins, located in the inner and outer membranes of *Brucella*. The 12 VirB proteins are encoded by genes that are located in the v*irB* operon (O'Callaghan, Cazevieille et al. 1999; Boschiroli, Ouahrani-Bettache et al. 2002; Sieira, Comerci et al. 2004). *Brucella virB* mutants are highly attenuated for survival in cultured cells, such as macrophages, or *in vivo* in mice and goats (O'Callaghan, Cazevieille et al. 1999; Hong, Tsolis et al. 2000; Sieira, Comerci et al. 2000; den Hartigh, Sun et al. 2004; Zygmunt, Hagius et al. 2006; den Hartigh, Rolan et al. 2008).

The T4SS of *B. abortus* is required for creating an intracellular niche, in which the bacteria can multiply. This is achieved by redirecting the trafficking of *Brucella*-containing phagosomes to the Endoplasmic Reticulum (ER) (Celli, de Chastellier et al. 2003; Celli, Salcedo et al. 2005; Starr, Ng et al. 2008). The effect of the T4SS on *Brucella* survival in host cells is most likely mediated by the multiple substrates this system translocates into the host cells. To date several substrates of the *Brucella* T4SS have been identified, including VceA, VceC, BPE123, BPE005, BPE275 and BPE043, and RicA (de Jong, Sun et al. 2008; de Barsy, Jamet et al. 2011; Marchesini, Herrmann et al. 2011). In order to manipulate host cell pathways, *Brucella* effectors probably interact with host proteins, interfering with their function or altering their intracellular localization. For example, RicA was shown to interact with the GDP-bound version of the host trafficking GTPase Rab2 and also to recruit this GTPase to the *Brucella*-containing vacuole (BCV) (de Barsy, Jamet et al. 2011). The function of other *Brucella* effectors in the host has yet to be determined.

In the present study we identified a new *Brucella* effector named VceB by screening the *B. abortus* genome for genes encoding proteins with similarity to VceC. The putative function of VceB was characterized in host cells, showing that VceB interacts with the host protein Lyric. In turn, this seems to lead to an

inhibited activation of NF-κB when cells were stimulated with TLR4 or TLR5 ligands.

Experimental Procedures

Bacterial strains and plasmids

The *B. abortus* and *Escherichia coli* strains used in this study are listed in Table 1. *B. abortus* 2308 was used as a wild-type strain. *Brucella* 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, Guigue-Talet 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 37ºC. 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.

Table 1. Plasmids and strains used in this study.

Strain or Plasmid	Genotype and antibiotic resistance phenotype	Reference or source
E. coli strains		
Top10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZ ΔM15 ΔlacX74 recA1 ara Δ139 Δ(ara-leu)7697 galU galK rpsL (StrR) endA1 nupG*	Invitrogen
B. abortus strains		
2308	Wild-type	Deyoe
MDJ68	*vceB*::*kan* in 2308	This study

Plasmids		
pFLAGTEM1	Beta-lactamase reporter vector (CmR)	Raffatellu at al. 2005
pFT-BAB1_1035C	BAB1_1035 (abortus) aa 51-250 fused to C-terminus of FLAGTEM1	This study
pFT-BAB1_1035NC	BAB1_1035 (abortus) aa 1-50 fused to N-terminus and aa 51-250 fused to C-terminus of FLAGTEM1	This study
pFT-VceB-C	BAB1_0735 aa 56-262 fused to C-terminus of FLAG-TEM1	This study
pFT-VceB-N	BAB1_0735 aa 1-55 fused to N-terminus of FLAGTEM1	This study
pFT-VceB-NC	BAB1_0735 aa 1-55 fused to N-terminus and aa 56-**262** fused to C-terminus of FLAGTEM1	This study
pFT-VceB-NCd20	BAB1_0735 aa 1-55 fused to N-terminus and aa 56-**242** fused to C-terminus of FLAGTEM1	This study
pFT-VceB-N-1674C	BAB1_0735 aa 1-55 fused to N-terminus and BAB1_1674 aa 66-144 fused to C-terminus of FLAG-TEM1	This study
pFT-VceC303-418	B. abortus VceC303-418 fused to C-terminus of FLAG-TEM1	de Jong 2008
pUKDVceB	VceB-upstream and downstream regions flanking a kanamycin resistance cassette (CarbR/KanR; for knockout in B. abortus)	This study
pEGFP-C1	Empty vector for EGFP-protein fusions	Clontech
pEGFP-N1	Empty vector for EGFP-protein fusions	Clontech
pEGFP-C1-VceB	BAB1_0735 fused to C-terminus of EGFP	This study
pEGFP-N1-VceB	BAB1_0735 fused to N-terminus of EGFP	This study
pKH3-VceB1-261	Full length BAB1_0735 in pKH3 (HA tag fusion to C-term)	This study
pKH3-VceB45-261	aa 45-261 of BAB1_0735 in pKH3 (HA tag fusion to C-term)	This study
pKH3-VceB148-261	aa 148-261 of BAB1_0735 inpKH3 (HA tag fusion to C-term)	This study

Construction of plasmids

All plasmids that were constructed are listed in Table 1. Genes and inserts were PCR-amplified from *B. abortus* 2308 genomic DNA with the forward and reverse primers listed in Table 2. Using restriction sites in the primers listed in table 2 PCR products were then digested with the appropriate enzymes and cloned in destination vectors.

Table 2. Primers used in this study.

Purpose	Name	Sequence	In-sert size	Restric-tion site
Cloning 1035 aa 51-250 in pFT	BAB1_1035Xba1-F	TCC**TCTAGA**ACCAAGGCCCGCCTCGAC	603	XbaI
	BAB1_1035Pst1-R	AAA**CTGCAG**TTACGGGGCAGGCGCATG		PstI
Cloning 1035 aa 1-50 in pFT	BAB1_1035Nde1-F	TCC**CATATG**GCGATTATTTTTACAAAAAAAT	150	NdeI
	BAB1_1035Xho1-R	AAA**CTCGAG**AGGCGCAATCTGGCCAAC		XhoI
Cloning VceB aa 56-261 in pFT	BAB1-0735Xba1-F	TCC**TCTAGA**TTATCCGCGCTATTCCAGC	621	XbaI
	BAB1-0735PstI-R	AAA**CTGCAG**CTAGTTTTTAGCGCCGA-CAGC		PstI
Cloning VceB aa 56-241 in pFT	BAB1-0735Xba1-F	TCC**TCTAGA**TTATCCGCGCTATTCCAGC	561	XbaI
	BAB1-0735-20PstI-R	AAA**CTGCAG**CTACGTGGACGCGGGCGA		PstI
Cloning VceB aa 1-55 in pFT	BAB1-0735Nde1-F	TCC**CATATG**GCTGCAAGGAAACGAAGCTC	165	NdeI
	BAB1-0735Xho-R	AAA**CTCGAG**AGCCGCTTGTGGGCTTTT		XhoI
pUKDvceB	0735-up-F2	GCCTGGAAGCATGACAATTCA	810	-
	0735-up-R2	TCC**CCCGGG**GAGCTTCGTTTCCTTGCAGCC		SmaI
	0735-dn-F2	TCC**CCCGGG**CTGTCGGCGCTAAAAAC-TAGCTA	823	SmaI
	0735-dn-R2	AAA**CTGCAG**GCGGCAGGCCATAATGTCAT		PstI
pEGFP-C1-VceB	gfpC-BAB1-0735-F	T**TCCGGA**GCTGCAAGGAAACGAAGCTC	783	BspE1
	gfpC-BAB1-0735-R	A**CTGCAG**CTAGTTTTTAGCGCCGACAGC		Pst1
pEGFP-N1-VceB	gfpN-BAB1-0735-F	T**AAGCTT**ATGGCTGCAAGGAAACGAAGC	783	HindIII
	gfpN-BAB1-0735-R	A**CTGCAG**GTTTTTAGCGCCGACAGCCTT		Pst1
pKH3-YopP	YopP-HindIII-F	T**AAGCTT**ATGATTGGACCAATATCACAAA-TAA	868	HindIII
	YopP-XbaI-R	A**TCTAGA**TACTTTGAGAAGTGTTTTATATT-CAGC		XbaI
pKH3-VceB1-261	0735wt-HA-F	T**AAGCTT**ATGGCTGCAAGGAAACGAAGC	783	HindIII
pKH3-VceB45-261	0735-45-HA-F	A**AAGCTT**ATGACTGCGCAGCACAAAAGCC	648	HindIII
pKH3-VceB148-261	0735-148-HA-F	A**AAGCTT**ATGCGCGGCGTCAATACACCGG	341	HindIII
pKH3-ALL	0735all-HA-R	A**TCTAGA**GTTTTTAGCGCCGACAGCCTT		XbaI

Confocal microscopy

HeLa cells were seeded on 12 mm coverslips in 24 well plates at 5 x 10^4 cells per well. After 24 hours, cells were transfected with the different constructs containing *vceB* or *vceB*$_{45\text{-}261}$ fusions (Table 1) using 0.5 μg of plasmid DNA at a ratio of 2 μg of DNA to 4 μl of Fugene HD (Roche). After 24 hours cells were washed three times with PBS and fixed with 3% paraformaldehyde for 10 minutes at 37°C. Fixed cells were washed again twice and NH_4Cl was added for 10 minutes. Then coverslips were incubated for 30 minutes in blocking buffer (PBS with 10 % horse serum, 0.1% Saponin (Sigma)), 40 minutes in blocking buffer containing primary antibody (at 1:1000 dilution), washed 3 times in PBS and then incubated again for 40 minutes in blocking buffer containing secondary antibody. Then the coverslips were washed 3 times in PBS and once in water and mounted on glass slides using Mowiol (Calbiochem). Primary antibodies used were mouse anti-HA (Covance), rabbit anti-calreticulin (Thermo Scientific), rabbit anti-Giantin (Covance) and rabbit anti-MTDH (Lyric, from Sigma). Secondary antibodies used were donkey anti-mouse (Alexa 488 or Alexa 647 conjugated) or donkey anti-rabbit (Alexa 488 or Alexa 568 conjugated) (From Invitrogen).

Immunoprecipitation

For IP experiments 15 dishes containing 8 x 10^5 HeLa cells each were transfected with constructs containing VceB or VceB$_{45\text{-}261}$. For each dish 6 μg of plasmid DNA was added to 600 μl DMEM and 18 μl of Fugene HD (Roche) transfection reagent was added. After 15 minutes this mixture was added to each dish and 40 hours later cells were scraped from the dishes and washed twice with cold PBS. The cells were lysed by adding lysis buffer (PBS, 50 mM Tris-HCl pH 7.6, 150 mM NaCl 0.1% (v/v) NP-40, 1 mM EDTA), containing phosphatase inhibitor (1:100) and protease inhibitor III (1:500) (Calbiochem). After 15 minutes the lysate was centrifuged at 3000 x g, for 15 minutes. The supernatant was then precleared by incubation with 70 μl 50% Protein A agarose (Amersham) slurry for 1 h at 4°C on a rotator. To pellet the agarose the

lysate was centrifuged at 1000 x g for 2 minutes, and the supernatant was collected. To the supernatant 7.5 μl of anti-HA antibody (Covance) or anti-Lyric (MTDH) antibody was added (0.5 μg antibody per plate) and incubated while rotating for 1 h at 4°C. Then 70 μl protein A agarose slurry was added for 1-2 h. The agarose was pelleted by low speed centrifugation. Another 5 μl of anti-HA antibody or anti-MTDH antibody was added to the supernatant for a second round of IP similar as described previously. The agarose was washed 4 times with lysis buffer and then once with lysis buffer without NP-40. 30 μl hot 2x SDS sample buffer was added and eluates of both rounds of IP were pooled together and boiled for 5 minutes. For LC/MS IP was performed with Dynabeads protein G (Invitrogen), which were washed an additional 7 times with ammonium bicarbonate prior to on-bead digestion of peptides.

Infection of mice

Female C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and used at an age of 4-6 weeks. For infection experiments, groups of five mice were inoculated intraperitoneally (i.p.) with 0.2 ml of PBS containing 1 x 10^5 CFU of wild-type *B. abortus* and MDJ68 (*vceB*) or ADH3 (*virB2*) (den Hartigh, Sun et al. 2004). Infected mice were held in microisolator cages in a Biosafety Level 3 facility. At the appropriate time points, mice were euthanized by CO_2 asphyxiation and the spleens and livers collected aseptically at necropsy. Spleens and livers were homogenized in 3 ml of PBS and serial dilutions of the homogenate plated on TSA for enumeration of CFU. All animal experiments were approved by the University of California, Davis, Institutional Laboratory Animal Care and Use Committee and conducted in accordance with institutional guidelines.

Macrophage killing assay

J774A.1 mouse macrophages were seeded in 24-well plates and infected 24 hours later with *B. abortus* 2308 or *vceB* mutant (MDJ68) at a multiplicity of in-

fection of 100:1. Plates were centrifuged for 5 min at 250 x 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 0.5 ml fresh DMEM containing 50 µg/ml gentamicin was added to each well and plates were incubated at 37°C in 5% CO_2. At appropriate time points cells were washed 2 times with PBS and lysed in 0.5% Tween 20. Viable bacteria were quantified by serial dilution in sterile PBS and plating on TSA.

Western Blotting

Western blotting was performed as follows. Protein samples were loaded and run on a 12% SDS-PAGE gel and then transferred to a nitrocellulose membrane. The membranes were blocked in 2% non-fat skim milk powder (Safemart) in PBS for 1 h and probed with appropriate antibodies, anti-HA (Covance) or anti-Lyric (MTDH, from Sigma), at a dilution of 1:5000 in blocking buffer. Goat anti-mouse (Jackson ImmunoResearch) or goat anti-rabbit antibodies (Biorad) conjugated to horseradish peroxidase (HRP) were used (1:5000) as secondary antibodies and HRP activity was detected with a chemiluminescent substrate (Perkin-Elmer).

Luciferase assay

HEK293 cells were seeded in 48 well plates at 40% confluency. The next day cells were transfected with 200 ng of pKH3 plasmid (empty, *yopP*, *vceB* or *vceB*45-261) using Fugene HD (Roche). Cells were also transfected with 25 ng pNFkB luciferase reporter construct and 25 ng pLacZ (a LacZ reporter to correct for transfection efficiency). Then cells were incubated for 48 hours at 37°C in 5% CO_2, prior to addition of 100 ng flagellin (FliC) from *Salmonella enterica* serotype Typhimurium for another 6 hours. For experiments using LPS, HEK293 cells seeded in 48 well plates were transfected with 100 ng of pKH3 plasmid (empty, *yopP*, *vceB* or *vceB*45-261), 25 ng each of pLacZ and pNFkB

and 33 each ng of pCD14, pTLR4 and pMD2. After 48 hours cells were stimulated with 10, 50 or 100 ng of LPS from *E. coli* (Sigma) for 6 hours.

Cells were washed 3 times in PBS and lysed by freezing at -80°C and defrosting. 10 μl of the lysate was tranferred into a 96 wells white OptiPlate (Perkin Elmer) and 50 μl of luciferase assay solution (Promega) was added shortly before luciferase bioluminescence was measured with the luciferase assay system (Promega) using a luminometer. For normalization of transfection efficiency, β-galactosidase activity (measured with assay from Promega) was used to adjust luciferase values.

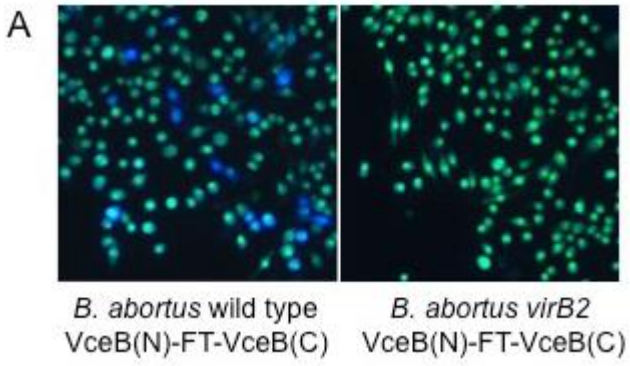
Results

Identification of VceB as a substrate of the B. abortus T4SS

Previously we have identified VceC as a substrate of the *B. abortus* T4SS (de Jong, Sun et al. 2008). The VceC protein is unique to *Brucella* and *Ochrobactrum* species and contains no domains that are conserved in other species. When examining the amino acid sequence of VceC, we identified two characteristic regions namely a predicted transmembrane (TM) domain and a proline-rich region. The TM domain was implicated in the localization of VceC to the ER of the host cell, and the proline-rich region might be required for interaction of VceC with host proteins (Chapter 3). We therefore hypothesized that similar domains could potentially exist in other yet unidentified *Brucella* effectors. We scanned the *B. abortus* genome and candidate effector proteins were selected based on the following properties: the predicted function of the protein is unknown, the amino acid sequence contains a proline-rich region, and the protein is not conserved in bacteria other than *Brucella* and *Ochrobactrum* species. We found two such candidates, BAB1_0735 and BAB1_1035, both of which are encoded by genes located on Chromosome I of *B. abortus*. BAB1_1035 contains an additional coiled-coil domain. This domain has been implicated in protein-protein interactions or interaction with host membranes in T4SS and T3SS effector proteins (Derre and Isberg 2005; Shohdy, Efe et al. 2005; Knodler, Ibarra et al. 2011). Similar to VceC, BAB1_0735 contains an N-terminal TM

domain. Furthermore, analysis of the N-termini of both candidate effectors using SIGNALP 3.0 suggested that they contain a potential N-terminal signal peptide (SignalP-HMM cleavage probability BAB1_0735: 0.512, BAB1_1035: 0.863 (Nielsen and Krogh 1998; Bendtsen, Nielsen et al. 2004)). Notably, the TM domain identified in BAB1_0735 corresponds to the hydrophobic region of the predicted signal peptide of this protein. To monitor the possible translocation of these candidate effectors into host cells, we fused them to the C-terminus of FLAGTEM-1 (FT) β-lactamase. Translocation of the β-lactamase fusion proteins by *B. abortus* into host cells that are loaded with the fluorescent β-lactamase substrate CCF2-AM will result in cleavage of this substrate and a change of fluorescence from green to blue (Charpentier and Oswald 2004). This method was previously utilized successfully to visualize translocation of the *Brucella* effectors VceA, VceC and RicA into J774A.1 macrophages (de Jong, Sun et al. 2008; de Barsy, Jamet et al. 2011). However, using this approach we were not able to detect translocation of the FT-BAB1_1035 fusion into the macrophages, and we observed only a low level of translocation for the FT-BAB1_0735 fusion (data not shown). The signal peptide could be important for translocation by the T4SS and, if so, it might only be functional when present on the N-terminus of our constructs. In addition, for VceC we found that the C-terminus is required for optimal translocation (de Jong, Sun et al. 2008). Although CagA of *Helicobacter pylori* does not have a predicted N-terminal signal sequence, we hypothesized that, like CagA, both N- and C-termini could be required for translocation of some *Brucella* effectors (Hohlfeld, Pattis et al. 2006). To test whether this is the case for BAB1_1035 or BAB1_0735, we created constructs containing a fusion of an N-terminal part of these candidates to the N-terminus of FLAGTEM1 and the C-terminal part to the C-terminus of FLAGTEM1. The BAB1_1035 fusion was unfortunately not expressed in *Brucella.* In contrast, the BAB1_0735 fusion protein was expressed and it was even translocated into J774A.1 macrophages at levels similar to those observed for the positive control FT-VceC (Figure 1). Notably, translocation of the BAB1_0735 fusion protein into macrophages was not observed when it was expressed in the *B. abortus virB2* mutant. BAB1_0735 was therefore designated as VceB, a new *Brucella*

T4SS effector. In order to determine the relative contribution of the N- and C-termini of VceB to translocation, several fusion proteins were constructed (Figure 1B). VceB(N)-FT-VceB(C) is the fusion described above, FT-VceB(C) does not contain the VceB N-terminus, and VceB(N)-FT does not contain the VceB C-terminus. VceB(N)-FT-1674 contains the N-terminus of VceB and the C-terminus of the *B. abortus* protein BAB1_1674, which we found not to be translocated into host cells and which thus served as a negative control (de Jong, Sun et al. 2008). A Western blot performed, using anti Flag antibodies, indicated that all the fusion proteins were expressed in both the wild-type and a Δ*virB2 B. abortus* strain (Figure 1C). Furthermore, results from translocation experiments showed that both the N- and C-termini of VceB are important for this protein's translocation into J774A.1 cells by the *Brucella* T4SS (Figure 1D). However a deletion of the last 20 amino acids at the C-terminus (ΔC20) of VceB did not affect translocation efficiency suggesting that, unlike VceC, the secretion signal is not located in this part of the C-terminus.



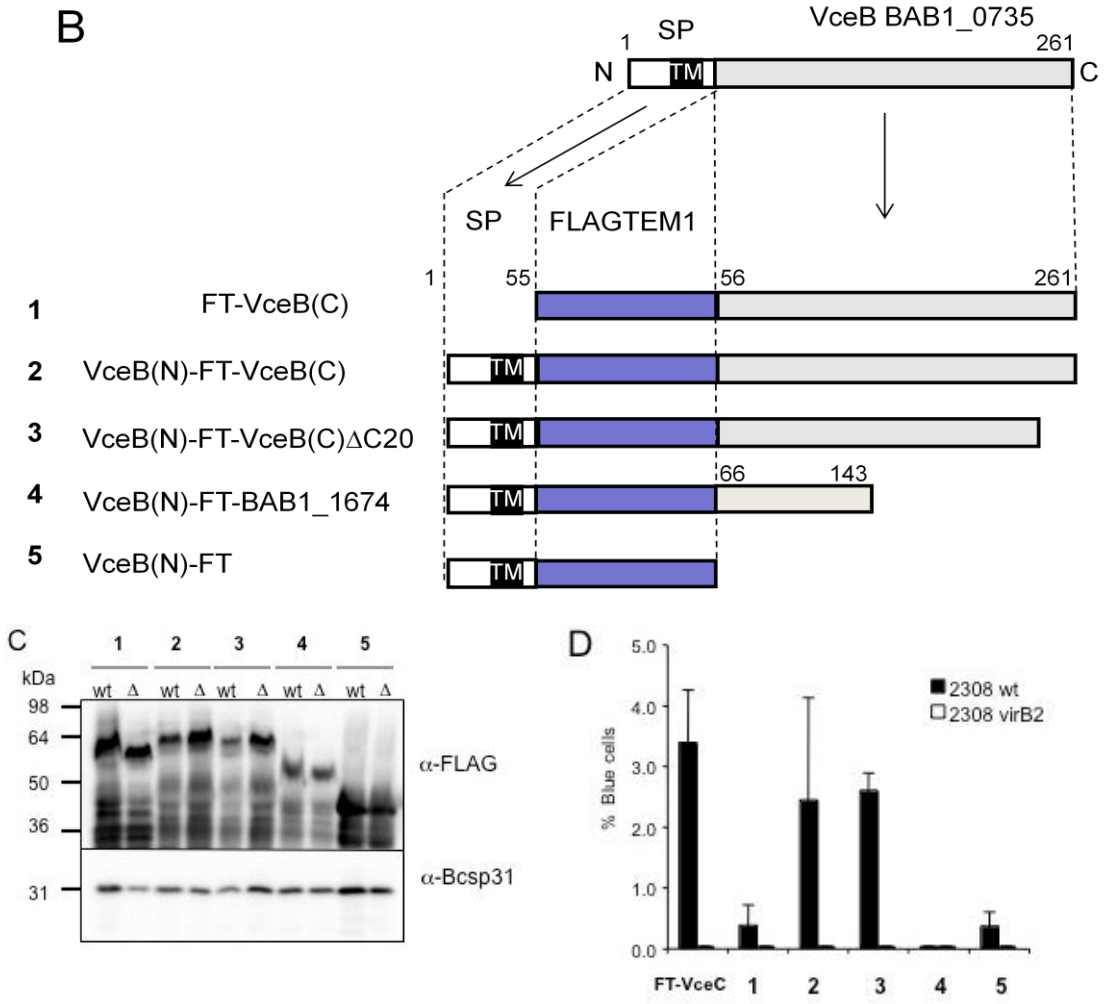


Figure 1. Translocation of VceB into J774A.1 macrophages by *B. abortus.* (**A**) VceB(N)-FT-VceB(C) is translocated into macrophages by wild-type, but not by *virB2* mutant *B. abortus*. (**B**) Schematic representation of the FT fusion proteins. Tested fusions are (1) FT-VceB(C), (2) VceB(N)-FT-VceB(C), (3) VceB(N)-FT-VceB(C)ΔC20, (4) VceB(N)-FT-BAB1_1674, (5) VceB(N)-FT. (**C**) Western blot stained with anti FLAG showing expression of FT fusion proteins in *B. abortus* wild-type (wt) and Δ*virB2 (*Δ) strains. (**D**) Translocation into J774.A1 macrophages is shown as the percentage of blue cells. Results shown are the average from three different experiments.

A B. abortus vceB mutant is attenuated for survival in J774A.1 cells but not in mice

Since *Brucella* strains lacking a functional T4SS are attenuated in both cultured cells and mice, we tested the survival of a *B. abortus vceB* mutant in both these models. We constructed a *B. abortus* Δ*vceB* strain by replacing the whole *vceB* gene with a kanamycin resistance cassette (Figure 2A). In J774A.1 mouse ma-

crophages infected at a multiplicity of infection (MOI) of 100 bacteria to 1 cell, the *B. abortus vceB* mutant strain survived approximately 2- to 3-fold less well than the wild-type strain at 48 hours post infection (Figure 2B). In these cells the initial killing of the wild-type and *ΔvceB* strains was similar, as bacterial numbers were equal at 24 hours post infection. However, compared to wild-type *Brucella*, the replication of the Δ*vceB* cells in the macrophages appeared to be less efficient between 24 and 48 hours post infection. In contrast, in C57BL/6 mice infected with either wild-type or Δ*vceB* cells of *B. abortus*, we observed no difference in the recovered bacterial load from the spleen and liver (Figure 2C).

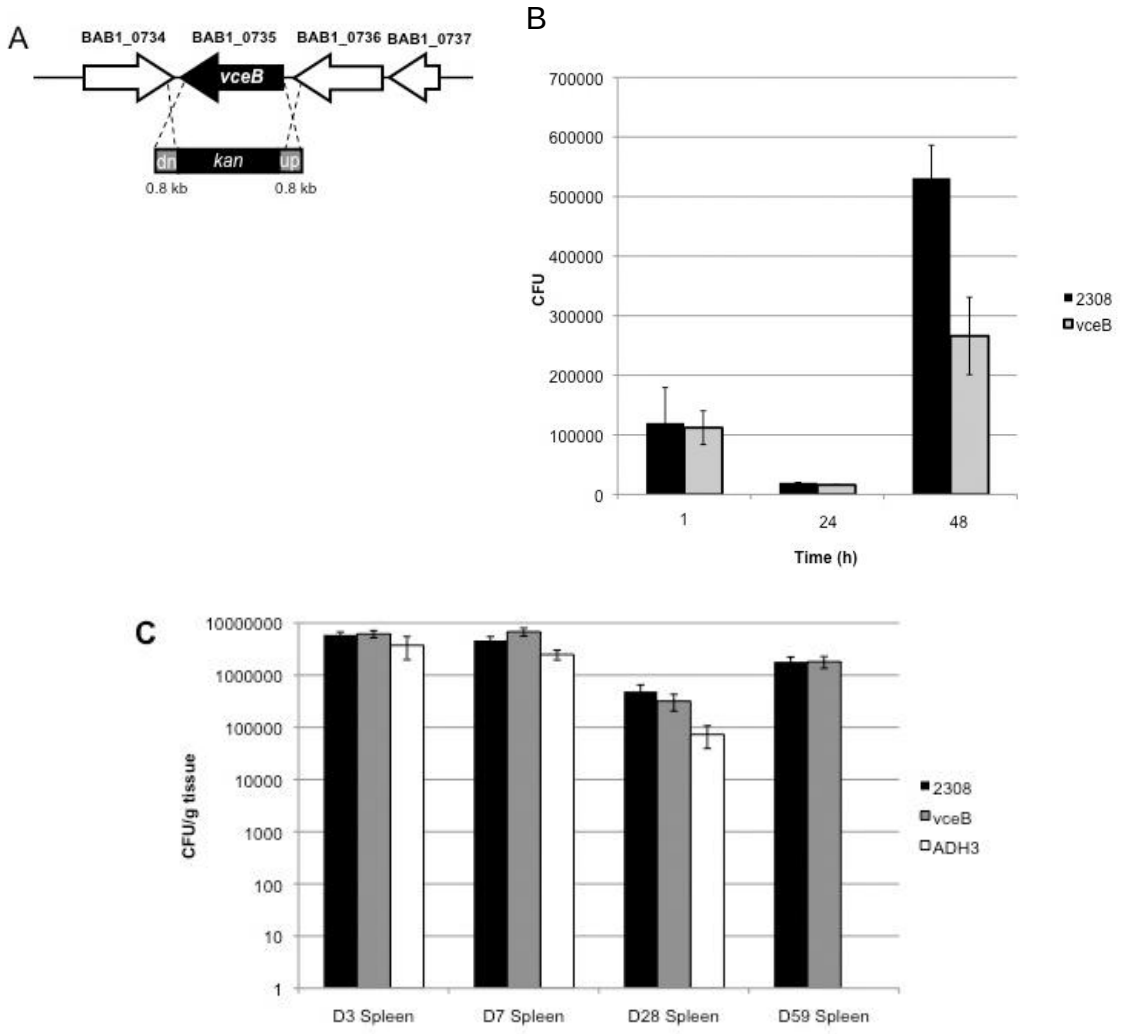
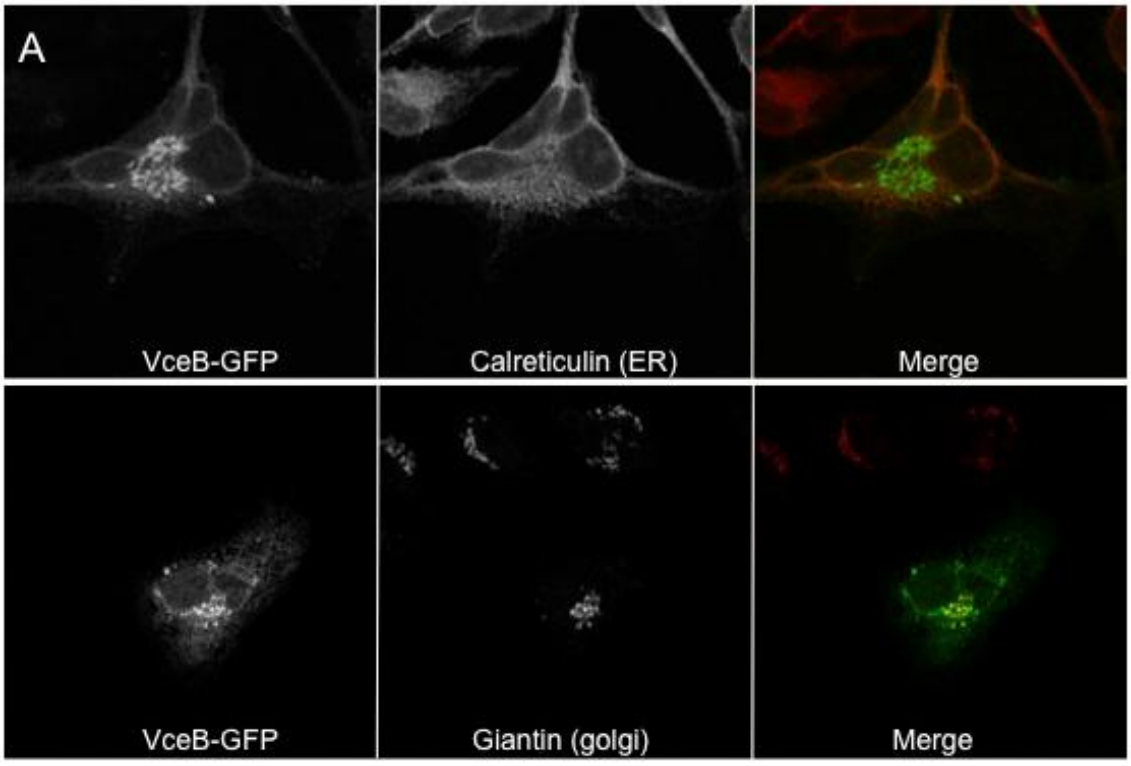


Figure 2. Construction and characterization of a *B. abortus vceB* mutant. (A) Schematic representation of the strategy used to create *B. abortus* ΔvceB. (B) Survival of *B. abortus* wild-type (2308) and Δ*vceB* strains in J774A.1 macrophages. (C) Survival of *B. abortus* wild-type (2308), Δ*vceB* and Δ*virB2* (ADH3) strains in the spleens of C57BL/6 mice at days 3, 7, 28 and 59 post infection.

VceB-GFP or VceB-HA localize to Golgi and ER of HeLa cells

In order to characterize the function of VceB inside host cells, we examined the localization of this protein in HeLa cells. HeLa cells grown on coverslips were transfected with a construct containing a VceB fusion to EGFP. Intracellular localization of the VceB-GFP protein was visualized using a confocal fluorescent microscope. This revealed that VceB-GFP was localized in a fine network throughout the cells, which resembles the ER. To examine whether VceB is targeted to this compartment, transfected cells were stained for the ER marker calreticulin. In these cells VceB-GFP clearly colocalized with calreticulin, showing that VceB is indeed targeted to the ER (Figure 3A). In cells with high expression of VceB-GFP, we also observed localization of VceB-GFP in the Golgi, as shown by colocalization of the GFP signal with the Golgi marker Giantin. However, it is possible that targeting of VceB-GFP to the Golgi of HeLa cells is a result of overexpression of this fusion protein. To ensure the observed localization of VceB-GFP in HeLa cells was not due to the GFP-tag we repeated the experiments with a VceB-HA fusion protein. Ectopically expressed VceB-HA in HeLa cells localized to the ER and Golgi (Figure 3B). Thus, it can be concluded that the VceB moiety mediates the observed ER and Golgi targeting of the VceB-GFP and VceB-HA fusion proteins in HeLa cells.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Calreticulin (ER)
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VceB-GFP
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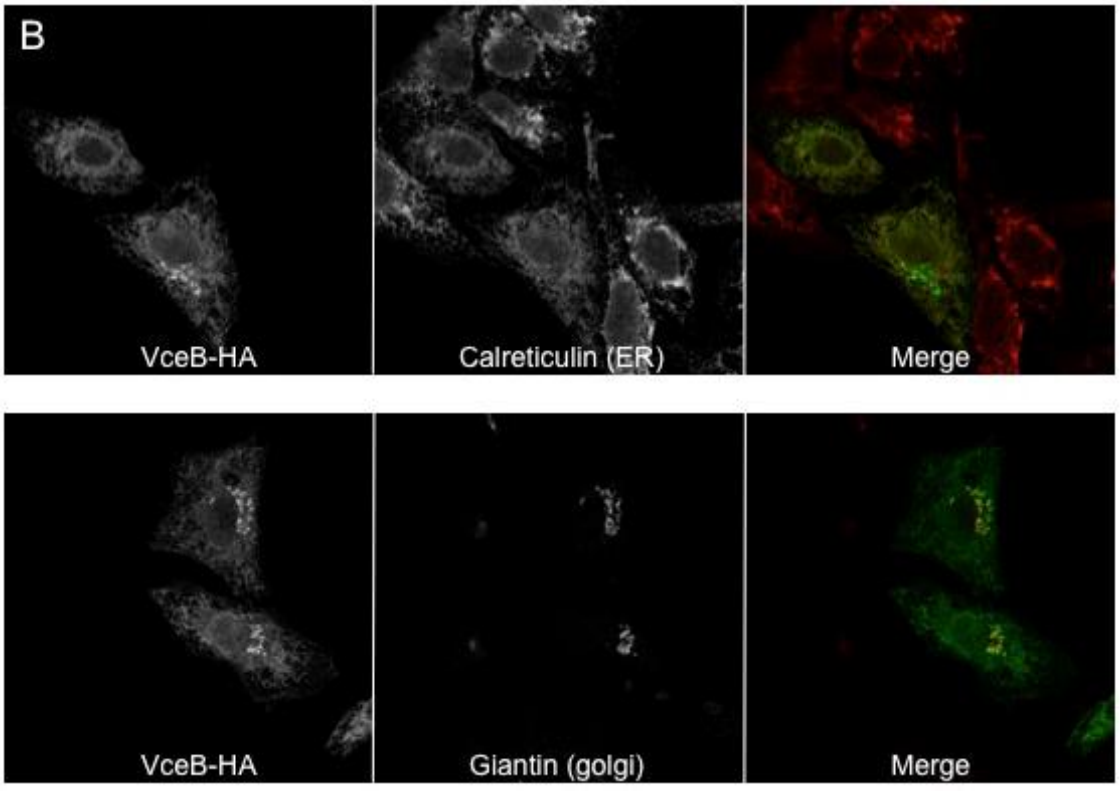
B
VceB-HA
Calreticulin (ER)
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VceB-HA
Giantin (golgi)
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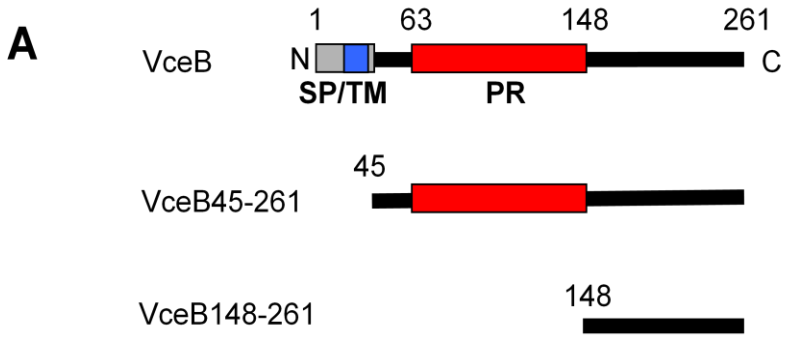
Figure 3. Localization of VceB in Hela cells. (A) Hela cells expressing VceB-GFP (green) were stained with anti-calreticulin (ER, red; top row) or anti-giantin (golgi, red; bottom row). (B) Hela cells expressing VceB-HA were stained with anti-HA and with anti-calreticulin (ER; top row) or anti-giantin (Golgi; bottom row). Secondary antibodies used were anti-mouse conjugated with Alexa 488 (for HA staining, green) and anti-rabbit conjugated with Alexa 568 (for calreticulin or giantin staining, red).

VceB requires its predicted signal peptide for targeting to ER and Golgi

To identify the domain of VceB responsible for targeting to the ER and Golgi of host cells, we created truncations of VceB fused to the HA tag (Figure 4A). The first construct, $VceB_{45\text{-}261}$-HA, lacks the N-terminal signal peptide or TM domain, which we hypothesized to be required for the localization of VceB in ER or Golgi membranes. In the second construct, $VceB_{148\text{-}261}$-HA, both the TM domain and the proline-rich region were deleted. Both constructs localized to the cytoplasm of the HeLa cells, from which it can be concluded that the first 44 amino acids containing the TM domain are required for VceB targeting (Figure 4B, and data not shown).



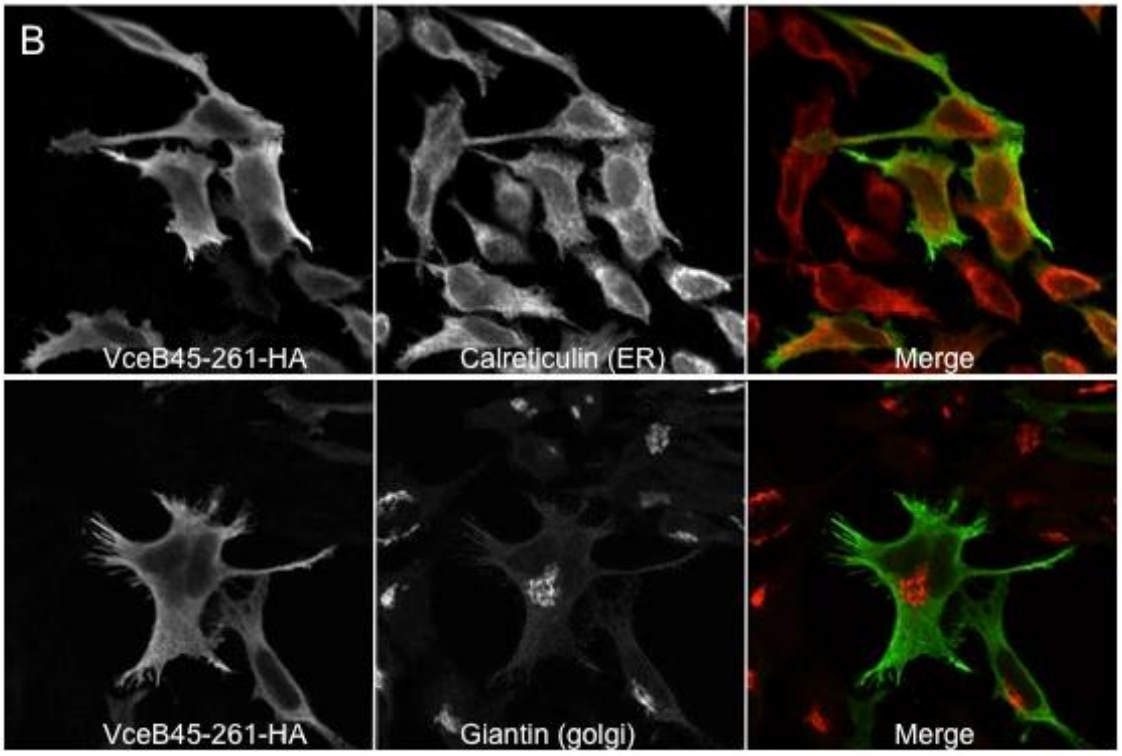


Figure 4. VceB requires its N-terminus for targeting to the ER or Golgi of cells. (A) Schematic representation of VceB truncations that were fused to the HA tag. The putative signal peptide (SP), transmembrane (TM) domain and proline-rich (PR) domain are shown. (B) HeLa cells expressing VceB45-261-HA were stained with anti-HA and with anti-calreticulin (ER; top row) or anti giantin (Golgi; bottom row). Secondary antibodies used were anti-mouse conjugated with Alexa 488 (for HA staining) and anti-rabbit conjugated with Alexa 568 (for calreticulin or giantin staining).

VceB Interacts with Lyric in HeLa cells

To gain insights into the function of VceB in host cells, we performed immuno-precipitation (IP) experiments using anti-HA antibodies to pull down VceB-HA together with interacting proteins from lysates of HeLa cells transfected with the VceB-HA construct. As a control, the same IP procedure was performed on HeLa cells that were not transfected or were transfected with a construct encoding an unrelated HA-tagged protein. The resulting IP fractions were then analysed with LC/MS. One protein, identified as Lyric (also known as AEG-1 or MTDH), was exclusively present in the VceB-HA IP fraction. To confirm the pulldown of Lyric by VceB-HA, the IP was repeated and a Western blot was

performed using anti-Lyric antibodies. This experiment showed that Lyric was only present in the IP fraction of the VceB-HA-expressing cells (Figure 5). Lyric has been described to localize in the ER, and since VceB is also localized in this organelle, we hypothesized that the VceB-Lyric interaction only occurs if both proteins are present in the ER. To test this and to confirm the VceB-Lyric interaction in a different experiment, we transfected HeLa cells with either full length VceB-HA or the truncated $VceB_{45\text{-}261}$-HA, which is no longer targeted to the ER. IP on the HeLa cell lysates was then performed with the anti-Lyric antibodies. As expected, Lyric was pulled down from both lysates. However, only VceB-HA, but not $VceB_{45\text{-}261}$-HA, was co-immunoprecipitated with Lyric, indicating that Lyric only interacts with VceB when it is targeted to the ER (Figure 5).

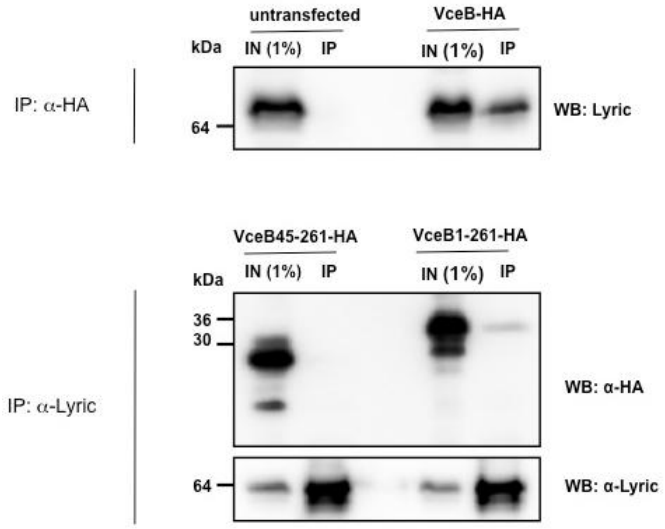


Figure 5. Interaction of VceB with Lyric. Pulldown of Lyric by VceB-HA from transfected HeLa cells with anti-HA antibodies or pulldown of VceB-HA from transfected HeLa cells with anti-Lyric antibodies. IN, 1% of input fraction (lysate). WB, Western blot.

VceB co-localizes with Lyric in HeLa cells

The above results suggested that both VceB and Lyric are located in the ER. To confirm co-localization of these two proteins, HeLa cells expressing VceB-GFP or VceB-HA were stained with anti-Lyric and anti-HA antibodies and examined by confocal fluorescence microscopy. Indeed, the results showed that Lyric and

GFP or HA-tagged VceB colocalized in HeLa cells. This colocalization depended on the presence of the N-terminus of VceB, as $VceB_{45-261}$-HA, which is cytosolic, did not colocalize with Lyric (Figure 6).

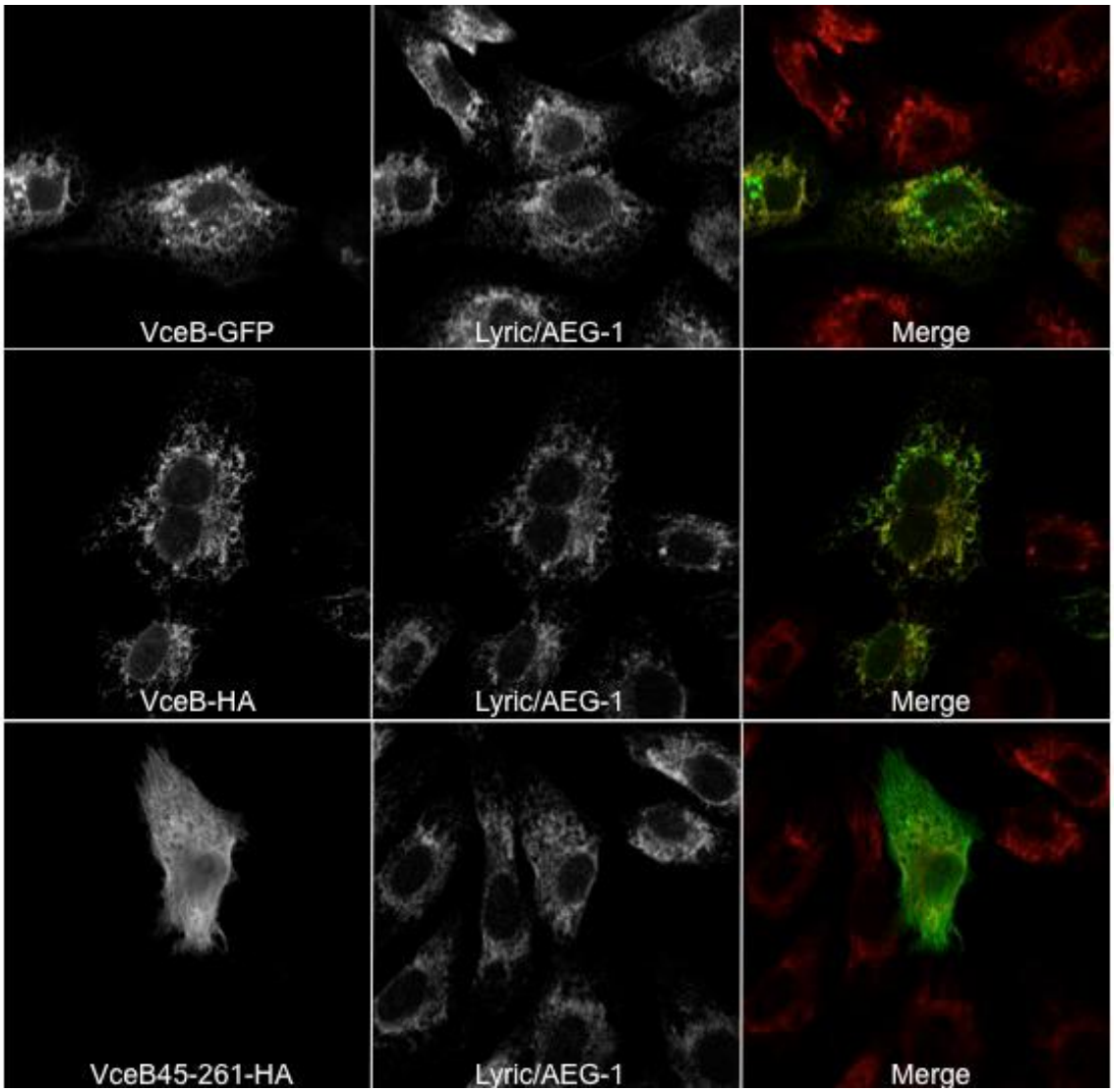


Figure 6. Co-localization of VceB with Lyric. Hela cells expressing VceB-GFP were stained with anti-Lyric and Hela cells expressing VceB-HA or VceB45-261-HA were stained with anti-HA and with anti-Lyric. Secondary antibodies used were anti-mouse conjugated with Alexa 488 (for HA staining) and anti-rabbit conjugated with Alexa 568 (for Lyric staining).

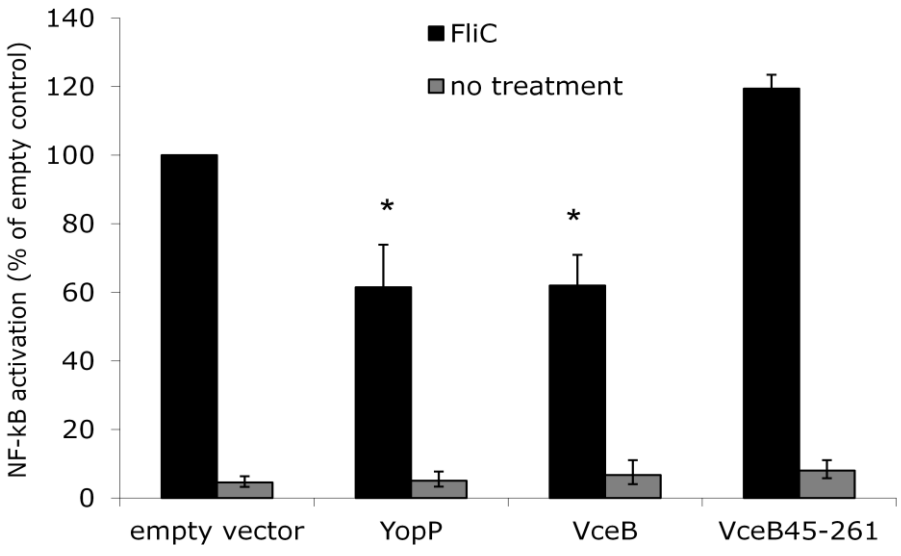
NF-κB activation is inhibited by VceB

The results shown above indicate that *B. abortus* VceB is targeted to the ER where it interacts with the host protein Lyric. Lyric contains a TM domain and is located predominantly in the ER and perinuclear space (Sutherland, Lam et al. 2004). Lyric also contains 3 nuclear localization signals and has been shown to

translocate to the nucleus and nucleoli of cells upon TNFα stimulation or when overexpressed (Sutherland, Lam et al. 2004). In the nucleus, Lyric activates NF-κB by interaction with the p65 subunit (Emdad, Sarkar et al. 2006; Sarkar, Park et al. 2008). We hypothesized that by interacting with Lyric in the ER, VceB could interfere with Lyric's function as an activator of NF-κB. To test this idea, we measured NF-κB luciferase reporter activity in HEK293 cells that were transfected with an empty vector or the vector encoding either VceB-HA or $VceB_{45-261}$-HA. As a positive control we used YopP, a T3SS effector from *Yersinia enterocolitica*, which similarly to its homolog YopJ in *Y. pseudotuberculosis*, is known to inhibit NF-κB activation (Schesser, Spiik et al. 1998; Ruckdeschel, Mannel et al. 2001). Treatment of the transfected HeLa cells with flagellin, a Toll-like receptor (TLR) 5 ligand from *Salmonella enterica* serotype Typhimurium, resulted in activation of NF-κB, which relates to the fact that HEK293 cells express TLR5. This activation was significantly reduced in cells expressing YopP compared to cells transfected with the empty plasmid. A similar reduction in NF-κB activation was observed for cells expressing full length VceB, but not for cells expressing $VceB_{45-261}$ (Figure 7A). To determine whether VceB can also inhibit activation of NF-κB in cells treated with the TLR4 ligand LPS, the experiments were repeated with HEK293 cells that had been transfected with constructs for the expression of components of the LPS receptor complex, namely TLR4, CD14, and MD-2. These cells were then also transfected with the constructs expressing YopP-HA, VceB-HA or $VceB_{45-261}$-HA. Upon treatment of the cells with different concentrations of LPS, the NF-κB luciferase reporter activity was measured. Similar to results shown in Figure 7A, VceB-HA, but not $VceB_{45-261}$-HA, was able to inhibit activation of NF-κB through TLR4 (Figure 7B).

Altogether, it can be concluded from these results that VceB is able to inhibit NF-κB activation. This inhibition occurs only if VceB can be targeted to the ER or Golgi

A



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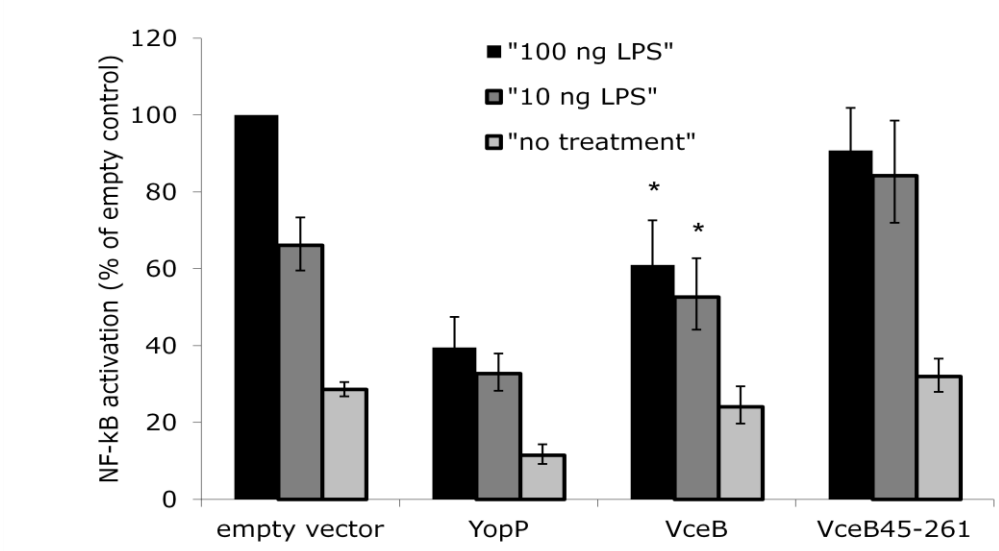

Figure 7. Inhibition of NF-κB activation by VceB. (A) HEK293 cells transfected with vectors for the expression of full-length YopP from *Y. enterocolitica*, full length VceB or truncated $VceB_{45\text{-}261}$, or HEK293 cells transfected with the empty vector were treated with 100 ng FliC and NF-κB activation was measured with a luciferase assay. As a contol cells were incubated without FliC. The results shown are from three independent experiments (* $p<0.05$). (B) HEK293 cells expressing TLR4, CD14, and MD-2 were transfected with vectors for the expression of YopP, VceB, or $VceB_{45\text{-}261,}$ or with the empty vector. Next the cells,were treated with 10 or 100 ng of *E. coli* LPS. As a control cells were inubated without LPS (no treatment). NF-κB activation was measured with a luciferase assay (* $p<0.05$, compared to VceB45-261).

Discussion

In this study we identified VceB, a novel effector that was translocated by the *Brucella* VirB T4SS into J774A.1 mouse macrophages. VceB was identified through a bioinformatic screen for hypothetical *Brucella* proteins containing features that are similar to those of the previously identified *B. abortus* effector VceC (de Jong, Sun et al. 2008). VceB is a protein of 261 amino acids that is specific to *Brucella* and *Ochrobactrum* species. Similar to VceC, VceB contains a proline-rich region. Translocation of VceB into host cells depended on both the N- and C-termini of this protein. The N-terminus of VceB contains a predicted Sec-type signal peptide, suggesting that the Sec system could be involved in the translocation of VceB into host cells by the T4SS. The maximum cleavage site probability of the signal peptide of VceB is 0.512 and this site is predicted between amino acids 45 and 46 ((Nielsen and Krogh 1998; Bendtsen, Nielsen et al. 2004)). The predicted signal peptide was shown to be important for VceB localization in the ER and, more generally, the function of this protein inside host cells. It thus seems unlikely that this signal peptide is processed during export of VceB from *Brucella* into a host cell. Furthermore, it should be noted that a long signal peptide of 45 amino acids is atypical for Gram-negative bacteria, with the exception of autotransporter signal peptides (Hiss and Schneider 2009). Further experiments are required to determine whether the Sec system of *Brucella* is involved in VceB secretion.

As a first step to characterize the function of intracellular VceB, the localization in HeLa cells of both full-length VceB and VceB missing its first 45 N-terminal amino acids ($VceB_{45-261}$) was determined by fluorescence microscopy. As mentioned above, the results from these experiments indicated that full-length VceB but not $VceB_{45-261}$ was targeted to the ER. In some cells with high VceB expression levels, the full length VceB was also targeted to the Golgi apparatus. Furthermore, in this work we show that VceB interacted with the protein Lyric. In the same experiments it was shown that co-localization of VceB and Lyric in the ER is required for the interaction, as $VceB_{45-261}$ did not interact with Lyric.

Lyric has been described as a protein that is localized in the ER and perinuclear space of human cells. It as shown to translocate to the nucleus and nucleoli of

host cells upon TNFα treatment or overexpression (Sutherland, Lam et al. 2004; Kang, Su et al. 2005). In the nucleus, Lyric interacts with the NF-κB p65 subunit and enhances the NF-κB regulation of downstream genes (Emdad, Sarkar et al. 2006; Sarkar, Park et al. 2008). Although, Lyric has been studied mostly for its involvement in tumor progression, the respective gene was initially identified through a transcript that was induced upon HIV-1 infection in human fetal astrocytes (Su, Kang et al. 2002; Kang, Su et al. 2005). Another report that indicated that Lyric may be involved in the host inflammatory response to microbial pathogens showed that Lyric is induced by LPS through TLR4 signaling and in turn increases TLR4 expression through its effect on NF-κB activation (Emdad, Sarkar et al. 2006; Khuda, Koide et al. 2009).

The co-localization and interaction of Lyric and VceB in the ER raised the question whether VceB could be interfering with Lyric-mediated NF-κB activation. In HEK293 cells ectopically expressing VceB or $VceB_{45-261}$, only full length VceB was able to reduce flagellin-mediated induction of NF-κB activity. Similar results were obtained in LPS-treated HEK293 cells that ectopically expressed the LPS receptor complex (TLR4, CD14 and MD-2). The results from these experiments suggest that the interaction of VceB with Lyric in the ER interferes with Lyric-mediated NF-κB activation.

To further characterize the function of VceB a *B. abortus vceB* mutant was constructed. After infection of macrophages the initial killing of both wild-type *B. abortus* and *vceB* mutant was at comparable levels, however replication of the *vceB* mutant between 24 and 48 hours was slower than that of the wild-type bacteria. This suggests that VceB is involved in later stages of *Brucella* infection of host cells and not in preventing the killing of *Brucella* in phagolysosomes. Thus, it is possible that the reduction in NF-κB activation caused by VceB during *Brucella* infection allows *Brucella* to replicate to higher numbers in the ER. Future experiments are required to show directly that inhibition of NF-κB activation by VceB occurs during *Brucella* infection of cells.

The finding of a *Brucella* effector that is involved in reduction of inflammation by inhibition of NF-κB is highly relevant as *Brucella* is known to be a stealthy pa-

thogen. *Brucella* has been found to passively and actively reduce its recognition by innate immune cells, for example through a reduced recognition of its LPS by host TLR4. Furthermore, *B. abortus* and *B. melitensis* actively prevent recognition by innate immune cells through the protein Btp1/TcpB. This protein promotes the degradation of the adapter protein MAL, thereby inhibiting signaling of TLR2 and TLR4 (Cirl, Wieser et al. 2008; Salcedo, Marchesini et al. 2008; Radhakrishnan, Yu et al. 2009; Sengupta, Koblansky et al. 2010). Notably, Btp1/TcpB is not conserved in all *Brucella* species, whereas VceB is conserved in all *Brucella* species. It thus seems that VceB is a more general effector that is employed by *Brucella* to inhibit NF-κB activation.

Acknowledgements
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5

Chapter 5 *Summary and discussion*

Brucella species are the causative agents of the disease brucellosis in humans and animals. These organisms require several virulence factors to infect and persist in their host. One of these virulence factors is the VirB T4SS, which is a complex of 12 proteins localized in the cell envelope of *Brucella*. The *Brucella* T4SS is known to translocate multiple effector proteins into infected host cells (reviewed in chapter 1 of this thesis). The effect of T4SS on proliferation in host cells and persistence in the host, is most likely mediated by the combined repertoire of translocated T4SS effectors. By studying the function of single T4SS effectors, more detailed insights can be gained into host cell pathways that are manipulated by *Brucella*.

Identification of the first *Brucella* effectors

The second chapter of this thesis describes the identification of the first two effectors of the *Brucella* VirB system, VceA and VceC. These effectors were found through a screen for genes co-regulated with the *virB* operon. This screen was initially performed in the heterologous host bacterium *E. coli*. A plasmid containing a *Brucella* promoter region transcriptionally fused to *lacZ* was introduced together with a plasmid containing an IPTG-inducible copy of a *Brucella* regulator. In the first screen, using this system, it was found that the *Brucella* regulator VjbR was able to activate the main promoter of the *virB* operon (P_{virB}) and the promoter upstream of *tetR* and *vjbR* genes ($P_{tetR\text{-}vjbR}$). Activation of P_{virB}-*lacZ* by VjbR in *E. coli* suggested a direct binding of VjbR to P_{virB}, as no other *Brucella*-specific genes were present in the *E. coli* model. Using EM-

SAs, VjbR was indeed shown to bind the *virB* promoter. Analysis of P_{virB} resulted in the identification of an 18 bp palindromic motif named the P_{virB} box, which is centered at position -37 relative to the transcription start site. A similar motif was shown to be present in $P_{tetR\text{-}vjbR}$, suggesting that this motif could be important for promoter activation by VjbR. This was indeed shown to be the case as activation of a mutant P_{virB}-*lacZ* fusion by VjbR was reduced. In this mutant P_{virB}-*lacZ* fusion, 6 nucleotids of the P_{virB} box were substituted with a HindIII site. However, in EMSAs we were unable to show reduced binding of VjbR to a fragment of P_{virB} containing this mutation (data not shown). This implies that the P_{virB} box is important for promoter recognition by VjbR, but that it is not the actual binding site of VjbR. These results and ideas were recently confirmed by the finding that the VjbR binding site in P_{virB} is centered at position -94 relative to the transcription start site (Arocena, Sieira et al. 2010). In our work described in chapter 2 the P_{virB} boxes found in P_{virB} and $P_{tetR\text{-}vjbR}$ were used to create a consensus box, which was then used to search all intergenic regions in the *B. abortus* and *B. suis* genomes for similar boxes. Candidate promoters containing a putative P_{virB} box were fused to *lacZ* and tested for activation by VjbR in *E. coli*. A total of 144 *Brucella* promoters containing a predicted P_{virB} box were identified, including 15 promoters that were activated by VjbR in *E. coli*. Thus, although the P_{virB} box was shown not to be the binding site of VjbR in P_{virB}, it was required for full activation of P_{virB}-*lacZ* in *E. coli* and, thus, allowed us to identify additional *Brucella* promoter regions that were activated by VjbR in *E. coli*. To identify actual *Brucella* effectors, proteins encoded by genes downstream of the 144 promoter regions that were predicted to contain the putative P_{virB} box, were examined. Candidate effectors were selected based on predicted unknown or hypothetical functions of the respective proteins. Thirteen candidate effectors were fused to TEM1 β-lactamase and tested for secretion into J774A.1 mouse macrophages, which resulted in the identification of the effectors VceA and VceC. Interestingly, we were also able to detect T4SS-dependent translocation of VceC expressed by *L. pneumophila* into host cells. Similar to *Brucella*, *L. pneumophila* is an intracellular pathogen, which relies on a T4SS for survival and replication in host cells. Although, the T4SS of the two

bacteria are only distantly related, these results suggest that the mechanism of T4SS substrate recognition and secretion is conserved in these different pathogens. In conclusion, the identification of genes co-regulated with the *virB* operon, resulted in the identification of two novel *virB* co-regulated effectors, VceA and VceC.

Characterization of two proline-rich *Brucella* effectors

The screen described in chapter 2 was designed to reduce the number of potential effector candidates. Nevertheless, it was conceivable that many effectors were missed in this screen. For example, the expression of certain effector genes could be under the control of different regulators, or certain effectors might even be expressed constitutively. In fact, other groups employed different screens, resulting in the identification of six additional effector proteins (de Barsy, Jamet et al. 2011; Marchesini, Herrmann et al. 2011). The identification of VceC as an effector of the VirB system, allowed us to screen for *Brucella* proteins with features that were similar to those of VceC, including a proline-rich region. This resulted in the identification of VceB as a novel *Brucella* effector protein as was described in chapter 4 of this thesis.

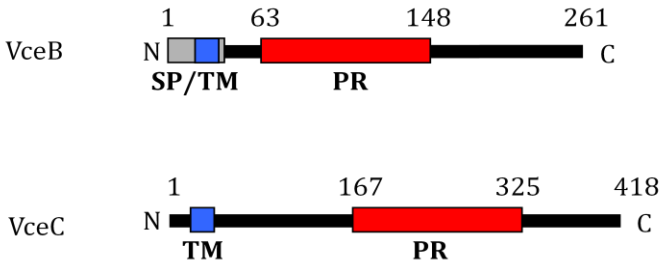

Figure 1. Schematic representation of the *B. abortus* proline-rich effectors, VceB and VceC. Shown are the relative positions of the putative signal peptide (SP), transmembrane domain (TM) and proline-rich domain (PR).

As described in chapters 3 and 4, the intracellular function of VceC and VceB was characterized in more detail. VceC was shown to be expressed in *B. abortus* and *B. melitensis*, but not in several other *Brucella* species including *B. ca-*

nis, *B. ovis*, *B. neotomae* and a *Brucella* isolate from baboons (chapter 3). In contrast, the *vceB* gene is highly conserved in all *Brucella* species. Even so, to date we have no data on *vceB* regulation and expression in *Brucella*. To gain insights into the intracellular functions of VceB and VceC, several different experimental approaches were utilized. These included the localization of VceB and VceC in HeLa cells by fluorescent confocal microscopy, and immunoprecipitation to identify potential host interaction partners. Both VceB and VceC contain a predicted N-terminal TM domain and a central proline-rich region (Figure 1). Although the proline-rich region appears to be a common feature of at least two *Brucella* effectors, the intracellular function of this region remains to be assessed. Localization studies revealed that both VceB and VceC were targeted to the ER of host cells, which required their N-terminal region including the TM domain. In addition, the N-terminus containing the TM domain was shown to be required for VceB translocation into host cells. Since the N-terminus is also required for VceB localization in host cells, we suggest that this region is not cleaved from VceB during translocation into host cells.

Furthermore, chapter 4 describes the finding that VceB interacts with the host protein Lyric in the ER and is able to inhibit the activation of NF-κB through TLR4 and TLR5 signaling. Since Lyric is known to act as an enhancer of NF-κB activation of downstream genes, these results suggest that VceB could interfere with Lyric function or localization. However, further experiments are required to determine the precise mechanism of the observed VceB-mediated inhibition of NF-κB activation. It will also be interesting to investigate which danger signal leading to NF-κB activation is triggered during host cell infection by *Brucella* and inhibited by VceB.

Chapter 3 describes a detailed characterization of VceC. The results show a direct interaction of VceC with the host protein Snapin. In addition we found an interaction of VceC with the ER chaperone Bip. However, due to the chaperone function of Bip, the binding to VceC might reflect Bip's general function in protein binding rather than a specific interaction between Bip and VceC. For example, the targeting of overexpressed VceC to the ER might result in an accumulation of malfolded VceC, which could be recognized and bound by Bip.

Based on the present findings, four models for intracellular function of VceC can be proposed: (1) inhibition of host cell exocytosis and protein secretion by interfering with Snapin function, (2) reduction of the Brucella containing vacuole (BCV) fusion with lysosomes by interfering with Snapin function, (3) enhancement of BCV trafficking to the ER by recruiting Snapin, and (4) induction of ER stress through interfering with Bip function. Further experiments should provide more insights into the role of VceC during *Brucella* infection. To test the hypothesis, described in model 1, that VceC inhibits host cell exocytosis, cytokine secretion by cultured macrophages infected with wild-type or *⊿vceC B. abortus* strains could be determined. Furthermore, the trafficking of wild-type or *⊿vceC B. abortus* strains in wild-type or Snapin -/- host cells could be monitored in more detail by using confocal microscopy. These experiments could reveal a possible role of VceC in intracellular trafficking of *Brucella*, as proposed in models 2, 3 and 4.

In conclusion, from the data presented in this thesis and and data from other groups, two main functions of *Brucella* effectors are emerging: (1) subversion of innate immune responses and (2) promotion of the trafficking of the BCV to the ER. These two functions could actually be overlapping, as prevention of an inflammatory response to *Brucella* by effectors likely increases the persistence of *Brucella* in the host. At the same time this might also serve to increase *Brucella* intracellular survival and proliferation. It will be an important goal for future research to determine how the manipulation of the host immune response by *Brucella* leads to increased intracellular survival and persistence of this pathogen.

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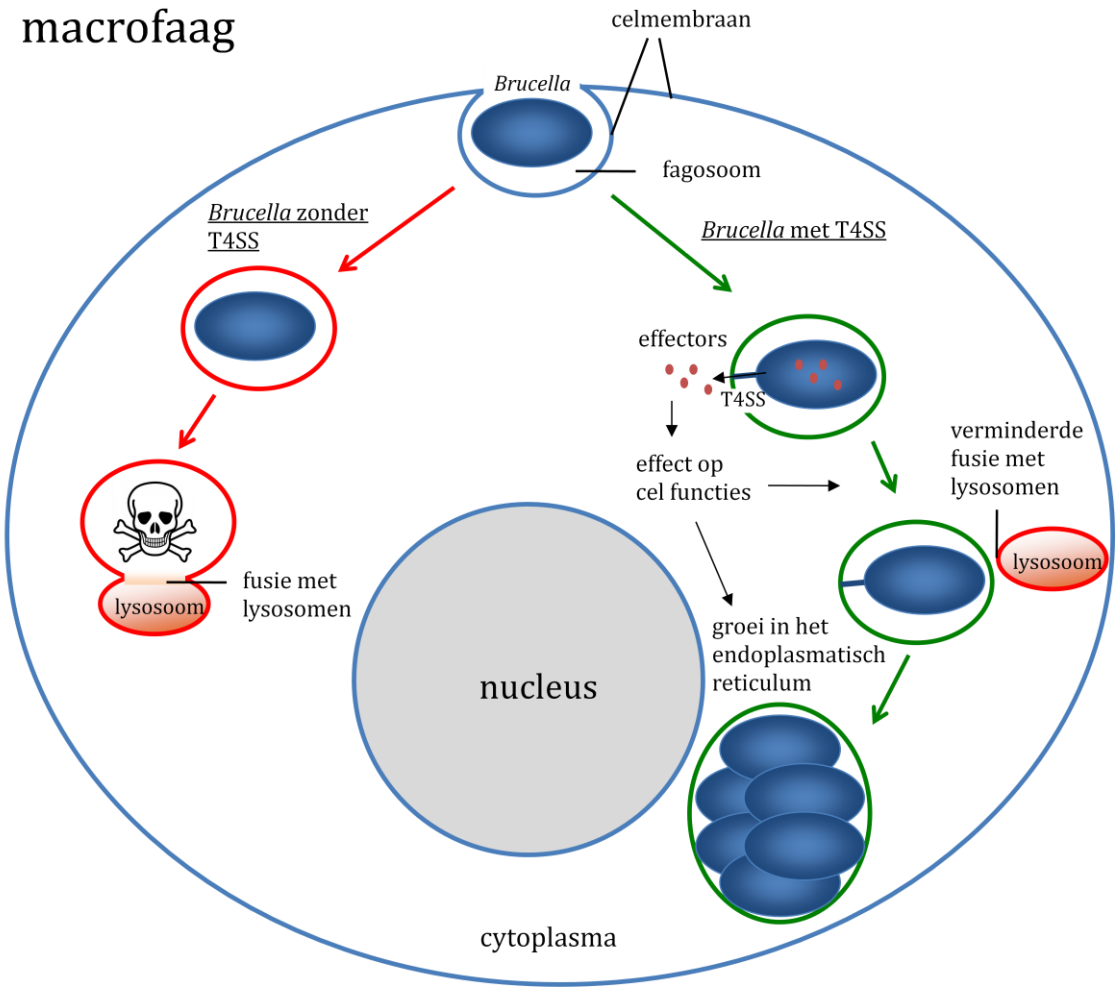
Nederlandse samenvatting

Zoönosen zijn ziektes die van dieren op de mens overdraagbaar zijn. Een van de meest voorkomende zoönotische infecties is Brucellose. Dit is een wereldwijd voorkomende ziekte van zoogdieren, die wordt veroorzaakt door *Brucella* bacteriën. Hoewel mensen niet de natuurlijke gastheren zijn voor de *Brucella* bacteriën komt infectie van mensen toch in veel landen voor. Het bacteriegeslacht *Brucella* is vernoemd naar de Schotse legerchirurg en microbioloog Sir David Bruce, die de bacterie voor het eerst isoleerde uit de milt van een patiënt met brucellose in Malta in 1886. Er zijn inmiddels verschillende *Brucella* soorten bekend, die zijn geklassificeerd naar hun natuurlijke gastheer, waaronder *B. melitensis* (geiten en schapen), *B. abortus* (runderen), *B. suis* (varkens), *B. ovis* (schapen), *B. canis* (honden), *B. neotomae* (woestijnratten), *B. microti* (woelmuizen), *B. ceti* (dolfijnen en walvissen) en *B. pinnipedialis* (zeehonden).

Het onderzoek dat beschreven is in dit proefschrift werd voornamelijk uitgevoerd met *B. abortus*. Brucellose in de mens wordt vooral veroorzaakt door *B. melitensis* en *B. abortus.* In de meeste gevallen krijgen mensen brucellose door de consumptie van ongepasteuriseerde zuivelproducten van besmette geiten of runderen. Direct contact met besmette geiten, schapen, runderen, varkens of honden is ook een belangrijke route van transmissie van diverse *Brucella* soorten naar de mens. *Brucella* infecties in mensen worden gekenmerkt door veel niet-specifieke symptomen, waardoor een accurate diagnose vaak moeilijk is. De symptomen zijn onder andere koorts, koude rillingen, nachtzweten, verlies van gewicht en kracht, hevige hoofdpijn en een vergrote milt. De ziekte kan vele jaren aanhouden, waarbij periodes van koorts en herstel elkaar vaak afwisselen. Zonder behandeling met antibiotica kunnen infecties met *Brucella* lang blijven voortduren, doordat *Brucella* soorten goed in staat zijn zich tegen het immuunsysteem van de gastheer te verweren. Dit is van cruciaal belang voor de *Brucella* bacteriën, omdat ze langdurig in de gastheer aanwezig moeten zijn voor hun transmissie naar andere gastheren. De belangrijkste route van transmissie van *Brucella* bij dieren verloopt namelijk via het veroorzaken van abortus van een foetus tijdens de dracht. Dit is een relatief infrequente gebeurtenis in

het leven van een dier en dientengevolge dienen de *Brucella* bacteriën zich langdurig in hun gastheer te handhaven. De *Brucella* bacteriën bevinden zich tijdens infectie van de gastheer zelfs voornamelijk in cellen van het immuunsysteem, waaronder macrofagen en dendritische cellen. *Brucella* bacteriën zijn daardoor ook voornamelijk aan te treffen in organen met veel van deze cellen zoals lymfeklieren, de milt, het beenmerg en de lever.

Macrofagen (Grieks voor grote eters) zijn witte bloedcellen, die gespecialiseerd zijn in het opnemen en vernietigen van vreemde microbiële indringers ter voorkoming van ziekte. Macrofagen doden opgenomen bacteriën door het intracellulaire compartiment, fagosoom genaamd, waarin de opgenomen bacteriën zich bevinden te laten fuseren met zogenaamde lysosomen. Lysosomen zijn intracellulaire compartimenten van macrofagen, die een hoge zuurgraad hebben en enzymen bevatten die bacteriën kunnen afbreken. Macrofagen zijn helaas niet goed in staat om opgenomen *Brucella* bacteriën te doden. Deze bacteriën zijn namelijk in staat om de fusie van de fagosomen, waarin ze zijn opgenomen, met lysosomen te verhinderen. Bovendien zorgen ze ervoor dat de fagosomen eigenschappen krijgen van het endoplasmatisch reticulum (ER), een ander intracellular compartiment van macrofagen. In het ER zijn *Brucella* bacteriën in staat voedingsstoffen te verkrijgen, waardoor ze zich goed in dit compartiment kunnen vermenigvuldigen (Figuur 1).



Figuur 1. Schematische weergave van een macrofaag en het lot van *Brucella* na opname door deze macrofaag. *Brucella* zonder een Type 4 secretiesysteem (T4SS) kan niet groeien in de macrofagen en wordt gedood. *Brucella* met een actief T4SS injecteert effector-eiwitten in het cytoplasma van de macrofaag, die er waarschijnlijk voor zorgen dat *Brucella* een veilige 'haven' in het endoplasmatisch reticulum kan opzoeken. Hier kan de bacterie zich vervolgens vermenigvuldigen. *Brucella* injecteert de effector-eiwitten via een kanaal rechtstreeks vanuit zijn eigen cytoplasma in het cytoplasma van de macrofaag. Dit kanaal wordt gevormd door het T4SS en het overspant de bacteriële cytoplasmamembraan, de bacteriële buitenmembraan en de membraan van het compartiment (groen), dat uit het fagosoom is ontstaan. Uiteindelijk leidt dit er toe dat deze vacuole gaat lijken op of fuseert met het Endoplasmatisch Reticulum (ER), waarin de *Brucella* bacteriën zich ongehinderd kunnen vermenigvuldigen.

Brucella bacteriën gebruiken verschillende virulentiefactoren om in cellen van hun gastheer te kunnen overleven, te groeien en zich te vermenigvuldigen, zodat ze een langdurige infectie in deze gastheer kunnen veroorzaken. Een van

deze factoren is het VirB type IV secretie systeem (T4SS). Dit systeem wordt gevormd door een complex van 12 eiwitten in de celenvelop van *Brucella.* Zonder het T4SS systeem kan deze bacterie niet overleven in cellen van zijn gastheer. Een mutant van *Brucella*, die een functioneel T4SS mist, kan daardoor geen ziekte veroorzaken in geïnfecteerde proefdieren, zoals muizen. Tijdens infectie van cellen zoals macrofagen, injecteert het T4SS eitwitten (ook wel effectors genoemd) vanuit het cytoplasma van een *Brucella* bacterie rechtstreeks in het cytoplasma van de gastheercel (zie Figuur 1). De combinatie van de verschillende effector-eiwitten is waarschijnlijk verantwoordelijk voor het groeibevorderende effect van het T4SS op *Brucella* in gastheercellen. Door de functie van deze effectors afzonderlijk te bestuderen is het mogelijk om uit te zoeken hoe verschillende functies van de gastheercel gemanipuleerd worden door de *Brucella* bacterie.

Voordat het in dit proefschrift beschreven onderzoek begon, waren de effectors die *Brucella* in geïnfecteerde cellen injecteert nog onbekend. Het experimentele gedeelte van dit proefschrift begint dan ook met de identificatie van de eerste twee *Brucella* effector-eiwitten, VceA en VceC (**hoofdstuk 2**). Deze effector-eiwitten werden gevonden door te zoeken naar genen, die co-gereguleerd worden met de *virB* genen, die coderen voor het T4SS. De hypothese achter deze methode was dat *Brucella* waarschijnlijk de genen, die coderen voor het T4SS, rond dezelfde tijd (namelijk na infectie van een macrofaag) zal activeren als de genen die coderen voor effector-eiwitten. De identificatie van het effector-eiwit VceC resulteerde vervolgens in de identificatie van een derde effector, VceB (**hoofdstuk 4**). Dit nieuwe effector-eiwit kon gevonden worden doordat eigenschappen in het VceC eiwit gebruikt konden worden om te zoeken naar andere *Brucella* eiwitten, die dezelfde eigenschappen bezitten. Dit betrof onder andere de aanwezigheid van een eiwitdomein (een transmembraan-domein), dat het eiwit kan gebruiken om zich te verankeren in de membraan van een gastheercel. Ook bevatte VceB net als VceC een domein met een groot aantal proline aminozuren. De functie van dit domein is nog niet precies opgehelderd, maar het zou door de effector gebruikt kunnen worden om interacties aan te gaan met andere eiwitten van de gastheercel. Na de identificatie van de effector-

eiwitten volgde de karakterisering van de intracellulaire functies van VceB en VceC (**hoofdstukken 3 en 4).**

Door verschillende experimenten kon aangetoond worden dat zowel VceB als VceC gelocaliseerd zijn in het endoplasmatisch reticulum van gastheercellen. Dit zijn interessante resultaten, omdat dit het compartiment van de gastheercel is waarin *Brucella* zich vermenigvuldigt. Ook zijn de gastheereiwitten waarmee VceB en VceC interacties aangaan bestudeerd en de verkregen resultaten suggereren, dat zowel VceB als VceC waarschijnlijk door *Brucella* gebruikt worden om de immuunrespons van de geïnfecteerde gastheer te manipuleren. Meer onderzoek is echter nodig om de precieze bijdrage van deze effector-eiwitten aan het infectieproces van *Brucella* in individuele gastheercellen en het geïnfecteerde gastheerorganisme op te helderen.