An essential virulence protein of *Brucella abortus*, VirB4, requires an intact nucleoside-triphosphate-binding domain

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***Brucella abortus* is a facultative intracellular bacterium capable of surviving inside macrophages. The VirB complex, which is highly similar to conjugative DNA transfer apparatuses, is required for intracellular replication. A conserved NTP-binding domain in VirB4 suggests that one or both proteins couple energy by NTP hydrolysis to transport of putative effector molecule(s). Here it is shown that a mutant strain of *B. abortus* that contains an in-frame deletion in *virB4* is unable to replicate in macrophages and survives in mice. Intracellular replication and virulence in mice are fully restored by expressing *virB4* in trans, indicating that VirB4 is essential for intracellular replication and virulence in mice. An alteration within the NTP-binding region of VirB4 by site-directed mutagenesis abolished complementation of a *virB4* mutant, demonstrating that an intact NTP-binding domain is critical for VirB4 function. Intracellular replication was inhibited in wild-type *B. abortus* after introducing a plasmid expressing a mutant VirB4 altered in the NTP-binding region. The dominant negative phenotype suggests that VirB4 either functions as a multimer or interacts with some other component(s) necessary for intracellular replication. Wild-type *B. abortus*-containing phagosomes lack the glycoprotein LAMP-1, which is an indicator of the normal endocytic pathway. Mutant strains were found in phagosomes that co-localized with LAMP-1, indicating that VirB4 containing the intact NTP-binding region is essential for evasion of fusion with lysosomes.**

Keywords: type IV secretion, macrophage

INTRODUCTION

Brucella spp. are Gram-negative bacteria that cause abortion and infertility in numerous domestic and wild mammals, and a disease known as undulant fever in humans (Acha & Szylres, 1980). The bacterium is endemic in many underdeveloped countries and is responsible for large economic losses and chronic infections in human beings (Zavala *et al.*, 1994). *Brucella* spp. are facultative intracellular pathogens that survive in a variety of cells, including macrophages, and their virulence and ability to cause chronic infections are thought to be due to their ability to avoid the killing mechanisms within macrophages (Baldwin & Winter, 1994; Sangari & Aguero, 1996). The molecular mechanisms of their virulence and infection are incompletely understood. Studies with HeLa cells have confirmed the observations that *Brucella* inhibits phagosome-lysosome fusion and transits through an intracellular com-

partment that resembles an autophagosome. Bacteria replicate in a different compartment, containing protein markers normally associated with the endoplasmic reticulum, as shown by confocal microscopy and immunogold electron microscopy (Comerci *et al.*, 2001; Pizarro-Cerda *et al.*, 1998a, b).

Genetic loci encoding export mechanisms specializing in transferring a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells have been described. These complexes, named type IV secretion systems, have been reported in organisms such as *Agrobacterium tumefaciens* (*virB* genes) (Kulda *et al.*, 1990; Stachel & Nester, 1986), *Bordetella pertussis* (*ptl* genes) (Kotob *et al.*, 1995; Weiss *et al.*, 1993), *Escherichia coli* (*tra* genes) (Pohlman *et al.*, 1994; Winans & Walker, 1985), *Legionella pneumophila* (*dot/icm* genes) (Segal *et al.*, 1998; Vogel *et al.*, 1998) and *Helicobacter pylori* (*cag* genes) (Covacci *et al.*,

1999). Recently, the *virB* operon of *Brucella* has been identified (O'Callaghan *et al.*, 1999; Sieira *et al.*, 2000). This operon comprises 13 ORFs, designated *virB1* to *virB11*, *orf12* and *orf13*, that share homology with components of other bacterial type IV secretion systems involved in the intracellular trafficking of pathogens. Polar mutations introduced in the first gene of the operon, *virB1*, abolish the ability of *Brucella* to replicate intracellularly, indicating that this system is essential for the intracellular lifestyle of this pathogen. Mice infected with polar and non-polar mutations in *virB10* demonstrated that the *virB* operon is a major determinant of *Brucella* virulence (Sieira *et al.*, 2000). Thus, *Brucella abortus* VirB proteins are thought to be constituent elements of the secretion apparatus, but their specific molecular functions are unknown.

Two *virB* proteins, VirB4 and VirB11, contain the putative NTP-binding site (Sieira *et al.*, 2000) first described by Walker *et al.* (1982). Nucleotide-binding proteins might have several roles in the secretion process, including providing energy for transport or signalling the opening of a gate or channel by a kinase activity. In this study, we examined one of these putative nucleotide-binding proteins, VirB4, to determine its importance in intracellular replication and the role that the putative NTP-binding site might play in the *Brucella* virulence.

METHODS

Bacterial strains and media. All *Bru. abortus* derivatives are from strain 544, which is a smooth virulent *Bru. abortus* biovar 1 strain (Table 1). *Bru. abortus* strains were maintained

as frozen glycerol stocks and were cultured on Brucella broth (BBL) or Brucella broth containing 1.5 % agar.

Initial isolation of replication-proficient plasmids was from *E. coli* strain DH5 α . For the propagation of suicide plasmids requiring R6K π protein, *E. coli* strain DH5 α (λ pir) (Kolter *et al.*, 1978) was used. Kanamycin was used at 40 μ g ml⁻¹.

Construction of in-frame deletion mutant of *virB4*. pMAW14 (Δ *virB4*) was constructed by cloning two PCR fragments into *Sal*I/*Sac*I-cleaved pSR47s (Andrews *et al.*, 1998). Fragment 1 was a 1640 bp *Sal*I–*Bam*HI fragment spanning a site located 1620 nt upstream of the 5' end of *virB4* to 20 nt downstream from the 5' end and was amplified by PCR using primers 5'-GTCGACCAAGTCAACAGGTACAATCTG-3' (*Sal*I site underlined) and 5'-GGATCCGCCCATTTATGATTCTCTT-TTG-3' (*Bam*HI site underlined) (nucleotide positions 690 and 2290 in GenBank accession no. AF226278, respectively; Sieira *et al.*, 2000). Fragment 2 was a 1617 bp *Bam*HI–*Sac*I fragment spanning the region starting 6 nt upstream of the 3' end of *virB4* to a position 1611 nt downstream from the 3' end and was amplified using primers 5'-GGATCCAGGTGAC-ACTATGAAGAAGAT-3' (*Bam*HI site underlined) and 5'-GAGCTCGCGGCTGAAGATTGCACTCGA-3' (*Sac*I site underlined) (nucleotide positions 4785 and 6385 in AF226278, respectively; Sieira *et al.*, 2000).

pMAW14 (Δ *virB4*) was introduced into DH5 α (λ pir), and subsequently the plasmid was transferred into *Bru. abortus* 544 by using electroporation (Gene Pulser; Bio-Rad). Cells were spread onto Brucella agar containing 5 % sucrose and incubated at 37 °C to isolate recombinants in which the entire plasmid integrated into the appropriate chromosomal site. The resulting sucrose-resistant colonies were tested for kanamycin sensitivity which indicates loss of the suicide vector. The kanamycin-sensitive colonies thus selected were analysed to confirm that in-frame deletion had occurred in the *virB4* gene by PCR amplification.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and relevant characteristic(s)	Source or reference
<i>Bru. abortus</i>		
544	Biovar 1 virulent strain	ATCC 23448
Ba598	544 Δ <i>virB4</i>	This study
Ba603	Ba598(pMAW15)	This study
Ba615	544(pMAW18)	This study
Ba616	Ba598(pMAW18)	This study
Ba620	544(pMAW15)	This study
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> AM15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Woodcock <i>et al.</i> (1989)
DH5 α λ pir	DH5 α (λ pir) <i>tet::Mu</i> <i>recA</i>	Kolter <i>et al.</i> (1978)
Plasmids		
pSR47s	<i>oriTRP4</i> <i>oriR6K</i> <i>kan</i> <i>sacB</i>	Andrews <i>et al.</i> (1998)
pMAW14	pSR47s Δ <i>virB4</i>	This study
pBBR1MCS-2	Broad-host-range cloning vector	Kovach <i>et al.</i> (1995)
pMAW15	pBBR1MCS-2 <i>virB4</i> ⁺	This study
pKF19k	Vector for site-directed mutagenesis	TAKARA
pMAW16	pKF19k <i>virB4</i> ⁺	This study
pMAW17	pKF19k <i>virB4</i> K463R	This study
pMAW18	pBBR1MCS-2 <i>virB4</i> K463R	This study

Fig. 1. Genetic map of the *virB4* flanking region and alteration of the putative nucleoside-binding region of VirB4. (a) Construction of an in-frame deletion mutation is shown for $\Delta virB4$. Nucleotides are numbered as described previously by Sieira *et al.* (2000). (b) The consensus Walker box A region is shown with the corresponding region from VirB4 (aa 457–464). The K463R alteration made by site-directed mutagenesis is underlined.

pMAW15 (*virB4*⁺) was constructed by cloning a PCR fragment into *EcoRI*/*Bam*HI-cleaved pBBR1MCS-2 (Kovach *et al.*, 1995). The 2786 bp *EcoRI*–*Bam*HI PCR fragment spanned a site located 270 nt upstream of the 5′ end of *virB4* to a position 20 nt downstream from the 3′ end (Sieira *et al.*, 2000) and was amplified using the primers 5′-GAATTCAGCCATGTTGTTGGGGTTCC-3′ (*EcoRI* site underlined) and 5′-GGATCCAGAATTATCTTCTTCATAGTG-3′ (*Bam*HI site underlined).

Construction of a point mutation in the NTP-binding domain of VirB4. A point mutation was made in the coding sequence (AAA; 1387–1389) for the invariant Lys₄₆₃ residue (K463) within the Walker-type NTP-binding motif of VirB4 (Fig. 1). pMAW16 was constructed by cloning a 2786 bp *EcoRI*–*Bam*HI fragment from pMAW15 into *EcoRI*/*Bam*HI-cleaved pKF19k for site-directed mutagenesis (TAKARA). The coding sequence for Lys₄₆₃ (AAA) was mutated to encode Arg (AGA) using the site-directed mutagenesis system Mutan-Express Km (TAKARA) and mutagenic oligonucleotide 5′-CAGGACAGTTCTACCAGCGCC-3′ which includes the codon that alters Lys₄₆₃ to Arg (K463R). The point mutation within this region of *virB4* was confirmed by DNA sequence analysis and the resultant plasmid was designated pMAW17. pMAW18 (*virB4* K463R) was constructed by cloning a 2786 bp *EcoRI*–*Bam*HI fragment from pMAW17 into *EcoRI*/*Bam*HI-cleaved pBBR1MCS-2.

Cell culture. Bone-marrow-derived macrophages from female BALB/c mice were prepared as described by Watarai *et al.* (2001). After culturing in L-cell conditioned medium, the macrophages were replated for use by lifting cells in PBS on ice for 5 to 10 min, harvesting by centrifugation and resuspending in RPMI 1640 containing 10% fetal bovine serum. The mouse macrophage-like cell line J774 was maintained in RPMI 1640 containing 10% fetal bovine serum. The macrophages were seeded (2×10^5 – 3×10^5 per well) in 24-well tissue culture plates for all assays.

Determination of efficiency of intracellular growth of bacteria. Bacteria were deposited onto macrophages at an m.o.i. of 20 by centrifugation at 150 g for 5 min at room temperature and were then incubated at 37 °C in 5% CO₂ for 1 h. The macrophages were then washed once with medium and incubated with 30 µg gentamicin ml⁻¹. At different time points, cells were washed and lysed with 1% Triton X-100 and the number of bacteria was counted in plates of a suitable dilution.

Detection of bacteria in macrophages. Mouse bone-marrow-derived macrophages were infected with *Bru. abortus* as described above. Infected monolayers were washed extensively with fresh medium to remove non-cell-associated *Bru. abortus*. Infected cells were fixed in periodate/lysine/paraformaldehyde (PLP) (McLean & Nakane, 1974) containing 5% sucrose for 30 min at 37 °C, washed three times in PBS and permeabilized in 0.1% Triton X-100 for 30 min at room temperature. Samples were then washed three times in blocking buffer (2%, v/v, goat serum in PBS) for 5 min and stained with anti-*Bru. abortus* rabbit serum (diluted 1:1000) in blocking buffer for 1 h at 37 °C. To visualize antibodies, samples were washed three times in blocking buffer, incubated with FITC-conjugated goat anti-rabbit IgG (Zymed) diluted 1:500 in blocking buffer for 1 h at 37 °C and visualized in mounting medium (90% glycerol containing 1 mg phenylenediamine ml⁻¹ in PBS, pH 9.0).

LAMP-1 staining. Infected macrophages were fixed in PLP-sucrose for 1 h at 37 °C. Samples were washed three times in PBS and wells were successively incubated three times for 5 min in blocking buffer (2% goat serum in PBS) at room temperature. All antibody-probing steps were carried out for 1 h at 37 °C in a humidified incubator. After blocking, samples were stained with anti-*Bru. abortus* polyclonal rabbit serum diluted 1:1000 in blocking buffer to identify extracellular bacteria. Samples were washed three times for 5 min with blocking buffer, stained with Cascade-Blue-conjugated goat anti-rabbit IgG diluted 1:500 in blocking buffer and incubated as above. Samples were washed three times in PBS for 5 min and then permeabilized in –20 °C methanol for 10 s. After incubating three times for 5 min with blocking buffer, samples were stained with anti-LAMP-1 rat mAb ID4B (obtained from Developmental Studies Hybridoma Bank of the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, USA, and the Department of Biology, University of Iowa, Iowa City, IA, USA) diluted 1:100 in blocking buffer (Swanson & Isberg, 1996). After washing three times for 5 min in blocking buffer, samples were stained simultaneously with TRITC-conjugated goat anti-rat IgG (Molecular Probes). Samples were placed in mounting medium and visualized by fluorescence microscopy.

Fluorescence microscopy. The specimens were analysed using an Olympus IX70 inverted phase microscope and the images were collected using a cooled CCD camera (CoolSNAP; Roper Scientific) and processed using Openlab software (Improvision) on a Power Macintosh G4 computer.

Virulence in mice. Virulence was determined by quantifying the survival of the strains in the spleen after 10 days. Six-week-old female BALB/c mice were injected intraperitoneally with approximately 10⁴ c.f.u. *brucellae* in 0.1 ml saline. Groups of five mice were injected with each strain. At 10 days post-infection the mice were sacrificed by decapitation and their spleens were removed, weighed and homogenized in saline. Tissue homogenates were serially diluted with PBS and plated on *Brucella* agar to count the number of c.f.u. in each spleen.

RESULTS

Effect of alteration of the putative NTP-binding region of VirB4 on intracellular replication

To investigate the role of the *virB4* gene in intracellular replication in macrophages, an in-frame deletion mutation was constructed in the *virB4* gene. This deletion mutation was designated $\Delta virB4$ and the mutant was designated Ba598 (Fig. 1a). As survival and multipli-

Fig. 2. Intracellular replication of *Bru. abortus* strains in J774 cells and mouse bone-marrow-derived macrophages. J774 cells (a) and mouse bone-marrow-derived macrophages (b) were infected with 544 (wild-type, □), Ba598 ($\Delta virB4$, ◇), Ba603 ($virB4^+$, ○) or Ba616 ($virB4$ K463R, △) for the indicated time. Data points and error bars represent the mean c.f.u. of triplicate samples from a typical experiment (performed at least four times) and their standard deviation.

cation in macrophages is an important virulence attribute of *Brucella*, we examined the intracellular replication of *Brucella* strains in two mouse macrophage models, using macrophage-like cell line J774 and bone-marrow-derived macrophages. J774 or mouse bone-marrow-derived macrophages were infected for various periods at an m.o.i. of 20. The results show that Ba598 ($\Delta virB4$) failed to replicate in both J774 and bone-marrow-derived macrophages (Figs 2 and 3). The mutation was complemented *in trans* by introducing plasmid pMAW15, which carries a wild-type copy of *virB4* (Ba603). Introduction of the complementing plasmid into Ba598 ($\Delta virB4$) restored replication to 544 (wild-type) levels (Fig. 2).

To determine whether the putative NTP-binding region of VirB4 plays a role in intracellular replication, we used site-directed mutagenesis to specifically alter *virB4* such that the encoded Lys₄₆₃ located in the Walker box A region of the protein is changed to an Arg residue (Fig.

1b). We introduced a plasmid, pMAW18, containing full-length *virB4* with that specific mutation into Ba598 ($\Delta virB4$) and the transformant was designated Ba616 (*virB4* K463R). Ba616 failed to restore replication to 544 (wild-type) levels (Fig. 2). These results implied that the putative NTP-binding region of VirB4 is necessary for intracellular replication of *Brucella*.

The VirB proteins have been proposed to interact with other VirB proteins to form a multicomponent membrane apparatus which can mediate export of putative substrate(s) (Christie, 2001). If VirB4 constitutes one of the transporter subunits, or if the protein functions as a multimer, then a merodiploid expressing both the wild-type and mutant allele may differ phenotypically from 544 (wild-type). To test this possibility, we introduced pMAW18 (*virB4* K463R) into the wild-type strain (Ba615) and then examined the ability of Ba615 to replicate in macrophages. Bone-marrow-derived macrophages were infected for 48 h at an m.o.i. of 20. Introducing a control plasmid containing either no *virB4* or a wild-type copy of *virB4* did not affect intracellular replication ($5.1 \times 10^5 \pm 0.5 \times 10^5$ or $5.4 \times 10^5 \pm 0.8 \times 10^5$ c.f.u., respectively). In contrast, introducing a plasmid containing the mutated *virB4* into 544 (wild-type) resulted in greatly reduced intracellular replication ($7.6 \times 10^4 \pm 0.7 \times 10^4$ c.f.u.). These data indicated that the *virB4* K463R mutant exhibits a dominant negative phenotype.

The putative NTP-binding region of VirB4 is required for inhibition of phagosome–lysosome fusion by *Bru. abortus* early in infection

Phagosomes containing virulent *Bru. abortus* are reluctant to fuse with lysosomes, whereas dead *Bru. abortus* phagosomes co-localize with endocytic compartments in the early stage of infection in macrophages (Arenas *et al.*, 2000). To test the ability of *Bru. abortus* to target properly within bone-marrow-derived macrophages early in infection, interaction of the mutants with the endocytic pathway was quantified by immunofluorescence localization of LAMP-1, a membrane protein of late endosomes and lysosomes (Chen *et al.*, 1988; Harter & Mellman, 1992).

As expected, most phagosomes containing 544 (wild-type) did not co-localize with the late endosomal and lysosomal marker: $14.3 \pm 3.2\%$ of the wild-type phagosomes were LAMP-1-positive (Fig. 4). In contrast, phagosomes containing Ba598 ($\Delta virB4$) with severe intracellular growth defects were frequently stained brightly by an antibody specific for LAMP-1 ($74.6 \pm 5.3\%$ LAMP-1-positive) (Fig. 4). Introduction of the complementing plasmid into Ba598 ($\Delta virB4$) restored evasion of the endocytic pathway to 544 (wild-type) levels ($17.0 \pm 2.9\%$ LAMP-1-positive). To determine whether the putative NTP-binding region of VirB4 plays a role in intracellular trafficking, interaction of Ba616 (*virB4* K463R) with the endocytic pathway was quantified. Similar results were obtained for the *virB4* mutant: $76.1 \pm 2.9\%$ of the Ba616 (*virB4* K463R) cells resided in

Fig. 3. Immunofluorescence micrograph of intracellular replicated *Brucella abortus*. Mouse bone-marrow-derived macrophages were infected with 544 (wild-type; a, c) or Ba598 ($\Delta virB4$; b, d) for 48 h, as described in Methods. (a, b) FITC-labelled intracellular bacteria; (c, d) phase-contrast images.

Fig. 4. Co-localization of *virB4* mutant with late endosomal and lysosomal marker LAMP-1 in mouse bone-marrow-derived macrophages by immunofluorescence microscopy. Macrophages were infected with 544 (wild-type; a, c) or Ba598 ($\Delta virB4$; b, d) for 1 h, fixed and stained for LAMP-1 co-localization (a, b) and intracellular bacteria (c, d).

a LAMP-1-positive compartment. Introducing a control plasmid containing either no *virB4* or a wild-type copy of *virB4* did not affect LAMP-1 staining (12.5 ± 2.7 or 13.5 ± 2.1 %, respectively). In contrast, introducing a plasmid containing the mutated *virB4* into 544 (wild-type) resulted in 73.9 ± 2.3 % LAMP-1-positive phagosomes.

The putative NTP-binding region of VirB4 is essential for virulence in mice

Groups of five mice were injected intraperitoneally with 10^4 c.f.u. of *Brucella abortus* 544 (wild-type), Ba598 ($\Delta virB4$), Ba603 (*virB4*⁺) or Ba616 (*virB4* K463R). At 10

days post-inoculation, mice were sacrificed and their spleens were weighed and examined for *Brucella* proliferation. The number of viable bacteria recovered from the spleens of mice injected with 544 (wild-type) and Ba603 (*virB4*⁺) were $3.8 \times 10^7 \pm 1.4 \times 10^7$ and $8.6 \times 10^7 \pm 0.7 \times 10^6$ c.f.u. per spleen, respectively. On the other hand, no viable bacteria were recovered from mice injected with Ba598 ($\Delta virB4$) and Ba616 (*virB4* K463R), based on counting the number of c.f.u. in each spleen. The weights of the spleens of mice injected with Ba598 ($\Delta virB4$) (58.3 ± 5.2 mg) and Ba616 (*virB4* K463R) (57.2 ± 4.9 mg) were markedly lower than those of mice injected with 544 (wild-type) (271.0 ± 18 mg) and Ba603 (*virB4*⁺) (216.6 ± 11 mg). These results indicated that the

putative NTP-binding region is involved directly or indirectly in the splenomegaly typically associated with infection by wild-type *Bru. abortus*.

DISCUSSION

In this study, we have examined the importance of VirB4 for *Bru. abortus* intracellular replication and the functional significance of NTP binding or hydrolysis by this protein. VirB4 is a component of the VirB secretion apparatus that has a high sequence similarity to members of the type IV secretion system (Sieira *et al.*, 2000). In *A. tumefaciens*, a type IV secretion system participates in delivering oncogenic T-DNA from the bacterium to the plant cell, whereas in *Bor. pertussis* it participates in the secretion of pertussis toxin (Christie, 2001; Christie & Vogel, 2000). Recently, O'Callaghan *et al.* (1999) and Sieira *et al.* (2000) have described the presence of a *virB* region in *Brucella suis* and *Bru. abortus* which is involved in intracellular growth in macrophages. We found that VirB4 is a critical component of the VirB secretion apparatus, as an in-frame deletion mutant of *virB4* exhibited greatly impaired intracellular replication in the macrophage-like cell line J774 and mouse bone-marrow-derived macrophages. The fact that this mutation could be complemented by introducing a plasmid containing a wild-type copy of *virB4* confirms that this defect was due to a mutation in *virB4* rather than to a polar effect of the mutation.

We have also demonstrated the importance of the putative NTP-binding motif of *virB4* by specifically altering the region of *virB4* that encodes a Walker box A motif. To date, two other organisms, *A. tumefaciens* and *Bor. pertussis*, have been shown to have a functionally important NTP-binding domain. The intact NTP-binding domain of VirB4 is essential for *A. tumefaciens* virulence (Berger & Christie, 1993) and the NTP-binding domain of PtlC, a member of a set of proteins necessary for the secretion of pertussis toxin from *Bor. pertussis*, is essential for transport of pertussis toxin across bacterial membranes (Cook *et al.*, 1999). We have shown that alteration of Lys₄₆₃ to Arg in *Bru. abortus* VirB4 protein had a marked effect on intracellular replication in macrophages and virulence in mice. As this mutation is in the putative NTP-binding region of VirB4, our results suggest that this region is critical for VirB4 function and support the idea that NTP binding is also an important aspect of VirB4 function. Three families of putative ATPases are associated with type IV transfer systems: (1) the TraG family of coupling proteins, (2) homologues of *A. tumefaciens* VirB4 protein, and (3) homologues of the RP4 TrbB and of *A. tumefaciens* VirB11 proteins (Christie, 2001). These proteins are ubiquitous among the type IV systems and are sometimes present in two or more copies. Further studies have provided evidence for transmembrane topology, self-association and a structural contribution to channel formation that is independent of VirB4 ATPase activity. Based on these properties, this family of ATPases might transduce information, possibly in the form of ATP-induced conformational

changes, across the cytoplasmic membrane to extracytoplasmic subunits (Dang *et al.*, 1999). Additional work needs to be done to determine the function of VirB4, since introduction of a plasmid containing an NTP-binding domain mutation into a wild-type strain of *Bru. abortus* resulted in drastically reduced intracellular replication. Our finding of a dominant negative phenotype for this mutation suggests that the altered protein may interfere with the action of the wild-type protein. Our study also indicates that VirB4 might interact with another component of the putative secretion system, possibly another molecule of VirB4, another VirB protein or unidentified substrate(s). These findings provide important information towards understanding the mechanism underpinning VirB function.

The mechanism of virulence of *Brucella* spp. is not yet fully understood. *Brucella* spp. infect their hosts through mucosae and wounds and initially enter into professional phagocytes where they survive and reproduce (Liautard *et al.*, 1996). It is known that intracellular pathogens have developed a series of strategies to survive inside cells (Sinai & Joiner, 1997). Alteration of the normal process of phagosome maturation has been described for several micro-organisms, such as *Mycobacterium*, *Legionella* and *Chlamydia* (Sinai & Joiner, 1997). The intracellular survival of *Brucella* spp. has been documented for several cell types. *Bru. abortus* shows a different intracellular trafficking pattern between professional and non-professional phagocytes. Multiple observations have been reported that *Bru. abortus* is incorporated into phagosomes and remains in membrane-bound compartments until the host cell dies (Comerci *et al.*, 2001; Pizarro-Cerda *et al.*, 1998a, b). In non-professional phagocytes, *Brucella* is located in structures that resemble the endoplasmic reticulum (Pizarro-Cerda *et al.*, 1998a, b). Other evidence has indicated that *Brucella* is transported through the autophagic pathway before accumulating in the endoplasmic reticulum (Comerci *et al.*, 2001; Pizarro-Cerda *et al.*, 1998a, b). Macrophages are particularly important for the survival and spreading of *Brucella* during infection (Liautard *et al.*, 1996) and these autophagosomes are not observed in macrophages. Arenas *et al.* (2000) monitored the intracellular transport of *Bru. abortus* in macrophages and observed the kinetics of the fusion of phagosomes with preformed lysosomes labelled with colloidal gold particles by electron microscopy. In that study, phagosomes containing live *Bru. abortus* delayed fusion with lysosomes and newly endocytosed material was not incorporated into these phagosomes (Arenas *et al.*, 2000). In this study, we tested the ability of a wild-type strain, a $\Delta virB4$ mutant and a mutant of the putative NTP-binding motif of *virB4* to target properly within bone-marrow-derived macrophages early in infection. Bacterial phagosomes were scored for acquisition of the lysosomal glycoprotein LAMP-1, an abundant transmembrane protein found predominantly in late endosomes and lysosomes (Chen *et al.*, 1988; Harter & Mellman, 1992). Our results, together with previous

evidence, indicate that *Bru. abortus* prevents phagosome-lysosome fusion after uptake by macrophages.

This report shows that *Bru. abortus* VirB4 plays a critical role in intracellular growth and virulence in mice. Moreover, VirB4 may be a nucleotide-binding protein that interacts with other members of the VirB secretion apparatus to facilitate transport of unidentified substrate(s) across the bacterial membrane. Future investigations directed toward the clarification of the nature of the effector molecules may shed light on the molecular mechanism underlying the infection process of *Bru. abortus*.

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