

Differential Requirements for VirB1 and VirB2 during *Brucella abortus* Infection

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The *Brucella abortus* *virB* operon, encoding a type IV secretion system (T4SS), is required for intracellular replication and persistent infection in the mouse model. The products of the first two genes of the *virB* operon, *virB1* and *virB2*, are predicted to be localized at the bacterial surface, where they could potentially interact with host cells. Studies to date have focused on characterization of transposon mutations in these genes, which are expected to exert polar effects on downstream genes in the operon. In order to determine whether VirB1 and VirB2 are required for the function of the T4SS apparatus, we constructed and characterized nonpolar deletion mutations of *virB1* and *virB2*. Both mutants were shown to be nonpolar, as demonstrated by their ability to express the downstream gene *virB5* during stationary phase of growth in vitro. Both VirB1 and VirB2 were essential for intracellular replication in J774 macrophages. The nonpolar *virB2* mutant was unable to cause persistent infection in the mouse model, demonstrating the essential role of VirB2 in the function of the T4SS apparatus during infection. In contrast, the nonpolar *virB1* mutant persisted at wild-type levels, showing that the function of VirB1 is dispensable in the mouse model of persistent infection.

Brucella spp. are found in association with a large number of wild and domesticated animal species, where they can cause persistent infection and abortion. Zoonotic transmission of the organism to humans can lead to a chronic febrile disease known as brucellosis or Malta fever. A characteristic of both natural and zoonotic forms of the disease is extended survival of the organism in tissues of the reticuloendothelial system, such as the spleen, lymph nodes, and bone marrow.

In a mutant screen for *Brucella abortus* virulence factors required for survival in the murine reticuloendothelial system, the *virB* operon, encoding homologs of *Agrobacterium tumefaciens* and *Bordetella pertussis* type IV secretion systems (24), was found to be essential for persistence in mice (18). The importance of these genes for intracellular survival was demonstrated in tissue culture models of infection as well (12, 15, 20, 24, 30). These genes were subsequently found to be expressed intracellularly by *Brucella suis* in macrophages (8), where they are required for localization of *B. abortus* to an intracellular niche that is associated with the endoplasmic reticulum (9), the same location where *B. abortus* was earlier shown to replicate in nonprofessional phagocytes (13, 14, 27) and in the ruminant placenta (2, 23). For *B. abortus*, the *virB* genes have been implicated in the initial interactions between the bacterium and the host cell during entry into macrophages (33).

Based on studies performed with *A. tumefaciens*, both VirB1 and VirB2 are predicted to be accessible on the surface of *B. abortus*. VirB2 is predicted to form a pilus-like structure, and VirB1 is predicted to have two domains—a lytic transgly-

cosylase and a second domain that is released to the extracellular medium and remains associated with the bacterial surface (4, 10, 22). These findings for *A. tumefaciens* suggested that both VirB1 and VirB2 could mediate interactions of *B. abortus* with host cells.

Evidence for a lytic transglycosylase function for VirB1 from *Brucella* spp. was provided by Höppner et al., who showed that *B. suis virB1* could complement an *A. tumefaciens virB1* mutant for tumor formation and for extracellular localization of VirB2 and that the complementation required the lytic transglycosylase active site (19). A polar *virB1* mutation in *B. abortus*, as well as transposon mutations in *Brucella melitensis virB2* and in the *virB1-B2* intergenic region of *B. melitensis* and *B. abortus*, has been reported to be defective for intracellular survival in vitro (12, 30, 32). However, for all of these mutant strains, the expected polar effect of the insertions on expression of the downstream genes in the *virB* operon did not allow any conclusions as to whether VirB1 or VirB2 are required for infection by *Brucella* spp. We therefore constructed and characterized defined deletion mutations of *virB1* and *virB2* in order to determine whether VirB1 and VirB2 are required for intracellular persistence of *B. abortus* in both tissue culture and in the mouse model of brucellosis.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. *B. abortus* strain 2308 was used throughout this study. Strains were cultured on tryptic soy agar (TSA; Difco/Becton-Dickinson, Sparks, Md.) or in tryptic soy broth (TSB) at 37°C on a rotary shaker. Bacterial inocula for infection of mice were cultured on potato infusion agar, as growth on this medium is described to prevent the appearance of spontaneous rough mutants (1). Antibiotics, when required, were added at the following concentrations: carbenicillin, 100 mg/liter; kanamycin, 100 mg/liter; chloramphenicol, 5 to 30 mg/liter. All work with live *B. abortus* was performed at biosafety level 3.

Strain construction and recombinant DNA techniques. For construction of strain BA1143 carrying a *virB4::lacZYA* fusion (Table 1), a fragment correspond-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s) ^a	Source or reference
<i>B. abortus</i> strains		
2308	Wild type	B. Deyoe
BA1143	<i>virB4::lacZYA</i>	This work
ADH1	$\Delta virB2::Km$ (polar)	This work
ADH3	$\Delta virB2$ (nonpolar)	This work
ADH4	$\Delta virB1-12::Km$	This work
ADH5	$\Delta virB1::Km$ (polar)	This work
ADH6	$\Delta virB1$ (nonpolar)	This work
ADH8	Knock-in complementation of ADH3	This work
<i>E. coli</i> strains		
DH5 α	<i>endA 1 hsdR17</i> ($r_c^- m_c^-$) <i>supE44 thi-1 recA1 gyrA relA1</i> $\Delta(lacZYA-argF)U169$ <i>deoR</i> ($\phi 80$ <i>dlac</i> $\Delta[lacZ]M15$)	34
BL21 (DE3)	<i>F'ompT gal [dcm] [lon] hsdS_B</i> ($r_B^- m_B^-$) DE3	31
Plasmids		
pBluescript KS	ColE1, <i>bla</i>	Stratagene
pCR2.1-TOPO	TA cloning vector	Invitrogen
pUC4-KSAC	Carries Tn903 kanamycin resistance cassette	Pharmacia
pAV1.4	<i>virB219-477</i> and <i>virB1154-1562</i> cloned into pBluescript	This work
pAV1.5	<i>virB219-477</i> and <i>virB1154-1562</i> separated by kanamycin resistance cassette KSAC cloned into pBluescript	This work
pAV2.1	PCR products of <i>virB988-1562</i> and <i>virB1796-2458</i> cloned into pBluescript	This work
pAV2.2	PCR products of <i>virB988-1562</i> and <i>virB1796-2458</i> separated by kanamycin resistance cassette cloned into pBluescript	This work
pAS1.1	<i>sacB</i> cloned into pAV2.1	This work
pAS1.2	<i>sacB</i> cloned into pAV1.4	This work
pMR10	RK2, <i>oriV</i> + <i>trfA</i> , <i>oriT</i> , <i>nptI</i> , <i>lacZα</i>	R. M. Roop II
pEX18Ap	<i>sacB</i>	17
pHB11	<i>virB140-1967</i> cloned into pMR10	This work
pFUSE	<i>oriR6K</i> plasmid containing promoterless <i>lacZYA</i>	5
pBA1143	<i>virB1644-2457</i> cloned into pFUSE	This work
pAVT1.4	PCR products of <i>B. abortus</i> using primer pairs 98736F-99854R and 112902F-114022R separated by kanamycin resistance cassette cloned into pBluescript	This work

^a Cloned fragments of the *virB* operon are numbered according to the sequence found under GenBank accession number AF226278 (30). Primer pairs 98736F-99854R and 112902F-114022R were designed using the *B. suis* genome sequence.

ing to bp 1644 to 2457 of the *B. abortus virB* locus was amplified by PCR using primers *virB-1644F* and *virB-2457R* (Table 2) and cloned into pCR2.1 (Invitrogen). The fragment was excised with XbaI and SmaI and ligated into pFUSE (5) digested with the same enzymes to yield pBA1143. This plasmid was introduced by electroporation into *B. abortus* 2308 and a recombinant resistant to chloramphenicol was designated BA1143. Integration of pBA1143 into *B. abortus* chromosome II of BA1143 was confirmed by Southern blot (data not shown).

For construction of plasmids to generate marked and unmarked deletions of *virB1*, fragments upstream and downstream of *virB1* were amplified by PCR using primer pairs *virB-219F-virB-477R* and *virB-1154F-virB-1562R*. The resulting fragments were cloned into pCR2.1-TOPO. The fragment *virB219-477* was excised with XhoI and ClaI and cloned into pBluescript KS. The fragment *virB1154-1562* was excised with EcoRI and XbaI and cloned into pBluescript KS containing *virB219-477*, generating plasmid pAV1.4 (Table 1). The Tn903 kanamycin resistance gene was amplified from pUC4-KSAC (Pharmacia) with primers KSAC707F1 and KSAC1638R, digested with EcoRI and ClaI, and cloned into EcoRI/ClaI-digested pAV1.4 to yield pAV1.5 containing a deleted copy of *virB1* replaced with the Tn903 kanamycin resistance gene. Plasmid pAS1.2, containing an unmarked deletion of *virB1* and the counterselectable marker *sacB*, was constructed by amplification of the *sacB* gene from pEX18Ap (17) using primers SacB-F and SacB-R. The product was digested with XhoI and ligated into XhoI-digested pAV1.4.

For construction of plasmids to generate marked and unmarked deletions of *virB2*, fragments upstream and downstream of *virB2* were amplified by PCR using primer pairs *virB-988F-virB-1562R* and *virB-1796F-virB-2458R*. The resulting fragments were cloned into pCR2.1-TOPO. The fragment *virB988-1562* was excised with XhoI and ClaI and cloned into pBluescript KS. The fragment *virB1796-2458* was excised with EcoRI and XbaI and cloned into pBluescript KS containing *virB988-1562*, generating plasmid pAV2.1 (Table 1). The Tn903 kanamycin resistance gene was amplified from pUC4-KSAC (Pharmacia) with primers KSAC707F1 and KSAC1638R, digested with EcoRI and ClaI, and cloned into EcoRI/ClaI-digested pAV2.1 to yield pAV2.2 containing a deleted copy of

virB2 replaced with the Tn903 kanamycin resistance gene. Plasmid pAS1.1, containing an unmarked deletion of *virB2* and the counterselectable marker *sacB*, was constructed by amplification of the *sacB* gene from pEX18Ap using primers SacB-F and SacB-R. The product was digested with XhoI and ligated into XhoI-digested pAV2.1.

For construction of a deletion encompassing the entire *B. abortus virB* operon, plasmid pAVT1.4 was generated as follows: fragments upstream and downstream of the *virB* operon were amplified using the primer pairs 98736F-99854R (upstream) and 112902F-114022R (downstream), designed using the preliminary *B. suis* genome sequence at The Institute for Genomic Research. The fragments were cloned in pCR2.1-TOPO and then excised with XhoI/SalI (upstream) and PstI/XbaI (downstream). The fragments were cloned sequentially into pBluescript KS digested with the same enzymes. The Tn903 kanamycin resistance gene was cloned with PstI/SalI ends and inserted between the upstream and downstream fragments to generate pAVT1.4. This plasmid was introduced into *B. abortus* strain 2308 by electroporation, and a kanamycin-resistant, carbenicillin-sensitive recombinant was selected. The resulting strain, ADH4, contains a deletion from 167 bp upstream of *virB1* to 1,133 bp downstream of *virB12* and was confirmed by Southern blot analysis to lack the *virB1-B12* genes (data not shown). The plasmids constructed in this work are listed in Table 1, and the primers used in the construction of the plasmids are listed in Table 2. Plasmid DNA was isolated using ion-exchange columns from QIAGEN. Standard methods were used for Southern blotting, PCR, restriction endonuclease analyses, and ligation and transformation of plasmid DNA into *E. coli* (3). PCR products were cloned into pCR2.1-TOPO using a TOPO-TA cloning kit (Invitrogen).

Cell lines. The mouse macrophage-like cell line J774 (28) was cultured in Dulbecco's modified Eagle's medium (Gibco, Rockville, Md.) supplemented with 10% heat-inactivated fetal bovine serum, 1% nonessential amino acids, and 1 mM glutamine (DMEMsup). Since this cell line can lose the ability to generate an oxidative burst after passage in culture (B. Bloom, personal communication), we routinely tested J774 cultures for generation of oxygen radicals via the hexose

TABLE 2. Primers used in this work^a

Primer name	Sequence (restriction enzyme)	Fragment
<i>virB</i> -1644F	5'-GCTCTAGAGCGGATTCTACCTCACCTACTGC-3' (XbaI)	<i>virB2</i> -B4
<i>virB</i> -2457R	5'-TCCCCGGGCTCCTTGTCTGCTGCTCTCG-3' (SmaI)	<i>virB2</i> -B4
<i>virB</i> -219F	5'-CTCGAGTGAATAACCCGCATACCAC-3' (XhoI)	<i>virB1</i> upstream
<i>virB</i> -477R	5'-ATCGATGAGGACAAGGAATGGCAC-3' (ClaI)	<i>virB1</i> upstream
<i>virB</i> -1154F	5'-ACGGCGTAGTTGTTTTCTAACCC-3'	<i>virB1</i> downstream
<i>virB</i> -1562R	5'-TCTAGACGGTCTGCTTGTCTCAATGTCTATTC-3' (XbaI)	<i>virB1</i> downstream
<i>virB</i> -988F	5'-CTCGAGGCTGCCAGTAAAAAACGAC-3' (XhoI)	<i>virB2</i> upstream
<i>virB</i> -1562R	5'-ATCGATTCTGCTTGTCTCAATGTCTAT-3' (ClaI)	<i>virB2</i> upstream
<i>virB</i> -1796F	5'-CCATCGCCATCATCTGGTCCG-3'	<i>virB2</i> downstream
<i>virB</i> -2458R	5'-TCTAGATCTCCTTGTCTGCTGCTCG-3' (XbaI)	<i>virB2</i> downstream
KSAC707F1	5'-ATCGATTAACCAATTCTGATTAGA-3' (ClaI)	Kan cassette
KSAC1638R	5'-GAATTCGGTGTGTCTCAAAATCTCTG-3' (EcoRI)	Kan cassette
<i>virB</i> -5063F	5'-GAATTCTACGAAGCGGTGATGACGC-3' (EcoRI)	GST-VirB5 fusion
<i>virB</i> -5549R	5'-CTCGAGGTGTAGATGATGTGTTGCGTG-3' (XhoI)	GST-VirB5 fusion
<i>virB</i> -629F	5'-GGAATTCTCCCCAGCAAGAAGTTCG-3' (EcoRI)	GST-VirB1 fusion
<i>virB</i> -1330R	5'-CTCGAGTGGTGTGCGACTGCCTCTATC-3' (XhoI)	GST-VirB1 fusion
98736F	5'-CTCGAGGCTGCGATTTCCATTCTCC-3' (XhoI)	<i>virB</i> upstream
99854R	5'-GTCGACTCTTGCCGTCATAATAGCCTGAG-3' (Sall)	<i>virB</i> upstream
112902F	5'-CTGCAGAAAGCCAACCCCTCGC-3' (PstI)	<i>virB</i> downstream
114022R	5'-TCTAGACACCTGATCCACGGAGTG-3' (XbaI)	<i>virB</i> downstream
KSAC707F2	5'-GTCGACTAACAATTCTGATTAGA-3' (Sall)	Kan cassette
KSAC1638R2	5'-CTGCAGGTTGTGTCTCAAAATCTCTG-3' (PstI)	Kan cassette
SacB-F	5'-GAGCTCGCTGGCTTAATACTATGCGGCA-3' (XhoI)	<i>sacB</i>
SacB-R	5'-GAGCTCTTCAAACAGGAGGGCTGGAAG-3' (XhoI)	<i>sacB</i>

^a Nucleotides in primers are numbered according to position in the *B. abortus virB* locus as indicated by the sequence found under GenBank accession number AF226278 (30). Primers 98736F, 99854R, 112902F, and 114022R were designed using the *B. suis* genome sequence (26).

monophosphate shunt (oxidative burst) after induction with phorbol myristate acetate (Sigma) as described elsewhere (11).

For macrophage killing assays, 24-well microtiter plates were seeded with macrophages at a concentration of 1×10^5 to 2×10^5 cells/well in 0.5 ml of DMEMsup and incubated overnight at 37°C in 5% CO₂. For preparation of the inoculum, *B. abortus* strains were grown for 24 h and then diluted in DMEMsup, and about 5×10^7 bacteria in 0.01 ml of DMEMsup was added to each well of macrophages. The microtiter plates were centrifuged at $250 \times g$ for 5 min at room temperature in order to synchronize infection. Cells were incubated for 20 min at 37°C in 5% CO₂, free bacteria were removed by three washes with phosphate-buffered saline (PBS), and the zero time point was taken as described below. DMEMsup plus 50 µg of gentamicin per ml was added to the wells, and the cells were incubated at 37°C in 5% CO₂. After 1 h, the DMEMsup plus 50 µg of gentamicin/ml was replaced with medium containing 25 µg of gentamicin/ml. Wells were sampled at 0 and 24 or 48 h after infection by aspirating the medium, lysing the macrophages with 0.5 ml of 0.5% Tween 20, and rinsing each well with 0.5 ml of PBS. Viable bacteria were quantified by dilution in sterile PBS and plating on TSA. All experiments were performed independently in triplicate at least three times, and the standard error for each time point was calculated. The statistical significances of differences observed were analyzed by analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) test (25).

Infection of mice. Female BALB/c ByJ mice were obtained from the Jackson Laboratory (Bar Harbor, Maine) and used at 6 to 10 weeks of age. For infection experiments, groups of four or five mice were inoculated intraperitoneally with 0.2 ml of PBS containing 1×10^5 to 5×10^5 CFU of *B. abortus*. Infected mice were held in microisolator cages in a biosafety level 3 facility. At the appropriate time points, the mice were euthanized by CO₂ asphyxiation, and the spleens were collected aseptically at necropsy. The spleens were homogenized in 3 ml of PBS, and serial dilutions of the homogenate were plated on TSA containing antibiotics, as appropriate, for enumeration of CFU. The statistical significances of differences observed were analyzed by ANOVA followed by Tukey's HSD test. All animal experiments were approved by the Texas A&M University Laboratory Animal Care and Use Committee and conducted in accordance with institutional guidelines.

Generation of polyclonal antisera to VirB1 and VirB5. *virB1* and *virB5* genes were PCR amplified from *B. abortus* by use of the primer sets described in Table 2, cloned into pCR2.1 (Invitrogen), and subsequently cloned in frame with glutathione S-transferase (GST) in pGEX4T-1 (Pharmacia) using the enzymes EcoRI and BamHI. GST fusion proteins were overexpressed and purified according to standard protocols (3) and used to raise polyclonal antisera in rabbits at the Texas A&M University Laboratory Animal Research Resource facility. Nonspecific

antibodies to *Escherichia coli* proteins and GST were depleted from sera by incubation with killed *E. coli* Top10F'(pGEX4T-1) in which overexpression of GST had previously been induced with isopropyl-β-D-thiogalactopyranoside.

Detection of VirB proteins. *B. abortus* cultures inoculated in TSB to a starting optical density at 600 nm of 0.01 were incubated at 37°C with shaking at 200 rpm for 36 h. Bacteria were pelleted and resuspended in 1× Laemmli sample buffer at a concentration of 10^{10} CFU/ml and heated at 100°C for 5 min, and the total protein from 10^8 CFU (0.01 ml) was loaded into each well for separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21). Proteins were transferred to polyvinylidene difluoride membranes by electroblotting and were detected using polyclonal rabbit sera specific for VirB1 and VirB5, and goat anti-rabbit immunoglobulin G was conjugated to horseradish peroxidase. Horseradish peroxidase activity was detected with a chemiluminescent substrate (NEN).

RESULTS

Construction of marked and unmarked deletion mutations of *virB1* and *virB2*. In order to assess whether VirB1 and VirB2 are essential components of the *B. abortus* type IV secretion system (T4SS), we constructed marked and unmarked deletions in *virB1* and *virB2* by a two-step method. In the first step, the deletion mutation was marked with a kanamycin resistance cassette. In the second step, the kanamycin resistance determinant was deleted using sucrose counterselection. This strategy has the advantage over a one-step sucrose selection strategy that in each step gain or loss of antibiotic resistance can be monitored, facilitating screening for the desired recombinants from the background of sucrose-resistant, nonrecombinant colonies. For the first step in the mutation construction, plasmids pAV1.5, containing part of the *virB* locus, in which *virB1* was replaced by the kanamycin resistance cassette KSAC, and pAV2.2, in which *virB2* was replaced by KSAC, were constructed (see Materials and Methods for details). The resulting constructs pAV1.5 (pBluescriptKS-*virB1*::Km) and pAV2.2 (pBluescriptKS-*virB2*::Km) were introduced into *B. abortus* 2308 by electroporation (Table 1). Transformants were screened

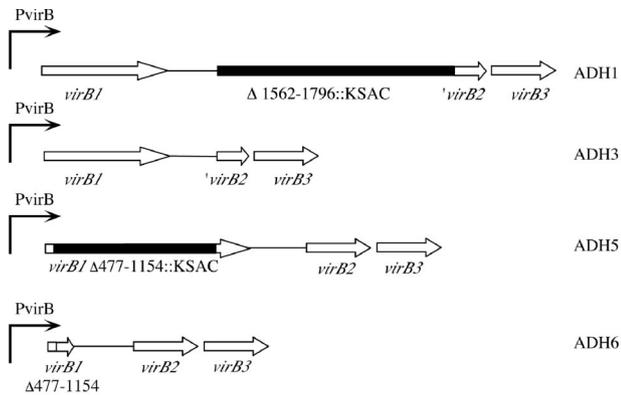
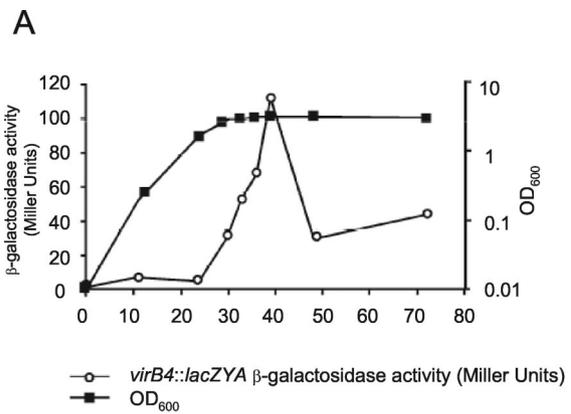


FIG. 1. Construction of marked and unmarked deletions of *virB1* and *virB2*. For details, see the text. The positions of deletions in the *B. abortus virB* locus are numbered according to the sequence found under GenBank accession number AF226278 (30).

for resistance to kanamycin and sensitivity to carbenicillin (the resistance determinant of pBluescript KS) to identify recombinants in which the allelic exchange had occurred. Kanamycin-resistant and carbenicillin-sensitive recombinants were tested for deletion of the *virB1* and *virB2* genes, respectively, by Southern blot analysis using probes containing the *virB1* and *virB2* genes (data not shown). The resulting marked deletion strains were designated $\Delta virB2::Km$ (ADH1) and $\Delta virB1::Km$ (ADH5). To delete the kanamycin resistance cassettes in these two strains, plasmids pAS1.1 and pAS1.2, carrying unmarked deletions of *virB1* and *virB2* and the *sacB* gene, were constructed and introduced into $\Delta virB2::Km$ (ADH1) and $\Delta virB1::Km$ (ADH5). Recombinants in which pAS1.1 and pAS1.2 had integrated into the *virB* locus were selected on carbenicillin and then subjected to sucrose counterselection in TSB containing sucrose. Sucrose-resistant recombinants were screened for loss of both the kanamycin resistance cassette and the *sacB* gene by screening for sensitivity to kanamycin and carbenicillin. Deletion of $\Delta virB1::Km$ and $\Delta virB2::Km$ was again confirmed by Southern blot analysis using probes containing *virB1* and *virB2* (data not shown). The resulting unmarked *virB1* and *virB2* deletion mutations (Fig. 1) were designated $\Delta virB2$ (ADH3) and $\Delta virB1$ (ADH6). The strains and plasmids constructed in this work are described in Table 1.

The $\Delta virB1$ and $\Delta virB2$ mutations do not eliminate expression of the downstream gene *virB5*. To test whether VirB1 and VirB2 are required for the function of the T4SS, it was first necessary to determine whether deletion of *virB1* and *virB2* affected the synthesis of proteins encoded by downstream genes. To this end, we raised polyclonal sera specific for VirB1 and VirB5, a product of a downstream gene in the *virB* operon, and determined in vitro conditions for synthesis of these proteins by *B. abortus*. Based on a previous finding that a *B. abortus virB10::lacZ* fusion was expressed in stationary phase (30), we performed an analysis of expression of the VirB1 and VirB5 proteins in wild-type *B. abortus* 2308 (Fig. 2). For this purpose, we constructed strain BA1143, containing a chromosomal *virB4::lacZYA* fusion, by introducing suicide vector pBA1143 into *B. abortus* 2308 and selecting for integration into *virB4* (Tables 1 and 2). We found that *virB* expression peaked after entry into stationary phase and then subsequently de-



B

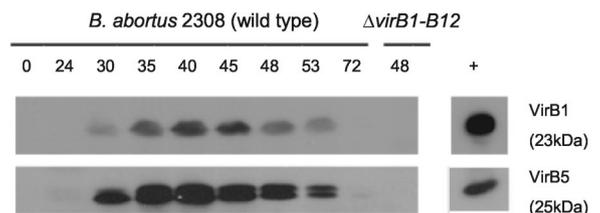


FIG. 2. Expression of the *virB* genes during growth of *B. abortus* in vitro. (A) Activity of a *virB4::lacZYA* fusion during growth in TSB. OD_{600} , optical density at 600 nm. (B) Western blot showing detection of VirB1 and VirB5 produced by *B. abortus* 2308 during growth in TSB at different times after inoculation. The positive controls shown at the right are GST-VirB1 and GST-VirB5.

clined. Analysis by Western blotting showed that VirB1 and VirB5 proteins could be detected starting at 30 h, with maximal levels detected between 35 and 40 h. Based on these findings, we used this time point to determine whether $\Delta virB1::Km$, $\Delta virB1$, $\Delta virB2::Km$, and $\Delta virB2$ exert polar effects on downstream *virB* genes. Figure 3 shows that while the marked *virB1* and *virB2* deletion mutations eliminate expression of the down-

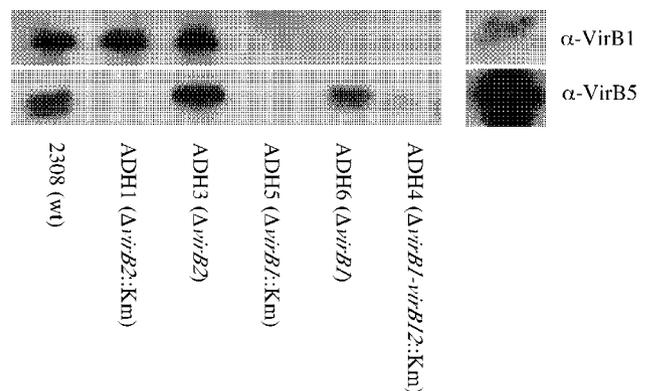


FIG. 3. Western blot showing detection of VirB1 and VirB5 produced by *B. abortus* mutants carrying marked or unmarked *virB1* or *virB2*. Marked mutations exert a polar effect on *virB5* expression, while VirB5 can still be detected in mutants carrying unmarked deletions. wt, wild type.

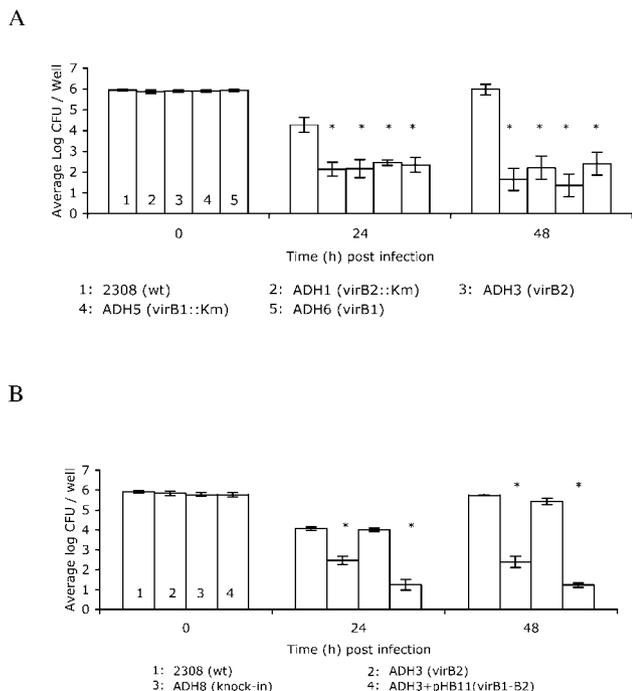


FIG. 4. (A) Survival in J774 macrophages of *B. abortus* mutants carrying marked or unmarked deletions of *virB1* or *virB2*. Data points represent the means of results from three independent experiments performed in triplicate \pm standard error of the mean. (B) Complementation of intracellular survival defect of the nonpolar *virB2* mutant by knock-in of *virB2*. Asterisks indicate significant ($P < 0.05$) differences in numbers of CFU of mutant strains recovered from J774 macrophages compared to the wild type (wt) by Tukey's HSD test following ANOVA.

stream gene *virB5*, the unmarked deletion mutations still produce VirB5, although the expression of VirB5 was reduced slightly compared to that in the other strains. We concluded from these data that deletion of *virB1* in ADH6 and deletion of *virB2* in ADH3 do not inhibit expression of downstream genes in the *virB* operon.

VirB1 and VirB2 are required for growth of *B. abortus* in macrophages. We found previously that polar transposon mutations in the 3' end of *virB1* or in *virB10* rendered *B. abortus* unable to grow intracellularly or persist in mice, which led us to conclude that the T4SS is essential for successful intracellular infection by *B. abortus* (18). To determine specifically whether VirB1 and VirB2 are essential to the function of the type IV apparatus in mediating intracellular growth, we tested whether the polar and nonpolar *virB1* and *virB2* mutants are able to grow within cells of the J774 macrophage-like line (Fig. 4A). Both polar and nonpolar deletions of *virB1* and *virB2* significantly decreased the ability of *B. abortus* to survive within macrophages as early as 24 h after infection, suggesting that both VirB1 and VirB2 proteins are required for intracellular survival of *B. abortus* in vitro. Based on data shown in Fig. 3, we knew that the unmarked deletion of *virB2* did not prevent expression of downstream genes in the *virB* operon. However, to eliminate the possibility that passaging of *B. abortus* in vitro to construct the *virB2* mutation could have resulted in other attenuating mutations, we complemented the *virB2* mutation

by two different methods. A plasmid-encoded copy of P_{virB} -*virB1-virB2* (pHB11) was introduced into ADH3 (*virB2*). The second method used was "knock-in" complementation of ADH3, in which an intact copy of *virB2* was reintroduced into its original location in the *virB* locus of ADH3, replacing the deleted allele by homologous recombination. The resulting strain was confirmed by Southern blot analysis (data not shown) to contain *virB2* and was designated ADH8. As an additional control, we analyzed expression of VirB5 in this strain by Western blotting, and it was unchanged compared to ADH3 (data not shown), showing that the knock-in of *virB2* did not disrupt any downstream genes. Introduction of *virB2* on pHB11 did not restore the ability of ADH3 to survive in J774 cells, but knock-in complementation of *virB2* restored intracellular replication of ADH3 to wild-type levels, thereby confirming that the intracellular replication defect of ADH3 was due to deletion of *virB2* (Fig. 4B).

VirB2 is required for persistence in the mouse model, but VirB1 is dispensable. In order to determine whether VirB1 and VirB2 are required for persistence of *B. abortus* in vivo, we tested the abilities of both polar and nonpolar *virB1* and *virB2* mutants to establish infection and persist in the mouse model. Groups of 12 to 15 mice were infected with either *B. abortus* 2308 or one of the isogenic *virB1* or *virB2* mutants, and the bacterial CFU in the spleens were enumerated in groups of 4 to 5 mice at 1, 3, and 8 weeks postinoculation. By 1 week after infection, the wild-type strain, 2308, was recovered in numbers 6- to 10-fold higher than the $\Delta virB2::Km$, $\Delta virB1::Km$, and $\Delta virB2$ mutants and in approximately 2-fold higher numbers than the nonpolar $\Delta virB1$ mutant (Fig. 5). At 3 weeks after infection, the same trend was observed, with $\Delta virB2::Km$, $\Delta virB1::Km$, and $\Delta virB2$ mutants recovered in numbers one-third to one-eighth of those of the wild type, while the nonpolar $\Delta virB1$ mutant was recovered in slightly higher numbers than the wild type. At 8 weeks postinoculation, the numbers of CFU of $\Delta virB2::Km$, $\Delta virB1::Km$, and $\Delta virB2$ mutants recovered were 3 to 4 logs lower than those of the wild type and the nonpolar $\Delta virB1$ mutant. These data suggest that VirB2 is essential for survival in vivo, while VirB1 is dispensable for persistent infection in the mouse.

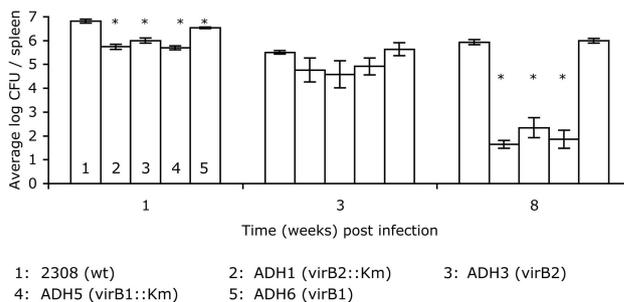


FIG. 5. Persistence of polar and nonpolar *virB1* and *virB2* mutants in spleens of BALB/c mice. Data are the averages of results from experiments with groups of five mice \pm standard error. Asterisks indicate significant ($P < 0.05$) differences in numbers of CFU of mutant strains recovered from mouse spleens compared to the wild type (wt) as determined by Tukey's HSD test following ANOVA.

DISCUSSION

The goal of this study was to determine whether *virB1* and *virB2* encode proteins required for the function of the T4SS apparatus. Based on studies performed in *A. tumefaciens*, these two proteins are predicted to be accessible on the surface of *B. abortus*. VirB2 is predicted to form a pilus-like structure, and VirB1 is predicted to have two domains—a lytic transglycosylase and a second domain that is released to the extracellular medium and associated with the bacterial surface (4, 10, 22). These findings in *A. tumefaciens* suggested that both VirB1 and VirB2 could be involved in *Brucella*-host cell interactions.

In order to examine the roles of VirB1 and VirB2 in pathogenesis, it was necessary to generate nonpolar mutants in their corresponding genes, since the *virB1* to *virB12* genes are transcribed as an operon in *B. abortus* and *B. suis* (8, 30). We constructed two unmarked deletion mutations ($\Delta virB1$ and $\Delta virB2$) and showed that in strains containing these mutations, the downstream gene *virB5* is expressed in stationary phase at levels comparable to those in the wild type (Fig. 2), which demonstrated that the $\Delta virB1$ and $\Delta virB2$ mutations did not eliminate expression of downstream genes in the *virB* operon. Our data showing stationary-phase expression of the *B. abortus virB* genes in vitro is in agreement with the work of Sieira et al. (30). Similarly, Rouot et al. were able to detect expression of VirB5 in stationary-phase *B. abortus* cultures grown in rich medium, but not in *B. suis* cultures, demonstrating that the regulation of the *virB* genes differs in these two *Brucella* species (29).

Nonpolar mutations of both *virB1* and *virB2* produced an intracellular survival defect in J774 macrophages that was similar to that seen for polar mutations (Fig. 3), suggesting that both VirB1 and VirB2 are required for survival in these cells. Therefore, it is likely that both of these T4SS components are required for assembly of a functional T4SS apparatus, as has been shown for *A. tumefaciens* (7).

Using the mouse model of persistent infection, we found that the $\Delta virB1$ mutation was not attenuated for persistence through 8 weeks postinoculation (Fig. 4). These data appear to be at odds with our results showing that *virB1* is required for survival in J774 macrophages (Fig. 3A). Since the lytic transglycosylase consensus motif of *B. suis* VirB1 was required for complementation of T4SS function in an *A. tumefaciens virB1* mutant (19), it is likely that this activity is also important for the function of *B. abortus* VirB1. One possible explanation for our finding that *B. abortus* VirB1 is dispensable during in vivo infection is that the predicted lytic transglycosylase activity of VirB1 can be compensated for by other proteins encoded in the *Brucella* genome that have similar functions. The presence of 10 additional open reading frames in the *B. melitensis* genome with sequence similarity to soluble or membrane-bound lytic transglycosylases appears to support this idea (<http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html>). One or a subset of these predicted lytic transglycosylases might function more efficiently during in vivo infection than in cultured macrophages to compensate for loss of VirB1. An alternate explanation is that in J774 cells, the C-terminal product VirB1* may contribute to intracellular survival. We have observed a smaller cross-reactive band with anti-VirB1 antisera (~12 kDa) in *B. abortus* lysates that is absent from

virB1 mutants (data not shown), which may represent the *B. abortus* form of VirB1*, as it is similar to the size reported by Baron et al. for *A. tumefaciens* VirB1* (4). The potential role of *B. abortus* VirB1* in intracellular growth could be addressed by complementation of the $\Delta virB1$ mutant with the C-terminal domain of VirB1. These results are an example of how tissue culture models of infection may not always reflect what occurs during host-pathogen interactions in vivo, and it will be interesting to determine whether the requirement for VirB1 in the natural bovine host is more similar to that in the mouse model or the macrophage model.

Unlike the $\Delta virB1$ mutant, the $\Delta virB2$ mutant ADH3 was attenuated for persistence in mice (Fig. 5). Intracellular replication of the $\Delta virB2$ mutant ADH3 could be restored by complementation (Fig. 4B), suggesting that the virulence defect of ADH3 ($\Delta virB2$) in mice is also due to deletion of *virB2*. Interestingly, we were able to complement ADH3 ($\Delta virB2$) only by reintroducing *virB2* back into chromosome II but not by expressing it from the *virB* promoter on low-copy-number plasmid pMR10, even though we were able to show that the complemented strain produced VirB1. Some of the possible explanations for this may be that the presence of multiple copies of *virB2* or *virB1* leads to elevated expression levels that are toxic to *B. abortus*. Overexpression of the lytic transglycosylase VirB1 could result in elevated degradation of the murein sacculus in *B. abortus* and lead to bacteriolysis, as has been shown for the lytic transglycosylase P19, which is involved in conjugation of plasmid R1 (6). Alternatively, multiple plasmid-encoded copies of the *virB* promoter in *B. abortus* may titrate out a repressor or activator of the *virB* genes and affect the regulation of the *virB* genes. Finally, although Berger and Christie were able to complement individual deletions of the *A. tumefaciens virB* genes with plasmid-encoded copies (7), it is possible that in *B. abortus*, VirB2 must be expressed coordinately with VirB3 or other VirB proteins in order to function correctly.

Our findings are consistent with an essential role for VirB2 in the assembly of the T4SS apparatus. In *A. tumefaciens*, VirB2 was shown to form a pilus structure that could be visualized by electron microscopy (16, 22). We have not reproducibly observed pili on the surface of *B. abortus* expressing the *virB* genes in laboratory media, but it is possible that pilus assembly occurs in vivo, or that the *B. abortus* T4SS has a different structure than its counterpart in *Agrobacterium*. Further experimentation will be required to determine how VirB2 interacts with other VirB proteins and secreted effectors to assemble a functional T4SS apparatus in *B. abortus*.

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