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Brucella abortus VirB12 Is Expressed during Infection but Is Not an Essential Component of the Type IV Secretion System

Yao-Hui Sun,† Hortensia G. Rolán,† Andreas B. den Hartigh,† David Sondervan, and Renée M. Tsolis*

Texas A&M University Health Science Center, Department of Medical Microbiology & Immunology, College Station, Texas 77843-1114

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The *Brucella abortus virB* operon, consisting of 11 genes, *virB1* to *virB11*, and two putative genes, *orf12* (*virB12*) and *orf13*, encodes a type IV secretion system (T4SS) that is required for intracellular replication and persistent infection in the mouse model. This study was undertaken to determine whether *orf12* (*virB12*) encodes an essential part of the T4SS apparatus. The *virB12* gene was found to encode a 17-kDa protein, which was detected in vitro in *B. abortus* grown to stationary phase. Mice infected with *B. abortus* 2308 produced an antibody response to the protein encoded by *virB12*, showing that this gene is expressed during infection. Expression of *virB12* was not required for survival in J774 macrophages. VirB12 was also dispensable for the persistence of *B. abortus*, *B. melitensis*, and *B. suis* in mice up to 4 weeks after infection, since deletion mutants lacking *virB12* were recovered from splenic tissue at wild-type levels. These results show that VirB12 is not essential for the persistence of the human-pathogenic *Brucella* spp. in the mouse and macrophage models of infection.

The type IV secretion system (T4SS) of *Brucella* spp. has been shown to be a major virulence factor, as *Brucella abortus*, *B. suis*, and *B. melitensis* mutants deficient in the T4SS are highly attenuated both in tissue culture models of intracellular survival and in the mouse model of persistent infection (3–6, 8, 9, 11, 12, 15, 22, 24, 28). The T4SS is encoded by the *virB* locus located on chromosome II, which in *B. suis* was shown to include *virB1* to *virB11* and *orf12*, with a second putative *orf13* predicted in *B. abortus* (15, 16, 22). It was subsequently shown that in *B. suis*, *orf12* is transcribed together with *virB11* on a common transcript and that insertional mutagenesis of *virB1* abrogated the transcription of *orf12* (3), suggesting that *virB1* to *virB12* form a transcriptional unit. Based on this evidence, *orf12* was designated *virB12* (3). The genes *virB1* to *virB11* are conserved among T4SSs from other bacterial pathogens, including *Agrobacterium tumefaciens*; however, homologues of *virB12* have not been found in association with T4SSs from pathogenic bacteria other than *Brucella* spp. The closest homologues of VirB12 have been identified on the mercury resistance plasmid pSB102 identified in the rhizosphere of alfalfa (20) and on a cryptic conjugative plasmid, pIPO2T, isolated from unidentified bacteria in the wheat rhizosphere (26). Other proteins with sequence similarities to VirB12 include the major outer membrane protein of *Pseudomonas* spp. (27, 30). Further analysis of the VirB12 protein sequence identified an OmpA homology domain and a lipoprotein signal sequence. Together, these characteristics suggest that VirB12 is a surface-localized protein of *Brucella* spp. that may play a role in interactions with host cells. We therefore sought to characterize VirB12 from *B. abortus* and to determine whether this protein is required for persistent infection.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Bacterial strains used were *Brucella abortus* 2308, *Brucella melitensis* 16 M, and *Brucella suis* 1330, and their characteristics are listed in Table 1. Strains were routinely 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 TSA plus 5% defibrinated sheep blood. Antibiotics, when required, were added at the following concentrations: carbenicillin, 100 mg/liter; kanamycin (Km), 100 mg/liter; and chloramphenicol, 5 to 30 mg/liter. All work with live *Brucella* strains was performed at Biosafety Level 3.

Strain construction and recombinant DNA techniques. For construction of *virB12* deletion mutants in *B. abortus* (AK/ORF12), *B. melitensis* (MK/ORF12), and *B. suis* (SK/ORF12), a pCR2.1 (Invitrogen)-based plasmid was generated using a three-step cloning strategy. This method takes advantage of the organization of the pCR2.1 TOPO vector. Plasmid pCR2.1 TOPO contains two PstI sites, and cleavage with PstI releases a fragment containing the 3′ end of the *lacZ* alpha fragment, the f1 origin, and the 5′ end of the kanamycin resistance gene (vector data are available from Invitrogen). Deletion of this 1.19-kb PstI fragment disrupts the kanamycin resistance gene but does not affect plasmid replication, and therefore pCR2.1 TOPO was used to generate a construct for the allelic exchange of *virB12* with a deleted copy marked with a kanamycin resistance gene. In the first cloning step, a fragment in the 5′ region of *virB12* (designated UP) was amplified from *B. abortus* genomic DNA using primers ORF12K1-F and ORF12K1-R (Fig. 1). A fragment in the 3′ region of *virB12* (designated DN) was amplified using primers ORF12K2-F (with SmaI) and ORF12K2-R. The resulting fragments were TOPO cloned into pCR2.1 to yield the two plasmids pUP/ORF12 and pDN/ORF12.

The orientations of the cloned inserts in pUP/ORF12 and pDN/ORF12 were determined by PCR and restriction analysis. Clones were identified that carry the SmaI site of the 5′ (UP)-*virB12* fragment located at the end of the insert opposite PstI in pUP/ORF12. For pDN/ORF12, clones were identified that carry the SmaI site of the 3′-*virB12* fragment located at the same end of the cloned insert as PstI. A 1,008-bp SmaI/PstI UP fragment of pUP/ORF12 was introduced into SmaI/PstI-cleaved pDN/ORF12 (the kanamycin resistance gene of pCR2.1 was truncated by this digestion) to link the UP and DN fragments of *virB12* together. The resulting plasmid, named pUD/ORF12, was selected for ampicillin resistance. In the third cloning step, a 1.3-kb SmaI fragment of pUC4-KIXX (Pharmacia)

* Corresponding author. Present address: Department of Medical Microbiology & Immunology, University of California at Davis, Davis, CA 95616. Phone: (530) 754-8497. Fax: (530) 754-7240. E-mail: rmtsolis@ucdavis.edu.

† Present address: Department of Medical Microbiology & Immunology, University of California at Davis, Davis, CA 95616.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
Brucella abortus		
2308	Wild type	B. Deyoe
BA41	Δ*virB*::mTn*5*Km2 (*virB1* 3′ region)	11
BA114	Δ*virB10*::mTn*5*Km2	11
ADH1	Nonpolar *virB2* deletion mutant	8
ADH3	Δ*virB2*::Km	8
ADH4.2	Δ*virB1-virB12*::Km	8
AK/ORF12	Δ*virB12*::Km	This study
Brucella melitensis		
16 M	Wild type	ATCC
MK/ORF12	Δ*virB12*::Km	This study
Brucella suis		
1330	Wild type	ATCC
SK/ORF12	Δ*virB12*::Km	This study
Escherichia coli		
DH5α	*endA1hsdR17* (r_K^- m_K^-) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169 deoR*(ϕ80d*lac*ZΔ*M15*)	29
BL21(DE3)	F^- *ompT gal* [*dcm*] [*lon*] *hsdS*$_B$ (r_B^- m_B^-) λDE3	23
Plasmids		
pCR2.1-TOPO	TA cloning vector	Invitrogen
pET101	Directional His-tagged fusion protein expression vector	Invitrogen
pET/1499	Gene *bcsp31* cloned in pET101 to express C-terminal His-tagged fusion protein	This study
pUC4-KIXX	Carries Tn*5* kanamycin resistance cassette	Pharmacia
pUKD/ORF12	PCR products of *virB*(988–1562) and *virB*(1796–2458) separated by a kanamycin resistance cassette cloned into pCR2.1-TOPO	This study
pIVEX2.4bNdel	Plasmid for generating His-tagged proteins	Roche
pDS1	pIVEX2.4bNdel::*virB12*	This study

containing the Tn*5* kanamycin resistance gene was cloned into the SmaI site of pUD/ORF12 to generate pUKD/ORF12.

Plasmid pUKD/ORF12 was introduced into *B. abortus* 2308, *B. melitensis* 16 M, and *B. suis* 1330 by electroporation, and recombinants resistant to kanamycin and sensitive to carbenicillin (the resistance encoded on the backbone of pUKD/ORF12) were screened. The resulting strains were designated AK/ORF12 (*B. abortus virB12*::*Km*), MK/ORF12 (*B. melitensis virB12*::*Km*), and SK/ORF12 (*B. suis virB12*::*Km*). 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, and the orientation of the cloned fragments in pUKD/ORF12 was confirmed by DNA sequencing. Standard methods were used for Southern blotting, PCR, restriction endonuclease analyses, and ligation and transformation of plasmid DNA into *Escherichia coli* (1). PCR products were cloned into pCR2.1-TOPO using a TOPO-TA cloning kit (Invitrogen).

Cell lines. The mouse macrophage-like cell line J774A.1 (18), obtained from ATCC, 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). For macrophage killing assays, 24-well microtiter plates were seeded with macrophages at concentrations of 1×10^5 to 2×10^5 cells/well in 0.5-ml portions of DMEMsup and incubated overnight at 37°C in 5% CO_2. The inocula were prepared by growing with shaking in TSB for 24 h and then subsequently diluting them in DMEMsup to concentrations of 4×10^7 CFU/ml. Approximately 2×10^7 bacteria in 0.5 ml of DMEMsup, containing a 1:1 mixture of the wild type and the isogenic mutant, were added to each well of macrophages. 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% CO_2, 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 gentamicin per ml was added to the wells, and the cells were incubated at 37°C in 5% CO_2. After 1 h, the DMEMsup plus 50 μg/ml gentamicin was replaced with medium containing 25 μg/ml gentamicin. Wells were sampled at 0 and 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 and TSA plus Km.

Infection of mice. Female BALB/c ByJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and used at ages of 6 to 10 weeks. For mixed-infection experiments, groups of four or five mice were inoculated intraperitoneally (i.p.) with 0.5-ml portions of PBS containing 2×10^5 CFU of a 1:1 mixture of *B. abortus*, *B. melitensis*, or *B. suis* wild type and the isogenic *virB12* mutant. Infected mice were held in microisolator cages in a Biosafety Level 3 facility. At 4 weeks postinfection, mice were euthanized by CO_2 asphyxiation, and the spleens were collected aseptically at necropsy. Spleens were homogenized in 3 ml of PBS and serial dilutions of the homogenate plated on TSA and TSA containing kanamycin for enumeration of mutant and wild-type CFU.

For assaying VirB12-specific antibody responses, mice were infected i.p. with 1×10^5 CFU of *B. abortus* 2308 or of the *B. abortus virB12* deletion mutant AK/ORF12. Blood samples were collected from the saphenous vein at various time points after infection. All animal experiments were approved by the Texas A&M University Laboratory Animal Care and Use Committee and were conducted in accordance with institutional guidelines.

Generation of polyclonal specific antisera. The gene encoding Bcsp31 (14) was PCR amplified from *B. abortus* using primers F1499-F and F1499-R (Table 2) and cloned into directional C-terminal His-tagged fusion protein expression vector pET101 (Invitrogen). For generation of VirB12-specific antiserum, a 504-bp fragment of the *virB12* gene was PCR amplified from *B. abortus* using primers VirB11084F and VirB11563R (Table 2), cloned into pCR2.1 (Invitrogen), and subsequently cloned in pIVEX2.4bNdeI (Roche) with restriction enzymes NotI and PstI to generate an N-terminal fusion with the six-His tag. Both Bcsp31-6xHis and 6xHis-VirB12 were overexpressed and purified according to standard protocols (1) and used to raise polyclonal antisera in rabbits at the Texas A&M Comparative Medicine Program facility. To eliminate background reactivity to whole *B. abortus*, the VirB12 immune rabbit serum was affinity purified using 6xHis-VirB12 bound to a HiTrap column (Amersham Pharmacia) according to the manufacturer's instructions.

Western blotting. *Brucella* cultures inoculated in tryptic soy broth to a starting optical density at 600 nm of 0.01 were incubated at 37°C with shaking at 200 rpm. After 18 h, the cultures' optical densities at 600 nm were 1.2 to 1.5, and bacteria were pelleted and resuspended in 1× Laemmli sample buffer and heated at 100°C for 5 min, and the total protein equivalent to 1×10^8 CFU per well was loaded for separation by sodium dodecyl sulfate-polyacrylamide gel electro-

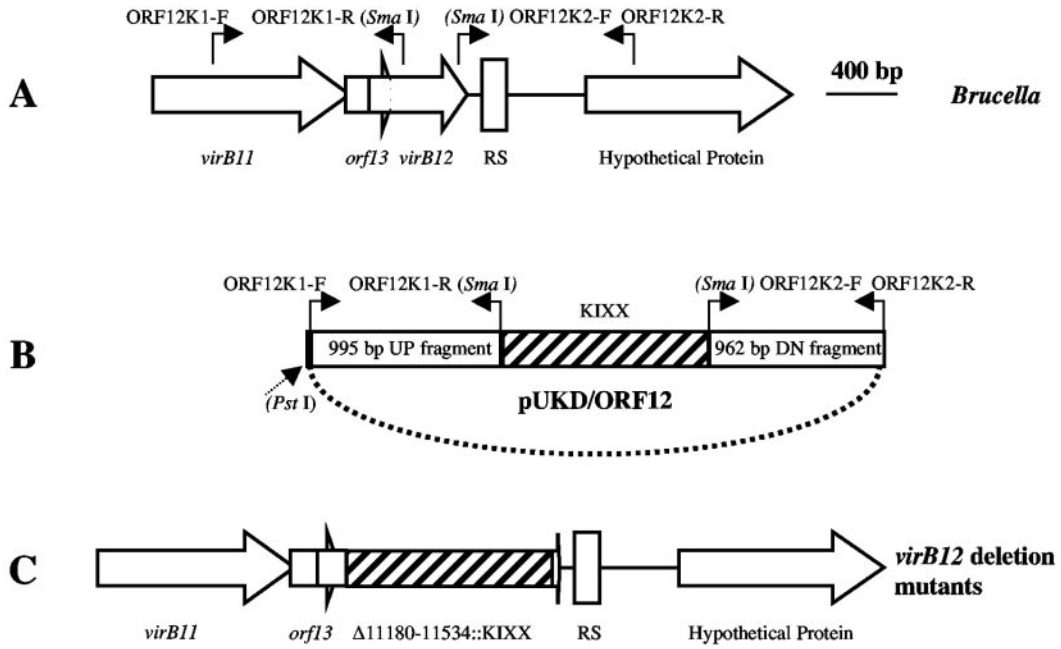

FIG. 1. Construction of *virB12* deletion mutants in *Brucella*. Top: Diagram showing overlap of a putative *orf13* with the 5′ end of *virB12*. Bent arrows indicate the primers used for construction of *virB12* deletion mutants. Middle: Map of pUKD/ORF12 showing KIXX flanked by upstream and downstream fragments cloned into the 2.7-kb pCR2.1 backbone (dotted line). The dotted arrow indicates the unique PstI site. Bottom: Replacement of an internal fragment of *virB12* with KIXX does not disrupt the predicted *orf13*. The numbering of the *virB12* deletion corresponds to that of the *B. abortus virB* operon in GenBank accession number AF226278 (22).

phoresis (SDS-PAGE) (13). Proteins were transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting and were detected using polyclonal rabbit serum and goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP). HRP activity was detected with a chemiluminescent substrate (NEN). To determine protein expression levels, immunoblots were quantified by measuring the relative optical densities and areas of the corresponding bands with a computerized image analysis system (AlphaImager 2200; Alpha Innotech Corporation). Data were expressed as integrated density values and calculated as ratios of VirB12 to Bcsp31 or VirB5 to Bcsp31.

Detection of VirB12-specific IgG in mouse serum. The presence of antibody reactivities against the recombinant protein VirB12 of *Brucella abortus* in the serum samples from 10 BALB/c mice infected with *B. abortus* 2308 and 5 mice infected with *B. abortus* AK/ORF12 (*virB12*) was determined by indirect enzyme-linked immunosorbent assay (ELISA). Ni-nitrilotriacetic acid HisSorb plates from QIAGEN (Valencia, CA) were coated with 100 ng of 6xHis-VirB12 per well in PBS with 0.2% BSA (PBS-B), and plates were incubated at 4°C overnight. After washing with PBS and 0.5% Tween 20 (PBS-T), the pooled serum samples were diluted 1:100 in PBS-B and incubated overnight at 4°C. After washing with PBS-T, the reactivity was measured using HRP anti-mouse IgG (1:1,000; BD Pharmingen) by incubating the plates at 37°C for 1 h. The reaction was developed with Sigma Fast *o*-phenylenediamine dihydrochloride tablet sets. The resulting color was read at 410 nm with an ELISA microplate reader (Dynatech MR5000). Data points are the averages of duplicate dilutions, with each measurement being performed twice.

TABLE 2. List of primers

Primer name	Primer sequence
F1499-F	5′-CACCATGAAATTCGGAAGCAAAATC-3′
F1499-R	5′-TTTCAGCACGCCCGC-3′
ORF12K1-F	5′-ACTGGAAGACTATGCGCAG-3′
ORF12K1-R	5′-ACCCGGGAAAACCTGCAAGCGCAG-3′
ORF12K2-F	5′-ACCCGGGATATCGAAATTTTACGC-3′
ORF12K2-R	5′-AGCATATCCTGCGACATGC-3′
ORF12C-F	5′-ACATATGCGCACATTGGTTATGGTC-3′
ORF12C-R	5′-AGTCGACCTTGCGTAAAATTTCGATATC-3′
ORF12I-F	5′-AGAACCCACCGCGCAAGC-3′
virB2C-F	5′-ACATATGAAAACCGCTTCCCCCAG-3′
virB2C-R	5′-ACTCGAGCCTAAGCAGGTAAGAGGC-3′
virB11084F	5′-GCGGCCGCTCCAGCCCGCCGAAGCCG-3′
virB11563R	5′-CTGCAGGAAAATGCGTGTCTTTGCGATAGGC-3′

Statistical methods. For macrophage killing assays, all experiments were performed independently in triplicate at least three times, and data were expressed as the geometric mean of the logs of CFU/well ± standard deviation. For competitive infection of mice, the mutant and wild-type CFU data were expressed as the mean of log-transformed CFU/spleen ± standard deviation for each group of four or five mice. For both in vitro and in vivo infections, competitive indexes were calculated as log(CFU mutant/CFU wild type) and adjusted in each case to the ratio of mutant to wild type in the inoculum. The statistical significance of differences between mutants and wild types was determined by paired Student's *t* test. A P value of <0.05 was considered significant.

RESULTS

Construction and characterization of *virB12* mutants. To determine whether *virB12* contributes an essential function to the T4SS apparatus, we constructed mutants of *B. abortus*, *B. melitensis*, and *B. suis*, each carrying an internal 334-bp deletion marked by the Tn*5* kanamycin resistance gene (see Materials and Methods for details on the construction). Since a predicted *orf13* overlaps the first 99 bp of *virB12* in *B. abortus* (22), we designed a deletion construct that left the first 143 bp of *virB12* intact. Expression of this fragment would be expected to result in a 48-amino-acid N-terminal fragment containing a predicted lipoprotein signal sequence, which after cleavage would be predicted to yield a 33-amino-acid N-terminal fragment of a putative VirB12 protein (Fig. 1).

Plasmid pUKD/ORF12, carrying a copy of *virB12* with an internal deletion of 334 bp replaced by the Tn*5* kanamycin resistance gene, was constructed (see Materials and Methods)

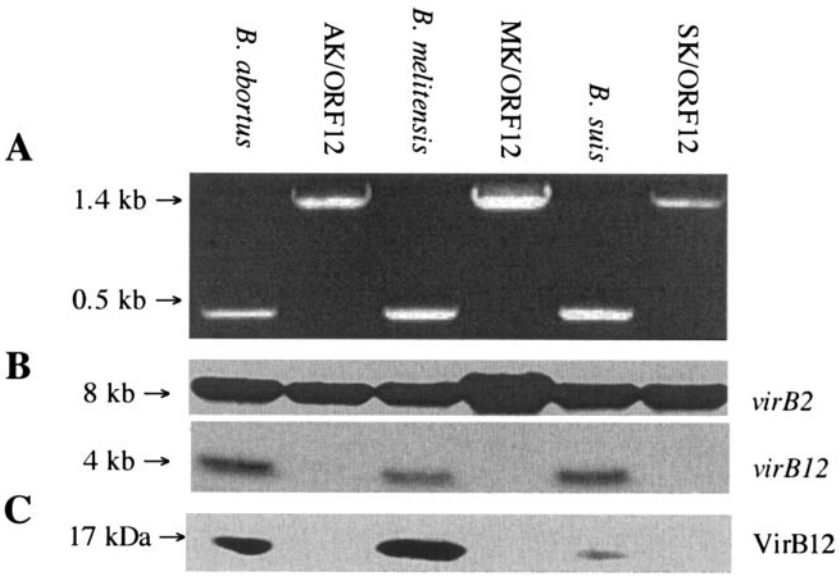

FIG. 2. Characterization of *virB12* mutants. (A) Confirmation of *virB12* deletion by PCR. Chromosomal DNAs of *B. abortus* 2308, *B. melitensis* 16 M, and *B. suis* 1330 and their respective *virB12* mutants were amplified with primer pair ORF12C-F and ORF12C-R (Fig. 1 and Table 2). Wild-type strains yield an amplicon of 520 bp of *virB12*, while mutants yield one of 1.3 kb, since they contain the Km resistance determinant KIXX. (B) Confirmation of *virB12* deletion by Southern blotting. EcoRI-digested genomic DNAs of wild-type *Brucella* strains and *virB12* mutants were hybridized with either a *virB2* probe (corresponding to the sequence from 1609 to 1926 of *B. abortus virB*) or an internal *virB12* probe (corresponding to the sequence from 11181 to 11554 of *B. abortus virB*). The *virB2* probe was generated by PCR using the primer pair virB2C-F and virB2C-R. The *virB12* probe was generated using primers ORF12I-F and ORF12C-R. Primer sequences are shown in Table 2. (C) Detection of VirB12 protein in stationary-phase cultures of *B. abortus* 2308, *B. melitensis* 16 M, and *B. suis* 1330. Proteins from 1×10^8 CFU of stationary-phase culture were separated on a 12% SDS-PAGE gel, transferred to a PVDF membrane, and probed with anti-VirB12 antiserum.

and introduced into *B. abortus* 2308, *B. melitensis* 16 M, and *B. suis* 1330 by electroporation. Recombinants resistant to kanamycin and sensitive to carbenicillin (the resistance encoded on pUKD/ORF12) were identified, and the resulting strains were designated AK/ORF12 (*B. abortus virB12*), MK/ORF12 (*B. melitensis virB12*), and SK/ORF12 (*B. suis virB12*) (Fig. 1). These strains were screened by PCR for the replacement of the 334-bp *virB12* fragment by the Tn*5* kanamycin resistance gene (1.4 kb) (Fig. 2A). This result was confirmed by a Southern blot of chromosomal DNA with a probe containing the deleted region of *virB12* (Fig. 2B). All three wild-type *Brucella* strains and all three *virB12* mutant strains hybridized with a *virB2* probe, but the three *virB12* deletion strains failed to hybridize with the *virB12* probe, demonstrating that this part of the *virB12* gene was deleted in all three *virB12* mutants. As a further control, we generated and purified a 6xHis-VirB12 fusion protein (see Materials and Methods) and raised a polyclonal rabbit serum specific for VirB12. On Western blots, the antiserum reacted with a protein of approximately 17 kDa in lysates of *B. abortus* 2308, *B. melitensis* 16 M, and *B. suis* 1330 (Fig. 2C) grown to stationary phase in TSB. No proteins were detected in lysates any of these three *virB12* mutants with this antiserum. Consistent with previous reports describing expression conditions for the *virB* genes, we found that *virB12* was expressed at lower levels by *B. suis* than by *B. abortus* or *B. melitensis* under these growth conditions (3, 19). These results provide the first evidence that *virB12* encodes a protein.

***B. abortus* mutants with polar insertions in the *virB* operon still express *virB12*.** In order to determine whether *B. abortus virB12* is coregulated with other genes in the *virB* operon, we assayed levels of VirB12 protein and compared them to levels of VirB5, whose expression was reduced by polar insertions in *virB1* and *virB2* (8). As a basis for comparison, we used the protein Bcsp31 (14). Bcsp31 is an immunogenic periplasmic protein that is not required for the growth of *B. abortus* in cultured epithelial cells or macrophages (10, 17). Our previous results (not shown) indicated that the abundance of Bcsp31 in *B. abortus* was not affected by mutations in the *virB* operon. Polar mutations in the *virB1* to *virB2* intergenic region (BA41) or in *virB2* (ADH1) reduced the detection of VirB5 to a greater extent than they did that of VirB12. A transposon insertion in *virB10* reduced the abundance of VirB12 but not of VirB5, suggesting that this insertion is polar on the *virB12* expression. As expected, no VirB12 was detected in the *virB12* mutant AK/ORF12, and neither VirB5 nor VirB12 was detected in the *virB1* to *virB12* operon deletion mutant, ADH4.2. These results suggest that polar mutations in the operon upstream of *virB5* exert a greater effect on the expression of *virB5* than they do on the expression of the downstream gene *virB12*.

***B. abortus* produces VirB12 protein during infection of mice.** If *virB12* is expressed during in vivo infection, then we would expect an infected host to develop an antibody response, since VirB12 was highly immunogenic in rabbits. To determine whether *virB12* is expressed during infections of a model host with *Brucella* spp., we assayed for VirB12-specific IgG in sera from mice infected with *B. abortus* 2308 or *virB12* mutant AK/ORF12. The results of ELISAs depicted in Fig. 3 show that the titer of IgG specific for 6xHis-VirB12 increases above the titer of naïve mice, starting at 21 days after infection. Titers of VirB12-specific IgG increased until 56 days postinfection, after

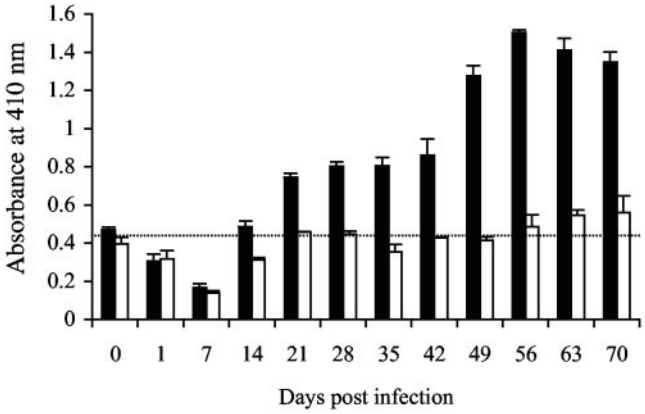

FIG. 3. VirB12-specific total IgG in serum samples of mice taken after infection of BALB/c mice with *B. abortus* 2308 ($n = 10$; filled bars) or its isogenic *virB12* mutant, AK/ORF12 ($n = 5$; open bars). Samples taken at the indicated time points postinfection were diluted and measured by ELISA using His-VirB12 bound to HisSorb plates (QIAGEN). Bars indicate the averages ± standard deviations of duplicate measurements. The dotted line represents the background reactivity in mice before infection with *B. abortus*.

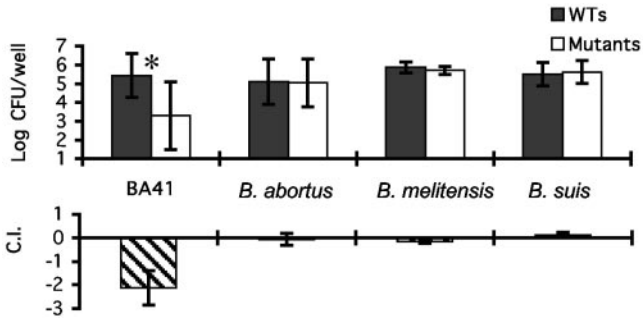

FIG. 4. Recovery of *B. abortus*, *B. suis*, and *B. melitensis virB12* mutants after coinfection of J774 macrophages with their respective wild-type strains (WTs). A mixed inoculum of the *virB12* mutant and the wild type was used to infect J774 cells, and intracellular bacteria were recovered after 48 h as described in Materials and Methods. Data shown are the means of three independent assays done in triplicate ± standard deviations and plotted logarithmically. Top: Log-transformed CFU of the wild type and *virB12* mutants recovered from J774 cells are given. Bottom: Competitive index (C.I.), calculated as log(CFU mutant/CFU wild type), is shown on the *y* axis. A significant difference (∗) was seen only in the control experiment using *B. abortus* mutant BA41 (*B. abortus virB1-virB2*::mTn*5*) in comparison with the wild type ($P = 0.04$).

which they remained at high levels through the end of the experiment at day 70. For mice infected with AK/ORF12, the IgG titers did not differ from the preinoculation titers over the course of the experiment, showing that VirB12-specific antibodies detected in mice infected with wild-type *B. abortus* are not elicited by other cross-reactive *B. abortus* proteins. These results indicate that VirB12 is synthesized and presented to the host immune system during infections of mice.

***Brucella* mutants lacking *virB12* survive in murine macrophages like their wild-type parent strains.** To determine whether VirB12 is required for intracellular survival of *B. abortus*, *B. melitensis*, or *B. suis*, we assayed the ability of *virB12* mutant strains to survive in J774A.1 macrophages. For these experiments, we performed a coinfection of each *virB12* mutant with its respective wild-type strain, as coinfection gives a sensitive measure of differences between the wild type and the mutant with regard to intracellular survival (Fig. 4). Inocula containing 1:1 mixtures of wild type and *virB12* mutant were used to infect J774A.1 cultures. Coinfection of *B. abortus* 2308 and *B. abortus* BA41 (*virB1-virB2*::mTn*5*Km2) showed that at 48 h after infection, the CFU of BA41 recovered was 1/100 of that of *B. abortus* 2308, demonstrating that under these conditions the wild type is not able to rescue a mutant lacking a functional T4SS apparatus (Fig. 4). In contrast, the *virB12* mutants AK/ORF12, MK/ORF12, and SK/ORF12 were recovered from J774A.1 cells in numbers indistinguishable from those of the wild type. These results show that VirB12 is not required for the survival of *B. abortus*, *B. melitensis*, or *B. suis* in this macrophage line.

***Brucella* mutants lacking *virB12* persist in murine spleens at wild-type levels.** To test the ability of *virB12* mutants to persist in an animal model of infection, we used the murine model to compare colonization levels of each *virB12* mutant and its virulent parent strain. To this end, we performed coinfection studies with each mutant and its respective wild-type strain. For mice, coinfection gives a sensitive readout of attenuation with small experimental groups of animals. In addition, this experimental design allowed us to assess whether VirB12 is required for infection in the context of the host response to wild-type *B. abortus*. Groups of four to five mice were infected i.p. with inocula of 2×10^5 CFU containing 1:1 mixtures of the mutant and the wild-type parent strain. While the *B. abortus virB* mutant BA41 was not detected in the spleen by 4 weeks, the numbers of CFU of *virB12* mutants AK/ORF12, MK/ORF12, and SK/ORF12 recovered from murine spleens were not significantly different from those of the parent strains, showing that *virB12* is not essential for persistence in this infection model (Fig. 5).

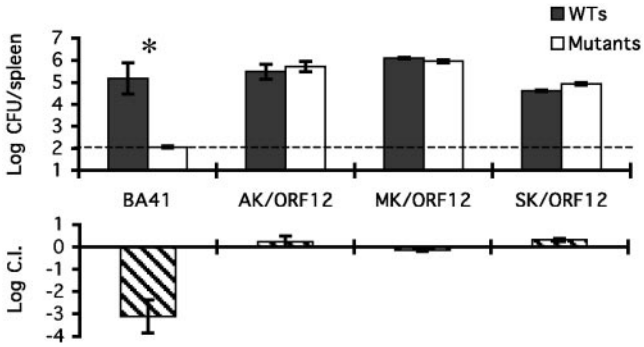

FIG. 5. Recovery of *B. abortus*, *B. melitensis*, and *B. suis virB12* deletion mutants from mouse splenic tissue after mixed infection with their respective wild-type strains (WTs). Bacteria were recovered 4 weeks after i.p. infections with 2×10^5 CFU of 1:1 mixtures of the wild type and the *virB12* mutant. Data shown are the averages of groups of four or five mice ± standard deviations and are plotted logarithmically. Top panel: Log-transformed CFU recovered from mouse spleens are given for wild-type and *virB12* mutant strains. The dashed line indicates the limit of detection in the spleen. Bottom: Competitive index (C.I.), calculated as log(CFU mutant/CFU wild type), is shown on the *y* axis. A significant difference (∗) was seen in the experiment using *B. abortus* mutant BA41 (*virB1-virB2*::mTn*5*) in comparison with the wild type ($P = 0.04$).

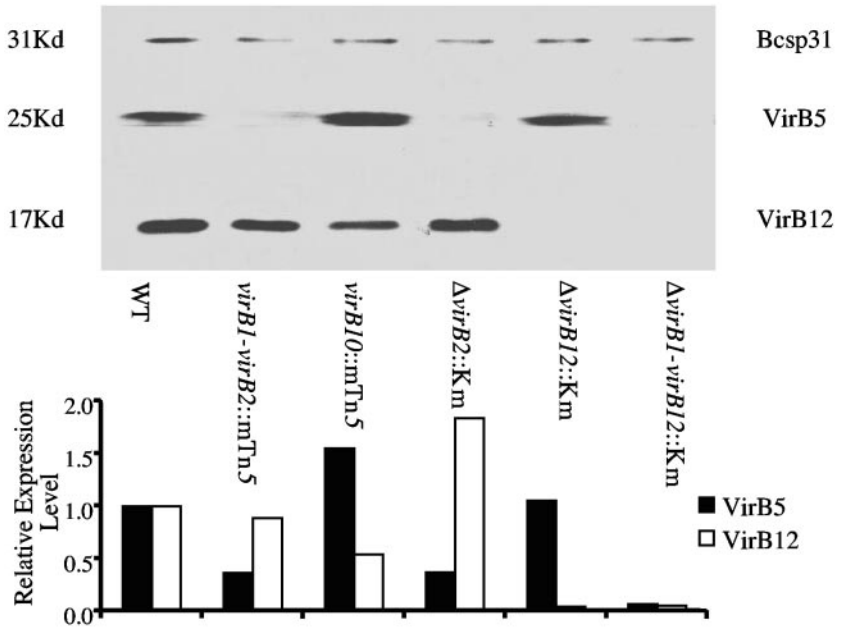

FIG. 6. Effect of mutations in *virB* genes on VirB12 protein levels in *B. abortus* 2308. Top: Western blots. Proteins from 1×10^8 CFU of stationary-phase culture were separated on a 12% SDS-PAGE gel and transferred to a PVDF membrane, which was followed by Western blotting with polyclonal anti-VirB5 (1:500), anti-VirB12 (1:50,000), or anti-Bcsp31 (1:5,000) serum. Bottom: To determine protein expression levels, immunoblots were quantified by measuring the relative optical densities and areas of the corresponding bands with a computerized image analysis system as described in Materials and Methods. Data were expressed as integrated density values and calculated as ratios of VirB5 to Bcsp31 or VirB12 to Bcsp31. Relative protein expression levels were plotted and compared with that of the wild type (WT), which was set to 1. The strain names corresponding to the genotypes shown are listed in Table 1. Data shown is from a single experiment that was representative of three independent experiments.

DISCUSSION

This study was undertaken to determine whether *virB12* encodes an essential part of the T4SS of *Brucella* spp. The results show that VirB12 is expressed during in vitro growth. As with VirB5, VirB12 was expressed more highly in stationary phase than in log phase (data not shown). It was shown previously that mutation of *virB1* abolishes expression of both *virB7* and *virB10* (21, 22), suggesting that polar mutations in the *virB* operon should also be polar on *virB12*, if it is in fact coregulated with the other *virB* genes. Furthermore, a *virB1* mutation was shown to eliminate the transcription of *virB12* in *B. suis* (3). However, the finding that *virB* mutations that decreased *virB5* expression exerted less of a polar effect on *virB12* expression (Fig. 6) suggests that in *B. abortus*, regulatory elements other than the *virB* promoter may influence VirB12 protein levels. The differences between our data and the data from *B. suis* may reflect different regulatory mechanisms, as the regulatory mechanisms of the *virB* genes have been shown to differ among the *Brucella* species (19, 21, 25), or they may reflect differences in the sensitivities of the various methods used to detect *virB12* expression.

Evidence of *virB12* expression was also detected in mice infected with *B. abortus* (Fig. 3), as an IgG response specific for VirB12 was elicited after infection with *B. abortus* 2308 but not with *virB12* mutant AK/ORF12. These results indicate that VirB12 is an immunogenic protein in the context of the response to *B. abortus* infection and provide indirect evidence for the expression of the *virB* genes during infection of mice. The cell surface localization of VirB12 may contribute to its immunogenicity. Cell surface proteins of *B. abortus* have previously been shown to elicit antibody responses in mice (17), and it was recently shown for *Salmonella enterica* serotype Typhimurium that T-cell responses are preferentially directed against surface antigens (2). It will be interesting to determine whether other components of the *Brucella* T4SS elicit antibody or T-cell responses during infection.

To determine whether the expression of *virB12* is required for persistent infection by *Brucella* spp., deletion mutants were constructed from *B. abortus*, *B. suis*, and *B. melitensis*. In designing these mutants, we considered that an *orf13* overlapping *virB12* was predicted in the *B. abortus virB* operon sequence (22). Although *orf13* has been annotated only for *B. abortus*, the DNA sequence of this region is identical in *B. abortus*, *B. suis*, and *B. melitensis* (7, 15, 16, 22). Since it is not yet known whether *orf13* encodes a functional protein in any of the *Brucella* species, the overlapping sequence was left intact in the *virB12* mutants.

Expression of *virB12* was not essential for growth in J774 cells or during the first 4 weeks of infection in mice. These results do not rule out a role for VirB12 in interaction with mucosal surfaces, since in our experiments this stage of infection was bypassed by using the i.p. route of inoculation. Further, since the mouse is not a model for interactions with the reproductive tract in ruminants, it is possible that VirB12 may play a role during infection in the natural hosts of *Brucella* spp. The ability of *virB12* mutants to survive within macrophages reported here differs from the phenotype reported by Boschiroli et al. for a *B. suis virB12* mutant (3) but is in agreement with the result reported by O'Callaghan et al. (15). Possible

explanations for the differences in these results include differences in the mutant construction (our mutant is a deletion mutant, whereas the *virB12* mutant reported by Boschiroli et al. is an insertion mutant). Since the previous studies of the *B. suis virB12* mutants were performed using the human-derived THP-1 macrophage line, we tested whether the intracellular survival defect of *virB12* mutants could be specific for human macrophages. However, as with the mouse macrophage line, none of the *virB12* mutants constructed in this study exhibited survival defects in THP-1 cells (data not shown).

Homologues of *virB12* are missing from other pathogenic bacteria with T4SS, but two conjugative plasmids from the rhizosphere of wheat, alfalfa, and tomato plants, pIPO2 and pSB102 (20, 26), contain highly conserved VirB12 homologues that are predicted to play a role in mating pair formation. Hence, it is possible that in *Brucella* spp., VirB12 may play a role in interactions between cells, such as would occur during conjugation, or, alternatively, that the *virB12* gene may be an evolutionary remnant of the acquisition of the *virB* genes by an ancestor of *Brucella* spp. Further experimentation will be required to determine whether VirB12 plays a role in DNA transfer or in infection of other animal hosts.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1994. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
2. **Bergman, M. A., L. A. Cummings, S. L. Barrett, K. D. Smith, J. C. Lara, A. Aderem, and B. T. Cookson.** 2005. $CD4^+$ T cells and toll-like receptors recognize *Salmonella* antigens expressed in bacterial surface organelles. Infect. Immun. **73:**1350–1356.
3. **Boschiroli, M. L., S. Ouahrani-Bettache, V. Foulongne, S. Michaux-Charachon, G. Bourg, A. Allardet-Servent, C. Cazevieille, J. P. Liautard, M. Ramuz, and D. O'Callaghan.** 2002. The Brucella suis virB operon is induced intracellularly in macrophages. Proc. Natl. Acad. Sci. USA **99:**1544–1549.
4. **Celli, J., C. de Chastellier, D. M. Franchini, J. Pizarro-Cerda, E. Moreno, and J. P. Gorvel.** 2003. Brucella evades macrophage killing via VirB-dependent sustained interactions with the endoplasmic reticulum. J. Exp. Med. **198:**545–556.
5. **Comerci, D. J., M. J. Martinez-Lorenzo, R. Sieira, J. P. Gorvel, and R. A. Ugalde.** 2001. Essential role of the VirB machinery in the maturation of the Brucella abortus-containing vacuole. Cell. Microbiol. **3:**159–168.
6. **Delrue, R. M., M. Martinez-Lorenzo, P. Lestrate, I. Danese, V. Bielarz, P. Mertens, X. De Bolle, A. Tibor, J. P. Gorvel, and J. J. Letesson.** 2001. Identification of Brucella spp. genes involved in intracellular trafficking. Cell. Microbiol. **3:**487–497.
7. **DelVecchio, V. G., V. Kapatral, R. J. Redkar, G. Patra, C. Mujer, T. Los, N. Ivanova, I. Anderson, A. Bhattacharyya, A. Lykidis, G. Reznik, L. Jablonski, N. Larsen, M. D'Souza, A. Bernal, M. Mazur, E. Goltsman, E. Selkov, P. H. Elzer, S. Hagius, D. O'Callaghan, J. J. Letesson, R. Haselkorn, N. Kyrpides, and R. Overbeek.** 2002. The genome sequence of the facultative intracellular pathogen *Brucella melitensis*. Proc. Natl. Acad. Sci. USA **99:**443–448.
8. **den Hartigh, A. B., Y.-H. Sun, D. Sondervan, N. Heuvelmans, M. O. Reinders, T. A. Ficht, and R. M. Tsolis.** 2004. Differential requirements for VirB1 and VirB2 during *Brucella abortus* infection. Infect. Immun. **72:**5143–5149.
9. **Foulongne, V., G. Bourg, C. Cazevieille, S. Michaux-Charachon, and D. O'Callaghan.** 2000. Identification of *Brucella suis* genes affecting intracellular survival in an in vitro human macrophage infection model by signature-tagged transposon mutagenesis. Infect. Immun. **68:**1297–1303.
10. **Halling, S. M., P. G. Detilleux, F. M. Tatum, B. A. Judge, and J. E. Mayfield.** 1991. Deletion of the BCSP31 gene of *Brucella abortus* by replacement. Infect. Immun. **59:**3863–3868.
11. **Hong, P. C., R. M. Tsolis, and T. A. Ficht.** 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. Infect. Immun. **68:**4102–4107.
12. **Kim, S., M. Watarai, Y. Kondo, J. Erdenebaatar, S. Makino, and T. Shirahata.** 2003. Isolation and characterization of mini-Tn*5*Km2 insertion mutants of *Brucella abortus* deficient in internalization and intracellular growth in HeLa cells. Infect. Immun. **71:**3020–3027.
13. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
14. **Mayfield, J. E., B. J. Bricker, H. Godfrey, R. M. Crosby, D. J. Knight, S. M. Halling, D. Balinsky, and L. B. Tabatabai.** 1988. The cloning, expression, and nucleotide sequence of a gene coding for an immunogenic *Brucella abortus* protein. Gene **63:**1–9.
15. **O'Callaghan, D., C. Cazevieille, A. Allardet-Servent, M. L. Boschiroli, G. Bourg, V. Foulongne, P. Frutos, Y. Kulakov, and M. Ramuz.** 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Mol. Microbiol. **33:**1210–1220.
16. **Paulsen, I., R. Seshadri, K. Nelson, J. Eisen, J. Heidelberg, T. Read, R. Dodson, L. Umayan, L. Brinkac, M. Beanan, S. Daugherty, R. Deboy, A. Durkin, J. Kolonay, R. Madupu, W. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S. van Aken, S. Riedmuller, H. Tettelin, S. Gill, O. White, S. Salzberg, D. Hoover, L. Lindler, S. Halling, S. Boyle, and C. Fraser.** 2002. The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. Proc. Natl. Acad. Sci. USA **99:**13148–13153.
17. **Pugh, G. W., Jr., L. B. Tabatabai, B. J. Bricker, J. E. Mayfield, M. Phillips, E. S. Zehr, and C. A. Belzer.** 1990. Immunogenicity of *Brucella*-extracted and recombinant protein vaccines in CD-1 and BALB/c mice. Am. J. Vet. Res. **51:**1413–1420.
18. **Ralph, P., and I. Nakoinz.** 1975. Phagocytosis and cytolysis by a macrophage tumour and its cloned cell line. Nature **257:**393–394.
19. **Rouot, B., M.-T. Alvarez-Martinez, C. Marius, P. Mentanteau, L. Guilloteau, R.-A. Boigegrain, R. Zumbihl, D. O'Callaghan, N. Domke, and C. Baron.** 2003. Production of the type IV secretion system differs among *Brucella* species as revealed with VirB5- and VirB8-specific antisera. Infect. Immun. **71:**1075–1082.
20. **Schneiker, S., M. Keller, M. Droge, E. Lanka, A. Puhler, and W. Selbitschka.** 2001. The genetic organization and evolution of the broad host range mercury resistance plasmid pSB102 isolated from a microbial population residing in the rhizosphere of alfalfa. Nucleic Acids Res. **29:**5169–5181.
21. **Sieira, R., D. J. Comerci, L. I. Pietrasanta, and R. A. Ugalde.** 2004. Integration host factor is involved in transcriptional regulation of the *Brucella abortus virB* operon. Mol. Microbiol. **54:**808–822.
22. **Sieira, R., D. J. Comerci, D. O. Sanchez, and R. A. Ugalde.** 2000. A homologue of an operon required for DNA transfer in *Agrobacterium* is required in *Brucella abortus* for virulence and intracellular multiplication. J. Bacteriol. **182:**4849–4855.
23. **Studier, F. W., and B. A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. **189:**113–130.
24. **Sun, Y. H., A. B. den Hartigh, R. L. Santos, L. G. Adams, and R. M. Tsolis.** 2002. *virB*-mediated survival of *Brucella abortus* in mice and macrophages is independent of a functional inducible nitric oxide synthase or NADPH oxidase. Infect. Immun. **70:**4826–4832.
25. **Taminiau, B., M. Daykin, S. Swift, M. L. Boschiroli, A. Tibor, P. Lestrate, X. De Bolle, D. O'Callaghan, P. Williams, and J. J. Letesson.** 2002. Identification of a quorum-sensing signal molecule in the facultative intracellular pathogen *Brucella melitensis*. Infect. Immun. **70:**3004–3011.
26. **Tauch, A., S. Schneiker, W. Selbitschka, A. Puhler, L. S. van Overbeek, K. Smalla, C. M. Thomas, M. J. Bailey, L. J. Forney, A. Weightman, P. Ceglowski, T. Pembroke, E. Tietze, G. Schroder, E. Lanka, and J. D. van Elsas.** 2002. The complete nucleotide sequence and environmental distribution of the cryptic, conjugative, broad-host-range plasmid pIPO2 isolated from bacteria of the wheat rhizosphere. Microbiology **148:**1637–1653.
27. **Ullstrom, C. A., R. Siehnel, W. Woodruff, S. Steinbach, and R. E. W. Hancock.** 1991. Conservation of the gene for outer membrane protein OprF in the family *Pseudomonadaceae*: sequence of the *Pseudomonas syringae oprF* gene. J. Bacteriol. **173:**768–775.
28. **Watarai, M., S. Makino, and T. Shirahata.** 2002. An essential virulence protein of *Brucella abortus*, VirB4, requires an intact nucleoside-triphosphate-binding domain. Microbiology **148:**1439–1446.
29. **Woodcock, D. M., P. J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S. S. Smith, M. Z. Michael, and M. W. Graham.** 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. Nucleic Acids Res. **17:**3469–3478.
30. **Woodruff, W. A., and R. E. Hancock.** 1989. *Pseudomonas aeruginosa* outer membrane protein F: structural role and relationship to the *Escherichia coli* OmpA protein. J. Bacteriol. **171:**3304–3309.

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