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VirJ Is a *Brucella* Virulence Factor Involved in the Secretion of Type IV Secreted Substrates*

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The VirB secretion apparatus in *Brucella* belongs to the type IV secretion systems present in many pathogenic bacteria and is absolutely necessary for the efficient evasion of the *Brucella*-containing vacuole from the phagocytic route in professional phagocytes. This system is responsible for the secretion of a plethora of effector proteins that alter the biology of the host cell and promote the intracellular replication process. Although many VirB substrates have been identified in *Brucella*, we still know very little about the secretion mechanism that mediates their translocation across the two membranes and the periplasmic space. In this manuscript, we describe the identification of a gene, *virJ*, that codes for a protein with periplasmic localization that is involved in the intracellular replication process and virulence in mice. Our analysis revealed that this protein is necessary for the secretion of at least two VirB substrates that have a periplasmic intermediate and that it directly interacts with them. We additionally show that VirJ also associates with the apparatus *per se* and that its absence affects the assembly of the complex. We hypothesize that VirJ is part of a secretion platform composed of the translocon and several secretion substrates and that it probably coordinates the proper assembly of this macromolecular complex.

Intracellular pathogenic bacteria have the capacity to circumvent the host defenses to establish a secure niche for replication. The mechanisms necessary to establish this "safe haven" are multiple, but in many cases they depend on the capacity of the bacteria to secrete and translocate effector molecules to the host cell (1). *Brucella* spp., the causative agent of brucellosis, is a zoonotic Gram-negative bacterium that still inflicts important economic losses in livestock and serious human health problems in endemic areas (2). *Brucella* is an intracellular pathogen with the capacity to avoid the bactericidal effects of its target cells, such as macrophages (one of the primary cell targets). To achieve this, the bacterium codes for a T4SS[5] that secretes and translocates effector proteins to the host cell that modulate the cellular response, avoiding the phagocytic process and favoring the infectious process (3–10). In *Brucella*, this secretion system is named *virB* because of its homology to the *Agrobacterium tumefaciens* conjugation-like system that translocates the T-DNA (transferred DNA) into the plant cell (11). The activity of the VirB system is necessary, in a first phase, to avoid the fusion of the BCV with the lysosomes and, in a second phase, to redirect its fate to an endoplasmic reticulum-derived membrane niche, where it actively replicates (3, 12).

Type IV secretion systems are macromolecular complexes that span the inner membrane, the periplasmic space, and the outer membrane, are present in many bacteria, and are ancestrally related to conjugation systems. Recently, the complete structure of a T4SS has been solved by electron microscopy, shedding light on several mechanistic aspects of the secretion process (13). One interesting finding was the fact that the outer membrane core component of the system is connected to the inner membrane complex by a periplasmic stalk, which could allow substrates present in the periplasm to be engaged by the system (13). This is an important difference to other secretion systems that either engage their substrates in the cytoplasm or the periplasm.

To date, in *Brucella*, several VirB substrates have been identified, but we still have almost no knowledge on what their targets are or what biological activities they have in the host cell. Additionally, very little is known about the secretion of T4SSs *per se*, the mechanism by which the substrates are selected, or how they are actually translocated through the system and into the host cells. Even though initially it was believed that the effectors are engaged by the T4SS in the cytoplasm, there has been increasing evidence that some proteins have a periplasmic localization or are inserted in the membrane (6, 7, 14–17). As indicated above, these observations are consistent with the recent structure of a type IV system and should encourage a

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[5] The abbreviations used are: T4SS, type IV secretion system; BCV, *Brucella*-containing vacuole; TSB, tryptic soy broth; BMDM, bone marrow-derived macrophage(s); BACTH, bacterial adenylate cyclase two-hybrid assay; IP, immunoprecipitation.

re-evaluation of the proposed secretion mechanism of these fascinating nanomachines.

The most studied T4SS is the *A. tumefaciens virB* system, a supramolecular structure with ~12 components, all codified in the Ti megaplasmid (11). Genetic and biochemical analyses from several groups over the past 30 years have given us a detailed picture of the functionality of this system (for a recent review, see Ref. 18). In 1995, a group identified a gene in the Ti plasmid that they named *virJ*, which codes for periplasmic protein with a role in the T-DNA transfer process (19). Work performed several years later by this same group additionally showed that VirJ associates with some VirB substrates and suggested that type IV secretion in *Agrobacterium* might by be a two-step process and that VirJ plays a role in the interaction of some secreted substrates with the T-pilus (17). To our knowledge, this is the only report identifying a protein involved in the type IV secretion process that participates in the translocation of substrates with periplasmic localization.

We have recently identified a VirB secretion substrate in *Brucella abortus* that we named SepA and that is involved in the early stages of the intracellular replication cycle (6). We showed that this new effector protein is secreted in a VirB-dependent manner, that its inactivation affects the intracellular trafficking particularly during the initial stages, and that its secretion involves a periplasmic intermediate (6). Although in *Brucella* this was the first report identifying a VirB substrate with a two-step secretion process involving a periplasmic intermediate, it was reported that other effectors have a predicted periplasmic signal peptide or putative transmembrane domains (5, 7, 8). We report here the identification in *Brucella* of a homologue of the *A. tumefaciens virJ* gene and characterized this gene genetically and biochemically. We showed that this gene is a virulence factor that codes for a protein that localizes in the periplasm and plays a central role in the secretion of two type IV secretion substrates (SepA and Bpe123). Moreover, our results indicate that VirJ directly interacts with both effectors and forms a complex with core components of the VirB apparatus, such as VirB5 and VirB8, strongly suggesting that some substrates might be part of a secretion platform of which VirJ could be a central core component.

Experimental Procedures

Bacterial Strains and Growth Conditions

Brucella melitensis bv. *abortus* 2308 was used as a wild-type strain. *B. abortus* strains were grown in tryptic soy agar (Difco/BD Biosciences) or in tryptic soy broth (TSB) at 37 °C on a rotary shaker for 16–24 h. Manipulation of *B. abortus* was performed at the biosafety level 3 laboratory facility at the Universidad Nacional de San Martín. *Escherichia coli* strains were grown on Luria-Bertani agar and broth at 37 °C or 2xYT at 18 °C. If necessary, media was supplemented with appropriated antibiotic at the indicated final concentrations: ampicillin, 100 μg/ml; kanamycin, 50 μg/ml; and nalidixic acid, 5 μg/ml.

Recombinant DNA Techniques

Construction of Ba Δvir J Mutant Strain—To construct a *Ba Δvir J* mutant strain, the regions flanking the *virJ* gene were amplified and ligated using a recombinant PCR technique (20). The primers used for PCR amplification were VirJ1 (5′-CCGGAATTCGCTGACCTTTTCCAAGCAAG-3′) and VirJ2 (5′-GATCGCCAATGCCTTTCTCAT-3′) to amplify a 500-bp upstream region and VirJ3 (5′-AAGGCATTGGCGATCGCAAATCACATGCCGTTACCG-3′) and VirJ4 (5′-CGCGGATCCGACAACACGTTCCAGCCTTA-3′) to amplify a 500-bp downstream region. VirJ-1 and VirJ-4 were used in an overlapping PCR. The resulting fragment was digested with EcoRI and BamHI and ligated to the pK18mobSacB plasmid digested with the same enzymes. The plasmid *pK18mobSacB/Δvir J* was introduced into the *B. abortus* strain by biparental mating using the *E. coli* S17λ*pir* strain. Double recombination events (Km^s Sac^r) were selected, and gene knockout was confirmed by genomic PCR.

Construction of Ba Δvir J/virJ-complemented Strain—To construct a C-terminal 3×FLAG-tagged version of VirJ the plasmid pBBR1-MCS4–3×FLAG was used (6). *VirJ* was amplified by PCR from *B. abortus* 2308 genomic DNA using primers VirJ5 (5′-CCCAAGCTTCCTTTGGAGTTCATTCGCAA-3′) and VirJ6 (5′-CGGAATTCGCGCGCAGGGCGCGG-3′). The PCR product was digested with EcoRI and HindIII, and the resulting fragment was cloned in pBBR1-MCS4–3×FLAG in the same sites, generating an in-frame fusion to the 3×FLAG epitope. The resulting plasmid, named *pBBR4/virJ-3×FLAG*, was introduced into the *B. abortus* strain by biparental mating. The expression of *virJ-3×FLAG* was confirmed by Western blotting.

Construction of the Ba sepA and Ba Δvir J/sepA Strains—To construct the *Ba sepA* and *Ba Δvir J/sepA* strains expressing SepA-3×FLAG, the plasmid *pBBR4/sepA-3×FLAG* (6) was introduced into the *Ba* wild-type and *Ba Δvir J* mutant strains by biparental mating. The expression of SepA-3×FLAG was confirmed by Western blotting.

Construction of the Ba bpe123 and Ba Δvir J/bpe123 Strains—To construct the *Ba bpe123* and *Ba Δvir J/bpe123* strains expressing Bpe123–3×FLAG, the plasmid pBPE123-$FLAG_{(1–153)}$ (7) was introduced in the *Ba* wild-type and *Ba Δvir J* mutant strains by biparental mating. The expression of Bpe123–3×FLAG was confirmed by Western blotting.

Construction of GST-tagged VirJ Protein—To construct glutathione S-transferase-tagged VirJ protein (VirJ-GST), the *virJ* gene was amplified by PCR from *B. abortus* 2308 genomic DNA using primers GST-VirJ FW (5′-cgggatccatggatgccatgttggcccg-3′) and GST-VirJ RV (5′-cggaattctcagcgcgcagggcgcgg-3′). The PCR products were digested with BamHI and EcoRI. The resulting fragments were cloned in pGEX-2T (GE Healthcare) in the same sites, generating an in-frame fusion to the GST. The expression of VirJ-GST was confirmed by Western blotting.

Protein Expression and Purification

Recombinant poly-histidine-tagged SepA (SepA-His_6) or Bpe123 (Bpe123-His_6) were expressed in *E. coli* and purified using nickel affinity chromatography under native conditions. Briefly, *E. coli* strains were grown at 37 °C at 250 rpm, and the expression was induced with isopropyl 1-thio-β-D-galactopyranoside (1 mM) at $A_{600\,nm}$ = 0.6. 3 h post-induction, cells were harvested and lysed by sonication. Supernatants were recovered and applied to a HisTrap™ HP column (GE Healthcare).

The recombinant proteins were eluted with an imidazole gradient (100–500 mM). For the GST pulldown assay, imidazole was eliminated by dialysis.

Recombinant GST or VirJ-GST was expressed in *E. coli* and purified using glutathione-Sepharose affinity chromatography under native conditions. Briefly, *E. coli* strains were grown in 2XYT broth at 37 °C at 250 rpm, and expression was induced with isopropyl 1-thio-β-D-galactopyranoside (0.2 mM) at $A_{600\,nm}$ = 0.8. Cultures were transferred to 18 °C, and 16 h post-induction cells were harvested and lysed by sonication. Supernatants were recovered and applied to a glutathione-Sepharose 4 Fast Flow column (GE Healthcare). The protein was eluted with 20 mM glutathione. For the GST pulldown assay, glutathione was eliminated by dialysis.

Intracellular Replication Assays

A standard antibiotic protection assay was performed in bone marrow-derived macrophages (BMDM) and murine macrophage-like J774 A.1 cells (10). To obtain BMDM, bone marrow cells were isolated from femora of 6- to 10-week-old C57BL/6 female mice and differentiated into macrophages as described in Ref. 12. Cells were seeded in 24-well plates in suitable culture medium at 10^5 cells/ml and incubated overnight at 37 °C. *Brucella* strains were grown in TSB with the appropriate antibiotics for 24 h and diluted in culture medium prior to infection. The suspension was added at the indicated multiplicity of infection (50:1 for J774 A.1 cells and 200:1 for BMDM) and centrifuged at 300 × *g* for 10 min. After 1 h of incubation at 37 °C, cells were washed, and fresh medium containing 100 μg/ml streptomycin and 50 μg/ml gentamicin was added. At 4, 24, or 48 h post-infection, cells were washed and lysed with 0.1% Triton X-100. The intracellular CFUs were determined by direct plating on TSB agar plates.

Immunofluorescence Microscopy

Cells were seeded on glass coverslips and infected as described above. At different times post-infection, cells were washed three times with PBS and fixed for 15 min in 4% paraformaldehyde and further processed for immunofluorescence labeling. Briefly, coverslips were washed three times with PBS and incubated for 15 min with PBS plus 50 mM NH_4Cl to quench free aldehyde groups. Coverslips were then incubated with the primary antibodies in PBS containing 10% horse serum, 5% bovine serum albumin, and 0.1% saponin solution (permeabilization) for 1 h at room temperature, washed in PBS, and incubated with the secondary antibodies in PBS containing 10% horse serum, 5% bovine serum albumin, and 0.1% saponin solution under the same conditions. The coverslips were mounted onto glass slides using FluorSave reagent (Calbiochem). Samples were examined on a Nikon microscope (Eclipse TE 2000) at a magnification of ×60 with a lens with a numerical aperture of 1.42. The software MBF ImageJ v1.43 m (Wayne Rasband, National Institutes of Health) was used to merge the microscopic images.

LAMP-1 Co-localization Assays

To determine the percentages of bacteria that co-localized with the lysosomal marker LAMP-1, BMDM cells were infected with *Ba SepA* or *Ba* Δ*virJ SepA* strains expressing SepA-3×FLAG (multiplicity of infection, 200:1). At 4 and 24 h post-infection, cells were washed three times with PBS, fixed for 15 min in 4% paraformaldehyde, and processed for immunofluorescence labeling. The primary antibodies used were rat anti-mouse LAMP-1 ID4B (Developmental Studies Hybridoma Bank, Department of Biological Sciences, University of Iowa; dilution, 1:4000) and rabbit anti-*Brucella* polyclonal antibody (dilution, 1:1500). The secondary antibodies used were goat anti-rat Alexa Fluor 568 antibodies or goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Invitrogen) at a 1:4000 dilution. For DNA staining, DAPI dye at 0.5 mg/ml (final concentration) was used. Co-localization was determined by counting the number of bacteria positive for both labels and expressed as the percentage of LAMP-1-positive BCVs. The assays were performed in triplicate, and a minimum of 100 intracellular bacteria (visualized by indirect immunofluorescence) were scored. Images were acquired with a confocal microscope (Olympus F100) at a magnification of ×60 with a lens with a numerical aperture of 1.42. The software MBF ImageJ v1.43 m was used to merge the microscopic images.

Secretion of SepA and Bpe123

To analyze the secretion of SepA, J774 A.1 cells were infected with *Ba SepA* or *Ba* Δ*virJ SepA* strains expressing SepA-3×FLAG (multiplicity of infection, 1000:1). At 4 h post-infection, cells were washed three times with PBS, fixed for 15 min in 4% paraformaldehyde, and processed for immunofluorescence labeling using rabbit anti-*Brucella* polyclonal antibody (dilution, 1:1500) and anti-FLAG M2 monoclonal antibody (dilution, 1:4000). The secondary antibodies used were goat anti-mouse Alexa Fluor 568 and goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Invitrogen) at a 1:4000 dilution. For DNA staining, DAPI dye was used. Co-localization was determined by counting the number of bacteria positive for both labels and expressed as the percentage of FLAG-positive BCVs. The assays were performed in triplicate, and a minimum of 100 intracellular bacteria (visualized by indirect immunofluorescence) was counted.

To analyze the secretion of Bpe123, J774 A.1 cells were infected with *Ba Bpe123* or *Ba* Δ*virJ Bpe123* strains expressing Bpe123–3×FLAG. At 4 h post-infection, cells were washed three times with PBS and fixed for 15 min in 4% paraformaldehyde. Immunofluorescence labeling and co-localization were determined as described above.

Analysis of the VirB Secretion System

Analysis of the assembly status of the VirB secretion system in the membrane was performed as described previously (21). *Brucella* whole-cell extracts and cell membranes were resuspended in Laemmli sample buffer and heated to 100 °C for 5 min. For cell membranes, non-reducing conditions were preserved. Samples were submitted to SDS-PAGE (12.5%) and transferred to nitrocellulose membranes. The presence and degree of assembly of the VirB5 and VirB8 proteins was carried out by immunoblot analysis using rabbit polyclonal antibodies specific for VirB5 and VirB8 (dilution, 1:2000) and IRDye secondary anti-rabbit antibody (LI-COR, Inc.). All antibodies were

diluted in TBS, 1% nonfat milk, 0.1% Tween solution. Detection was performed using the Odyssey Imaging System (LI-COR, Inc.).

Detection of 3×FLAG-tagged, GST-tagged, or His-tagged Proteins—*Brucella* or *E. coli* whole-cell extracts were resuspended in Laemmli sample buffer and heated to 100 °C for 5 min. Samples were submitted to SDS-PAGE (10% or 12.5% depending on the assay) and transferred to nitrocellulose membranes. The presence of 3×FLAG-tagged proteins was carried out by immunoblot analysis using mouse anti-FLAG M2 monoclonal antibody (dilution, 1:5000), mouse anti-GST monoclonal antibody, mouse anti-SepA or anti-Bpe123 polyclonal antibodies, and IRDye secondary anti-mouse or anti-rabbit antibody (LI-COR, Inc.). All antibodies were diluted in TBS, 1% nonfat milk, 0.1% Tween solution. Detection was performed using the Odyssey imaging system (LI-COR, Inc.).

Mouse Infections—Mice infections were performed as described previously (22). Groups of five 8- to 9-week-old female BALB/c mice were intraperitoneally inoculated with 1×10^5 CFUs of *B. abortus* 2308 wild-type, Δ*virJ*, or complemented strains in PBS. At 2 weeks post-infection, spleens from infected mice were removed and homogenized in 2 ml of PBS. Serial dilutions from individualized spleens were plated on tryptic soy agar with the appropriate antibiotics to quantify recovered CFUs.

Periplasmic and Cytoplasmic Localization Assay

Localization assays were performed as described previously (6). *B. abortus* strains were grown in TSB for 16–24 h at 37 °C until an A_{600} of 1 was reached, and 2.5×10^{10} bacterial cells were centrifuged for 10 min at 3300 × *g*. The pellets were washed with physiological solution, centrifuged for 10 min at 3300 × *g*, and resuspended in 1 ml of 0.2 M Tris-HCl (pH 7.6). One milliliter of 0.2 M Tris-HCl (pH 7.6), 1 M sucrose, and 0.25% Zwitterion 3-16 solution was added to the cell suspension and incubated for 10 min at room temperature. The samples were centrifuged for 30 min at 8000 × *g*, and the pellets were separated from the supernatants and stored at −20 °C until used. The pellets and supernatants were processed for Western blotting using an anti-FLAG M2 monoclonal antibody (1:5000), anti-GroEL (1:2000), and anti-OMP-19 (1:2000), provided by Dr. Axel Cloeckaert as primary antibodies, and IRDye secondary anti-mouse antibody (LI-COR, Inc.). All antibodies were diluted in TBS, 1% nonfat milk, 0.1% Tween solution. Detection was performed using the Odyssey imaging system (LI-COR, Inc.).

Glutathione S-transferase Pulldown Assay

For each GST pulldown reaction, 30 μl of glutathione-Sepharose 4 Fast Flow resin (GE Healthcare) was mixed with 250 μg of dialyzed VirJ-GST, GST, or TolT-GST (an immune-dominant protein of *Trypanosoma cruzi* (23)) recombinant proteins in binding buffer (Tris-HCl, 50 mM; NaCl, 250 mM; pH 7.6). After 1 h of incubation, the resin was extensively washed with the same buffer and incubated with blocking solution (1% BSA in binding buffer) for an extra hour. Without removing blocking solution, 200 μg of recombinant SepA-His or Bpe123-His was added and incubated overnight. Finally, the resin was extensively washed with Tris-buffered saline, 0.05% Triton solution, and bound proteins were eluted with 15 mM of reduced glutathione and processed for Western blotting. All incubations were performed at 4 °C on a rotating platform.

Bacterial Adenylate Cyclase Two-hybrid Assay (BACTH)

For the BACTH analysis, genetic fusions at either the amino or the carboxyl termini of the T18 or T25 fragments of the catalytic domain of *Bordetella pertussis* adenylate cyclase (CyaA) were constructed. The genes coding for VirJ and SepA were amplified by PCR from *B. abortus* 2308 genomic DNA using specific primers (5′-gcTCTAGATATGGATGCCATGTTGGCCCG-3′ and 5′-CGGGATCCCGCGCAGGGCGCGGCG-3′ for *virJ* and 5′-GCTCTAGATACCCCGAGCGAAACCATTGAC-3′ and 5′-cGGGATCCGCGGACGCCGGGCCAGAC-3′ for *sepA*) and cloned in the XbaI/BamHI sites of the BACTH vectors (pUT18, pUT18c, pKT25, and pKNT25). The expression of the fusion proteins was confirmed by immunoblot using a specific polyclonal antiserum against CyaA.

The BACTH analysis was performed using the bacterial adenylate cyclase two-hybrid system kit (Euromedex) according to the instructions of the manufacturer. VirJ and SepA proteins were fused to the complementary fragments (T25 and T18) of the catalytic domain of CyaA, as indicated above, and plasmids carrying the resulting fusions were co-transformed in an *E. coli cyaA* reporter strain (BTH101). To evaluate the functional complementation between hybrid proteins, transformants were plated on Luria-Bertani agar supplemented with 100 μg/ml of ampicillin and 50 μg/ml of kanamycin and incubated at 30 °C for 48 h. Afterward the selected clones were cultured at 30 °C overnight in Luria-Bertani broth supplemented with the same antibiotics and 0.5 mM of isopropyl 1-thio-β-D-galactopyranoside to induce the expression of hybrid proteins. Finally, 2 μl of cultures was spotted in MacConkey agar plates supplemented with 1% of lactose and antibiotics, and the presence of red colonies was evaluated.

Functional complementation of T25/SepA and T18/VirJ was confirmed by measuring the β-galactosidase activity as described previously (24). A protein interaction was considered positive when the β-galactosidase activity was at least three times higher than the values measured for the negative controls.

The plasmids pKT25/zip and pUT18C/zip were used as positive controls for complementation. They expressed the T25/zip and T18/zip fusion proteins that can associate as a result of dimerization of the leucine zipper motifs fused to the T25 and T18 fragments. When pKT25-zip and pUT18C-zip are co-transformed into BTH101, they restore a characteristic Cya^+ phenotype. The empty plasmids were used as negative controls.

Immunoprecipitation of VirJ-3×FLAG and SepA-3×FLAG

B. abortus strains expressing 3×FLAG-tagged proteins and the *B. abortus* control strain were cultivated in TSB for 16–24 h at 37 °C. Cells were harvested at 8000 × *g*, resuspended in 50 ml of buffer A (15 mM Tris-HCl (pH 8), 0.45 mM sucrose, 8 mM EDTA (pH 8), and 0.4 mg/ml lysozyme) and incubated for 15 min at 4 °C. Then, cells were centrifuged (8000 × *g*, 15 min) and

sonicated in 2 ml of buffer B (50 mM Tris-HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM PMSF, and DNase). The resulting lysate was centrifuged twice, and supernatants were recovered and mixed with IP buffer (150 mM NaCl, 1 mM EDTA, and 1% Triton X-100).

For immunoprecipitations, 40 μl of anti-FLAG M2 affinity gel (Sigma-Aldrich) pre-equilibrated with IP buffer was mixed with supernatants and incubated at 4 °C overnight on a rotating platform. The gel suspension was extensively washed with TBS, 1% Triton X-100, and bound proteins were eluted with 2× Laemmli sample buffer without reducing agent. The presence of 3×FLAG-tagged proteins and VirB5 and VirB8 proteins was evaluated by immunoblot as described above.

Results

VirJ Is a Periplasmic Protein Involved in the Intracellular Replication Cycle—The recent identification of a VirB effector protein with a two-step secretion mechanism involving a periplasmic intermediate (6) prompted us to search in the genome of *Brucella* for putative proteins that could participate in stabilizing this intermediate. Searching the literature, we found a 2002 report identifying a gene in *A. tumefaciens* that codes for a periplasmic virulence factor (VirJ) that associates with two VirB substrates and is also in complex with other core components of the secretion apparatus (17). A search for a homologue of *virJ* in the annotated genomes of *Brucella* spp. indicated that all species have a homologous gene (Bab2_0654 in the *B. melitensis* bv. *abortus* strain 2308 genome) that codes for a protein with 34% of identity and 52% of homology and with a predicted periplasmic signal peptide and transmembrane domain in the N-terminal (Fig. 1*A*). The gene is flanked by a putative transmembrane protein and a 14-kDa immunoreactive protein and distanced by more than 500 genes from the VirB cluster. To determine the subcellular localization of the protein encoded in this gene, we 3×FLAG-tagged the gene, expressed it from a plasmid in *B. abortus* 2308 (see "Experimental Procedures"), and performed a periplasmic extraction as described previously (6) with the resulting strain. Fig. 1*B* shows that, as expected, VirJ fractioned in both the periplasmic and the cytoplasmic fractions as the outer membrane OMP19, whereas the control GroEL, which measures that no contamination with cytoplasm occurs during the fractionation procedure, was only present in the corresponding compartment.

To determine whether *virJ* plays a role during the virulence process, we generated a deletion mutant strain and analyzed its intracellular replication capacity in J774 A.1 and BMDM in comparison with the wild-type parental and the complemented strains. As can be observed in Fig. 1, *C* and *D*, deletion of *virJ* significantly affected the intracellular replication cycle, particularly during the early stages of the process, as the mutant showed a marked decrease in the viable intracellular CFUs at 4 h post-infection. Despite the fact that, during the initial phases, the mutant showed less CFUs, the bacteria that survived replicated efficiently with a kinetic similar to the one observed with the wild-type strain. This phenotype is not the consequence of a general replication deficiency because the mutant strain replicated, *in vitro*, with a kinetic undistinguishable from that of the wild-type strain (Fig. 1*E*). To determine whether the strain also showed a defect at earlier time points, we performed an antibiotic protection assay and measured the intracellular CFU at 1, 2, 3, and 4 h post-infection. Fig. 1*D* shows that, even at very early time points post-infection, the mutant exhibited a significant defect. The decrease in the intracellular CFUs at early times points suggested that, as with the *virB* (3) and the *sepA* mutants (6), the Δ*virJ* could have a deficiency in excluding the lysosomal marker LAMP-1 from the BCV. To determine whether this was the case, we infected BMDM with the Δ*virJ*, the wild type, and the complemented strains and determined the capacity of these strains to acquire and afterward exclude LAMP-1 from the BCV by a co-localization assay. Figs. 2, *A* and *B*, shows that the Δ*virJ* mutant strain, as the Δ*sepA* mutant (6), was significantly less effective in excluding LAMP-1 from the BCVs at 24 h post-infection even though it acquired it equivalently at 4 h, indicating that the decrease in the intracellular CFUs observed in the antibiotic protection assays was the result of a higher degradation in the phagocytic route.

The results observed with the Δ*virJ* mutant in the intracellular replication curves as well as in the LAMP-1 co-localization assays prompted us to evaluate the role of the gene during the infectious process in virulence assays in the mouse model. For this, groups of five female BALB/c mice were intraperitoneally infected with 1×10^5 CFUs of the wild-type 2308, Δ*virJ*, and complemented strains, and, at 15 days post-infection, the numbers of viable bacteria in the spleens were determined by plating. As can be observed in Fig. 2*C*, deletion of *virJ* significantly affected the virulence of *B. abortus* because the mutant showed over 10-fold less bacteria than the parental wild-type strain. Together, these results demonstrate that *virJ* codes for a periplasmic protein necessary during the early stages of the intracellular replication cycle that participates in the inhibition of the BCV endosome-lysosome fusion event and is important during the virulence process in the mouse model.

VirJ Is Necessary for the Secretion of Two VirB Substrates—The reported literature on the *A. tumefaciens virJ*, the phenotypes observed with the Δ*virJ* mutant (intracellular replication deficiency, LAMP-1 exclusion from the BCV, and reduced virulence in the mouse model), as well as its periplasmic localization suggested that this gene might code for a protein necessary for the secretion of some VirB substrates. To explore whether this was the case, we decided to determine the secretion level of two VirB effectors recently identified: SepA and Bpe123. For this we generated *B. abortus* wild-type and Δ*virJ* strains expressing *sepA* and *bpe123* fused to a 3×FLAG epitope as described in Refs. 6, 7. With these strains, we infected J774 A.1 cells and determined, at 4 h post-infection, the level of secretion by immunofluorescence double staining with a monoclonal anti-FLAG and a rabbit polyclonal anti-*Brucella* antibody (see "Experimental Procedures"). Fig. 3 shows that secretion of both effectors was significantly reduced in the Δ*virJ* mutant because they showed a marked reduction in surface exposition even though they were found in the periplasm, indicating that VirJ is necessary for secretion through the outer membrane but not for translocation to the periplasm. It is of note that SepA was informed to have a periplasmic localization but Bpe123, even though it has a predicted signal peptide, was not formally dem-

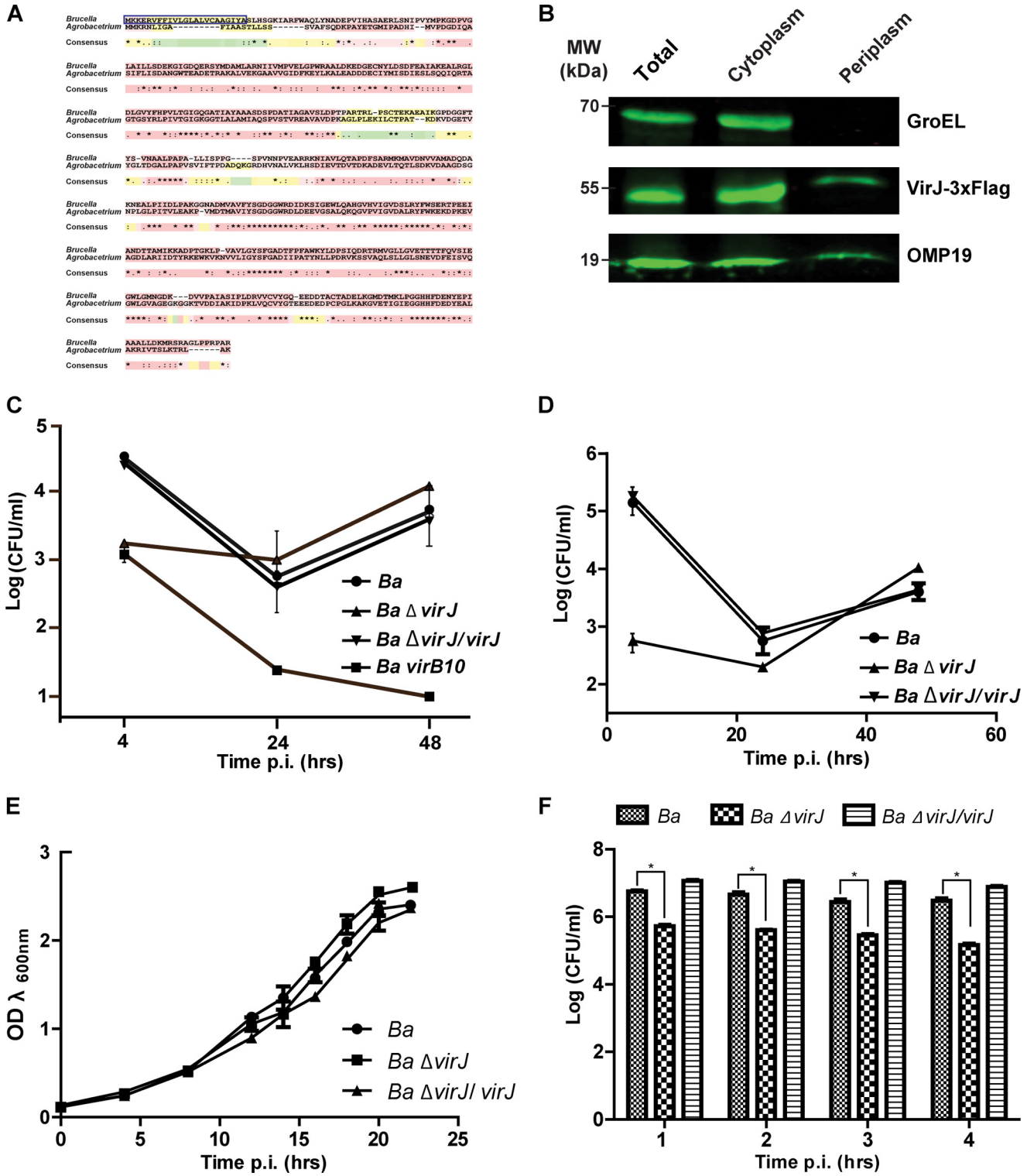

FIGURE 1. **VirJ is a periplasmic protein involved in intracellular replication in professional phagocytes.** *A*, protein alignment of the *B. a abortus* VirJ protein with the *A. tumefaciens* homologue. The *pink shadows* indicate identity or homology. The *blue box* indicates the putative signal peptide and transmembrane helix. The alignment was performed with T-Coffee. *B*, Western blotting analysis with anti-FLAG, anti-GroEL, and anti-OMP19 monoclonal antibodies of whole bacteria (*Total*) and periplasmic and cytoplasmic fractions of the *Ba virJ* strain expressing a VirJ-3×FLAG. *C*, intracellular multiplication of wild-type (*Ba*), mutant (*Ba* Δ*virJ*), complemented (*Ba* Δ*virJ/virJ*), and *virB10* mutant (*Ba virB10*) strains in J774 A.1 cells. *D*, intracellular multiplication of wild-type, mutant, and complemented strains in BMDM. *E*, *in vitro* growth curve of wild-type, mutant, and complemented strains in TSB medium. *F*, intracellular viable CFU of the wild-type, mutant, and complemented strains at early times post-infection in BMDM. *, $p < 0.005$. *p.i,* post infection.

onstrated as periplasmic. This VirJ-dependent secretion could be due to a direct interaction of VirJ with these substrates or an indirect effect. To rule out these possibilities, we produced SepA and Bpe123 in *E. coli* fused to a poly-histidine tag and purified them. VirJ was also produced fused to GST and purified (see "Experimental Procedures"). With these proteins, pull-

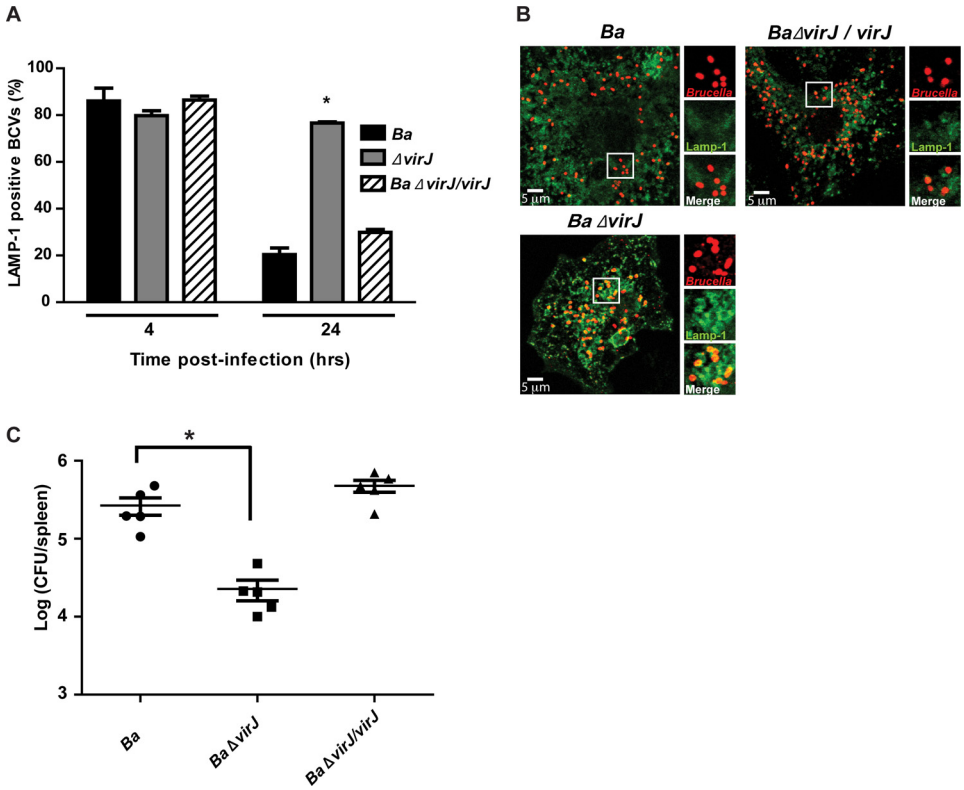

FIGURE 2. **VirJ is involved in the exclusion of LAMP-1 from the BCV and participates in the virulence process in mice.** *A*, determination of LAMP-1-positive BCVs at 4 and 24 h post-infection of the wild-type (*Ba*), Δ*virJ* mutant (*Ba* Δ*virJ*), and complemented (*Ba* Δ*virJ/virJ*) strains in BMDM. *, $p < 0.0001$. *B*, representative images of the LAMP-1-positive and -negative BCVs of the wild-type, mutant, and complemented strains at 24 h post-infection. *Red*, *Brucella*; *green*, LAMP-1. *C*, spleen bacterial load of intraperitoneal infections of groups of five BALB/c mice with 10^5 CFU of wild-type, mutant, and complemented strains at 2 weeks post-infection. *, $p < 0.01$.

down assays were performed, using as a negative controls GST alone or fused to a non-related protein (TolT-GST, see "Experimental Procedures"). Fig. 4*A* shows the results of these assays. As can be observed, SepA and Bpe123 were pulled down by the VirJ-GST fusion but not with GST alone or TolT-GST, an immunodominant protein of *T. cruzi* (23) fused to GST (see signal for SepA and Bpe123 in the VirJ-GST lanes but not in the control lanes), indicating that both VirB substrates specifically interacted with VirJ directly.

To further confirm the interaction of SepA with VirJ, we performed a bacterial two-hybrid assay, cloning both genes in the T25 and T18 vectors covering all possible combinations (see "Experimental Procedures"). As can be observed in Fig. 4*B*, the combination of T25-SepA and T18-VirJ confirmed the VirJ-SepA interaction, which gave a β-galactosidase activity ~15 times higher than the negative controls. In the case of Bpe123, we were not able to detect interaction using the bacterial two-hybrid system with all combinations tested.

The results presented above demonstrate that VirJ is necessary for the secretion through the outer membrane of, at least, two VirB secretion substrates, both of which have a periplasmic intermediate. VirJ is not needed for the translocation of these effectors to the periplasm and directly interacts with them.

VirJ Associates with VirB5 and VirB8, and Its Absence Affects the Assembly of the VirB Complex—The fact that VirJ interacts with at least two VirB effectors, together with the phenotypes of the mutant in cells and mice, raised the possibility that this protein might be an important constituent of the type IV secretion complex composed of many substrates as well as the VirB core components. To determine whether this is the case, we first analyzed the interaction of VirJ with VirB8 and VirB5 by co-immunoprecipitation assays using a strain expressing a VirJ-3×FLAG and, as a negative control, the wild-type parental strain. Immunoprecipitations were performed with a monoclonal anti-FLAG antibody, and the co-immunoprecipitates were probed with an anti-VirB8 or anti-VirB5 rabbit polyclonal antibody (see "Experimental procedures"). Fig. 5 shows that both VirB5 and VirB8 co-immunoprecipitated with VirJ in the strain expressing the FLAG-tagged version of the gene (*Ba VirJ* lane in the IP gel) but not in the negative control (*Ba* lane in the IP gel), indicating that they interact either directly or indirectly. The input gel shows that equivalent amounts of VirB5 and VirB8 were present prior to the immunoprecipitation. To further investigate whether SepA is also part of this complex, we performed the same co-immunoprecipitation assay but with a strain expressing a SepA-3×FLAG protein. As can be observed in Fig. 5, VirB5 and VirB8 also co-immunoprecipitated with SepA (*Ba SepA* lane in the IP gel), and no signal was detected in the negative control, indicating that this protein is also part of the same complex.

The results presented above suggested that the absence of *virJ* could have an effect on the proper assembly of the VirB secretion apparatus. To evaluate this possibility, we analyzed the presence/status of two proteins of the system in the membrane of the bacteria (VirB5 and VirB8) as we have described previously (21). Briefly, total cell and membrane extracts were

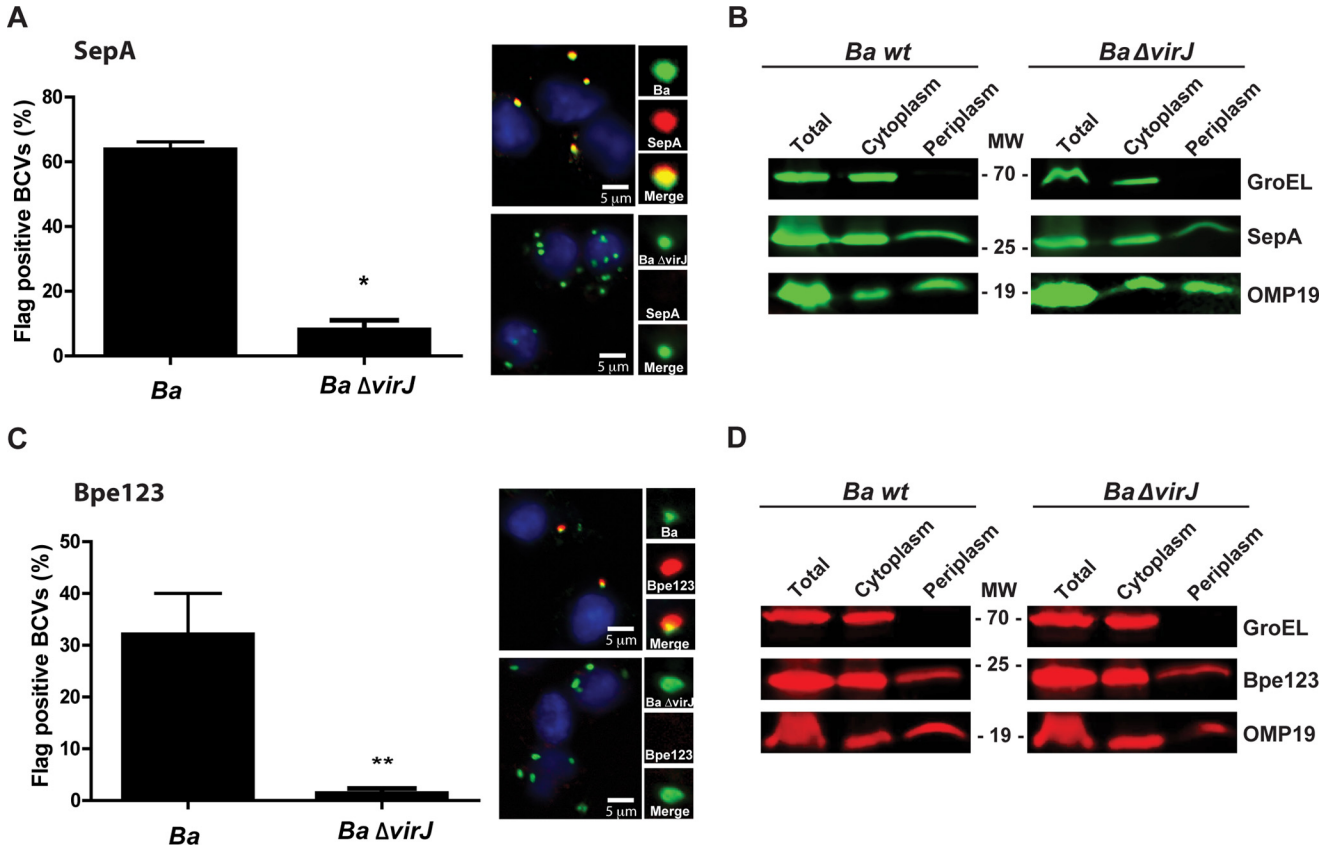

FIGURE 3. **VirJ is necessary for the secretion of at least two VirB effector proteins.** *A*, percent of bacteria positive for surface-exposed SepA in J774 A.1-infected cells with wild-type (*Ba sepA*) and Δ*virJ* mutant (*Ba ΔvirJ/sepA*) strains expressing SepA-3×FLAG at 4 h post-infection. *Right panel*, representative images of the quantification. *Top right panel*, wild-type strain. *Bottom right panel*, Δ*virJ* mutant. *Green*, *Brucella*; *red*, 3×FLAG. *B*, Western blot with anti-FLAG, anti-GroEL, and anti-OMP19 monoclonal antibodies of whole bacteria (*Total*) and periplasmic and cytoplasmic fractions of *Ba sepA* and *Ba ΔvirJ/sepA*. *MW*, molecular weight. *C*, percent of bacteria positive for surface-exposed Bpe123 in J774 A.1-infected cells with wild-type (*Ba bpe123*) and Δ*virJ* mutant (*Ba ΔvirJ/bpe123*) strains expressing Bpe123–3×FLAG at 4 h post-infection. *Right panel*, representative images of the quantification. *Top right panel*, wild-type strain. *Bottom right panel*, Δ*virJ* mutant. *Green*, *Brucella*; *red*, 3×FLAG. *D*, Western blot with anti-FLAG, anti-GroEL, and anti-OMP19 monoclonal antibodies of whole bacteria (*Total*) and periplasmic and cytoplasmic fractions of the *Ba bpe123* and *Ba ΔvirJ/bpe123* strains. *, $p < 0.0001$; **, $p < 0.005$.

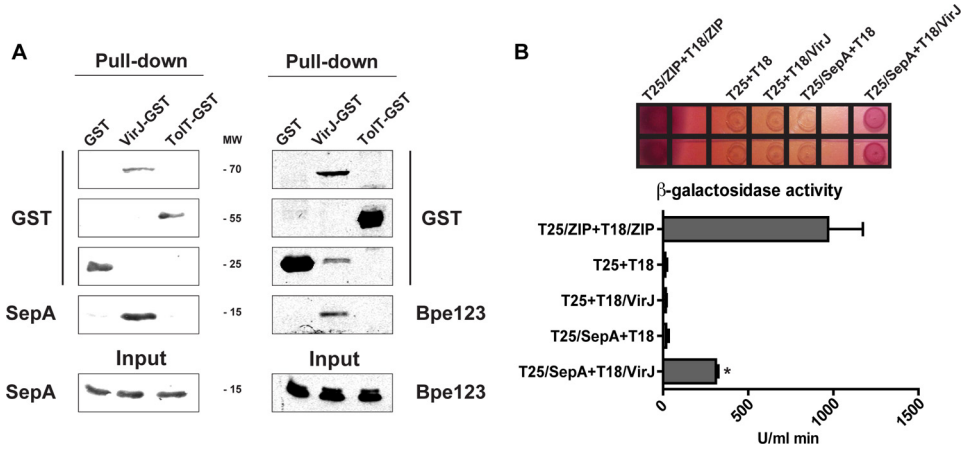

FIGURE 4. **VirJ interacts with SepA and Bpe123.** *A*, Western blotting analysis of a GST pulldown assay performed with recombinant SepA-His or Bpe123-His and VirJ-GST developed with an anti-GST monoclonal antibody and anti-SepA or Bpe123 mouse polyclonal antibodies. As negative controls, GST alone and TolT-GST were used. *Input*, prior to the pulldown assay, 20 μl of each sample was removed for analysis. *MW*, molecular weight. *B*, BACTH for the SepA-VirJ interaction. The plasmids T25-SepA and T18-VirJ were co-transformed in the *E. coli* BTH101strain, and the interaction was determined by the level of cAMP in MacConkey/lactose agar plates (*top panel*) and activity of the reporter β-galactosidase (*bottom panel*). *, $p < 0.0001$ with respect to the negative controls (*T18/ZIP*+*T25/ZIP*, positive control; *T25*+*T18*, *T25*+*T18/VirJ*, and *T25/SepA*+*T18*, negative controls).

prepared with the wild-type and Δ*virJ* strains, and the level as well as the banding patterns of both proteins were assessed by Western blotting. As can be observed in Fig. 6, although both proteins were expressed at similar levels in the wild-type and the mutant strain (whole bacteria lanes), when total membranes submitted to a SDS-PAGE were used, the wild-type and the mutant strains showed different band patterns for both the VirB5 and VirB8 proteins. More specifically, although the level

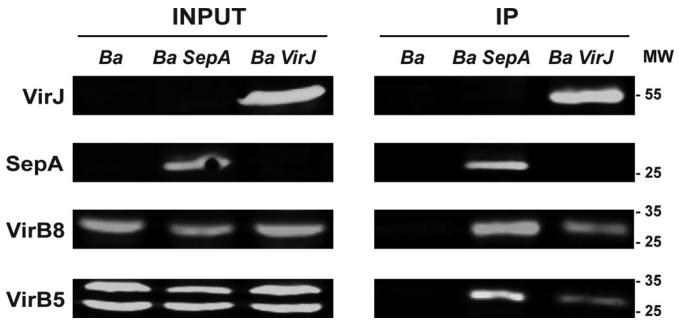

FIGURE 5. **VirJ and SepA interact with components of the VirB secretion system *in vivo*.** Immunoprecipitation assays were performed from the *B. abortus* strains expressing 3×FLAG-tagged proteins (VirJ or SepA) as indicated under "Experimental Procedures." Samples were subjected to SDS-PAGE, and the presence of VirB5 and VirB8 proteins was determined by Western blotting with the polyclonal antibodies anti-VirB5 and anti-VirB8. Negative controls were performed with the wild-type strain without the FLAG-tagged proteins. *Input*, samples before immunoprecipitation. *MW*, molecular weight.

of the VirB5 higher complex was found to be less intense in the membranes of the Δ*virJ* mutant, the lower molecular mass form was absent. On the other hand, we were unable to detect the higher molecular mass complexes of VirB8 in the mutant strain even though the monomer was equivalent between strains. Additionally, in the total cell extracts, this higher molecular complex migrated slightly less in the mutant as well. In both cases, the levels of OMP19, both in the total cell extracts and in membranes, were equivalent. Although we cannot understand at this stage the difference in the assembly status of the VirB complex between the wild type and the Δ*virJ* mutant, our results indicate that the complex is different between them, at least when assessed with the VirB5 and VirB8 components. This is consistent with the functional deficiency observed with the mutant: an altered intracellular replication curve, a deficiency in the secretion of two VirB effectors, and a diminished virulence in the mouse model of infection.

Discussion

Secretion of proteins in pathogenic bacteria is normally a key feature of the virulence process, and there are multiple secretion systems that have been identified and characterized in a wide range of pathogens (25). The different secretion mechanisms vary depending on the life style of the bacterium and the secretion systems they code. Additionally, each of these secretion systems has a unique mechanism by which it mediates the translocation of substrates across either one membrane or two membranes and the periplasmic space. Although some systems, like the type III, engage their substrates in the cytoplasm of the bacteria and translocate them to either the outer space or the cytoplasm of the infected cells, other secretion systems only secrete the proteins through the outer membrane when they have been translocated to the periplasm by the canonical *sec* system (25). Type IV secretion systems are widely distributed in pathogenic and non-pathogenic bacteria and are evolutionary related to conjugation systems (11). These membrane supramolecular complexes are normally composed of 12 proteins arranged through the inner membrane, the periplasmic space, and the outer membrane. Although much has been done to identify the substrates of T4SSs in several bacteria, we still have scarce information on the mechanistic properties of these systems. This is the mechanism by which they mediate secretion. An intriguing observation in several bacteria with T4SSs is that they are able to secrete and/or translocate substrates that are present in the cytoplasm, the periplasmic space, or even inserted in the inner membrane (6, 7, 11, 15–17, 26). These reports raise issues regarding the initial models that proposed that the T4SSs engage their substrates in the cytoplasm and should encourage the search for alternative models. Recently the structure of a T4SS was solved by electron microscopy. The analysis showed that the complex is composed of two substructures that have a certain degree of independence between them, one anchored in the inner membrane and one in the outer membrane, and that are connected through a flexible stalk (13). This model fits the observation that some substrates are engaged in the cytoplasm and others in the periplasm and raises very interesting questions regarding why, how, and when the apparatus secretes substrates in each of these compartments. Moreover, it has been proposed that these systems might switch between two modes: a pilus biogenesis mode and a substrates translocation mode with different mechanistic properties (25). This model might imply that the different modes have "preferences" for substrates in different compartments.

Virulence in the zoonotic pathogen *Brucella* is completely dependent on the presence of the type IV secretion *virB* system that translocates several effectors to the host cell which modulate the intracellular fate of the BCV (27). The VirB complex in *Brucella* has a high degree of similarity to the VirB system in *Agrobacterium*, and because much has been done at the biochemical and structural level with this bacterium, most of what we know about the system in *Brucella* is due to extrapolation. To date, 15 T4SS substrates have been identified (4–8, 28), but we do not know the molecular mechanism that mediates their secretion and/or translocation. Interestingly, some of these effectors, like SepA, Bpe123, Bpe275, Bpe043, VceC, BspC, BspE, and BspB, either have a predicted periplasmic signal peptide or a transmembrane domain (5–8), which highlights the complexity of the secretion process.

In this manuscript, we describe the identification in *B. abortus* of a gene, *virJ*, that codes for a protein necessary for the secretion of at least two VirB effectors that have a periplasmic intermediate: SepA and Bpe123. We demonstrated that a mutant in this gene has a defect in the intracellular replication cycle in macrophages because of a diminished capacity to exclude the lysosomal marker LAMP-1 from the BCV and is less virulent in the mouse model of infection. Our analysis of the secretion of Bpe123 and SepA in the Δ*virJ* mutant showed that both substrates need the presence of VirJ for an efficient secretion but not for a periplasmic localization. *In vitro* GST pulldown assays further demonstrated that VirJ directly interacts with SepA and Bpe123, indicating that, most probably, these proteins are part of the same complex in the periplasmic space prior to translocation through the outer membrane. The sum of these results allowed us to formulate the hypothesis that VirJ could be part of the T4SS complex composed of several effector proteins as well as the apparatus *per se*. To test this, we immunoprecipitated either SepA or VirJ and determined the co-immunoprecipitation of VirB5 and VirB8. Our results

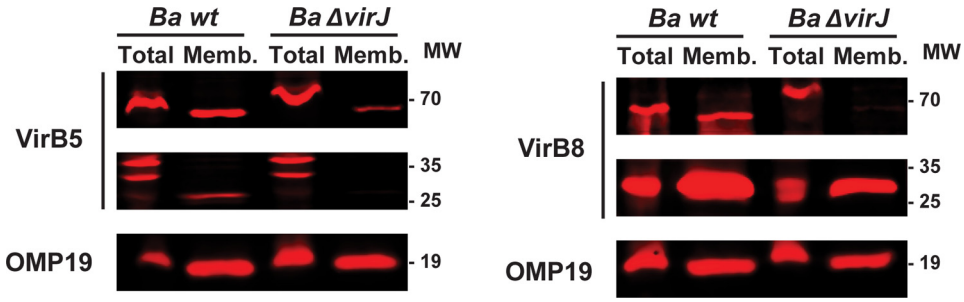

FIGURE 6. **Deletion of *virJ* reduces VirB5 and VirB8 levels in the membrane.** Western blotting analysis of VirB5 and VirB8 in whole bacteria (*Total*) and membranes (*Memb.*) of *Ba* wild-type and *Ba* Δ*virJ* strains. *Brucella* cell membranes were subjected to SDS-PAGE in the absence of any reducing agent and probed with anti-VirB5 and VirB8 antibodies. *MW*, molecular weight.

showed that both of these core components are associated with SepA and VirJ in *in vitro* cultures, strongly suggesting the existence of a secretion complex of which VirJ is probably a central organizational unit. Moreover, absence of VirJ affects the assembly of the secretion apparatus, thus reinforcing this hypothesis.

Our working hypothesis proposes that VirJ as well as several of the effectors are part of a periplasmic secretion platform of which the VirB apparatus is its main core. VirJ could be playing a central role in organizing this structure in the periplasm by stabilizing the complex and probably presenting the effectors to the secretion components. This hypothesis is consistent with the results obtained by one group for the *A. tumefaciens* homologue of *virJ* (17), which reported that this gene is a virulence factor and that its protein product interacts with several VirB substrates. Contrary, another group informed that they were unable to detect an interaction between VirJ and the T-DNA and that its absence did not affect the transfer of the substrate (29). We do not have an explanation for the discrepancies between these two reports in *Agrobacterium* but have to highlight that, regardless the homology between the systems in these two bacteria, the *Agrobacterium* VirB systems translocates DNA-protein complexes, whereas the *Brucella* system only translocates proteins, indicating that they probably have several differences. Our genetic, biochemical, and *in vitro* as well as *in vivo* results clearly show that VirJ plays a relevant role in the activity of the VirB secretion system in *Brucella*.

An interesting result was the fact that the *virJ* mutant, even though it showed a significant defect in the viable intracellular bacteria at early times post-infection, was able to replicate after this initial bottleneck with kinetics similar to the wild-type strain. This could imply that there might be a set of T4SS substrates involved in these early events that have a certain dependence on VirJ and a subset of effectors needed in the later stages of the intracellular life cycle that could be VirJ-independent. Our working hypothesis raises several interesting questions regarding the organization and mechanistic properties of the *Brucella* T4SS. How many additional effectors are also part of this secretion complex, and are they all periplasmic? How does the system shuttle between the secretion of substrates engaged in the cytoplasm or the periplasm, and is there a hierarchy in their secretion? Are the secretion signals of the cytoplasmic *versus* the periplasmic effectors different, and how does the system recognize these substrates in the different compartments? Answering these questions will be central to moving forward in understanding how this amazing nanomachine functions.

Author Contributions—M. G. D. G., C. C., and J. E. U. conceived and designed the research. M. G. D.G., P. H. D., J. M. S., F. T. L., and C. C. performed the experiments. M. I. M provided the Bpe123-His tagged construct. M. G. D. G., C. C., and J. E. U. wrote the paper.

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VirJ Is a *Brucella* Virulence Factor Involved in the Secretion of Type IV Secreted Substrates

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