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The *Brucella suis* Type IV Secretion System Assembles in the Cell Envelope of the Heterologous Host *Agrobacterium tumefaciens* and Increases IncQ Plasmid pLS1 Recipient Competence

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Pathogenic *Brucella* species replicate within mammalian cells, and their type IV secretion system is essential for intracellular survival and replication. The options for biochemical studies on the *Brucella* secretion system are limited due to the rigidity of the cells and biosafety concerns, which preclude large-scale cell culture and fractionation. To overcome these problems, we heterologously expressed the *Brucella suis virB* operon in the closely related α_2-proteobacterium *Agrobacterium tumefaciens* and showed that the VirB proteins assembled into a complex. Eight of the twelve VirB proteins were detected in the membranes of the heterologous host with specific antisera. Cross-linking indicated protein-protein interactions similar to those in other type IV secretion systems, and the results of immunofluorescence analysis supported the formation of VirB protein complexes in the cell envelope. Production of a subset of the *B. suis* VirB proteins (VirB3-VirB12) in *A. tumefaciens* strongly increased its ability to receive IncQ plasmid pLS1 in conjugation experiments, and production of VirB1 further enhanced the conjugation efficiency. Plasmid recipient competence correlated with periplasmic leakage and the detergent sensitivity of *A. tumefaciens*, suggesting a weakening of the cell envelope. Heterologous expression thus permits biochemical characterization of *B. suis* type IV secretion system assembly.

Brucella species are pathogens of mammals, which cause severe infections and abortions in animals and long-lasting febrile diseases in humans (65). They impact agriculture by causing zoonotic diseases of cattle (*Brucella abortus*), sheep (*B. melitensis*), and swine (*B. suis*), which cause substantial economic losses, and they pose a threat for those handling the animals (8, 28). The eradication of *Brucella* from livestock has succeeded in some parts of the world, but expensive control and surveillance systems are necessary due to the possibility of reinfection of livestock from wildlife. In addition to its threat to commercial agriculture, *Brucella* is considered as a potential category B bioterror agent (32). *Brucella* infections are very long-lasting, and current treatment regimens require 6 to 8 weeks of therapy with two antibiotics (61). Several live attenuated vaccines are effective for animals, but safe vaccines for humans are currently not available (28). The threat posed by *Brucella* infections gives research on the molecular basis of virulence and persistence in the mammalian body a high priority.

Brucella species survive and multiply inside mammalian cells, including cells of the immune system such as macrophages (12, 51). They inhibit apoptosis of infected cells and apparently evade the immune response of their hosts, causing long-lasting infections (48). After entering macrophages via lipid rafts, the *Brucella*-containing vacuole (BCV) does not fuse with the lysosomes, thus avoiding rapid cell destruction (13). Instead, the BCV follows a novel intracellular trafficking pathway, which interacts with the endoplasmic reticulum (ER), leading to the creation of a specialized vacuole in which the bacteria multiply (37). *Brucella* species are trophic for cells of the reproductive tissues in their natural animal hosts. Analysis of the genomes of three *Brucella* species has shown that they are devoid of "classical" virulence factors such as adhesins or toxins (21, 29, 50). One exception is the VirB type IV secretion system (T4SS) that has been identified in several transposon mutagenesis screens as a key virulence factor (20, 31, 49).

T4SSs are a family of multiprotein complexes, which serve to secrete macromolecules across the bacterial envelope. The *Brucella virB* operon encodes 12 proteins, of which VirB1 to VirB11 show significant similarity to those from other T4SSs. The similarity of the *Brucella* VirB proteins to components of other T4SSs, including that of the well-studied model organism, the plant pathogen *Agrobacterium tumefaciens* (11, 14, 15, 49), suggests that *Brucella* uses it as conduit for the translocation of virulence factors into mammalian cells (12, 48). It is currently unknown at which stage of the infection process the *Brucella* T4SS secretes virulence factors, how it assembles in the membranes, whether it forms a pilus-like structure, and whether and which host structures it contacts during this process. Analysis of gene regulation shed some light on the time frame of T4SS action. The *B. suis virB* operon was induced after uptake into mammalian cells, which is well in accord with a requirement for intracellular growth (9). In contrast,

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the *B. abortus virB* genes appear to be expressed constitutively (22, 52). In both *B. melitensis* and *B. suis*, *virB* expression is negatively regulated by quorum sensing and dependent on a quorum-sensing regulator (19, 59).

In contrast to work on bacterial uptake, trafficking within infected cells, and gene regulation, relatively little research has been done on the structure and function of the *Brucella* T4SS. Transposon insertions were likely polar so that conclusions on the effects of single genes could not be made (17, 20, 49). In-frame deletions of *B. abortus virB1* and *virB2* were shown to inhibit intracellular survival and multiplication in macrophages; however, only deletion of *virB2*, which encodes a protein similar to the main pilus component in other T4SS (23, 43), attenuated bacterial persistence in a mouse infection model (22). The VirB12 protein, which does not have homologs in other T4SSs, encodes a protein with similarity to outer membrane adhesin in *Pseudomonas* species (1). Whereas this suggested a role in host cell attachment, it was recently shown that *virB12* is dispensable for infections of J774 macrophage and mouse models (58). Work with purified *B. suis* VirB proteins has shown binding of the putative lytic transglycosylase VirB1 to VirB8, VirB9, and VirB11 (33). These interactions are believed to coordinate transmembrane assembly of the T4SS at the site of murein lysis by VirB1. Purified *B. suis* VirB5, which is similar to minor T-pilus components of other T4SSs (54, 64), interacts with VirB8 and VirB10, and these interactions are likely required for binding to VirB2, followed by pilus assembly (66). *B. suis* VirB4 fully complemented an *A. tumefaciens virB4* mutant in a plant tumor assay (66), and *B. suis* VirB1 partly complemented *virB1* gene defects in *A. tumefaciens*, showing that many protein-protein interactions are conserved (34).

Due to the pathogenicity of *Brucella* species and the requirement for biosafety level 3 containment, the options for biochemical studies on T4SS assembly in this organism are very limited. Based on our previous findings that some VirB components could be exchanged between the *B. suis* and the *A. tumefaciens* T4SS, we here expressed the entire *B. suis virB* operon in the heterologous host. Production of subsets of the *B. suis* VirB proteins increased the ability of *A. tumefaciens* to serve as recipient in T4SS-mediated plasmid conjugation experiments. Analyses of their membrane association and interactions further substantiated that the *B. suis* VirB proteins assembled into a T4SS with basic features similar to that of *A. tumefaciens* in the heterologous host.

MATERIALS AND METHODS

Cultivation of bacteria and yeast. Overnight cultures of *A. tumefaciens* wild-type A348 and C58 (62) or strains carrying pTrc300 or *virB* operon constructs were grown in YEB medium (0.5% beef extract, 0.5% peptone, 0.1% yeast extract, 0.5% sucrose, 2 mM $MgSO_4$) in the absence of antibiotics (wild-type strains) or with spectinomycin (300 μg/ml) and streptomycin (100 μg/ml) for plasmid propagation. The cells were then inoculated to an optical density at 600 nm (OD_{600}) of 0.1 in liquid AB minimal medium (10 g of glucose/liter, 4 g of MES [morpholinoethanesulfonic acid]/liter, 0.3 g $MgSO_4 \cdot 7\ H_2O$/liter, 0.15 g of KCl/liter, 0.01 g of $CaCl_2$/liter, 0.0025 g of $FeSO_4 \cdot 7\ H_2O$/liter, and 1 mM potassium phosphate [pH 5.5]) and grown for 5 h at 20°C, followed by plating of 1 ml on 15-cm-diameter AB agar plates with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for induction of the *trc* promoter or 200 μM acetosyringone (AS) for induction of the *Agrobacterium virB* promoter as indicated in individual experiments and further cultivation at 20°C for 3 days.

For the analysis of pLS1 recipient activity, donor A348 pLS1 (57) cells were cocultivated with UIA143 recipient cells (cured of Ti plasmid) carrying pTrc300 or *virB* operon plasmids in a 5:1 ratio for 3 days on AB minimal medium with 300 μM AS and 0.5 mM IPTG, followed by plating on YEB agar with antibiotics (carbenicillin at 150 μg/ml, streptomycin at 100 μg/ml, and spectinomycin at 300 μg/ml) for selection of donors, recipients, and transconjugants as described previously (34).

For the analysis of sodium dodecyl sulfate (SDS) sensitivity cells from overnight cultures grown in YEB medium were diluted to an OD_{600} of 0.1 in liquid AB minimal medium and cultivated for 2.5 to 3 h at 20°C, followed by aliquoting into wells of a 96-well microtiter plate in the presence or absence of 0.5 mM IPTG, the addition of SDS (0.025, 0.006, or 0.003%), and further cultivation and shaking for up to 60 h.

To study functional complementation of *Agrobacterium virB* defects by *B. suis* proteins in translocation of effector proteins we used the Cre reporter assay for translocation (CRAfT) (56). Here, we cocultivated *Agrobacterium* A348 containing plasmid pSDM3155, expressing a Cre-VirF fusion, with *Saccharomyces cerevisiae* strain LBY2 (56), in which Cre-mediated excision of a chromosomal *URA3* gene was scored as colony growth on medium containing 5-fluoroorotic acid. The excision efficiency was calculated as number of 5-fluoroorotic acid-resistant colonies per output yeast.

B. suis strain 1330 was grown in tryptic soy broth (1.7% peptone from casein, 0.3% peptone from soy meal, 0.5% NaCl, 0.25% glucose, and 0.25% K_2HPO_4) or on tryptic soy agar.

Construction of *B. suis virB manB* mutant. To construct *B. suis* 1330 (*virB2*::Tn*5-manB*), an internal fragment of the *B. suis pgm* gene was amplified by PCR (primers pgm5 [5′-TATGCGATGGGTGCGAAAGC-3′] and pgm3 [5′-GTTGGAGGTGACTGGCGTGA-3′]) and cloned into pGEM-T (Promega). Since ColE1-based vectors do not replicate in *Brucella*, this plasmid was introduced into *B. suis* 1330 *virB2*::Tn*5* (27) by electroporation to inactivate the gene with insertional mutagenesis by homologous recombination. The rough phenotype of the resulting strain *B. suis virB manB* was checked by slide agglutination with O-antigen-specific sera and acriflavin.

Construction of *B. suis virB* operon constructs. The *trc* promoter expression vector pTrc300 was constructed from pTrc200 (55), by cleavage at the NcoI site and removal of the overhanging 4-bp single-stranded DNA with mung bean nuclease, followed by blunt-end ligation. This modification permitted the expression of genes cloned into the polylinker without the need of directly fusing them to the NcoI site encoded ATG codon of pTrc200. For construction of *virB* operon vectors the following cloning strategy was used (Fig. 1A). First, *virB2-6* (primers virBsuis2-5 [5′-GGCAGAGCTCGACATAAGGAATAAAGATCA TGAAAAC-3′] and virBsuis6-3 [5′-GAGGTCTAGAAAGGCCCTAATCCC TGTTGAACTG-3′]) and *virB7-12* (primers virBsuis7-5 [5′-GGCATCTAG AAGGAAATCATAATGAAAAAGGTAATCC-3′] and virBsuis12-3 [5′-GAG CCTGCAGGTTACTTGCGTAAAATTTCGATATC-3′]) operon fragments were PCR amplified from pUCvirB (49) by using the Expand Long Template PCR System (Roche). Next, *virB2-6* and *virB7-12* fragments were excised by SacI/XbaI and XbaI/PstI, respectively, and cloned into pTrc300 to give pTrcB2-6 and pTrcB7-12, respectively. The *virB7-12* fragment was further excised from pTrcB7-12 with XbaI and PstI and cloned into the XbaI/PstI sites of pTrcB2-6, resulting in pTrcB2-12 and pTrcB3-12 (after detection of a missense mutation at the *virB2* start codon). DNA manipulations such as DNA isolation cloning and sequencing were performed according to standard techniques (47). Next, the *virB1* gene was PCR amplified from pUCvirB (primers virB1-5′ [5′-GCGCGA GCTCAGAAGGAGACGATCCTATGGTGCCA-3′] and virB1-3′ [5′-GCG CGAGCTCTTAGAAAACAACTACGCCGTCC-3′]), cloned into pCR2.1 by using the TOPO cloning system (Invitrogen), excised with SacI, and inserted into *virB* operon plasmids resulting in pTrcB1+3-12 and pTrcB1+2-12.

Generation of VirB protein-specific antisera. For the generation of VirB12-specific antiserum, a 471-bp fragment of the gene corresponding to the processed periplasmic form of the protein (156 amino acids [16 to 172]) was PCR amplified from pUCvirB with oligonucleotides (VirB12-5 [5′-CAGGGTACCCTCCAG CCCGCCGAAGCC-3′] and virB12-3 [5′-GAGCTGCAGTTACTTGCGTAA AATTTCGATATCCAC-3′]), cleaved with Acc65I and PstI (restriction sites are underlined), and ligated with similarly cleaved vector $pT7H_6TrxFus$ (39). The hexahistidyl-TrxA fusion protein was overexpressed and purified by immobilized metal affinity chromatography as described previously (66), and 500 μg was used to immunize rabbits for the generation of an antiserum (BioGenes). A 15-amino-acid peptide (NGGLDKVNTSMQKVC) was used for the immunization of rabbits to generate a VirB2-specific antiserum (BioGenes). The generation of antisera for the detection of *B. suis* VirB1, VirB5, VirB8, VirB9, VirB10, and VirB11 was described previously (33, 66).

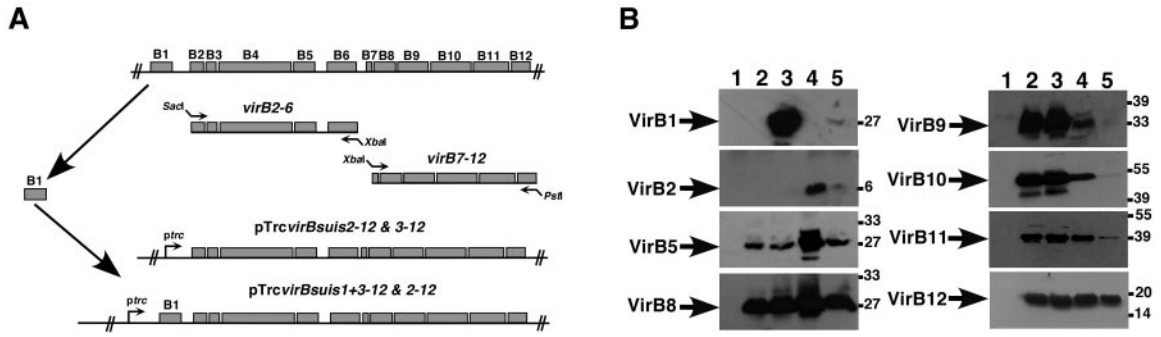

FIG. 1. Cloning of the *B. suis virB* operon and production of VirB proteins in *A. tumefaciens.* (A) Construction of different *virB* operon-containing pTrc300 derivatives for IPTG-induced expression. Portions of the *virB* operon containing *virB2-6* and *virB7-12* were PCR amplified and cloned separately, followed by the construction of pTrcB2-12. pTrcB3-12 resulted from a spontaneous change at the *virB2* start codon abolishing the expression of this gene. Cloning of the *virB1* gene 5′ to the *virB2-12* and *virB3-12* operon resulted in pTrcB1+2-12 and pTrcB1+3-12. (B) Detection of VirB protein production in cells of UIA143 carrying pTrc300 (lane 1), pTrcB3-12 (lane 2), pTrcB1+3-12 (lane 3), pTrcB2-12 (lane 4), and pTrcB1+2-12 (lane 5). Cells were cultivated on AB minimal medium plates at 20°C for 3 days in the presence of IPTG for induction of the *trc* promoter, followed by cell lysis, SDS-PAGE, Western blotting, and analysis with *B. suis* VirB protein-specific antisera. Arrows indicate VirB proteins and molecular masses of reference proteins are shown on the right.

Isolation of T pili and subcellular fractions. Cells were cultivated on AB minimal medium plates in the presence of AS or IPTG, followed by cell harvest and shearing for the isolation of T pili as described previously (54). Membrane fractions were separated from soluble fractions by cell lysis in a French press, followed by ultracentrifugation as described previously (66).

Analysis of protein-protein interactions by cross-linking. Cells were cultivated in liquid AB minimal medium in the presence of 0.5 mM IPTG; the OD_{600} was adjusted to 1; and aliquots of 1 ml were sedimented, washed three times with phosphate-buffered saline pH 6 (PBS; 0.08% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, and 0.024% KH_2PO_4 adjusted to pH 6), and suspended in 1 ml of the same buffer. The cross-linking agent bis(sulfosuccinimidyl)suberat (BS^3; Pierce) was added at a concentration of 1 mM, followed by incubation for 30 min at room temperature and stopping of the reaction by the addition of 200 μl of Tris-HCl buffer (pH 6). The cells were then sedimented, washed once with PBS (pH 6), and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

SDS-PAGE and Western blotting. *Agrobacterium* cells and subcellular fractions were incubated in Laemmli sample buffer for 5 min at 100°C, followed by SDS-PAGE using the Laemmli (for proteins larger 20 kDa) (42) or the Schägger and Jagow (for proteins smaller 20 kDa) system (53). Western blotting and detection with a chemoluminescence system (Amersham Biosciences) was done according to standard protocols with *A. tumefaciens* and *B. suis* VirB protein-specific antisera (30).

Immunofluorescence analysis and image processing. *A. tumefaciens* C58 carrying pTrcB3-B12 or pTrc300 grown on AB minimal medium plates was washed three times with PBS and fixed for 30 min in 4% paraformaldehyde, followed by three washes in PBS (0.08% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, 0.024% KH_2PO_4 [pH 7]). Samples (30 μl) were applied to 0.1% polylysine-coated cover slides (Sigma) and dried. For permeabilization of the cell envelope, the cover slides were immersed in GET buffer (20 mM Tris-HCl [pH 7.5], 10 mM EDTA [pH 8], 50 mM glucose) containing 8 mg of lysozyme (Sigma)/ml, followed by a 10-min incubation at room temperature and three washes with PBS. Nonspecific binding sites were blocked by incubation with 1% bovine serum albumin (BSA) in PBS for 30 min, followed by treatment with primary antisera (1:200 dilution in PBS–1% BSA) at 4°C for 12 h. Next, the cover slides were washed three times in PBS and then incubated in Oregon green goat anti-rabbit immunoglobulin G (IgG)-coupled secondary antiserum (Molecular Probes) at a 1:200 dilution in PBS–1% BSA for 3 h in the dark. The samples were washed three times in PBS, treated with antifade solution (AF1; Citifluor), sealed with VALAP (vaseline-lanoline-paraffin [1:1:1]) on a microscope slide, and analyzed.

B. suis virB manB carrying pTrcB3-B12 or pTrc300 was grown as shaken tryptic soy broth culture at 37°C to an OD_{600} of 0.4, followed by induction of the *trc* promoter with 0.5 mM IPTG for 5 h. Cells were subsequently washed three times in PBS, fixed for 30 min in 3% paraformaldehyde, sedimented, and incubated in 1% Triton X-100 for 5 min for permeabilization of the cell envelope, followed by blocking with 1% BSA as described above and two washes in PBS. The cells were next treated with primary antiserum diluted in PBS (monoclonal anti-Omp31 [1:5] and polyclonal sera anti-VirB5 or anti-VirB8 [1:50 each]) for 2 h, followed by three washes with PBS. Cells were then treated with secondary antibodies (anti-rabbit IgG-fluorescein isothiocyanate conjugate [Sigma] and goat anti-mouse IgG-Texas red conjugate [Molecular Probes]) for 2 h, followed by three washes with PBS, treatment with antifade solution, sealing on a microscopy slide, and analysis.

Samples were analyzed by fluorescence microscopy with a Zeiss Axioplan microscope (filter set 9, BP 450 to 490, LP 515), the images were digitalized with a Spot-RT camera (Visitron Systems) and Spot 3.02 and IPLab 3.5 software, followed by processing with Adobe Photoshop 6 and Canvas 7 software.

RESULTS

Cloning and expression of the *virB* operon from *B. suis* in *A. tumefaciens*. For the heterologous expression of the 11-kb *B. suis virB* operon, we followed a three-step procedure to assemble the operon from fragments. The reason for this procedure was to circumvent expression problems due to the intergenic regions between *virB1*/*virB2* (contains conserved *Brucella* repeat sequence, BruRS1) and *virB6*/*virB7* (49). In preliminary experiments we subcloned the entire *virB* operon including the intergenic regions, but this led to low-level constitutive expression of *virB8-virB10* (data not shown). To avoid this complication, the regions encoding *virB2-virB6* and *virB7-virB12* were PCR amplified separately, cloned into the broad-host-range vector pTrc300, sequenced, and subsequently joined, resulting in pTrcB2-12 (Fig. 1A). We noticed a spontaneous mutation at the start codon of *virB2* in one of our clones, and this vector was designated pTrcB3-12. The *virB1* gene was subsequently PCR amplified and inserted 5′ to the operon, resulting in vectors pTrcB1+3-12 and pTrcB1+2-12. The plasmids were transformed into Ti plasmid-free *A. tumefaciens* strain UIA143. Western blot analysis with the available antisera was used to detect VirB protein production in IPTG-induced cells. We detected *B. suis* VirB1, VirB2, VirB5, and VirB8-VirB12 as expected by the composition of the operons (Fig. 1B). We noticed low levels of most VirB proteins in pTrcB1+2-12-carrying cells, and for this reason the strain was not analyzed further. To analyze whether the *B. suis* T4SS is functional in the heterologous host, we determined its ability to substitute for the *A. tumefaciens virB* operon using virulence, plasmid, and protein transfer assays.

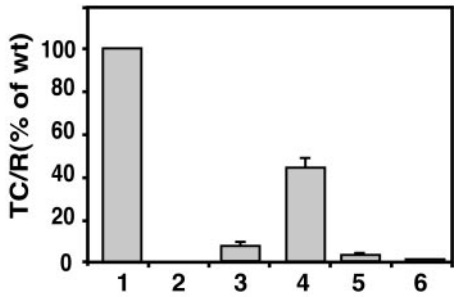

FIG. 2. Expression of the *B. suis virB* operon stimulates pLS1 transfer into *A. tumefaciens*. The recipient strain UIA143 carrying the Ti plasmid (bar 1), pTrc300 (bar 2), pTrcB3-12 (bar 3), pTrcB1+3-12 (bar 4), pTrcB2-12 (bar 5), and pTrcB1+2-12 (bar 6) were cultivated on AB minimal medium plates at 20°C for 3 days together with donor strain A348 pLS1 under virulence-inducing (+AS) conditions in the presence of IPTG. Exconjugants were identified by growth on selective agar media and the pLS1 transfer efficiency (transconjugants per recipient [TC/R]) into virulence gene-induced induced UIA143 pTiA6 (bar 1) was set to 100%. The standard deviation of results from three independent experiments is shown.

Expression of *B. suis virB* operon stimulates pRSF1010 recipient competence. We have previously shown that *B. suis* VirB1 and VirB4 can partially or fully complement the corresponding *Agrobacterium virB* gene deletion mutant for virulence in plant infections (34). However, introduction of the *B. suis virB* operon constructs pTrcB1+2-12 and pTrcB1+3-12 did not restore the virulence of the *virB* operon deletion strain PC1000 of *A. tumefaciens* (26) in plant infection assays (data not shown). Similarly, expression of the *B. suis virB* genes in this strain did not permit conjugation of the IncQ plasmid pLS1 to other bacteria. Since *Brucella* does not encode a VirD4 homolog, we also assessed the transfer of the small mobilizable plasmid CloDF13, which encodes a coupling protein, into yeast recipients, but expression of the *Brucella virB* genes did not mediate its transfer (24). In line with these findings, using the CRAfT (63) we found that the *B. suis* VirB1 protein complemented an *Agrobacterium virB1* mutant for translocation of a Cre recombinase-VirF fusion protein into yeast but saw no indication of complementation of the individual *Agrobacterium virB* gene deletions by pTrcB1+3-12 (data not shown). These results showed that the *B. suis* VirB proteins cannot fully substitute for all functions of the *A. tumefaciens* T4SS in transfer of virulence proteins or plasmid substrates. We next used an alternative plasmid conjugation assay based on the phenomenon that a subset of VirB proteins expressed in *A. tumefaciens* cells can increase recipient activity by 3 orders of magnitude (7, 46). Although the mechanism of this increase is not understood, it is dependent on the function of apparent subcomplexes of the VirB apparatus and therefore may serve as an assay for assembly of T4SS components. Using this assay, we found that conjugation of the IncQ plasmid pLS1 to *Agrobacterium* strain UIA143 expressing its native T4SS from pTiA6 was 3,330-fold more efficient than to the Ti plasmid-free strain UIA143 containing pTrc300 (Fig. 2 and Table 1). Expression of the *Brucella* VirB proteins also increased the recipient activity. The low-level expression from pTrcB1+2-12 increased the transfer efficiency nearly fourfold compared to UIA143 containing pTrc300. Higher levels of expression gave much greater increases in recipient activity, with a 265-fold increase of transfer to UIA143 pTrcB3-12 and a 1,460-fold increase to UIA143 pTrcB1+3-12, showing a clear role for VirB1. The increase in recipient activity was strictly dependent on *trc* promoter induction by IPTG in both cases. We need to emphasize that UIA143 does not carry a Ti plasmid, so that these results cannot be explained by the action of *Agrobacterium* VirB proteins in the recipient. These data suggest the assembly of at least a subset of *Brucella* VirB proteins in a T4SS-like complex in *Agrobacterium*. Interestingly, the presence of pTrcB2-12 only increased recipient activity by 55-fold, suggesting that the presence of VirB2 does not make the strains better recipients. In the next set of experiments, we characterized the molecular basis of this phenomenon.

The *B. suis* VirB proteins localize in the *A. tumefaciens* membranes. As a first step toward the characterization of *B. suis* T4SS assembly in *A. tumefaciens*, we studied the membrane association of VirB proteins. Strain UIA143 carrying pTrcB2-12, pTrcB1+3-12, and pTrcB3-12, respectively, was cultivated on AB minimal medium in the presence of IPTG and lysed, and the total cell lysate (Fig. 3, lanes T) was subjected to ultracentrifugation to separate the membranes (lanes M) from the soluble fraction (lanes S, cytoplasm and periplasm). Subsequent analysis of the subcellular fractions with specific antisera for VirB1, VirB2, VirB5, and VirB8-VirB12 showed that all VirB proteins were detected in the membranes but that most of them were also present to some extent in the soluble fraction (Fig. 3). We have not attempted to quantify the degree of membrane association, but it was evident that the *B. suis*

TABLE 1. Conjugative transfer of pLS1 from *A. tumefaciens* donor A348 into recipient UIA143 carrying Ti plasmid pTiA6, pTrc300, pTrcB3-12, pTrcB1+3-12, pTrcB2-12, or pTrcB1+2-12[a]

Donor	Recipient	No. of recipients (10^7)	TC	TC/recipient (frequency)	TC/recipient (%)	Fold increase relative to UIA143 pTrc300	SD[b]
A348 pLS1	UIA143 pTiA6	104	156,000	1.5×10^{-4}	100	3,300	0
A348 pLS1	UIA143 pTrc300	62	28	4.52×10^{-8}	0.03	1	0.01
A348 pLS1	UIA143 pTrcB3-12	150	18,000	1.2×10^{-5}	8.0	265	0.51
A348 pLS1	UIA143 pTrcB1+3-12	108	71,000	6.57×10^{-5}	43.8	1460	5.46
A348 pLS1	UIA143 pTrcB2-12	82	2,020	2.46×10^{-6}	1.64	55	0.15
A348 pLS1	UIA143 pTrcB1+2-12	117	200	1.71×10^{-7}	0.114	4	0.03

[a] TC, transconjugants. The ratio of donors to recipients mixed for conjugation was 5 to 1.
[b] Results are from three independent experiments.

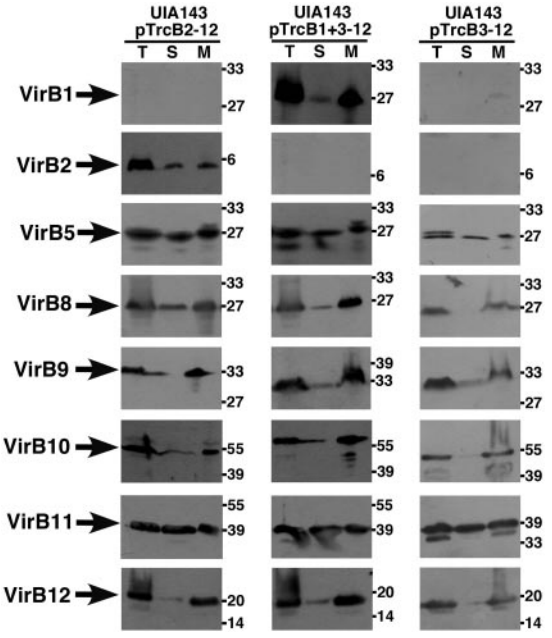

FIG. 3. Subcellular fractionation of *B. suis* VirB proteins produced in *A. tumefaciens*. Cells of UIA143 carrying pTrcB2-12, pTrcB1+3-12, and pTrcB3-12, respectively, were cultivated on AB minimal medium plates at 20°C for 3 days, followed cell lysis and membrane isolation. The protein content of subcellular fractions (total cell lysate [T], supernatant [S], and membrane fraction [M]) was analyzed by SDS-PAGE and Western blotting with *B. suis* VirB protein-specific antisera. Molecular masses of reference proteins are shown on the right.

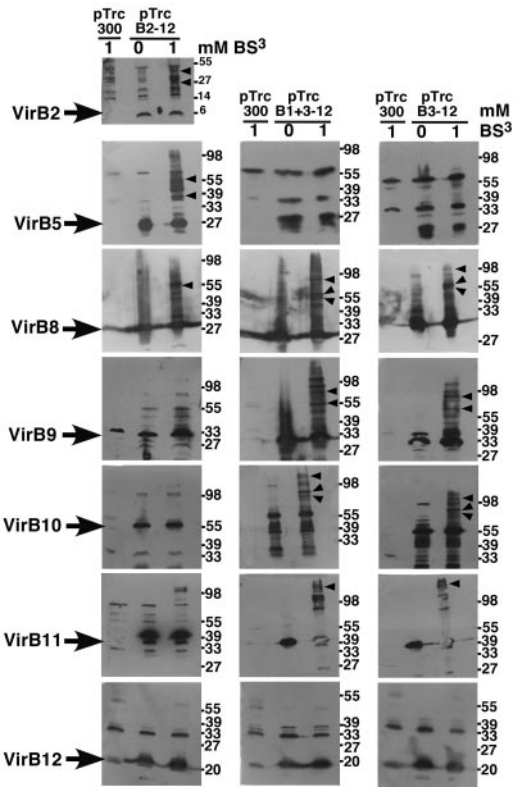

FIG. 4. Cross-linking monitors protein-protein interactions between *B. suis* VirB proteins in *A. tumefaciens*. Cells of UIA143 carrying pTrc300, pTrcB2-12, pTrcB1+3-12, and pTrcB3-12, respectively, were cultivated on AB minimal medium plates at 20°C for 3 days, followed by cross-linking with BS^3 (1 mM). Cell lysates from cross-linked and non-cross-linked samples were analyzed by SDS-PAGE and Western blotting with *B. suis* VirB protein-specific antisera. Arrows indicate monomeric proteins, and arrowheads indicate higher-molecular-mass cross-linking products differentiating interactions in UIA pTrcB2-12 from those in pTrcB1+3-12 and pTrcB3-12. Molecular masses of reference proteins are shown on the right.

VirB proteins did not associate as strongly with the membranes as their *A. tumefaciens* counterparts (38, 60). This indicates that assembly of the T4SS complex may not be as efficient as that of the *A. tumefaciens* system. We used different methods next to assess interactions between *B. suis* VirB proteins in the heterologous host.

Cross-linking reveals differential interactions between *B. suis* VirB proteins in the cell envelope of *A. tumefaciens*. Cross-linking agents have been used extensively to characterize interactions between *A. tumefaciens* VirB proteins and to determine the effects of *virB* gene deletions or amino acid changes in individual proteins on T4SS assembly (2, 3, 5, 10, 46). We here used the cross-linking agent BS^3, which primarily cross-links proteins via Lys residues, to monitor VirB protein interactions in strain UIA143 carrying the different *B. suis virB* operon constructs. Cells carrying cloning vector pTrc300 and *virB* operon constructs pTrcB2-12, pTrcB1+3-12, and pTrcB3-12, respectively, cultivated on AB minimal medium were incubated with the cross-linking agent, followed by SDS-PAGE separation of cell lysates and Western blot detection with specific antisera. Similar to previous reports on the *A. tumefaciens* T4SS (46), multiple cross-linking products were detected with most antisera (Fig. 4). Due to the large number of putative interaction partners, it was not possible to unambiguously assign cross-linking products to pairwise interactions. However, we noted a striking correlation between the formation of cross-linking products and the ability to increase pLS1 transfer. As expected, VirB2 was only present in UIA143 pTrcB2-12, and its cross-linking products had molecular masses similar to those observed in the case of VirB5 in this strain. In contrast, cross-linking products of VirB5 were not observed in lysates from UIA143 carrying pTrcB3-12 and pTrcB1+3-12, indicating that VirB5 undergoes different interactions in the presence and in the absence of VirB2. Analysis with VirB core complex component-specific antisera (VirB8, VirB9, and VirB10) revealed substantial differences in the cross-linking patterns. The results indicate that the core components undergo certain interactions only in strains with strongly increased recipient competence, and similar observations were made with VirB11-specific antiserum (Fig. 4). No cross-linking products were detected with VirB12-specific antiserum, suggesting that it does not associate with the other *B. suis* T4SS components in *A. tumefaciens*. The results of the cross-linking experiments suggest that the T4SS core components form multiple interactions when they assemble into a complex competent to increase plasmid transfer. We used fluorescence microscopy next to localize this complex in *A. tumefaciens* and *B. suis*.

Immunofluorescence microscopy localizes *B. suis* VirB proteins in foci in the cell envelope. Several VirB proteins have been shown to localize in foci on the surface of *A. tumefaciens*, which are believed to represent complexes of multiple T4SS

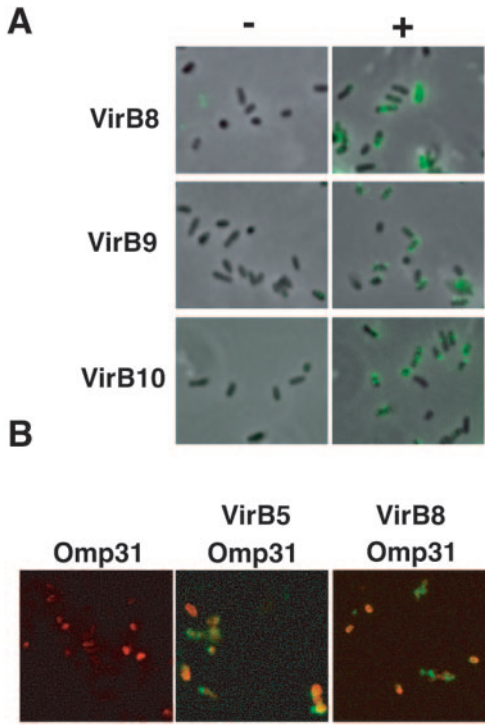

FIG. 5. Immunofluorescence analysis localizes *B. suis* VirB proteins in the cell envelope of *A. tumefaciens.* (A) *A. tumefaciens* strain C58 carrying pTrc300 (−) or pTrcB3-12 (+), respectively, was cultivated on AB minimal medium plates for 3 days in the presence of IPTG for *trc* promoter induction, followed by immunofluorescence analysis with primary VirB8-, VirB9-, or VirB10-specific antisera, and secondary Oregon green anti-rabbit antiserum. (B) Analysis of *B. suis virB manB* pTrcB3-12. Cells were cultivated in tryptic soy broth, and *virB* gene expression was induced with IPTG for 5 h. Immunofluorescence analysis was conducted with primary mouse Omp31-specific and rabbit VirB5- or VirB8-specific antisera, followed by the addition of secondary antibodies anti-rabbit IgG-fluorescein isothiocyanate conjugate and anti-mouse IgG-Texas red conjugate.

components (35, 36, 40, 41). Although the functionality of these complexes has not been proven, it is likely that they constitute assembly sites of the T4SS in the cell envelope. To assess whether the *B. suis* T4SS components form similar foci when expressed in a heterologous host, we subjected *A. tumefaciens* strains carrying pTrcB3-12 and the control plasmid pTrc300 to immunofluorescence analysis with *B. suis* VirB8-, VirB9-, and VirB10-specific antisera. Analysis by fluorescence microscopy showed that, similar to their *A. tumefaciens* core protein homologs, they localized in the cell envelope and VirB8 was detected in the characteristic spot-like pattern (Fig. 5A). We next analyzed the localization of these proteins after expression from pTrcB3-12 in the natural host *B. suis*. Preliminary experiments with a *B. suis* 1330 *virB2*::Tn*5* insertion mutant (27) (abolished expression of the native *virB* operon) gave very low levels of surface labeling with the anti-VirB sera. This suggested that the smooth lipopolysaccharide (LPS) blocked access of the antibodies as previously reported with monoclonal antibodies recognizing *Brucella* outer membrane proteins (16). To overcome this problem, we constructed a rough *manB* derivative (*B. suis virB manB*), followed by immunofluorescence analysis. Similar to the heterologous host, VirB8 and VirB5 were detected in the cell envelope in a spot-like pattern (Fig. 5B). The cell biological data show that the *B. suis* T4SS components localize in the cell envelope in complexes similar to those from *A. tumefaciens*. Taken together, the expression of the *B. suis virB* operon from an IPTG-inducible promoter on a broad-host-range plasmid leads to the production of *B. suis* VirB proteins, and they assemble in the cell envelope in defined regions, which is in accord with the results of the recipient assay and the cross-linking experiments. The following experiments were aimed at addressing the molecular basis for the ability of the *B. suis* T4SS to increase recipient activity.

Assembly of *B. suis* T4SS components weakens the cell envelope of *A. tumefaciens*. To analyze whether the *B. suis* T4SS assembles pilus-like structures in the heterologous host, we isolated extracellular high molecular mass structures by shearing of the cells, followed by ultracentrifugation. In samples from the *A. tumefaciens* wild-type control we detected the T-pilus major component VirB2a (VirB*a* indicating *A. tumefaciens* VirB protein) and the minor component VirB5a in the cells (lanes C) and in the sediment obtained after ultracentrifugation (lanes P), indicating pilus assembly as expected (Fig. 6A) (54). Other T4SS components such as VirB10a, VirE2a, and the periplasmic protein AcvB were only detected in the cells and not in the pilus fractions or in the supernatant after ultracentrifugation (lanes S) (Fig. 6A and B). The same fractionation procedure was applied to UIA143 carrying pTrc300 and the *B. suis virB* operon vectors, and the VirB proteins were detected in the subcellular fractions with specific antisera (Fig. 6B). The results were substantially different from observations made with *A. tumefaciens* VirB proteins, especially in case of UIA143 pTrcB1+3-12. Pilus fractions from this strain contained most VirB proteins and in addition the periplasmic AcvB. AcvB was also detected in the supernatant obtained after ultracentrifugation. The results indicated that AcvB was released from the periplasm during the shearing procedure. In pilus fractions isolated from UIA143 pTrcB3-12 only VirB12 was detected and AcvB was not present in the supernatant, indicating that the presence of VirB1 accounts for the major changes observed between the two strains. Pilus fractions from UIA143 pTrcB2-12 contained only VirB5 and VirB12. Since VirB5 is the minor pilus component in other T4SS, this suggested the formation of a pilus-like structure, but we did not detect VirB2 in these fractions. In addition, we did not detect pili by transmission electron microcopy in any of the *B. suis virB* operon-carrying strains (data not shown). Thus, in spite of the many similarities shown above, the *B. suis* T4SS expressed in the heterologous host did not share all of the structural features of the native *Agrobacterium* T4SS.

To monitor the weakening of the cell envelope permitting the leakage of periplasmic proteins, we tested the growth of cells carrying the different operon plasmids in the presence of various concentrations of the detergent SDS (at 0, 0.025, 0.006, or 0.003%) over a period of 3 days. This assay monitors the integrity of the cell envelope, and similar assays have previously been used to assess the effects of *Rhizobium leguminosarum exo5* and *Sinorhizobium meliloti bacA* (25, 45). The addition of SDS to recipient strain UIA143 with or without cloning vectors at concentrations 0.003 and 0.006% successively inhibited growth, and cells did not grow at concentrations of 0.025% and higher (Fig. 7). The presence of pTrcB2-12 did not affect the sensitivity of strain UIA143

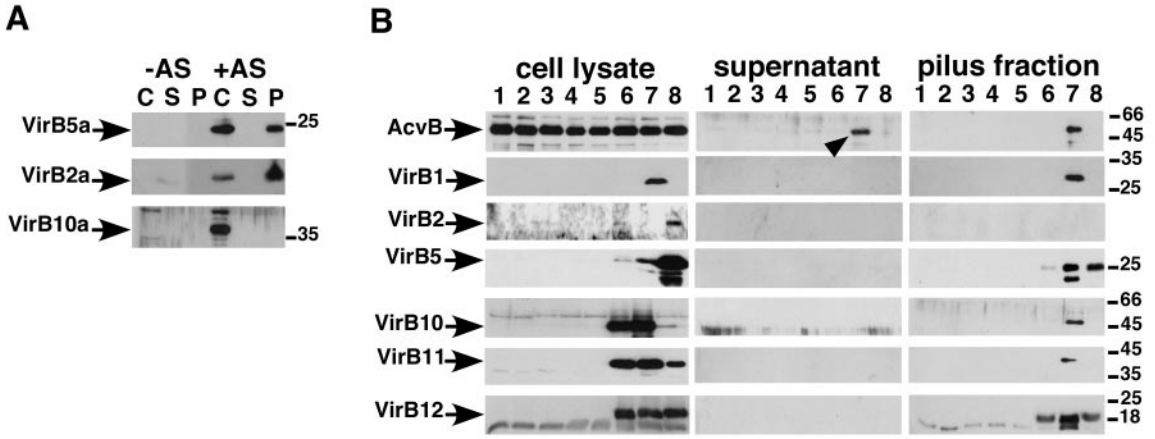

FIG. 6. Expression of the *B. suis virB1+3-12* operon induces periplasmic leakage in *A. tumefaciens*. Cells were cultivated on AB minimal medium plates at 20°C for 3 days, followed by shearing of the cells and analysis of protein content of subcellular fractions after SDS-PAGE and Western blotting: cell lysate (C), ultracentrifugation supernatant (S), and pellet (P [pilus fraction]). (A) *A. tumefaciens* wild-type C58 grown under noninducing (−AS) or virulence gene-inducing conditions (+AS). (B) *A. tumefaciens* wild type grown under noninducing (lanes 1) or virulence gene-inducing conditions (lanes 2) and Ti plasmid-free strain UIA143 grown without IPTG (lanes 3) or with IPTG (lanes 4). Strain UIA143 was grown with different *virB* operon plasmids in the presence of IPTG as follows: lanes 5, pTrc300; lanes 6, pTrcB3-12; lanes 7, pTrcB1+3-12; and lanes 8, pTrcB2-12. An arrowhead indicates periplasmic protein AcvB released into the supernatant by shearing. Antisera detected *A. tumefaciens* VirB*a* proteins or *B. suis* VirB proteins. The molecular masses of reference proteins are shown on the right.

to the detergent. In contrast, the growth of UIA143 carrying pTrcB3-12 was slightly reduced in the presence of 0.003 and 0.006% SDS, and this effect was very obvious in the case of UIA143 pTrcB1+3-12 (Fig. 7). These results correlate with the release of periplasmic AcvB and suggest that production of the *B. suis* T4SS functional in the recipient assay reduces the integrity of the cell envelope. In the absence of ITPG induction of the *trc* promoter, we did not note any reduction of growth, showing that the sensitization of *A. tumefaciens* to SDS was strictly dependent on the expression of *virB1+B3-12* and *virB3-12* (Fig. 7).

DISCUSSION

The experiments reported here suggest that the *B. suis* VirB proteins assemble into a T4SS-like complex in the heterologous host *A. tumefaciens*. All VirB proteins were detected in the membranes, some of them localized in characteristic patterns in the cell envelope and the cross-linking patterns were reminiscent of those observed in case of the *A. tumefaciens* VirB homologs (2, 3, 5, 10, 46). Expression of subsets of *B. suis* VirB proteins in an *Agrobacterium virB* deletion mutant increased their competence as recipients in a conjugation assay, but the *virB* operon constructs did not fully complement T4SS functions. These results may indicate principal differences between the T4SSs of *A. tumefaciens* and *B. suis* or merely reflect the fact that some but not all VirB proteins can be exchanged. A principal difference between the two organisms is that *B. suis* does not encode a VirD4 homolog (50), suggesting that the coupling of substrate transport may follow a different mechanism and may be similar to that of the *B. pertussis* T4SS. VirD4 is required only on the donor but not on the recipient side in plasmid transfer experiments (7), and it is also dispensable for T-pilus formation (44), suggesting that it may not be required for functional assembly of the other T4SS components. We have previously shown that *B. suis* VirB4 and to some extent VirB1 could replace their *Agrobacterium* counterparts (34, 66), but others, such as VirB5, VirB6, and VirB11 could not (unpublished observations). This suggests that full T4SS functionality in *A. tumefaciens* requires interactions with specific sets of VirB and non-VirB assembly factors, DNA substrates, and/or coupling proteins, which cannot be conducted by the *B. suis* VirB proteins. Given that the sequence conservation between *B. suis* and *A. tumefaciens* VirB proteins is not high (amino acid identities of 18 to 32%, similarities of 46 to 65%) (49), it is not surprising that full complementation is not possible, but the results of the recipient assay suggest the correct assembly of a T4SS (7, 46). An alternative explanation is that overexpression of the *B. suis* VirB proteins in the heterologous host from the strong *trc* promoter and alteration of the operon structure may lead to protein production in a stoichiometry, which does not permit functional assembly. In the future, we will use alternative promoters, such as the *A. tumefaciens virB* and the arabinose-inducible pBAD promoter, to assess this possibility.

One of the interesting features of the different subsets of *B. suis* proteins is that strains carrying pTrcB1+2-12 and pTrcB2-12 were poorer recipients than those carrying pTrcB1+3-12 and pTrcB3-12. This suggests that the expression of *Brucella* VirB2 has a negative effect on the recipient assay. Similarly, the absence of VirB2 had an effect on the protein-protein interactions of VirB5, as well as of VirB8-VirB11 identified in the cross-linking experiments. This demonstrates that VirB proteins in recipient-competent (UIA143 pTrcB3-12 and pTrcB1+3-12) and less-competent (pTrcB1+2-12, pTrcB2-12) strains undergo different sets of interactions, which correlate with their assembly, and similar results were obtained in recipient assays with the *A. tumefaciens* T4SS (46). Also, marked variations of the levels of different VirB proteins were observed, which is in line with different overall structure and stability of the complexes. For example, the level of VirB5 was markedly increased in UIA143 pTrcB2-12 compared to the other strains, and the results of the cross-linking experiments indicated that this

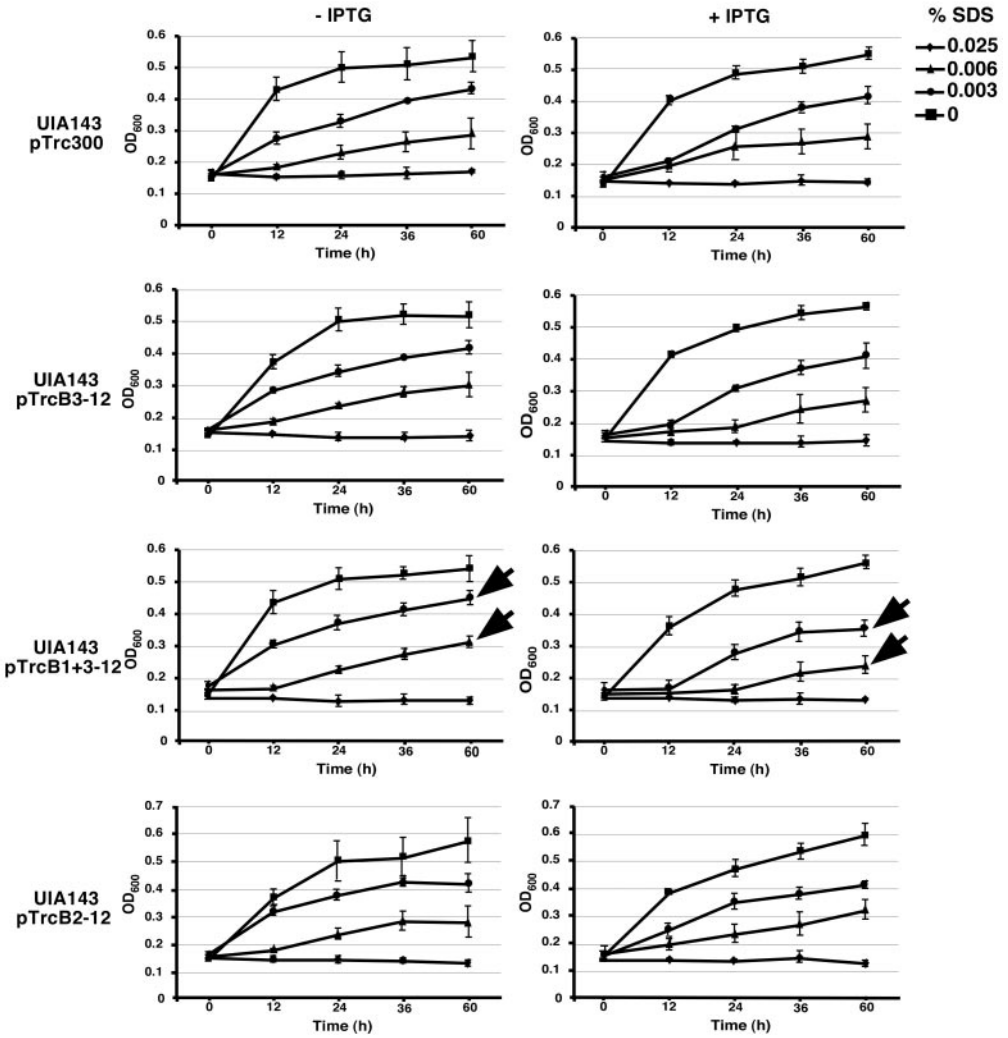

FIG. 7. Expression of *B. suis virB* operon constructs increases the sensitivity of *A. tumefaciens* to SDS. Cells of strain UIA143 carrying pTrc300 or different *virB* operon plasmids (pTrcB3-12, pTrcB1+3-12, or pTrcB2-12) were cultivated in liquid AB minimal medium at 20°C with or without IPTG for various times as indicated in the presence of various concentrations of SDS. The average of three independent experiments is given, and error bars show the standard deviations. Arrows indicate reduction of growth of UIA143 pTrcB1+3-12 upon induction of the *trc* promoter with IPTG.

could rely on a direct interaction with and stabilization by VirB2. However, expression of *virB2-virB12* did not lead to the assembly to T-pilus-like structures on the surface of *A. tumefaciens*, in spite of the fact that VirB5 was detected in high-molecular-mass extracellular fractions. One explanation of the negative effect of VirB2 on pLS1 recipient competence is that this protein may not undergo proper processing (signal peptide removal followed by cyclization [23]) due to the absence of the matching cofactors in *A. tumefaciens*. Incorrectly processed VirB2 may bind to VirB5 in nonproductive complexes and thereby negatively impact T4SS assembly. As an alternative explanation we hypothesize that production of VirB2 completes the assembly and "seals" the T4SS so that it cannot increase recipient competence. The molecular basis of the recipient stimulation phenomenon is currently unknown. It may be based on interactions between T4SS components in the donor and the recipient, and the exposed VirB2 pilus components may mediate this process. *A. tumefaciens* VirB2 is an important positive contributor in this assay, but if homologs from *A. tumefaciens* and *B. suis* did not interact it would explain why pTrcB2-12-carrying strains had a lower increase in recipient competence. In future, we will separately express VirB2 homologs from *A. tumefaciens* and *B. suis* and hybrid proteins in the recipient to directly test this hypothesis. Yet another interpretation becomes apparent if the levels of VirB9 and VirB10 in the different *virB* operon-carrying strains are compared. The levels of these proteins are most elevated in UIA143 carrying pTrcB3-12 and pTrcB1+3-12, respectively, suggesting that these T4SS core proteins may be principle factors for the increased recipient competence.

An interesting finding reported here is the weakening of the cell envelope, which is most pronounced in UIA143 pTrcB1+3-12. Cells carrying this plasmid had increased sensitivity to low concentrations of the detergent SDS, the periplas-

mic AcvB protein was released by shearing, and all VirB proteins were detected in extracellular high-molecular-mass fractions. Whereas the cell envelope was apparently more fragile, growth in YEB and AB minimal media was not reduced. This indicates that, in contrast to previous reports on cell lysis induced by overproduction of the plasmid R1 VirB1 homolog ORF169, the cells did not lyse (4). These results constitute an illustration of the cell envelope-permeabilizing potency of the lytic transglycosylase VirB1 (67), since the SDS sensitivity of UIA143 pTrcB3-12 was much less pronounced and release of VirB proteins and AcvB was not detected. We suggest that the release of high-molecular-mass VirB protein complexes from UIA143 pTrcB1+3-12 is a consequence of the absence of VirB2, which leads to an "open" recipient-competent complex, which is not stable and can thus be removed from the cells by shearing. It is intriguing to speculate that this "open" complex may reflect a natural status of the *B. suis* T4SS during the substrate translocation process. Thus far, there are few reports describing the actual channel properties of T4SSs, which may be due to the fact that this system is well sealed by VirB2 and other VirB proteins. The presence of the plasmid RP4 T4SS was shown to increase leakage of ATP from *E. coli* and to increase their permeability to certain lipophilic agents, but effects on growth of the cells have not been reported (18). We have analyzed the growth of 11 *virB* gene deletion variants of A348 (6) in the presence of various concentrations of SDS but did not determine any growth defects (not shown). Thus, the opening of the cell envelope in UIA143 pTrcB1+3-12 may either be due to the overexpression from the *trc* promoter or reflect a unique property of the *B. suis* T4SS.

Taken together, the findings presented here constitute the first comprehensive approach to study the *Brucella* T4SS with biochemical methods. The establishment of a heterologous system in a nonpathogenic host was an essential prerequisite for this strategy. The analysis of different *virB* operon constructs revealed novel features of this T4SS machinery, which may be generally applicable to those from other bacteria. We have not detected the translocation of substrates from *A. tumefaciens* cells carrying the *B. suis* T4SS, and this may reflect the fact that they do not undergo all protein-protein interactions necessary for translocation into the host. An alternative explanation is that the *Agrobacterium* VirD4 coupling protein, which is thought to be essential for recruitment of substrates, does not interact with the *Brucella* VirB proteins. However, a *virB1* mutant could be complemented, suggesting that the *Brucella* transglycosylase activity is able to functionally complement for this deficit. As we have demonstrated assembly of the *B. suis* T4SS, it may be capable of translocation of *Brucella* substrates between cells. Translocated substrates have not been identified in *Brucella*, but the heterologous system may provide opportunities to study this process as well without the requirement for biosafety level 3 pathogen containment. We envisage that the expression of T4SSs from other organisms in heterologous hosts will permit similar insights into their specific features in the future.

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