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 α₂-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* 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,
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 T4SSs (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 adhesion 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 VirB13 and VirB23 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 murine 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 CS6 (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 MgSO4) in the absence of antibiotics (wild-type A348 and C58 (62) or strains carrying pTrc300 or virB/H9262 strains) or with spectinomycin (300 μg/ml) and grown for 5 h at 20°C, followed by plating of 1 ml on an 15-cm-diameter AB agar plates with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for induction of the trc promoter or 200 μM acetoxyrin (GOS) for induction of the Agrobacterium virB promoter as indicated in individual experiments and further cultivation at 20°C for 3 days.

For the construction of pLS1 recipient activity, donor A348 pLS1 (57) cells were cocultivated with UA143 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 carbenicillin 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 500 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 OD600 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 (CRAT) (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.

**Construction of B. suis virB plasmid.** To construct B. suis 1330 (virB2::Tn5-manB), an internal fragment of the B. suis ppsm gene was amplified by PCR (primers ppsm5 [5'-TATTGGCAATGTTGCGAAGAC-3'] and ppsm3 [5'-GTTGGAGGTGACTGGCGTGA-3']) and cloned into pGem-T (Promega). Since CoElI-based vectors do not replicate in Brucella, this plasmid was introduced into B. suis B2-12 (27) by electroporation to activate 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 overlapping 4-bp single-stranded DNA with mung bean nuclease, followed by blunt end ligation. This selection 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, virB-2 (primers virB2-5 [5'-GGCGCAAGGCTCGAGAATTTGCTGAGC-3'] and virB2-3 [5'-GGCCTCGAGCTGCTATTTTGACTTG-3']) were PCR amplified from pUVirB (49) by using the Expand Long Template PCR System (Roche). Next, virB2-6 and virB7-12 fragments were excised by ScaI/XbaI and XbaI/PstI, respectively, and cloned into pTrc300 to give pTrcB2 and pTrcB7-12, resulting in pTrcB2 and pTrcB3-12 (after deletion of a nonsense 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 pUVirB (primers virB1-5 [5'-GGCGCGAAGCTGCTAGAATTTGCTGAGC-3'] and virB1-3' [5'-GGCCTCGAGCTGCTATTTTGACTTG-3']) using the TOPO cloning system (Invitrogen), excised with SacI and inserted into virB operon plasmids resulting in pTrcB1-12 and pTrcB1-2-12.

**Generation of VirB protein-specific antisera.** For the generation of Brucella virB antisera, a 471 bp fragment of the gene encoding the translocated periplasmic form of the protein (156 amino acids [16 to 172]) was PCR amplified from pUVirB with oligonucleotides (VirB2-5 [5'-CAGGGTAGCCTCCCCAGCCCCTGGAACAACGTGGGGGGGGCAG-3'] and virB2-3 [5'-GGGCTCGAGCTGCTATTTTGACTTG-3']) cloned into pCR2.1 by using the Topo cloning system (Invitrogen), excised with SacI, and inserted into virB operon plasmids resulting in pTrcB1-12 and pTrcB1-2-12.

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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 OD600 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% Na2HPO4, and 0.024% KH2PO4 adjusted to pH 6), and suspended in 1 ml of the same buffer. The cross-linking agent bis(sulfosuccinimidyl) suberate (BS3; 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 (for proteins smaller 20 kDa) system (53). Western blotting and detection with a chemiluminescence system (Amersham Biosciences) was done according to standard protocols with A. tumefaciens and B. suis VirB proteins-specific antisera (30).

Immunofluorescence analysis and image processing. A. tumefaciens CS8 carrying pTrcB3-B12 or pTrc300 grown on AB minimal medium plates was washed three times with PBS and fixed at 30 min in 4% paraformaldehyde, followed by three washes in PBS (0.08% NaCl, 0.02% KCl, 0.14% Na2HPO4, 0.024% KH2PO4, pH 6). 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 antisera (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 (AFC; Citifluor), sealed with VALAP (vaseline-lanoline-paraffin [1:1:1]) on a microscope slide, and analyzed.

RESULTS

Cloning and expression of the virB operon from B. suis in A. tumefaciens. For the heterologous expression of the 11-κb 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.
Expression of the *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* VirB protein complemented an *Agrobacterium virB* 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).

**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) into virulence gene-induced UIA143 pTiA6 (bar 1) was set to 100%. The standard deviation of results from three independent experiments is shown.

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

<table>
<thead>
<tr>
<th>Donor</th>
<th>Recipient</th>
<th>No. of recipients (10^4)</th>
<th>TC</th>
<th>TC/recipient (frequency)</th>
<th>TC/recipient (%)</th>
<th>Fold increase relative to UIA143 pTrc300</th>
<th>SD</th>
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<tr>
<td>A348 pLS1</td>
<td>UIA143 pTiA6</td>
<td>104</td>
<td>156,000</td>
<td>1.5 × 10^-4</td>
<td>100</td>
<td>3,300</td>
<td>0</td>
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<tr>
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<td>UIA143 pTrc300</td>
<td>62</td>
<td>28</td>
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<td>8.0</td>
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<td>UIA143 pTrcB1+3-12</td>
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<tr>
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<td>200</td>
<td>1.71 × 10^-7</td>
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* TC, transconjugants. The ratio of donors to recipients mixed for conjugation was 5 to 1.

* Results are from three independent experiments.
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 BS3, 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, were cultivated on AB minimal medium plates at 20°C for 3 days, followed by cross-linking with BS3 (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. 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 antisera (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 that is 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
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. 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 assemblies 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 A. tumefaciens wild-type control we detected the T-pilus major component VirB2a (VirBa 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 microscopy 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
DISCUSSION

The experiments reported here suggest that the \textit{B. suis} VirB proteins assemble into a T4SS-like complex in the heterologous host \textit{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 \textit{A. tumefaciens} VirB homologs (2, 3, 10, 46). Expression of subsets of \textit{B. suis} VirB proteins in an \textit{Agrobacterium virB} deletion mutant increased their competence as recipients in a conjugation assay, but the \textit{virB} operon constructs did not fully complement T4SS functions. These results may indicate principal differences between the T4SSs of \textit{A. tumefaciens} and \textit{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 \textit{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 \textit{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 \textit{B. suis} VirB4 and to some extent VirB1 could replace their \textit{A. tumefaciens} counterparts (34, 66), but others, such as VirB5, VirB6, and VirB11 could not (unpublished observations). This suggests that full T4SS functionality in \textit{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 \textit{B. suis} VirB proteins. Given that the sequence conservation between \textit{B. suis} and \textit{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 \textit{B. suis} VirB proteins in the heterologous host from the strong \textit{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 \textit{A. tumefaciens virB} and the arabinose-inducible pBAD promoter, to assess this possibility.

One of the interesting features of the different subsets of \textit{B. suis} proteins is that strains carrying \textit{pTrcB1+2-12} and \textit{pTrcB2-12} were poorer recipients than those carrying \textit{pTrcB1+3-12} and \textit{pTrcB3-12}. This suggests that the expression of \textit{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 (\textit{UIA143 pTrcB3-12} and \textit{pTrcB1+3-12}) and less-competent (\textit{pTrcB1+2-12}, \textit{pTrcB2-12}) strains undergo different sets of interactions, which correlate with their assembly, and similar results were obtained in recipient assays with the \textit{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 \textit{UIA143 pTrcB2-12} compared to the other strains, and the results of the cross-linking experiments indicated that this
could rely on a direct interaction with and stabilization by VirB2. However, expression of \textit{virB2-virB12} did not lead to the assembly to T-pilus-like structures on the surface of \textit{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 \cite{23}) due to the absence of the matching cofactors in \textit{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. \textit{A. tumefaciens} VirB2 is an important positive contributor in this assay, but if homologs from \textit{A. tumefaciens} and \textit{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 \textit{A. tumefaciens} and \textit{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 \textit{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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