STRUCTURE/FUNCTION ANALYSIS OF THE MINOR T-PILUS COMPONENT VIRB5 FROM *AGROBACTERIUM TUMEFACIENS*

STRUCTURE/FUNCTION ANALYSIS OF THE MINOR T-PILUS COMPONENT VIRB5 FROM *AGROBACTERIUM TUMEFACIENS*

By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2010) McMaster University
(Biology) Hamilton, Ontario

TITLE: Structure/Function analysis of the minor T-pilus component VirB5 from *Agrobacterium tumefaciens*

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NUMBER OF PAGES: xii, 113

ABSTRACT

Type IV Secretion Systems (T4SS) are machineries required for the virulence of many Gram-negative pathogens. They contribute to bacterial competence, conjugation and the translocation of toxins from bacteria into eukaryotic hosts. In the plant pathogen *Agrobacterium tumefaciens*, the T4SS complex is composed of 11 VirB proteins (VirB1-VirB11) and VirD4. The VirB/D4 complex spans the bacterial envelope and assembles filamentous T-pili, which extend into the extracellular environment and mediate inter-bacterial conjugation as well as the formation of Crown Gall tumors on plants.

In my Ph.D. project, the role of VirB5 during the T4SS process occupied the major focus. VirB5 is required for inter-bacterial conjugation and to incite tumors on plant surfaces. Previous research has demonstrated that VirB5 is indispensable for the assembly of the outermost T4SS section, the T-pilus. In addition, detailed analysis of the VirB5 ortholog, TraC, from the *Escherichia coli* plasmid system pKM101 revealed that this putative pilus protein might be exploited during Type IV-dependent phage binding prior to entrance into bacterial cells. Collectively, previous research findings led to the hypothesis that VirB5 is a minor T4SS component with a T-pilus associated form, which contributes to agrobacterial pathogenesis (Schmidt-Eisenlohr, Domke et al. 1999). Towards addressing this hypothesis, several approaches were undertaken such as forced localization of VirB5 to various sub-cellular compartments, PCR mutagenesis, C-terminal truncation and alanine replacements. An optimized immuno-electron microscopy (immuno-EM) procedure was also applied during the course of my studies.

Forced localization of VirB5 to the outer membrane or the periplasmic space using an inner trans-membrane domain fused to its amino- or carboxyl-terminus led to a nonfunctional T4SS and abolished T-pilus assembly. Using immuno-EM, VirB5 was found to associate with the T-pilus tips, bacterial surface and the ends of detached pili. Characterization of VirB5 C-terminal deletion and alanine replacement variants revealed that VirB5 may be assigned an important role in host cell specificity.

As result of other investigation of the necessity of VirB5 for agrobacterial pathogenesis, a former Ph.D. candidate in Dr. Baron's laboratory in Munich (Dr. Lilian Krall) found that

VirB5 interacts with the agrobacterial host range factor *trans*-zeatin synthesizing enzyme, Tzs. My follow-up work showed that, similar to VirB5, Tzs localizes to the bacterial surface. Unlike the T4SS transglycosylase VirB1, Tzs is not secreted into the culture supernatant. Using Blue Native Electrophoresis (BNE), it was shown that the association of Tzs with agrobacterial protein complexes of diverse molecular masses was significantly altered in case of the *virB5* deletion mutant CB1005. In immuno-EM experiments, it was shown that Tzs distribution to the cell surface was reduced in case of *virB2* and *virB8* deletion mutants CB1002 and CB1008, respectively. Tzs association with the cell surface was strongly reduced in case of the *virB5* deletion mutant CB1005. Taken together, these results highlighted that VirB5 is a key protein required for the cell surface localization of Tzs.

The results of my Ph.D. study have significantly contributed towards better understanding the role of VirB5 during the T4SS process and have opened numerous research avenues to better understand the method(s) by which T4SS machineries from the source pathogen can recognize the recipient.

ACKNOWLEDGEMENTS

يٰٓأَيُّهَا النَّاسُ اتَّقُوْا رَبَّكُمْ وَاخْشَوْا يَوْمًا لَّا يَجْزِىْ وَالِدٌ عَنْ وَّلَدِهٖ وَلَا مَوْلُوْدٌ هُوَ جَازٍ عَنْ وَّالِدِهٖ شَيْـًٔا اِنَّ وَعْدَ اللّٰهِ حَقٌّ فَلَا تَغُرَّنَّكُمُ الْحَيٰوةُ الدُّنْيَا وَلَا يَغُرَّنَّكُمْ بِاللّٰهِ الْغَرُوْرُ

I am indebted to my supervisor, Dr. Christian Baron for support and guidance. Alongside with my project outlines, Dr. Baron has never discouraged me from exploring my own ideas. His contribution covered every essential chapter required to graduate an independent Ph.D. holder who knows how life can be tough for an extended period of time. Counting the benefits of being trained in Dr. Baron's laboratory is a real challenge.

I am also very thankful for the useful discussions and support of my committee members, Drs. Turlough Finan, Justin Nodwell and Suleiman Igdoura. Over many years of supervision, they helped me narrowing down my project objectives into an achievable plan. I am grateful to Dr. Marrie Elliot for being an important member of my comprehensive examination committee, and Dr. John Lott for useful discussions and the continuous supply of interesting reading materials. Drs. André Bédard and Patricia Chow-Fraser are thanked very much for the insightful discussions. I deeply appreciate Dr. Herbert Schellhorn for his useful advices. Dr. Kevin Kelly is thanked for the exchange of research ideas. The outlines of his thesis served as an excellent guide while writing my own.

Without the ongoing support of my family members, I would have never pursued my Ph.D. education. I can't remember how many times I did upset my mother when I was a child trying to explore the unknowns around me using the terms and ideas of a kid, or even as a grownup who didn't align well with some of her views. Regardless of all that, hers and my father's support were unconditional, especially during the down times. I am very honored and blessed by having them as my parents. I also dedicate this work to Professor Taha Ibrahim Zaghloul, the first scientist I have ever met in my life who triggered my desire to learn more about the hidden facts of microbes. I also hope that this written document can inspire my friends and direct family members to always value scientific research but more importantly, to value dedication to any noble cause ..... because dedication is the actual path to success in today's world.

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LIST OF ABBREVIATIONS

Abbreviation	Full form
Acetosyringone	4-Hydroxy-3',5'-dimethoxyacetophenone
BNE	Blue native electrophoresis
BTI	B2-interacting proteins
Immuno-EM	Immuno-electron microscopy
IPTG	Isopropyl β-D-thiogalactopyranoside
PCR	Polymerase chain reaction
PMSF	Phenylmethanesulphonylfluoride
TEM	Transmission electron microscopy
Tzs	*trans*-zeatin synthesizing protein

Chapter One
INTRODUCTION

1.1 Overview

Gram-negative bacteria employ at least six different secretion systems for protein export across the cytoplasmic membrane and secretion into the extracellular environment. Secretion systems range from simple machineries, such as type I secretion systems composed of three subunits that only secrete one substrate protein, to complex machines such as type III and IV secretion systems composed of ~20 subunits that can translocate large sets of effector proteins into eukaryotic target cells (Hensel, Gerlach et al. 2007). An intriguing feature of various secretion systems resides in their evolution pattern. For example, studies have shown that the basal structure of Type III secretion systems (T3SS) has considerable similarities with the structure of flagellar core proteins (Blocker, Komoriya et al. 2003). These findings confirm that bacterial T3SS and flagellar systems are evolutionary linked. More recently, it was found that the flagellar assembly and/or mobility antagonizes the T3SS and that a negative cross talk exists between these two systems (Soscia, Hachani et al. 2007). Furthermore, it is believed that Type II secretion systems, Type IV pili and archeal flagella are evolutionary related (Peabody, Chung et al. 2003). Among the many secretion systems assembling on the surface of Gram-negative bacteria, our laboratory studies the biogenesis mechanism(s) of Type IV Secretion Systems (T4SS) and their role in bacterial pathogenesis. They assemble an inner and outer membrane spanning apparatus composed of 7 to 12 conserved VirB proteins in addition to VirD4. In the plant pathogen *Agrobacterium tumefaciens*, T4SS is composed of two interlinked sub-complexes of distinct masses. The larger sub-complex is 500-900 kDa and comprises the basal structure of the apparatus. This sub-complex energizes the assembly of the second and outermost sub-complex of a smaller

mass of around 140 kDa. Some VirB proteins might link both sub-complexes together. The outermost small sub-complex is composed mainly of the T-pilus proteins, VirB2 and VirB5. At least two activities are mediated by T4SSs in different pathogens. First, the translocation of macromolecular toxins from the bacteria into their eukaryotic hosts and second, plasmid transfer between bacterial cells as it is believed that T4SSs have evolved from conjugation machineries (Christie 2004) (Seubert, Hiestand et al. 2003). They also control the transfer of single stranded nucleoprotein particles and the mechanism of mating junction disassembly upon substrate translocation (Christie 2004).

1.2 Core complex components of T4SS

The T4SS complex is best understood in *A. tumefaciens* where it is composed of 12 proteins. The core structure is likely composed of 8 proteins: VirB4, VirB6, VirB7, VirB8, VirB9, VirB10, VirB11 and VirD4. A model of T4SS structure is represented (Fig. 1) (Baron 2006). Three proteins: VirD4, VirB11 and VirB4 are inner membrane bound/spanning hexameric NTPases with Walker-A nucleotide binding motif signatures. VirD4 directly interacts with the translocated T-DNA substrate and links it to the inner membrane bound VirB11 during the early stages of substrate trafficking and thus required for the *A. tumefaciens* virulence (Atmakuri, Ding et al. 2003). The T-DNA substrate is single stranded DNA fragment translocated via T4SS into plant hosts and becomes integrated onto plant genomes prior to tumor formation. Tumor formation results from the expression of oncogenes transferred on the T-DNA fragment. The products of the oncogenes lead to a severe hormonal imbalance in infected plant tissues (Ulker, Li et al. 2008). The average length of the T-DNA fragment is 25 kb, depending on the agrobacterial species. But a minimal fragment of 5 kb can

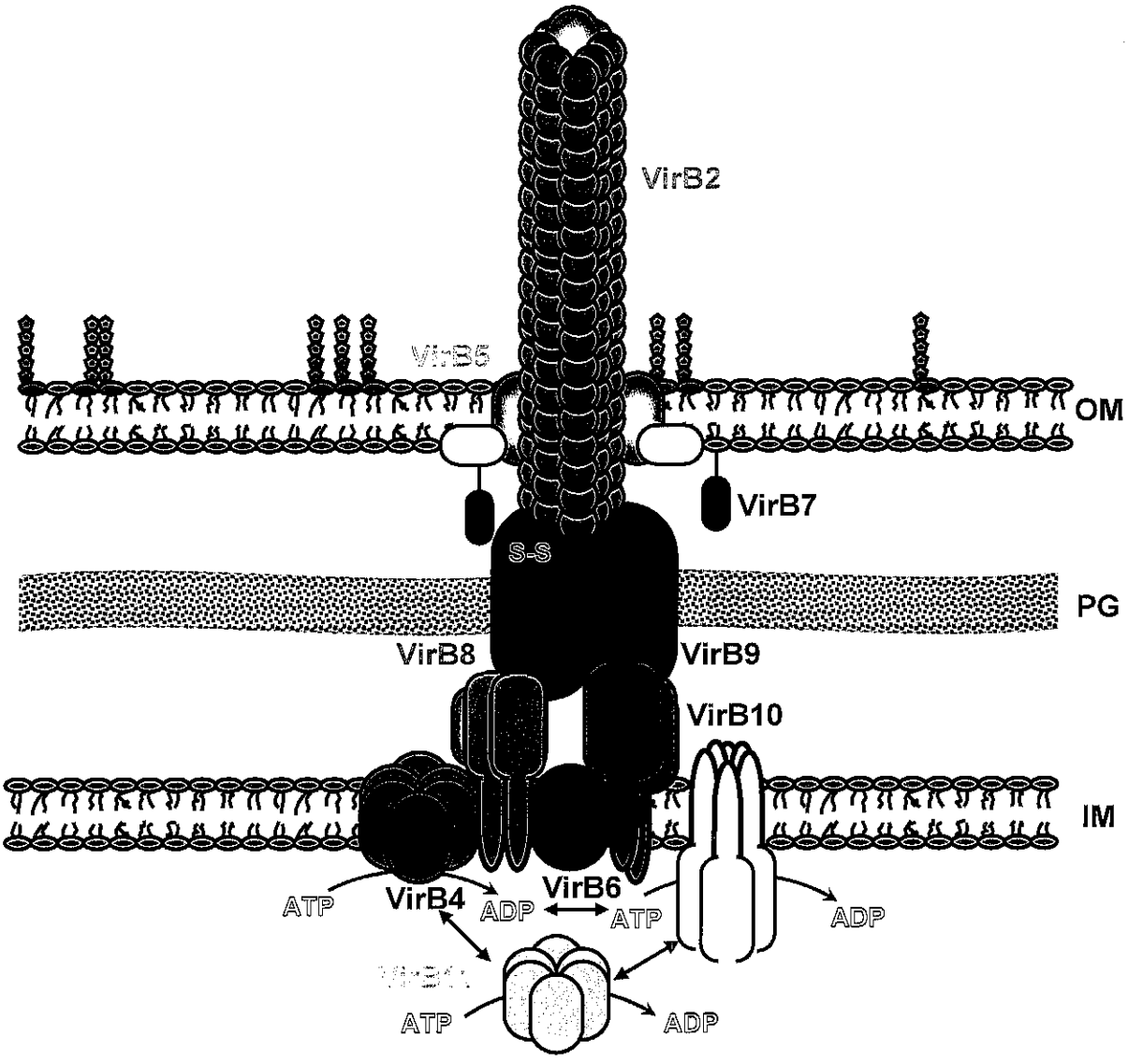
be sufficient to disrupt the growth pattern of infected plants (Krysan, Young et al. 1999). Two 25-bp imperfect direct repeats, termed border sequences, define the T-DNA. In the presence of the VirD1 protein, VirD2 nicks the border sequence in a strand-specific manner and covalently attaches to the 5' end of the nicked DNA. The nicked DNA is thought to be displaced from the plasmid to yield single-stranded T-DNA. This single-stranded T-DNA–VirD2 complex is next transferred to the plant cell and is coated with the single-stranded DNA (ssDNA) binding protein VirE2, forming the so-called T-DNA complex. The T-DNA complex enters the nucleus, and the T-DNA is finally integrated into the plant cell genome (Ziemienowicz, Merkle et al. 2001).

Although some T4SS encoding pathogens such as the genus *Brucella* do not have a VirD4 ortholog, their virulence for the mammalian hosts remains T4SS dependent (O'Callaghan, Cazevieille et al. 1999). This result can be possibly explained by the finding that substrate recruitment towards the T4SS apparatus may depend on more than VirD4 (Guo, Jin et al. 2007).

VirB11 is an inner membrane bound cytoplasmic protein with a hydrophilic nature and is considered one of the most conserved ATPases in Gram-negative virulence secretion systems (Rashkova, Zhou et al. 2000). It reaches its functional destination at the cell pole without the involvement of other VirB proteins (Judd, Kumar et al. 2005). Mutagenesis studies revealed that VirD4 guides the T-DNA substrate to VirB11 and VirB4 independently of other VirB proteins or ATP hydrolysis (Atmakuri, Cascales et al. 2004). However, substrate trafficking to the rest of the T4SS core proteins requires the NTPase activities of the three proteins. This proposed a collective role for the three NTPases for T-DNA passage to the rest

of the T4SS proteins. Different VirB11 mutations in *Legionella pneumophila* arrest the translocation of subsets of effectors suggesting a vital role for VirB11 in substrate selectivity (Sexton, Yeo et al. 2005).

Figure 1. Schematic presentation of a Type IV Secretion System (T4SS) model. Based on the role of individual VirB proteins in complex assembly, a model apparatus was drawn and classifies VirB proteins into 3 sections. The first section represents the inner most proteins which include 3 NTPases (VirB4, VirB11 and VirD4). The second section is composed of likely periplasmic proteins such as VirB3, VirB6, VirB7, VirB8, VirB9 and VirB10. The last section is composed of the outermost T-pilus components VirB2 and possibly VirB5. IM, inner membrane; PG, peptidoglycan layer; OM, outer membrane.


VirB2
VirB5
OM
VirB7
S-S
PG
VirB8
VirB9
VirB10
IM
ATP
VirB4
ADP
VirB6
ATP
ADP
ATP
ADP

Although a direct interaction between VirB4 and the T-DNA substrate was not proven, the VirB4 NTPase activity is necessary for T-DNA transfer to the rest of the T4SS proteins (Atmakuri, Cascales et al. 2004). Apart from that, it was shown that VirB4 protein stabilizes VirB8, a core polytopic inner membrane protein partner as well as VirB3, which is believed to have an outer membrane associated form (Yuan, Carle et al. 2005). In addition, VirB2 and VirB5 complex formation prior to incorporation into extracellular pili (T-pili) was found to be VirB4 dependent. This indicates that VirB4 -the largest protein member of the T4SS apparatus- is a dynamic multifunctional protein. The T4SS function of the wild-type *A. tumefaciens* strain C58 lacking *virB4* can be functionally complemented by the *B. suis* VirB4 (Yuan, Carle et al. 2005).

Previous studies have suggested that VirB4 spans the inner membrane by at least two C-terminal domains (Dang, Zhou et al. 1999). Recently, the VirB4 ortholog TrwJ from the conjugative plasmid system R388 was shown to undergo ATP hydrolysis and it was also shown that the catalytically active form of that protein is likely homo-hexameric (Arechaga, Pena et al. 2008). These findings might be system-specific and are not generally accepted for all VirB4-like proteins until further investigation.

The cell-pole localized VirB6 is a polytopic inner membrane protein comprising a cytoplasmic C-terminus, five inner trans-membrane segments probably in the middle of its sequence and a periplasmic N-terminus. Different VirB6 domains are believed to play different roles in substrate trafficking through the T4SS apparatus. While the C- and the N-termini enhance substrate transfer into the periplasm and the outermost T4SS proteins respectively, the trans-membrane segments might facilitate the substrate translocation to

VirB8 in the inner membrane (Jakubowski, Krishnamoorthy et al. 2004; Judd, Kumar et al. 2005). Polar localization of VirB6 requires a subset of VirB proteins such as VirB7-VirB10 as minimal components. Deletion and mutagenesis studies have also shown that VirB6 stabilizes VirB5 and VirB3 (Hapfelmeier, Domke et al. 2000). This has been demonstrated upon the deletion of *virB6*. Study found that cellular levels of VirB3 and VirB5 were reduced in the absence of VirB6 (Hapfelmeier, Domke et al. 2000). Taken together, the data suggests that VirB6 is a key T4SS component required for substrate translocation as well as a necessary stabilizing protein for the entire T4SS complex. In the human pathogen *Ehrlichia chaffeensis*, 4 paralogs of VirB6 have been discovered (Bao, Kumagai et al. 2009). Far-Western and co-immunoprecipitation analysis revealed interactions between more than one of these paralogs as well as their interaction with VirB9. In some Gram-negative genomes, gene duplication during the course of evolution led to the presence of several copies of one *virB* gene (Dehio 2008). The biological significance of this phenomenon and its contribution to pathogenesis remain poorly understood.

VirB8 is a protein required for T4SS function and apparatus assembly. It was previously shown that VirB8 interaction with VirB9 is necessary for substrate passage to the rest of the T4SS proteins (Ward, Draper et al. 2002). Homo- and hetero-dimer clusters of VirB8 have been recently observed using a bacterial two-hybrid technique (Bourg, Sube et al. 2009). VirB8 interacts with VirB10 and it was shown that deletion of *virB10* abolished substrate transfer from VirB8 to the external portion of the T4SS complex. Little is known about the exact role of the VirB8-VirB10 interactions for T4SS apparatus assembly. However, the crystal structures of VirB8 from *B. suis* and of a VirB10-like protein from *H. pylori* were both solved (Terradot, Bayliss et al. 2005). This provided crucial information on both dimeric

proteins for future structure-driven functional studies of the nature of VirB8-VirB10 interaction. It was shown that the interaction of the *B. suis* VirB8 with VirB4 and VirB10 is crucial for its biological activity. This was shown through the construction of several point mutations in *virB8*, followed by the analysis of T4SS recipient competence supported by these variant-producing mutants (Paschos, Patey et al. 2006). In addition to its direct interaction with VirB10, VirB8 also interacts with VirB9. The formation of clusters of VirB9 and VirB10 in the cell envelope was abolished in *virB8* mutants, suggesting that VirB8 mediates the formation of this complex, perhaps in a process which involves the translocated bacterial substrates (Kumar and Das 2001).

VirB9 plays a key role in T-pilus biogenesis and might be also important for substrate selectivity (Jakubowski, Cascales et al. 2005). This however, does not exclude the hypothesis that VirB9 and VirB10 may be core complex components of the T4SS. Its role in T-pilus biogenesis has been suggested after introducing dipeptide insertion mutations. Some of the mutations in the gene sequence resulted in a loss of T-pilus biogenesis (Jakubowski, Cascales et al. 2005). In addition to its role as an important member of the substrate translocation channel, research has revealed direct interactions between VirB9 and VirB10, VirB11 as well as VirB7. The most directly proven VirB7-VirB9 interaction evidence is a recent NMR-based study which shows that the C-terminus of the VirB9 ortholog, TraO, from the *E. coli* plasmid system pKM101 binds to the VirB7-like protein TraN (Bayliss, Harris et al. 2007). VirB9 might also function as a secretin and becomes required for the last stages of substrate translocation into the host (Das and Xie 2000; Lawley, Klimke et al. 2003).

VirB10 has a proline rich region similar to the extensively studied energy transducer TonB protein (Llosa and O'Callaghan 2004). VirB10 undergoes conformational changes depending on the energy status of the *Agrobacterium*. This unique feature might allow VirB10 to link energy providing components from the cell cytoplasm to the periplasm and also to interact with several coupling proteins in plasmid conjugation systems (Gilmour, Gunton et al. 2003; Llosa, Zunzunegui et al. 2003). In addition, detailed analysis of the importance of various VirB10 domains for the T4SS process revealed that this protein has a dynamic structure (Jakubowski, Kerr et al. 2009). This has been shown upon conducting protease susceptibility tests and it was concluded that VirB10 undergoes structural transitions depending on energy utilization by VirB11 and VirD4 (Cascales and Christie 2004). Whereas VirB10 remains tightly integrated into the bacterial inner membrane, its proline rich region largely occupies the periplasmic space (Jakubowski, Kerr et al. 2009), leaving its β-barrel C-terminal domain as an interaction site with the VirB7-VirB9 heterodimeric complex. VirB10 thus links different VirB proteins together in the course of T4SS assembly.

VirB7 was long thought to be localized at the outer membrane as a lipoprotein. It was interestingly shown that a *virB7* deletion blocked substrate transfer from VirD4 to VirB11 at the inner membrane (Cascales and Christie 2004). This suggests that VirB7 may play role in maintaining the structural integrity of the T4SS complex. VirB7 exists in three different forms in the complex: Monomers, disulphide-cross-linked homodimers as well as heterodimers with VirB9. In a recent study which confirms this hypothesis, a core complex T4SS structure composed of VirB7-VirB9-VirB10 has been reconstructed using cryo-electron microscopy (Fronzes, Schafer et al. 2009). The cryo-EM study documented a 15-A° resolution VirB7-VirB9-VirB10 T4SS core complex structure that forms a channel. Structural analyses indicate

that this channel is open on the cytoplasmic side but significantly constricted on the extracellular side. In a different study, homodimer and heterodimer forms of VirB7 were shown to co-fractionate with T-pili. VirB7 might thus link core and periplasmic components to the protruding T-pilus (Krall, Wiedemann et al. 2002).

1.3 Periplasmic and outer membrane T4SS components

This category may include two proteins: VirB1 and VirB3. The N-terminal segment of VirB1 has significant sequence similarity to goose-egg white lysozyme (Llosa, Zupan et al. 2000). Although its C-terminal sequence is important for interaction with VirB9, it was suggested by yeast two-hybrid analysis that VirB1 further interacts with VirB4, VirB8, VirB10 and VirB11 (Ward, Draper et al. 2002). Taken together, the available data substantiate the role of VirB1 as a lytic transglycosylase. Lytic transglycosylases are bacterial muramidases that cleave β-1,4-glycosidic bonds in the peptidoglycan cell wall polymer, resulting in the formation of 1,6-anhydromuropeptides. This cleavage generated by VirB1 is believed to create openings in the bacterial cell wall that permit proper assembly of the T4SS apparatus in the periplasm (Mushegian, Fullner et al. 1996; Blackburn and Clarke 2001). Deletion of any virB gene except virB1 completely abolishes T4SS virulence. In contrast, virB1 mutants are still able to initiate plant tumors, but virulence is decreased to 10% or less, likely because cellular lytic transglycosylases can partly compensate for VirB1 functions (Hoppner, Liu et al. 2004). The role of VirB1 during the T4SS process might be more complicated than previously thought. In addition to the established view of VirB1 as a lytic transglycosylase, a C-terminal fragment of this protein was found to be secreted into the

extracellular environment (Baron 1997) (Zupan, Hackworth et al. 2007; Aly, Krall et al. 2008). In addition, VirB1 may also cofractionate with T-pili.

VirB3 is an outer membrane bound protein that is stabilized by VirB4 (Yuan, Carle et al. 2005). It was shown that VirB3 interacts with VirB5 (Shamaei-Tousi, Cahill et al. 2004), however, little is known about the function of VirB3 during the T4SS process.

1.4 T-pilus proteins

A. tumefaciens assembles 3 different types of surface-exposed appendages, which can be easily differentiated based on their different diameters (flagella, 15 nm; T-pili, 10 nm; common pili, 3 nm) (Eisenbrandt, Kalkum et al. 1999; Lai, Chesnokova et al. 2000). VirB proteins only contribute to the assembly of one type of appendages, T-pili. Two proteins, VirB2 and VirB5 are believed to be T-pilus components. Since VirB7 and VirB1 are also detected by Western blot analysis of sheared T-pili sedimented by high-speed centrifugation, they might as well be T-pilus associated proteins.

1.4.1 VirB2, the major T-pilus protein

The T-pilus likely functions in plant host recognition and/or attachment and possibly, as substrate injection channel. The diameter of the T-pilus is 10 nm and represents one of three different types of appendages assembled by *Agrobacterium*. In addition, *A. tumefaciens* cells assemble flagella (15 nm) and common pili (3 nm) in diameters respectively (Lai and Kado 1998). Neither flagella nor common pili were previously reported to play any role in T4SS-mediated virulence. VirB2 is the T-pilus major protein (Lai and Kado 1998). Its N-

terminal signal peptide is 47 amino acids long and the C-terminal portion comprises 74 amino acids. Mass spectrometry was applied to show that the processed VirB2 (pilin) (C-terminal segment) carries a peptide linkage between its amino- and carboxyl- terminal residues as explained in detail (Lai, Eisenbrandt et al. 2002). Briefly, cell suspension or pilus preparation was cocrystallized with trans-3-indolylacrylic acid and analyzed with a Reflex-II time-of-flight spectrometer. There was a significant change in mass when VirB2 was overproduced in *E. coli* versus *A. tumefaciens*. The difference in mass of 18 Da is explained by the loss of one molecule of H_2O, caused by the formation of a cyclized T-pilus subunit (Lai, Eisenbrandt et al. 2002). This cyclized form of VirB2 was detected in *A. tumefaciens* but not when produced in *E. coli*. This indicates that inspite of the conserved T4SS function among bacterial species, pilus assembly mechanisms may be species-specific. T-pilus biogenesis requires all T4SS proteins except VirD4, but VirB1 and VirB3-VirB11 are not required for the pre-pilin processing or pilin cyclization (Lai, Eisenbrandt et al. 2002).

ClustalW is a reliable sequence alignment tool that is widely used for sequence comparison between various genes or proteins (Chenna, Sugawara et al. 2003). In a typical multiple sequence alignment experiment, ClustalW assigns specific score for each nucleotide or amino acid residue. The more the conserved is the "point", the higher the score it receives and vice versa. When a group of residues adjacently lined next to each have low consecutive scores, the alignment tool introduces a gap so that a new alignment process is initiated for the purpose of improving the overall alignment score. Using ClustalW, multiple sequence alignment of VirB2 proteins from select organisms: The *A. tumefaciens* VirB2 of strain C58 (accession number: NP_396488), the *B. suis* VirB2 (accession number: AAD56612), the *Sinorhizobium meliloti* VirB2 (accession number: AAK65376), the *Bartonella henselae* VirB2

(accession number: AAD48919) and the *Ochrobactrum anthropi* VirB2 (accession number: YP_001373080), reveals that amino acid sequence similarity is around 23%. However, the leader peptide sequence is extremely divergent and this divergence may affect the overall sequence alignment pattern (Fig. 2). With regards to the size of the mature pilin, the VirB2 leader peptide sequence is considered one of the longest in known secretion systems. For example, in *A. tumefaciens*, the leader peptide comprises around 40% of the total VirB2 protein length. Many pre-pilins from other pilus systems are known to have significantly shorter leader peptides (Nunn and Lory 1992). This observation suggests that VirB2 signal peptides in functional T4SS machineries may contribute to more than protein targeting towards the bacterial envelope.

In *A. tumefaciens*, it was shown that when used as bait, the C-terminus of VirB2 bound to three B 2 Interacting proteins (BTI) in an *Arabidopsis thaliana* plant cDNA expression library (Hwang and Gelvin 2004).

Figure 2. Multiple sequence alignment of VirB2 proteins from select T4SS-encoding organisms. Multiple sequence alignment of VirB2 proteins from select organisms: The *A. tumefaciens* VirB2 of strain C58 (accession number: NP_396488), the *B. suis* VirB2 (accession number: AAD56612), the *Sinorhizobium meliloti* VirB2 (accession number: AAK65376), the *Bartonella henselae* VirB2 (accession number: AAD48919) and the *Ochrobactrum anthropi* VirB2 (accession number: YP_001373080), reveals low amino acid sequence similarity (~23%). In the *A. tuemfaciens* VirB2, signal peptide cleavage occurs between alanine (A47) and glutamine (Q48) (shown in red color). (*), identical amino acid residue; (:), conserved amino acid substitution ; (.), partially conserved amino acid substitution.

```
meliloti        -----------------MTFSSRIRPIAASTVMATAIMVTMVEPAFAQAAG--------I
anthropi        -----------------MNFYCRLRPIVASCVMAAAIVPIVSEPALAQAAG--------I
henselae        -----------------MTDTISRNIIFIIIMLLLTALVVSDPSYAAAATGSASGLGNV
C58             MRCFERYRVHLNRLSLSNAVMRMVSGYAPSVVGAMGWSIFSSGPAAAQSAGGG----TDP
suis            ------------MKTASPSKKSLSRILPHLLLALIVSIAAIEPNLAHANGG----LDKV
                                                :           *  * :

meliloti        ETVLQNIVDMLTGNIAKLLAVIAVIVICIAWMFGYMDLRRAGFWIIGIGGIFGATELVNT
anthropi        ETILQNIVDLLTGNIFRLLATIAVIVIAIAWMFGYMDLRRAGYWIIGIGIIAGSSELVGT
henselae        DNVLQSIVTMMTGTTAKLIATICVAAVGIGWMYGFIDLRKAAYCLIGIGIVFGASALVSK
C58             ATMVNNICTFILGPFGQSLAVLGIVAIGISWMFGRASLGLVAGVVGGIVIMFGASFLGKT
suis            NTSMQKVLDLLSG-VSITIVTIAIIWSGYKMAFRHARFMDVVPVLGGALVVGAAAEIASY
                 . ::.:  :: *     :..: :        :    :  .   : *   : .:: :

meliloti        IVGS-
anthropi        IVGS-
henselae        LTSAS
C58             LTGGG
suis            LLR--
                :
```

However, the BTI proteins did not interact with the minor T-pilus component VirB5 or any of the *A. tumefaciens* substrates. Interestingly, over-expression of BTI1 (one of the three BTI proteins) in transgenic *A. thaliana* results in hyper-susceptibility to *A. tumefaciens*-mediated transformation. These results suggest that the BTI proteins could provide an initial interaction between *A. tumefaciens* and plant hosts (Hwang and Gelvin 2004).

1.4.2 VirB5, the minor T-pilus protein

VirB5 is a minor T-pilus component and might be the outermost protein in the T4SS complex (Schmidt-Eisenlohr, Domke et al. 1999). VirB5 localization in the T4SS complex remained a long-standing question in T4SS research. In *A. tumefaciens*, VirB5 is 220 amino acid residues long. Sequence comparisons between different VirB5 proteins of select organisms: The *A. tumefaciens* VirB5 of strain C58 (accession number: NP_396492), the *B. suis* VirB5 (accession number: AAD56615), the *Sinorhizobium meliloti* VirB5 (accession number: NP_435961) and the *Ochrobactrum anthropi* VirB5 (accession number: YP_001369232) is shown (Fig. 3). The crystal structure of the VirB5 ortholog TraC from the plasmid system pKM101 in *E. coli* was solved upon the refinement of an electron density map at 3.0-A° resolution (Yeo, Yuan et al. 2003). 7 α-helical domains largely comprise the TraC structure. These helices can be divided into 2 groups: The backbone structure which is composed of 3 α-helices and supports a loose appendage composed of 4 other relatively short helices (Yeo, Yuan et al. 2003). This leaves a C-terminus which can be best described as a loose appendage that was hypothesized by our group to be required for VirB-VirB, VirB-substrate or VirB-host interactions. This hypothesis is attributed to the fact that the C-terminal amino acid sequence of the VirB5 family of proteins is divergent and it might have a role in host cell recognition. VirB5 was also shown to interact with the inner membrane protein

VirB8 (Yuan, Carle et al. 2005) and hypothesized to interact with the possible outer membrane protein VirB3 in addition to VirB2, the major T-pilus protein.

Figure 3. Multiple sequence alignment of VirB5 proteins from select T4SS-encoding organisms. Sequence comparisons between different VirB5 proteins of select organisms: The *A. tumefaciens* VirB5 of strain C58 (accession number: NP_396492), the *B. suis* VirB5 (accession number: AAD56615), the *Sinorhizobium meliloti* VirB5 (accession number: NP_435961) and the *Ochrobactrum anthropi* VirB5 (accession number: YP_001369232). In the *A. tumefaciens* VirB5, the signal peptide cleavage occurs between alanine (A23) and glutamine (Q24) (shown in red color). (*), identical amino acid residue; (:), conserved amino acid substitution ; (.), partially conserved amino acid substitution.

```
meliloti        MAFFRINCVVIASALILSATGAAGQGIPVIDQTAIAKQIES-IAQLKAQLDALNQQIEQA
anthropi        MASYRCSCAIMAAVLTLSTGAAMGQGIPVIDQTAIAKHMES-IAQLKSQLDALHQQIEKA
suis            -----MKKIILSFAFALTVTSTAHAQLPVTDAGSIAQNLANHLEQMVKFAQQIEQLKQQF
C58             --MKIMQLVAAAMAVSLLSVGPARAQFVVSDPATEAETLAT----------ALETAANL
                      .    : .. *   ..    : * *  : *: : .            :   :

meliloti        QQLHGSLNKLTDMSDVASVLNDPAIRKALPADFSAIEGLFKGNGTGVFADSASKFLDGNT
anthropi        EELYTSFNKLTDMADVAKVLNDPAIRKALPPEFAAVEGLFEGNGSGALNDAATKFLDKNT
suis            EEQKMQFDALTGYRGLGDILRDPTLRSYLPHNWRDLYEAVMSGGYLAAAGETANLLRKSQ
C58             EQTITMVAMLTSAYGVTGLLTSLNQKNQYPSTRDLDTEMFSPRMPMSTTARAITTDTDRA
                ::    .  **.  .:  :* .   :.  *          .           : .

meliloti        TYQTNAADDFYAQELSRIQ--NKNAGQMSLGQQIYDAATKRIDGIDQLREKISTAGDAKD
anthropi        TYKT-SADDFYAKELSRVQ--NKNAGQMGIGQKIYDTATKRIEGIDELRQKISTASEAKE
suis            VYDPCASISDKDQRIACEAKVVKPVQDKVMTSKAYDATDKRLQEIESLMQEINKTGDPKA
C58             VVGGDAEADLLRSQITGSANSAGIAADNLETMDKRLTANAETSTQLSRSRNIMQATVTNG
                .    :  .   ..::        . :     .   ::  . .   .  .:*  :  .:

meliloti        IADLQARLQAEQAFLQTDVLRMEGLRMVQQAQEQVDEQR----KAEDWRQRMDAIKAALQ
anthropi        IADLQARLLAEQAFVQTDVLRMEGLRMVQQAQTQVDDQR----KAEDWRRRMDAMKGALK
suis            IAELQGRIESENAMIQNEDTRLHLYQQMAEAQDKLLDERQHELDAKDNARRGYPQPKALE
C58             LLLKQIHDAMIQNVQATSLLTMTTAQAGLHEAEEAAAQR-------KEHQKTAVIFGAVP
                :   * :    : .  ..   :   :   .   :   :*       .  ::      *:

meliloti        ---
anthropi        ---
suis            AAY
C58             ---
```

1.4.3 Is VirB5 a surface exposed adhesin?

Several lines of evidence suggest that VirB5 is exposed on the bacterial surface presumably as an adhesion (Schmidt-Eisenlohr, Domke et al. 1999). Bacterial adhesins are often involved in recognition of host cells and contribute to the initial stages of host cell attachment. Their ability to attach to biotic and abiotic surfaces has a significant impact on bacterial virulence and therefore, adhesins are attractive candidates for the development of vaccines. Numerous reasons led to proposing that VirB5 may be a potential adhesin. For example, Dr. Christian Baron's laboratory has optimized a protocol for extracting T-pili by shearing and ultracentrifugation without cell permeation. Only VirB2 (the major T-pilus protein) as well as VirB5 (a possible minor T-pilus protein) are detected by Western blot analysis of sheared and sedimented pili (Schmidt-Eisenlohr, Domke et al. 1999). This indicates that VirB5 is surface exposed or that it has a surface exposed form in addition to other forms that might exist elsewhere in the T4SS complex. In addition, the VirB complex from the agrobacterial membranes has been successfully extracted using mild detergents (Krall, Wiedemann et al. 2002). Those experiments revealed that VirB5 co-fractionates with the T-pilus major component VirB2. They also support the possibility that VirB5 is exposed on the cell surface probably as an adhesin. The adhesin hypothesis of VirB5 has been brought into mind after structural superposition studies of the VirB5 ortholog, TraC, with the focal adhesion targeting region of the focal adhesion kinase which revealed significant structural similarity (Yeo, Yuan et al. 2003). This finding suggests that VirB5 family of proteins may be assigned an adhesive role during the T4SS process.

1.5 Summary of intent

The VirB complex machinery significantly contributes to the pathogenesis of different bacterial pathogens. The outermost portion of that complex is termed the T-pilus. Whereas it is largely established that VirB2 is the major T-pilus component, direct evidence for that hypothesis has not been demonstrated prior to my thesis studies. I have shown during the course of my studies that VirB2 is indeed the major component of the T-pilus by performing extensive electron microscopy studies (**Chapter 3**). I showed that VirB5 localizes to the tips of cell bound T-pili (**Chapter 3**). From the solved crystal structure of the VirB5 ortholog TraC, the C-terminal domain of VirB5 was hypothesized to be a site of protein-protein interactions. Detailed analysis of the VirB5 C-terminus and its contribution to bacterial pathogenesis led to discovering that it may be required for host cell specificity. In addition, the T-pilus strength and less susceptibility to breakage might also be dependent on the VirB5 C-terminus (**Chapter 3**).

The role of VirB5 during bacterial pathogenesis is hypothesized to be significantly broader than its contribution to the T-pilus assembly process. I found that the surface exposure of the host range factor Tzs is T4SS-dependent with VirB5 significantly contributing towards this process (**Chapter 4**). The co-fractionation of Tzs with many complexes of diverse molecular masses in the bacterial membranes is VirB5-dependent (**Chapter 4**).

I contributed in the establishment of an assay for testing the functionality of the *B. suis* T4SS components in the heterologous host *A. tumefaciens* (**Chapter 5**). Further characterization of that assay has shown that various VirB proteins contribute to recipient competence.

I found that the *B. suis* VirB4 can substitute the agrobacterial VirB4 in T-pilus assembly (**Chapter 6**). I also studied the possibility that VirB5 may be functional when forced to localize to various subcellular compartments using membrane-standing domains (**Chapter 7**).

The systematic approaches explored throughout my Ph.D. studies have advanced our knowledge regarding T-pilus assembly and the role of VirB5 during the T4SS process.

Chapter 2
MATERIALS AND METHODS

Cultivation of bacteria

All cloning experiments were conducted using cultures of *E. coli* JM109. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl) liquid medium or in Petri dishes containing medium solidified with 2% agar. In case of *A. tumefaciens*, cells were grown on YEB (0.5-1% tryptone, 0.1% yeast extract, 0.5% sucrose, 2 mM $MgSO_4$) liquid medium or in Petri dishes containing medium solidified with 2% agar. For virulence gene inductions, cells were grown on AB minimal medium (1% glucose, 0.4% MES [morpholineethanesulfonic acid], 0.2% NH_4Cl, 0.3 g of $MgSO_4 \cdot 7H_2O$ per liter, 0.15 g of KCl per liter, 0.01 g of $CaCl_2$ per liter, 0.0025 g of $FeSO_4 \cdot 7H_2O$ per liter, and 1 mM potassium phosphate [pH 5.5]) by the addition of acetosyringone (AS) at a final concentration of 200 μM and isopropyl-β-thiogalactopyranoside (IPTG) at a final concentration of 0.5 mM both when needed.

When required for plasmid propagation, media were supplemented with streptomycin (50 μg/ml for *E. coli* and 100 μg/ml for *A. tumefaciens*), spectinomycin (50 μg/ml for *E. coli* and 300 μg/ml for *A. tumefaciens*), ampicillin (100 μg/ml), or erythromycin (150 μg/ml).

Analysis of T4SS functions: T-pilus isolation, conjugation and virulence assays

For T-pilus isolation, cells were grown overnight in YEB medium with/without antibiotics for plasmid propagation. Next day, cell cultures where adjusted to a final optical density (OD_{600}) 0.1 in a liquid minimal medium and cells were grown for 4-5 hours at 26°C. 1 ml of cells was plated per large (15 cm^2) AB medium plate (20 g of agar/liter) with or without 200 μM AS and the plates were further incubated at 20°C for 4 days. T-pili were isolated from the cells grown on 2 plates each with and without AS by shearing and ultracentrifugation as follows. Cells were suspended in 10 ml of buffer P (50 mM potassium phosphate, pH 5.5), and then centrifuged at 10,000 rpm in an SS34 rotor in an RC-5B centrifuge (Sorvall) for 60 min. Cell pellets were suspended in 1 ml of buffer P, passed eight times through a 26-gauge needle to remove surface-associated high-molecular-weight structures, and then centrifuged in a

microcentrifuge for 60 min at 15,000 rpm. The supernatant was subjected to high-speed centrifugation at 40,000 rpm for 90 min in a 90 Ti rotor to separate high-molecular-weight structures, like flagella and pili, in the pellet from soluble constituents removed from the cells by shearing (Schmidt-Eisenlohr, Domke et al. 1999; Hoppner, Liu et al. 2004).

For plant infection experiments, agrobacterial cells were grown overnight in YEB liquid medium supplemented with antibiotics when necessary. Cells were then inoculated into AB liquid medium to a final optical density (OD_{600}) of 0.1 and grown for 5 hours at 20°C while shaking at 200 rpm. *Kalanchöe diagremontiana* leaves mildly scratched with 26.5 gauge needle were next infected with 20 µl of each of the bacterial strains under examination. Plants were constantly incubated at 20°C for an average time interval of 5 weeks until tumors were detected on the plant leaves.

For the analysis of pLS1 donor activity, A348 and PC1005 carrying pTrc200 with and without VirB5 encoding genes were co-cultivated with UIA143(pTiA6) recipient cells in a 1:5 ratio for 3 days on AB minimal medium agar containing 500 µM AS and 500 µM IPTG followed by plating on selective agar media (CAR, 150 µg/ml; ERY, 150 µg/ml) and quantification of recipient and donor cells. For the analysis of pLS1 recipient activity, donor A348 pLS1 cells were co-cultivated with UIA143 recipient cells carrying pTrc200 or other *B. suis* various *virB* constructs in a 5:1 ratio for 2 days on AB induction medium plus 500 µM AS and 500 µg of IPTG/ml followed by plating on Luria-Bertani agar plus antibiotics (150 µg of CAR/ml and/or 100 µg of SPC/ml) as necessary for quantification of donors/recipients, and transconjugants (Hoppner, Liu et al. 2004).

SDS/PAGE and Western blotting

SDS-PAGE was conducted according to Laemmli (for proteins larger 20 kDa) or Schägger and von Jagow (for proteins smaller 20 kDa) (Schagger, Aquila et al. 1988). Western blotting was performed in a tank blot apparatus. Detection was performed with a chemiluminescence-based system with *A. tumefaciens* strain C58 VirB protein-specific antisera was performed following standard protocols with VirB protein-specific antisera.

Cloning and plasmid constructs

The *A. tumefaciens virB5* gene in pTrcB5 was amplified by polymerase chain reaction (PCR) with oligonucleotides to introduce changes into the gene product and cloned into expression vector pTrc200. The sequences of PCR-amplified genes were confirmed by DNA sequencing. Various constructs were PCR amplified using the oligonucleotides shown (Table 1).

Table : 1

Name/restriction	Sequence	Constructed plasmid
virB5-5'/*Afl*III	CCACATGTCGATCATGCAACTTGTTGC	pTrcB5
virB5-3'/ScaI	GAAAGTACTCAGGGGACGGCCC	
VirB5Δ1-3'/*Hin*dIII	GCAAGCTTAGACGGCCCCAAAGATG	pTrcB5Δ1
VirB5Δ2-3'/*Hin*dIII	GCAAGCTTAGGCCCCAAAGATGACC	pTrcB5Δ2
VirB5Δ3-3'/*Hin*dIII	GGAAGCTTACCCAAAGATGACCGCA	pTrcB5Δ3
VirB5Δ4-3'/*Hin*dIII	GGGAAAGCTTAAAAGATGACCGCAGTC	pTrcB5Δ4
VirB5Δ5-3'/*Eco*RI	CGGGAATTCAGATGACCGCAGTCTT	pTrcB5Δ5
VirB5Δ10-3'/*Hin*dIII	CCGAAGCTTATTGATGCTCCTTACG	pTrcB5Δ10
VirB5Δ15-3'/HindIII	CGGAAGCTTATTGAGCGGCCGCCTC	pTrcB5Δ15
VirB5Δ20-3'/HindIII	CGGAAGCTTATTCCGCCTCATGAAG	pTrcB5Δ20
VirB5Δ25-3'/HindIII	CGGAAGCTTTCAGCCAGCCTGCGCGGT	pTrcB5Δ25
VirB5Δ30-3'/*Hin*dIII	CCTAAGCTTAGGTCATCGTTAACAA	pTrcB5Δ30
VirB5AAA-3'/*Hin*dIII	CCGAAGCTTTCAGGCGGCGGCCCCAAA GATGACC	pTrcB5ΔAAA
VirB5AVA-3'/*Hin*dIII	CCGAAGCTTTCAGGCGACGGCCCCAAA GATG	pTrcB5ΔAVA
VirB5AAP-3'/*Hin*dIII	CCGAAGCTTTCAGGGGGCGGCCCCAAA GATG	pTrcB5ΔAAP
VirB10sN-5'/*AflIII*	CCACATGTCGCAGGAAAACATTCCGGT GCAGC	pTrcB5N
VirB10sN-3'/*EcoRI*	GCGAATTCATTGCCCCTCATGTGAAAC ACG	
VirB5N-5'/*EcoRI*	CCGAATTCCAGTTCGTTGTCAGCGATCCG	
VirB5N-3'/*HindIII*	CCAAGCTTTCAGGGGACGGCCC	
VirB5C-5'/*AflIII*	CCACATGTCGATCATGCAACTTGTTGC	pTrcB5C
VirB5C-3'/*PstI*	GGCCTGCAGGGGGACGGCCCCAAAG	
VirB10sC-5'/*PstI*	CACTGCAGAGGATGCACGTGTTGCTCTT TCTCTTTGTCGTGGGCTTCAT	
VirB10sC-3'/*HindIII*	CCAAGCTTAGCGGGTGCGCTTAAACACG AGCAGCCA CAGCAGCAC	

VirB5SP-5'/*AflII*	CCACATGTCGATCATGCAACTTGTTGCTG CGGCCATGGCCGTCAGCCTTCTTGGGGGG TGCCAGTTCGTTGTCAGCGATCCGGCGAC	pTrcB5SP
VirB5SP-3'/*HindIII*	CGAAGCTTTCAGGGGACGGCCCCAAAGAT GACCGCAGTCTTTTGATGCTCCTTACGTTG AGCGGCC	

* Restriction sites underlined

For strain constructions/in-frame deletions, 4 oligonucleotides (Table 2) were designed first for PCR amplification of the gene of interest with 500 base pair (bp) overhangs, followed by deleting the gene using an inverse PCR procedure, leaving a fragment of 1000 bp which only includes the overhanging regions. Next, the 1000 bp fragment was excised from the temporary cloning vector and further sub-cloned into the suicide vector pK*moBsacB* (Schafer, Tauch et al. 1994). The vector was introduced to wild-type *A. tumefaciens* competent cells by electroporation. Cells were grown on LB kanamycin (LB Km) plates followed by screening for the desired mutant upon homologous recombination.

Select purified colonies grown on LB Km plates were subsequently grown in LB liquid medium for 12 hr at 26°C. Cultures were then diluted 1:1000 into LB 10% sucrose liquid medium and further grown for 16 hr at 26°C. Cells were then plates onto LB 10% sucrose plates followed by screening the purified colonies for the desired kanamycin sensitive mutant.

Table : 2

Application	Name	Sequence and restriction enzyme
Cloning of *virB2*	B2-5	CCACACGAATTCCAAGTCGTGATGGACCGT CTCGA
	B2-3	CCACACGAATTCGACGGCAACGTGCATTGCG CATTT
Deletion of *virB2*	ΔB2-5	AGGAGGTCCGCAATAATGAATGATCGTCTGG AAGCAACCCTT
	ΔB2-3	TTATTGCGGACCTCCTTGATTTAAGTCGAACA AGAGTTGATCGTC
Cloning of *virB8*	B8-5	CGCAGTCTAGAGCAAAGTGGATCGGGCAACT TAT
	B8-3	CGCAGTCTAGACCTCTGCTCTCTGTTGATATTG CGCTT
Deletion of *virB8*	ΔB8-5	CCGTGCTCGAGTTATTCAGACCCCTTCATGGCG ACCACCT
	ΔB8-3	CCGTGCTCGAGATGACGAAAAAAGCATTTCTCA

* Restriction sites underlined

T-pilus purification by gel filtration

T-pili from different *A. tumefaciens* strains were isolated by shearing of the cells and ultracentrifugation. For further purification, the pilus-containing sediments obtained after ultracentrifugation were resuspended in 100 µl 50 mM sodium potassium phosphate (NaKP) buffer, pH 5.5, and applied to a Superdex 200 column (GE Healthcare), followed by separation with a flow rate of 0.5 ml min^{-1}. The fractions eluting from the column were concentrated by the addition of three volumes of acetone and incubation on ice for 4 h. The samples were then subjected to ultracentrifugation for 2 h at 270,000 *g* for precipitation of the pili, followed by re-suspension in 40 µl NaKP buffer. Thirty microlitres of these samples were mixed with equal volumes of Laemmli sample buffer followed by SDS-PAGE and Western blot analysis. The remaining samples were loaded on electron microscopy (EM) grids for further visualization of purified T-pili.

Transmission electron microscopy (TEM) and immuno-EM

Negative staining for visualization of T-pili was conducted as follows. Virulence-induced *A. tumefaciens* were collected with 5 ml of 50 mM sodium potassium phosphate

buffer, pH 5.5, and the optical density (OD_{600}) adjusted to 1.5–2. 10 µl samples were applied onto UV-sterilized 200 mesh carbon-coated formvar copper grids and air-dried. The grids were then stained with 1% phosphotungstic acid-0.01% glucose, pH 6, for 15 s prior to examination. For quantification of T-pilus formation, experiments were conducted as described, followed by more intense staining with 2 % phosphotungstic acid, 0.01 % glucose, pH 6, for 15–20 s to efficiently label the pili. Depending on the experiment, T-pili from 60–400 cells from three independent virulence-induction experiments of each strain were counted, and 10 cells per visual field were analysed.

For immuno-EM, strains were cultivated on AB minimal medium agar plates as above, collected with liquid AB minimal medium supplemented with AS and the cell density was adjusted to OD_{600} of 0.25. 15 µl were applied onto UV-sterilized 200 or 300 mesh carbon-coated formvar nickel electron microscopy grids and cultivated for 10-12 h at 20°C in a humid chamber. The grids were then fixed for 1 h with 2% formaldehyde and 0.5% glutaraldehyde in 50 mM sodium cacodylate buffer at pH 5.5 as described (Jin, Hu et al. 2001). After fixation, the grids were subjected to immuno-gold labeling largely as described (Quintero, Busch et al. 1998). Grids were blocked with 5% skim milk in TBST (20 mM Tris/HCl, 137 mM NaCl, 0.1% Tween-20, pH 8) for 20 min, followed by incubation an a drop of 1:250 diluted anti-VirB5 in 5% skim milk and TBST for 45 min at room temperature. Grids were then washed 3x5 min, incubated with the secondary antibodies for 45 min at room temperature (1:10 diluted anti-rabbit 10 nm. gold conjugate, Sigma-Aldrich) in TBST with 5% skim milk, 0.1% BSA and 5% fetal bovine serum. Finally, the grids were washed and negative stained with 1% of phosphotungstic acid in 0.01% glucose (pH 6) for 15 s prior to examination. Images were recorded with a JEOL JEM-1200EX or JEOL 1200EX II transmission electron microscopes.

Extraction of T4SS protein complexes using the mild detergent dodecylmaltoside

Agrobacterial cells were grown overnight at 26°C by adding antibiotics when necessary to allow propagation of the overexpression pTrc vector. Next day, cells were inoculated in AB liquid minimal medium to a final OD_{600} value of 0.1 and incubated 4-5 h at 20°C. 1 ml was spread on 16x15 cm^2 large Petri dishes in the presence of 200 µM of

acetosyringone and IPTG when necessary for the induction of *trc* promoter in strains carrying the pTrc overexpression vector. On the sixth day of growth, cells were harvested from each plate using 10 ml of 50 mM NaKP buffer. Cells were next pelleted using table centrifuge at 12,000 rpm for 45 min and resuspended in 20 ml of NaKP buffer. Cells were adjusted to equal densities of a final OD_{600} value of around 20 maintaining a total volume of 20 ml per 50 ml standard sterile plastic 50 ml falcon tubes. Phenylmethanesulphonylfluoride (PMSF) was added to a final concentration of 0.5 mM and cells were lysed by passage through French press (3 times, 18,000 psi).

Cell debris was next pelleted for 1 h at 12,000 rpm, followed by transferring the supernatant (soluble proteins and membranes) to high-speed centrifugation tubes. Samples were subjected to high-speed centrifugation for 2 h at 40,000 rpm followed by discarding the supernatant and storing the pellet (membranes) on ice overnight. Membrane pellet was then transferred to new tube and suspended in 1 ml of ACA buffer (750 mM 6-amino-caproic-acid and 50 mM BisTris, pH 7). 1 µl of membrane suspension was diluted in 9 µl of ACA buffer and 1 µl was used to determine the total protein concentration using a standard Bradford assay. 1-10 µl of bovine serum albumin (BSA) were used to construct a standard curve. For detergent extraction, a protein concentration of 10 mg/ml was adjusted in the reaction mixture for extraction with 2% of the non-ionic detergent dodecyl-β-D-maltoside. Samples were shaken for 2 h at 4 °C followed by a second step of high speed centrifugation at 40,000 rpm. The supernatant was then collected as the solubilized membrane protein complexes for further analysis using Blue Native Electrophoresis (BNE) and gel filtration.

Blue Native Electrophoresis (BNE) and Western blot analysis

The purpose of BNE is the separation of detergent solubilized proteins and protein complexes under non-denaturing conditions. The ratio of detergent to Coomassie stain was kept as 4:1 because the dye will exchange with the detergent used in the complex extraction experiment. Upon mixing the extracted complexes with Coomassie Blue G-250, gels were loaded with samples and followed by Western blot analysis using standard procedures.

Gel filtration experiments

Gel filtration columns were used either for the purification of extracted pili or for monitoring the co-fractionation pattern of extracted complexes from various variant carrying mutants. These studies employed Superdex 200 gel filtration column and a total volume of 100 µl from each sample was injected into the column using a 100 µl injection loop.

Chapter 3

The VirB5 protein localizes to the T-pilus tips in *Agrobacterium tumefaciens*

Preface

This chapter consists of the following article, in its originally published format: **Aly, K. A.** and Baron, C. **The VirB5 protein localizes to the T-pilus tips in *Agrobacterium tumefaciens.*** (2007). Microbiology 153 (11): 3766-3775. A copy of the original manuscript is included in my thesis with permission from the publisher.

I performed all the experiments within this article, assembled the results and generated the figures together with Dr. Baron. Dr. Christian Baron has helped improving the display of data in all figures so that they become easier for the reviewer to understand. Dr. Baron has written the entire manuscript. Dr. Baron and I made some revisions to the manuscript, and Dr. Baron contributed significant intellectual input and support.

The rationale behind this study was based on various lines of evidence suggesting that VirB5 is a T-pilus associated protein. The crystal structure of the VirB5 ortholog, TraC, from the *E. coli* plasmid system pKM101 suggested that the C-terminus may be a domain involved in protein-protein interactions. To test this hypothesis, an immuno-EM approach was undertaken with the purpose of immuno-gold labeling of both, the major T-pilus protein VirB2 and the minor T-pilus component VirB5 in cell-bound as well as detached T-pili. C-terminal deletion and alanine replacement variants of VirB5 were introduced into the *virB5* deletion strain CB1005 and T-pilus assembly and T4SS function were monitored.

I found that in cell-bound T-pili, VirB2 can not be labeled by immune-gold methods. This could be attributed to the fact that intact T-pili may have a structure that rendered the VirB2 epitope inaccessible. In addition, VirB5 was detected on the tips of cell-bound pili, ends of broken pili as well as on the cell surface. VirB5 C-terminal deletions (up to 4 amino acid deletions) resulted in the assembly of non-functional pili (except for Δ4 which was partially functional in donor inter-bacterial conjugation assay). Pili assembled on the variant-producing strains are perhaps less susceptible to breakage. In addition, alanine replacement variants were also non-functional except for the AAP variant (instead of an AVP wild-type C-terminal sequence) which was partially functional in inter-bacterial conjugation.

Taken together, the data presented here have advanced our understanding on the localization of VirB5 in the T4SS apparatus. This manuscript represented the first documentation in T4SS research regarding the location of VirB5 in any T4SS and the importance of its C-terminus in host cell specificity.

Microbiology (2007), 153, 3766–3775 DOI 10.1099/mic.0.2007/010462-0

The VirB5 protein localizes to the T-pilus tips in *Agrobacterium tumefaciens*

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The *Agrobacterium tumefaciens* VirB/D4 type IV secretion system (T4SS) mediates the transfer of single-stranded DNA and protein virulence factors into plant cells, and also determines the assembly of the T-pilus, which is believed to play a role in host recognition. The T-pilus is composed of the major component VirB2 and the minor component VirB5. Using immuno-electron microscopy we detected the major component VirB2 along the entire length of detached T-pili, but not on cell-bound T-pili or on the cell surface. In contrast, the minor T-pilus component VirB5 was detected on the tips of cell-bound T-pili as well as on the ends of detached T-pili and on the cell surface. To gain further insights into the role of VirB5 we introduced changes at its C terminus. C-terminal deletions of up to four amino acids and alanine replacements did not abolish T-pilus formation and incorporation of the VirB5 variants at the tip, although they did impact the length of T-pili. Also, these changes differentially affected the ability of the T4SS to transfer DNA into plant and bacterial recipients, suggesting differential effects on host-cell specificity. The data presented here suggest that VirB5 localizes at the T-pilus tip, and provide novel insights into its role during the type IV secretion process.

Received 7 June 2007
Revised 22 July 2007
Accepted 6 August 2007

INTRODUCTION

Many Gram-negative bacteria assemble extracellular high-molecular-mass filamentous structures such as pili on their surface, which are typically composed of one protein or a small set of proteins. Binding is the principal function in the case of adhesive pili, such as P and type-1 pili, which mediate the attachment of bacteria to host tissues to initiate colonization (Kau *et al.*, 2005; Sauer *et al.*, 2004). In contrast, type IV secretion system (T4SS)-determined surface structures are involved in a complex cascade of events that lead to the inter-bacterial or trans-kingdom delivery of effectors, mostly proteinaceous virulence factors, but also DNA molecules (Baron, 2005; Christie *et al.*, 2005; Yeo & Waksman, 2004). T4SSs are essential for the virulence of many pathogens, such as *Agrobacterium tumefaciens* (gene transfer and tumour formation on plants), *Bartonella henselae* (cat scratch disease), *Bordetella pertussis* (whooping cough), *Helicobacter pylori* (gastritis and stomach cancer) and *Legionella pneumophila* (Legionnaires' disease) (Backert & Meyer, 2006; Baron, 2005; Christie *et al.*, 2005; McCullen & Binns, 2006; Schröder & Dehio, 2005).

VirB2-like proteins are the major pilus components of the *A. tumefaciens*, plasmid RP4 and F-determined T4SSs, and homologues exist in most other systems (Fullner *et al.*, 1996; Kalkum *et al.*, 2002; Lai & Kado, 1998). VirB2 proteins are small and hydrophobic, and after cleavage of their signal peptides, those from *A. tumefaciens* and RP4 are processed to cyclic peptides (Eisenbrandt *et al.*, 1999; Kalkum *et al.*, 2002). Interaction partners of *A. tumefaciens* VirB2 pilin have been identified in an *Arabidopsis thaliana* cDNA expression library, implying that the major T-pilus protein may directly interact with host-cell components (Hwang & Gelvin, 2004).

Whereas the presence of VirB2-like major pilin proteins is well established, knowledge of minor pilus components and their function(s) is relatively limited. Minor components may play a role in pilus elongation, but in a similar manner to tip components of adhesive pili, they may also initiate contact with host cells. There is some indirect evidence for the existence of an adhesin at the F-pilus tip (Anthony *et al.*, 1994), but so far, minor pilus components have not been identified in this system. VirB5-like proteins have been shown to be minor components of pili determined by the *A. tumefaciens*, IncN plasmid pKM101 and IncP plasmid pJP4 T4SSs (Schmidt-Eisenlohr *et al.*, 1999a, b, 2001), and VirB5 is essential for the incorporation of the major component VirB2 into T-pili (Lai *et al.*, 2000; Schmidt-Eisenlohr *et al.*, 1999a). The X-ray structure of the pKM101 VirB5 homologue TraC has been solved, and structure–function analysis has identified residues important for DNA transfer and binding to pilus-specific bacteriophages (Yeo *et al.*, 2003). These results constitute

Abbreviations: AS, acetosyringone; EM, electron microscopy; immuno-EM, immuno-electron microscopy; T4SS, type IV secretion system; T-DNA, transferred DNA; TEM, transmission electron microscopy.

Supplementary data are available with the online version of this paper.

indirect evidence for a role of externally localized TraC in cell adhesion, a possibility that was originally proposed based on the discovery that *traC* deletion variants of pKM101 can be complemented extracellularly (Winans & Walker, 1985). However, earlier work has not directly assessed the localization of VirB5-like proteins in T4SS-determined pili and the possibility that they may play a role in host-cell contact formation.

Apart from VirB5, there is no firm evidence for other minor T-pilus components. The lipoprotein VirB7 co-fractionates with extracellular high-molecular-mass structures such as T-pili after shearing from *A. tumefaciens*, indicating that it may be a part of this structure (Sagulenko *et al.*, 2001), although this fractionation is not dependent on pilus formation. These results suggest that upon lipidation, VirB7 may become a T-pilus assembly factor, and its cofractionation with VirB2 and VirB5 after detergent extraction accords with this view (Yuan *et al.*, 2005). The fact that its fractionation does not depend on T-pilus formation indicates that VirB7 may associate with high-molecular-mass membrane blebs sheared from the cell, but that it is not likely to be an integral pilus component (Sagulenko *et al.*, 2001). Another externally localized VirB component is VirB1*, the C-terminal processing product of VirB1 (Baron *et al.*, 1997). This fragment of VirB1 is secreted into the supernatant, and it is possible that it contributes to host-cell recognition (Llosa *et al.*, 2000), but so far, it has not been found to associate with T-pili.

A pilus assembly sequence has recently been proposed, and according to this model the inner membrane-bound NTPase VirB4 stabilizes VirB8, which then mediates interactions between VirB2 and VirB5 followed by T-pilus incorporation (Yuan *et al.*, 2005). VirB3 is also stabilized by VirB4, it fractionates with VirB2 and VirB5 after extraction with the mild detergent dodecyl-β-D-maltoside, and it interacts with VirB5; these results indicate that it may also play a role in T-pilus assembly (Jones *et al.*, 1994; Shamaei-Tousi *et al.*, 2004). A key feature of this model is the formation of a VirB2–VirB5 T-pilus preassembly subcomplex (Krall *et al.*, 2002; Yuan *et al.*, 2005), but further understanding of this process requires clarification of the localization of VirB2 and VirB5 in the assembled structure. VirB5 may function as an outer-membrane usher, similar to PapC of the P-pilus system, and the outer membrane would be its final localization in this case (Sauer *et al.*, 2004). Alternatively, VirB5 may initiate T-pilus formation after its export into the periplasm; it may become incorporated at the T-pilus tips and also localize inside the pilus. In order to decide between these different models of T-pilus assembly, it is crucial to localize T-pilus components in the assembled structure.

To this end, we have analysed the localization of VirB2 and VirB5 in the T-pilus by immuno-electron microscopy (immuno-EM). Using a specific antiserum we detected VirB2 in T-pili isolated by shearing and ultracentrifugation but not in cell-bound T-pili, indicating that these structures may undergo conformational changes after removal from the cells. We have also determined the functionality of the T4SS and the length of T-pili in the wild-type strain C58 and in strains expressing VirB5 variants with changes at the C terminus. These analyses showed that the VirB5 C terminus impacts T-pilus incorporation of the protein and T-pilus length, and that it differentially affects DNA transfer to bacterial versus plant cells. The experiments reported here suggest that the minor component VirB5 localizes at the T-pilus tip, whereas VirB2 localizes along its entire length. Combined with the results of our analysis of the effects of C-terminal variants on host-cell specificity, these data suggest that tip-localized VirB5 impacts host-cell recognition.

METHODS

Cultivation of bacteria, and strain and plasmid construction. *Escherichia coli* JM109 for cloning experiments and *A. tumefaciens* for virulence gene induction were cultivated on AB minimal medium with acetosyringone (AS) and 0.5 mM IPTG for induction of plasmid-encoded genes as described previously (Höppner *et al.*, 2004; Yuan *et al.*, 2005). DNA manipulations followed standard procedures (Maniatis *et al.*, 1982). The *A. tumefaciens virB5* gene in pTrcB5 (Schmidt-Eisenlohr *et al.*, 1999a) was amplified by PCR with oligonucleotides to introduce C-terminal deletions or changes into the gene product (Supplementary Table S3) and cloned into expression vector pTrc200. The sequences of PCR-amplified genes were confirmed by DNA sequencing. The *virD4* deletion strain CB2004 was generated from the wild-type C58 by PCR-amplification of the *virD4* gene and deletion of an internal fragment, followed by integration into the chromosome by established methods (Berger & Christie, 1994).

Analysis of T4SS functions: T-pilus isolation, conjugation and virulence assay. Assays for T4SS functionality (T-pilus isolation, conjugation and virulence assays) were performed as previously described (Höppner *et al.*, 2004; Yuan *et al.*, 2005).

SDS-PAGE and Western blotting. SDS-PAGE was conducted according to Laemmli (1970) for proteins >20 kDa, or Schägger & von Jagow (1987) for proteins <20 kDa. Western blotting was performed following standard protocols with VirB protein-specific antisera, as described previously (Harlow & Lane, 1988; Yuan *et al.*, 2005).

T-pilus purification by gel filtration. T-pili from different *A. tumefaciens* strains were isolated by shearing of the cells and ultracentrifugation, according to established procedures (Höppner *et al.*, 2004; Yuan *et al.*, 2005). For further purification, the pilus-containing sediments obtained after ultracentrifugation were resuspended in 100 µl 50 mM sodium potassium phosphate (NaKP) buffer, pH 5.5, and applied to a Superdex 200 column (GE Healthcare), followed by separation with a flow rate of 0.5 ml min^{-1}. The fractions eluting from the column were concentrated by the addition of three volumes of acetone and incubation on ice for 4 h. The samples were then subjected to ultracentrifugation for 2 h at 270 000 *g* for precipitation of the pili, followed by resuspension in 40 µl NaKP buffer. Thirty microlitres of these samples were mixed with equal volumes of Laemmli sample buffer followed by SDS-PAGE and Western blot analysis. The remaining samples were loaded on electron microscopy (EM) grids for further visualization of purified T-pili.

Transmission electron microscopy (TEM). *A. tumefaciens* strains were grown under virulence-induction conditions on solid AB agar, as previously reported (Yuan *et al.*, 2005), and collected with liquid AB minimal medium supplemented with AS, and the cell density was adjusted to OD_{600} 0.25. Next, 15 µl was applied to UV-sterilized 200 or 300 mesh carbon-coated Formvar nickel EM grids, and the cells were cultivated for 10–12 h at 20 °C in a humid chamber to permit T-pilus elongation and to minimize their breakage. The cells were then stained with 1 % phosphotungstic acid in 0.01 % glucose (pH 6) for 15–45 s, followed by EM examination.

For T-pilus length analysis, we measured 88, 77 and 67 cell-bound T-pili of strains C58, CB1005 pTrcB5Δ3 and CB1005 pTrcB5Δ4, respectively. The length of the T-pili was measured directly from the micrographs and scaled using a standard EM correction grid. Similarly, we also measured the length of 300 cells from pole to pole. Micrographs from three independent virulence-induction experiments were analysed. For quantification of T-pilus formation, experiments were conducted as described above, followed by more intense staining with 2 % phosphotungstic acid, 0.01 % glucose, pH 6, for 15–20 s to efficiently label the pili. Depending on the experiment, T-pili from 60–400 cells from three independent virulence-induction experiments of each strain were counted, and 10 cells per visual field were analysed.

For immuno-EM, strains were cultivated under virulence gene-inducing conditions on EM grids as above, and the grids were then fixed for 1 h with 2 % formaldehyde, 0.5 % glutaraldehyde in 50 mM sodium cacodylate buffer, pH 5.5, as described by Jin *et al.* (2001). After fixation, the grids were subjected to immunogold labelling, largely as described by Quintero *et al.* (1998). The grids were blocked with 5 % skimmed milk in TBST (20 mM Tris/HCl, 137 mM NaCl, 0.1 % Tween-20, pH 8) for 20 min, followed by incubation on a drop of either 1 : 250 diluted anti-VirB5 or 1 : 250 anti-VirB2 antiserum in 5 % skimmed milk and TBST for 45 min at room temperature. The grids were then washed three times for 5 min, incubated with the secondary antibodies for 45 min at room temperature (1 : 10 diluted anti-rabbit 10 nm gold conjugate; Sigma-Aldrich) in TBST with 5 % skimmed milk, 0.1 % BSA and 5 % fetal bovine serum. Finally, the grids were washed and negative-stained with 1 % phosphotungstic acid in 0.01 % glucose (pH 6) for 15 s prior to examination. For quantification of the percentage of VirB5-labelled tips on T-pilus carrying C58, CB1005 pTrcB5Δ3 and CB1005 pTrcB5Δ4 cells, we analysed 300 T-pilus-carrying cells from three independent experiments.

The electron microscopic images were recorded with a JEOL JEM-1200EX or a JEOL 1200EX II transmission electron microscope.

RESULTS

Deletion of C-terminal amino acids leads to gradual loss of T-pilus incorporation of VirB5

A. tumefaciens assembles three different types of surface-exposed appendages, which can be easily differentiated based on their different diameters (flagella, 15 nm; T-pili, 10 nm; common pili, 3 nm) (Fig. 1a) (Eisenbrandt *et al.*, 1999; Lai *et al.*, 2000). The approach reported here was inspired by the analysis of the X-ray structure of TraC, which had suggested that the C-terminal 15–20 amino acids are likely exposed on the surface of the protein and may therefore be available for protein–protein interactions (Yeo *et al.*, 2003). To assess the functional importance of this region, we deleted the gene to express VirB5 variants truncated by blocks of five amino acids (deletion of five to

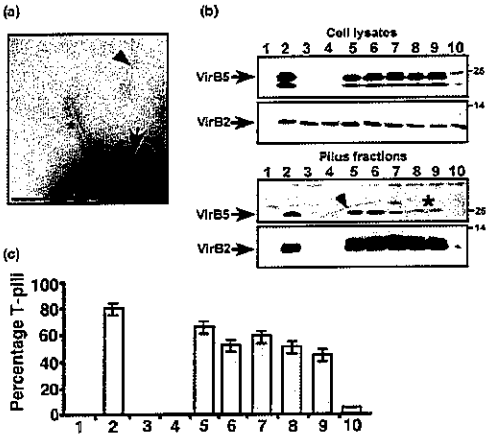

Fig. 1. Effects of C-terminal deletions of VirB5 on T-pilus formation. (a) Representative micrograph of the different surface appendages assembled by *A. tumefaciens*. The asterisk points to a flagellum (diameter 15 nm), the arrow points to a common pilus (diameter 3 nm) and the arrowhead points to a T-pilus (diameter 10 nm). (b) Samples from subcellular fractions were separated by SDS-PAGE, followed by Western blot analysis with VirB2- and VirB5-specific antisera. The analysis is shown of wild-type C58, CB1005 (Δ*virB5*) and CB1005 complemented with pTrc200 expressing VirB5 and its variants grown on AB minimal medium under virulence-inducing (+AS, lanes 2–10) and non-inducing (–AS, lane 1) conditions. IPTG (0.5 mM) was added to induce transcription of the pTrc200-encoded *virB5* genes. Lanes: 1, C58 –AS; 2, C58 +AS; 3, CB1005; 4, CB1005 carrying pTrc200; 5, pTrcB5; 6, pTrcB5Δ1; 7, pTrcB5Δ2; 8, pTrcB5Δ3; 9, pTrcB5Δ4; 10, pTrcB5Δ5. The arrowhead indicates pilus incorporation of VirB5 in complemented CB1005 and the asterisk indicates the detection of low amounts of VirB5Δ3 and VirB5Δ4 in pilus fractions (lanes 8 and 9). Numbers on the right indicate molecular masses of reference proteins. (c) Quantification of results of the TEM analysis of pilus formation (percentage detection of one or more T-pili per cell) [numbering of bars as in (b); representative images shown as Supplementary Fig. S1]. Forty different fields (10 cells each) from three different inductions were counted for each strain and the SD is shown.

30 amino acids) in the *A. tumefaciens* strain C58 *virB5* gene-deletion mutant CB1005 (Schmidt-Eisenlohr *et al.*, 1999a). The genes were cloned into the broad-host-range plasmid pTrc200, followed by analysis of the localization of the gene products. Analysis of VirB5 in cell lysates showed that all the deletion variants accumulated in strongly reduced amounts, and pili containing VirB2 and VirB5 were not formed (not shown). We next created deletions in *virB5* to direct synthesis of VirB5 variants truncated in one-amino-acid steps from the C terminus. Analysis of the lysates (from CB1005 pTrcB5Δ1, pTrcB5Δ2, etc.) showed

that deletion of up to four amino acids did not affect the stability of VirB5 in the cell, but we observed a gradual loss of T-pilus incorporation. Whereas deletion of one amino acid (VirB5Δ1) did not notably affect the levels of VirB5 in T-pili, successive deletions led to reduced amounts, although the proteins were still detected in T-pilus fractions (Fig. 1b). In contrast, the incorporation of VirB2 into T-pili was not notably affected by truncations of VirB5 of up to four amino acids, which further supported the notion that T-pili were still formed (Fig. 1b).

T-pilus formation was next visualized and quantified by TEM of virulence gene-induced agrobacteria (Supplementary Fig. S1). One or more T-pili were detected on the surface of 79 % of the cells in virulence-induced strain C58 and of 63 % in the case of CB1005 pTrcB5 (Fig. 1c). Deletions at the C terminus of up to four amino acids led only to minor reductions of the amounts of surface-exposed T-pili, but the number was strongly reduced in CB1005 pTrcB5Δ5, as this VirB5 variant was not stable. Similarly, no T-pili were observed in CB1005 producing VirB5 variants with further deletions that were not stable in the cell (not shown).

The amounts of VirB5Δ3 and VirB5Δ4 variants associated with T-pili were significantly reduced (Fig. 1b), and it was therefore necessary to conduct additional purification steps to assess their association with T-pili. To this end, T-pili isolated from the cells by shearing and ultracentrifugation as above were resuspended in a small volume of buffer and separated by gel filtration over a Superdex 200 size-exclusion column. The eluted fractions were concentrated by acetone precipitation, followed by SDS-PAGE and Western blotting to determine the elution volume of VirB2, and of VirB5 and its truncated variants. This analysis showed that VirB5 wild-type as well as VirB5Δ3 and VirB5Δ4 co-eluted with VirB2 in the high-molecular-mass range, as in our previous work (Schmidt-Eisenlohr *et al.*, 1999a), and similar to the analysis above, the amounts of C-terminally truncated variants were reduced as compared to the wild-type (Fig. 2a). Electron microscopic analysis of these fractions confirmed the presence of T-pili (Fig. 2b). Thus, in spite of the reduced amounts of C-terminally truncated VirB5 variants, our data suggest that they associate with T-pili in a similar manner to wild-type VirB5.

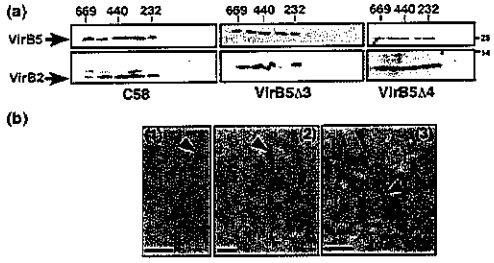

Fig. 2. Gel-filtration analysis of T-pili containing VirB5 and C-terminal deletion variants. Surface structures isolated by shearing and high-speed centrifugation from agrobacteria grown under virulence gene-inducing conditions were further separated by gel-filtration chromatography. Column fractions were subjected to SDS-PAGE followed by Western blotting with specific antisera for VirB2 or VirB5. (a) VirB2 and VirB5 detected in the high-molecular-mass fractions eluted from the column after separation of samples from strains C58, CB1005 pTrcB5Δ3 and CB1005 pTrcB5Δ4. Numbers on the top indicate molecular masses of reference proteins thyroglobulin (669 kDa), ferritin (440 kDa) and catalase (232 kDa). (b) TEM micrographs of T-pili (indicated by arrowheads) from strains C58 (1), CB1005 pTrcB5Δ3 (2) and CB1005 pTrcB5Δ4 (3) after gel-filtration chromatography. Bars, 100 nm.

The VirB5 C terminus impacts T-pilus length

When we monitored T-pilus production in CB1005 producing C-terminal deletion variants of VirB5 we noticed that the pilus length differed from that of the wild-type in all cases. CB1005 pTrcB5Δ1–Δ3 produced notably shorter T pili, whereas those of CB1005 pTrcB5Δ4 appeared on average to be longer than those of the wild-type. To quantify this phenomenon we conducted virulence-gene induction of the wild-type C58, CB1005 pTrcB5Δ3 and CB1005 pTrcB5Δ4 as above, and measured the length of cell-bound T-pili after negative staining and TEM. The mean T-pilus length in strain C58 was 1390 nm, and we occasionally observed pili with a length of up to 4500 nm (Fig. 3). As a comparison, the mean length of virulence gene-induced wild-type C58 cells was 1480 nm (maximum length, 2250 nm; minimum length, 1020 nm). In contrast to the results obtained for the wild-type, the mean T-pilus lengths observed for cells of CB1005 pTrcB5Δ3 and CB1005 pTrcB5Δ4 were 455 and 1750 nm, respectively. Whereas these results confirmed our initial observations, we also noticed that the maximum lengths of pili assembled on strains CB1005 pTrcB5Δ3 and CB1005 pTrcB5Δ4 were 1500 and 3,500 nm, respectively. These results show that in spite of the increased mean length of T-pili formed on CB1005 pTrcB5Δ4, they do not reach the maximum length of those on the wild-type, indicating a more complex effect of the C-terminal deletion than variation in length. Thus, C-terminal deletions of VirB5 do have differential effects on T-pilus length as measured by EM, and we next assessed the functionality of these structures during the type IV secretion process.

Truncations of the C terminus differentially affect the functionality of VirB5 in gene transfer

The *A. tumefaciens* T4SS transfers substrates to different hosts (Lacroix *et al.*, 2006), and we here used bacteria and plants as recipients to assess the functionality of C-terminal VirB5 deletion variants. First, we tested transferred DNA (T-DNA) transfer in a plant infection assay leading to

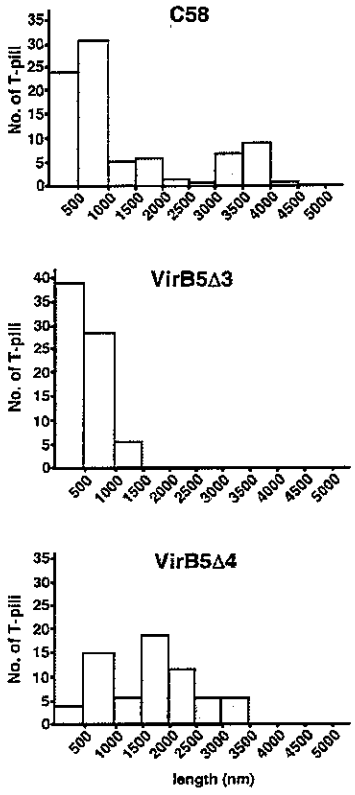

Fig. 3. C-terminal deletions of VirB5 impact the length of T-pili. *A. tumefaciens* strains C58, CB1005 pTrcB5Δ3 and CB1005 pTrcB5Δ4 were grown under virulence-inducing conditions, followed by negative straining and TEM to visualize the surface-exposed T-pili. The lengths of 88, 77 and 67 cell-bound T-pili on strains C58, CB1005 pTrcB5 B5Δ3 and CB1005 pTrcB5 B5Δ4, respectively, were measured using a magnification-correction grid. T-pili on cells from three independent virulence-induction experiments were analysed.

tumour formation on wounded *Kalanchoë diagremontiana* leaves. Second, as a more quantitative assay, we exploited the ability of the T4SS to direct conjugative transfer of the IncQ plasmid pLS1 between agrobacteria (Stahl *et al.*, 1998; Ward *et al.*, 1991). The results of both assays were comparable in most cases, but we noted a few intriguing differences. *The virB5* deletion strain CB1005 did not incite tumours after wounding and infection of *K. diagremontiana* leaves; this defect was complemented by expression of wild-type VirB5, but the CB1005 strains expressing C-terminal deletion variants (VirB5Δ1–Δ5) were all avirulent (Supplementary Fig. S2A). Using the conjugation assay, we found that transfer of pLS1 from the *virB5* deletion strain was reduced to 1.5–10 % in different independent series of experiments (Tables 1 and 2). Complementation with VirB5 increased pLS1 transfer to 50–75 % of the wild-type. Expression of VirB5Δ1–Δ3 and VirB5Δ5 did not complement, but in contrast to the results of the plant infection experiments, production of VirB5Δ4 fully complemented pLS1 transfer to *A. tumefaciens* (Table 1). These results showed that truncation of the C terminus differentially affected gene transfer into the recipients *K. diagremontiana* and *Agrobacterium*.

C-terminal amino acid changes differentially affect the stability and functionality of VirB5

As the C-terminal amino acids apparently have a major impact on the functionality of VirB5 we next mutagenized the gene to change the sequences of the last two amino acids from AVP to AAA, AVA and AAP. The variants were produced in CB1005, and analysis of their subcellular localization showed that VirB5AAA and VirB5AAP were detected in the cells and T-pili in levels similar to those of the wild-type (Fig. 4a). In contrast, VirB5AVA was unstable and was not detected in the cells or the T-pili, which may be due to recognition of this sequence by a protease in the bacterial cytoplasm or periplasm. Analysis by TEM confirmed that T-pili were formed on CB1005 pTrcB5AAA and CB1005 pTrcB5AAP, but not on the strain carrying pTrcB5AVA (Fig. 4b). Following the analysis of their subcellular localization, we next analysed the functionality of C-terminal VirB5 alanine variants in gene transfer, and they all proved to be avirulent in plant infection assays (Supplementary Fig. S2b). In contrast, in the pLS1 plasmid transfer assay, VirB5AAP was partly functional (69 % of the wild-type), whereas VirB5AAA and VirB5AVA did not complement (Table 2). Thus, similar to the results obtained with C-terminal deletion variants, subtle changes of the C-terminal amino acid sequences differentially affected gene transfer into the two recipients used here. As the localization of VirB2 and VirB5 is key to understand their role in T-pilus function, we addressed this question next.

Immuno-EM detects VirB2 in detached but not in cell-bound T-pili

The *A. tumefaciens* virulence genes were induced as above and the cells fixed on EM grids, followed by detection with VirB2-specific primary and 10 nm gold-labelled secondary antisera. Analysis by TEM revealed that gold grains did not label VirB2 on cell-bound intact T-pili, and we also did not detect any label on the cell surface (Fig. 5a, b). However, we did notice gold grain label on pilus-like structures that were detached from the cells (Fig. 5b). These structures often had an irregular shape unlike the straight and elongated T-pili, and may represent pili that were partly degraded during cultivation or the EM preparation

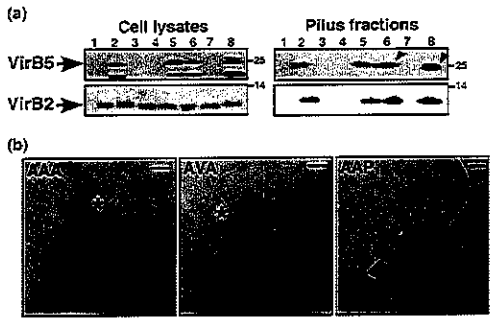

Fig. 4. Effects of C-terminal alanine changes of VirB5 on T-pilus formation. The analysis is shown of wild-type C58, CB1005 and CB1005 complemented with pTrc200 expressing VirB5 and its variants cultivated under virulence-inducing (+AS) and non-inducing (−AS) conditions. IPTG (0.5 mM) was added to induce the transcription of pTrc200-encoded *virB5* genes. (a) Samples from subcellular fractions were separated by SDS-PAGE, followed by Western blot analysis with VirB2- and VirB5-specific antisera. Lanes: 1, C58 −AS; 2, C58 +AS; 3, CB1005; 4, CB1005 pTrc200; 5, pTrcB5; 6, pTrcB5AAA; 7, pTrcB5AVA; 8, pTrcB5AAP. Arrowheads indicate detection of VirB5 variants in pilus fractions. Numbers on the right indicate molecular masses of reference proteins. (b) TEM analysis of C58 and CB1005 producing C-terminal alanine variants of VirB5 (C-terminal amino acids given: VirB5AAA, VirB5AVA and VirB5AAP). Asterisks show flagella and arrowheads point to T-pili. Bars, 100 nm.

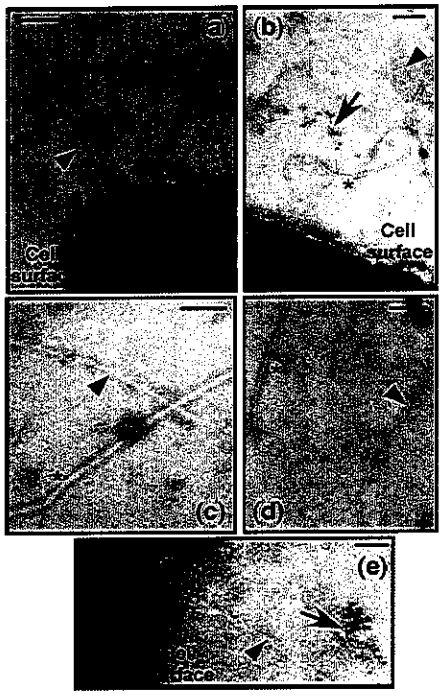

Fig. 5. Immuno-EM analysis of cell-bound and detached T-pili with VirB2-specific antiserum. The wild-type strain C58 was cultivated under virulence-inducing conditions followed by immuno-EM analysis of cell-bound T-pili (a, b). Alternatively, T-pili were detached from the cells by shearing, concentrated by ultracentrifugation and analysed by immuno-EM (c, d). (e) Analysis of virulence-induced *virD4* deletion strain CB2004. Detection with VirB2-specific primary and 10 nm gold-coupled secondary antisera is shown; bars, 100 nm. Arrowheads show T-pili, asterisks point to flagella, and arrows indicate the detection of the VirB2 antigen in detached T-pilus-like structures in (b) and (e).

pTrcB5Δ3 and CB1005 pTrcB5Δ4, and gold grains were detected on the pilus tips in both cases (Fig. 6d, e). When these data were quantified it became apparent that compared to the wild-type (26 %), a higher percentage of T-pilus tips was labelled by gold grains in the case of CB1005 pTrcB5Δ3 (55 %), and a lower percentage in the case of CB1005 pTrcB5Δ4 (7 %); we made similar observations with isolated T-pili (Supplementary Table S2). Thus, in spite of the reduced levels detected by Western blotting in detached T-pili, these results show that both VirB5Δ3 and VirB5Δ4 localize to the tips of T-pili.

DISCUSSION

The work presented here provides direct evidence for the localization of the major component VirB2 and of the minor component VirB5 in T-pili. We first analysed the localization of VirB2 in T-pili using intact cells and immuno-EM, but the VirB2-specific antiserum did not target gold-labelled secondary antibodies to cell-bound pili or to the cell surface. However, we occasionally noticed the labelling of curled structures by multiple gold grains, and these had the approximate diameter of T-pili, though not their mostly straight appearance. These curled structures are reminiscent of T-pili treated with detergents, acids or bases (Lai & Kado, 2002). They may correspond to T-pili that were detached from the cell and partly degraded during growth under virulence gene-inducing conditions or by the EM fixation procedure. These observations indicated that the VirB2 epitope may not be accessible in cell-bound pili, and to assess this possibility, we isolated T-pili from the cells that were fragmented by shearing and then sedimented by ultracentrifugation. Analysis of isolated T-pili by immuno-EM with VirB2-specific antiserum targeted gold grain-labelled secondary antibodies to almost 100 % of the isolated T-pili, and we typically observed several grains per pilus. These results suggest that the epitope recognized by the VirB2-specific antiserum, which

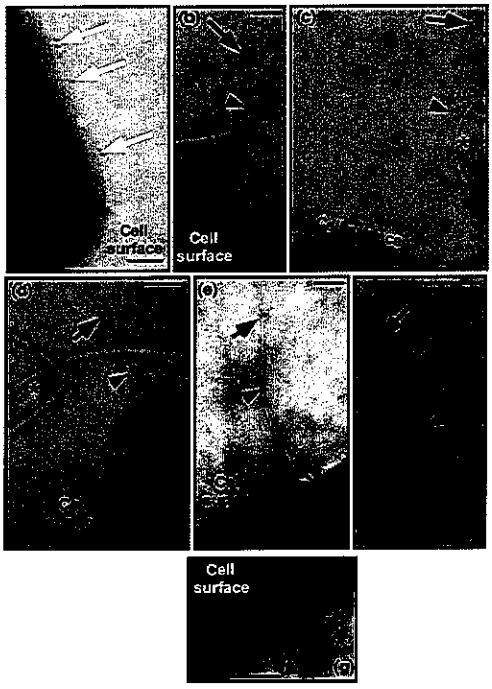

Fig. 6. Immuno-EM analysis of cell-bound and isolated T-pili with VirB5-specific antiserum. Cells were cultivated under virulence-inducing conditions, followed by immuno-EM analysis (a–e) or T-pilus isolation by shearing and ultracentrifugation followed by immuno-EM analysis (f). Analysis of the wild-type strain C58 (a–c, f), CB1005 pTrcB5Δ3 (d), CB1005 pTrcB5Δ4 (e) and the *virD4* deletion strain CB2004 (g) is shown. Detection with VirB5-specific primary and 10 nm gold-coupled secondary antisera is shown; bars, 100 nm. Arrowheads show T-pili, asterisks point to flagella, and black arrows show the detection of gold grains on T-pilus tips (b–f). (a) An *Agrobacterium* cell; white arrows point to gold grains that indicate the localization of VirB5 on the cell surface.

was generated against a 15 amino acid peptide immediately adjacent to the processing and cyclization sequence (Carle *et al.*, 2006), may be hidden in intact T-pili. A conformational change upon detachment from the cells, possibly triggered by experimental manipulation, makes it accessible. It is not clear whether this conformational change has any biological significance, but it is tempting to speculate that it reflects a change of the pilus conformation upon host-cell binding and/or during substrate translocation. To address this question we analysed the localization of VirB2 and VirB5 in the *virD4* deletion strain CB2004, which does not couple the translocated T-complex to the T4SS. The results were identical to those of the wild-type, showing that substrate coupling does not have an effect on T-pilus conformation that is detectable with the methods used here. High-resolution structural studies, e.g. by cryo-electron microscopy and image reconstruction, will be required in future to investigate the nature of this conformational change (Craig *et al.*, 2006). We will also conduct immuno-EM experiments that include host cells (plant or bacterial) in order to assess whether host recognition by T-pili leads to conformational changes that unmask the epitope recognized by the VirB2-specific antiserum.

We next analysed the localization of the minor T-pilus component and detected VirB5 on the tips of T-pili and on the cell surface. VirB5 was detected on the tips of 26 % of the pili in the wild-type strain C58, and one possible explanation for this incomplete labelling is that surface-exposed structures such as T-pili may break off during the preparation for EM. This is in accord with a model that implies that VirB5 initiates T-pilus assembly in the cell envelope and remains at the tip upon pilus elongation. According to this model, the major pilus subunit may be delivered to the pilus base by the VirB2–VirB5 complex that we described previously (Yuan *et al.*, 2005), followed by the degradation of VirB5 and successive addition of VirB2 subunits at the base. As a consequence, VirB5 may not be present inside pili and was therefore not detected on the ends of broken pili. This model would explain why VirB5 was detected on only 26 % of the cell-bound pili and why a similar degree of labelling (34 %) was also observed on detached pili. The analysis of VirB5 variants with C-terminal deletions lent further support to this model.

Analysis of CB1005 expressing VirB5Δ3 resulted in a higher number of tip-labelled pili on cells (55 %), and a similar amount of label was detected on detached T-pili (56 %). This elevated level of labelling in comparison to the wild-type may be due to the fact that these pili are shorter and may therefore not break off and fragment as easily. A similar reasoning may explain the fact that only 7 % of the tips on cells of CB1005 expressing VirB5Δ4 were labelled (11 % in the case of detached T-pili). These pili are on average longer than those from C58, and they may break off more easily and further break into pieces, which may explain the lower level of tip labelling. In spite of having longer pili on average, the maximum length of pili on CB1005 expressing VirB5Δ4 was shorter than in the wild-type, which is also in accord with a structure that may break off more easily. Nevertheless, the overall structure of these pili appears to be similar to that of the wild-type, and the detached pili from CB1005 expressing VirB5Δ3 and VirB5Δ4 were labelled with the VirB2-specific antiserum along their entire length, like those from the wild-type. There is little precedent for the length regulation of surface appendages, but that of type III secretion system-determined needles from *Yersinia enterocolitica* is determined by a domain of YscP in a molecular ruler-like fashion (Journet *et al.*, 2003; Mota *et al.*, 2005). The

discovery that minor changes of VirB5 lead to notable variations of pilus length opens up a fascinating area of research for future work.

The above-mentioned model does not exclude alternative mechanisms of T-pilus assembly, e.g. the incorporation of VirB2 subunits at the tip after transfer via the pilus lumen, as in the case of the subunits of bacterial flagella. Also, VirB5 may be present inside the T-pilus, where it cannot be readily detected by antisera, but the facts that it is only a minor pilus component (Schmidt-Eisenlohr *et al.*, 1999a) and that we did not detect higher levels of labelling on detached in comparison to cell-bound T-pili argue against this possibility. An alternative explanation for the localization of VirB5 at the tips may be that it can be sheared from the cell surface in the form of membrane blebs, which may bind to the tips of broken T-pili. Although this possibility cannot be ruled out, there is currently no published evidence for the shearing of VirB5-containing membrane blebs from *A. tumefaciens*, and we have not observed such structures in the course of our electron microscopic studies. In any case, the detection of VirB5 at the pilus tip as well as on the cell surface argues in favour of a sequence of interactions that lead to T-pilus assembly following initiation by a cell-bound form of VirB5 in the periplasm and/or on the surface. As discussed below, the effects of C-terminal deletions and changes on the efficacy of DNA transfer to different hosts are also consistent with the notion that the tip is one of the natural localizations of VirB5.

In addition to providing interesting clues for future research on T-pilus assembly, the analysis of C-terminal VirB5 variants provided novel insights into the host specificity of the DNA translocation process. None of the C-terminal VirB5 variants analysed here (C-terminal deletions as well as alanine replacements) complemented the defect of the CB1005 mutant in plant-infection assays. The variants with deletions of up to four amino acids and with two of the three alanine changes were stable in the cell, indicating that the avirulence of these strains was not due to degradation of the protein but that the C terminus of VirB5 is important for T-DNA transfer to plants. The finding that relatively minor changes at the C terminus abolished DNA transfer to plants was unexpected, and there are three possible explanations. First, many of our assumptions are based on the X-ray structure of TraC, which suggests a surface-exposed C terminus. VirB5 and TraC have only limited sequence similarity (14 % identical and 33 % similar amino acids; see alignment in Supplementary Fig. S4), but this portion of VirB5 may localize inside the protein, and even minor changes may have caused drastic conformational changes. We do not consider this possibility very likely, as most of the non-complementing variants were stable in the cell, which would not be expected if their overall structure were changed. Also, a variant with a C-terminal extension of eight amino acids (StrepII tag) was fully functional (not shown) and this is also in accord with an accessible C terminus. Second, VirB5 may interact via its C terminus with translocated substrates so that changes directly block the translocation of DNA and/or of proteins via the T4SS. This is indeed an interesting possibility that warrants further study, but so far, there is no evidence for an interaction between VirB5 and the translocated substrates. The third explanation is that VirB5 may be involved in binding to plant cells, and this hypothesis is in accord with the proposed role of the VirB5 homologue TraC in phage adhesion and plasmid transfer (Yeo *et al.*, 2003). This notion is supported by our data that show that two of the VirB5 variants that did not complement T-DNA transfer to plants (VirB5Δ4 and VirB5AAP) did complement the defect of a *virB5* deletion strain in transfer of the IncQ plasmid pLS1 to recipient agrobacteria. The fact that VirB5Δ4 and VirB5AAP complemented the *virB5* defect in this assay indicates that the T-pili engaged fully in DNA- and protein-transfer processes and that recognition of the recipient by VirB5 may play a role during the transfer process. This view is consistent with the results of our EM work, which showed that VirB5 localized at the pilus tip, which is suggestive of a role in host-cell recognition. Nevertheless, in spite of the evidence provided here and in previous work (Yeo *et al.*, 2003), a function of VirB5 as an adhesin is still hypothetical. Future work will directly test this possibility, e.g. by screening for interaction partners with the yeast two-hybrid system or by using biochemical approaches to isolate VirB5-binding proteins from plants and TraC/VirB5-binding proteins from bacteria.

ACKNOWLEDGEMENTS

We are indebted to Patricia Zambryski (University of California, Berkeley) for continued support and discussions, Marcia Reid (McMaster University) for help with EM and John Lott (McMaster University) for help with pilus-length measurements. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC; grant 262104), the Canada Foundation for Innovation (CFI) and the Ontario Innovation Trust (OIT) to C.B.

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Edited by: M. S. Ullrich

Chapter 4

The type IV secretion system component VirB5 binds to the *trans*-zeatin biosynthetic enzyme Tzs and enables its translocation to the cell surface of *Agrobacterium tumefaciens*

Preface

This chapter consists of the following article, in its originally published format: **Aly, K. A.**, Krall, L, Lottspeich, F. and Baron, C. **The type IV secretion system component VirB5 binds to the *trans*-zeatin biosynthetic enzyme Tzs and enables its translocation to the cell surface of *Agrobacterium tumefaciens*** (2008). Journal of Bacteriology 190(5):1595-1604. A copy of the original manuscript is included in my thesis with permission no. 2270920496670 from the publisher.

I performed the experiments shown in figures 4, 5, 6 and 7. I generated the figures. Dr. Christian Baron has helped improving the display of data in all figures so that they become easier for the reviewer to understand. Dr. Baron wrote the entire manuscript and he and I made some revisions to the manuscript, and Dr. Baron contributed significant intellectual input and support.

Dr. Krall is a co-first author on this article and she found by a gel overlay assay that VirB5 interacts with Tzs. Following up on her work, I found that Tzs signal can be detected in the pellet of extracted and sedimented bacterial appendages by high speed centrifugation. Unlike the lytic transglycosylase VirB1, Tzs is not secreted into the bacterial culture supernatant. These findings led to our hypothesis that Tzs might be exposed on the bacterial surface.

Using immuno-EM, Tzs was found to be homogeneously distributed on the cell surface in case of wild-type strain C58. In this study, I constructed both, *virB2* and *virB8* deletion mutants (*virB8* deletion mutant was constructed with the help of a former summer student, John Morala). I found that surface association of Tzs was less in case of both, *virB2* and *virB8* deletion mutants and was largely reduced in the *virB5* deletion mutant CB1005. This led to our conclusion that whereas VirB5 might be essential for Tzs surface localization, Tzs localization seemed dependent on more than one T4SS component.

Blue Native Electrophoresis analysis of the distribution pattern of Tzs with protein complexes of variable molecular masses showed that in case of the *virB5* and *virB8* deletion mutants, this pattern was significantly altered. We did not construct a Tzs deletion mutant in this study which will be of a paramount importance to further investigate the nature of VirB5-Tzs interaction. We also do not know which domain of VirB5 is required for Tzs interaction.

JOURNAL OF BACTERIOLOGY, Mar. 2008, p. 1595–1604 Vol. 190, No. 5
0021-9193/08/$08.00+0 doi:10.1128/JB.01718-07

The Type IV Secretion System Component VirB5 Binds to the *trans*-Zeatin Biosynthetic Enzyme Tzs and Enables Its Translocation to the Cell Surface of *Agrobacterium tumefaciens*▽

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Received 26 October 2007/Accepted 14 December 2007

VirB5 is a minor component of the extracellular T pilus determined by the *Agrobacterium tumefaciens* type IV secretion system. To identify proteins that interact with VirB5 during the pilus assembly process, we purified VirB5 as a recombinant fusion protein and, by using a gel overlay assay, we detected a 26-kDa interacting protein in *Agrobacterium* cell lysates. The VirB5-binding protein was purified from *A. tumefaciens* and identified as the cytokinin biosynthetic enzyme Tzs. The VirB5-Tzs interaction was confirmed using pulldown assays with purified proteins and the yeast two-hybrid system. An analysis of the subcellular localization in *A. tumefaciens* showed that Tzs was present in the soluble as well as the membrane fraction. Tzs was extracted from the membranes with the mild detergent dodecyl-β-D-maltoside in complexes of different molecular masses, and this association was strongly reduced in the absence of VirB5. Using immunoelectron microscopy, we also detected Tzs on the *Agrobacterium* cell surface. A functional type IV secretion system was required for efficient translocation to the surface, but Tzs was not secreted into the cell supernatant. The fact that Tzs localizes on the cell surface suggests that it may contribute to the interaction of *Agrobacterium* with plants.

Agrobacterium tumefaciens is a gram-negative plant pathogen that incites crown gall tumors after infecting plant wounds (34, 54, 55). Tumor formation is a consequence of the transfer of a piece of single-stranded DNA, the T-DNA (transferred DNA), and of its integration into the plant genome. The T-DNA encodes proteins that direct the synthesis of plant hormones, such as cytokinin (Tmr) and indole acetic acid (Tms), and the subsequent deregulation of the plant phytohormone balance leads to tumor formation (1, 2). The Tmr protein exerts its function after the uptake into the chloroplasts, where it diverts an intermediate of the methyl-erythritol phosphate (MEP) pathway of isoprenoid biosynthesis for the production of hydroxylated *trans*-zeatin ribotides (40). Cytokinin biosynthesis in plants predominantly uses isoprenoid metabolites from the MEP pathway as well, but the primary products are isopentenyl ribotides and those are subsequently hydroxylated to *trans*-zeatin ribotides (36, 39). Alternatively, isopentenyl ribotides are synthesized from metabolites of the cytoplasmic mevalonate pathway or they are produced as products of the degradation of modified tRNAs resulting in *cis*-zeatin ribotides.

A subgroup of *A. tumefaciens*, the nopaline strains, encodes the Tmr homologous *trans*-zeatin synthesis (Tzs) protein on the tumor-inducing (Ti) plasmid (9, 20, 51). In contrast to the T-DNA gene *tmr*, the *tzs* gene is not translocated into plant cells and its gene product Tzs catalyzes the last step of the biosynthesis of the *trans*-zeatin ribotides inside *A. tumefaciens* (24, 27, 37). The biological significance of Tzs action is believed to be that the produced cytokinins stimulate plant cell growth in the wound callus. This stimulation may increase the efficacy of T-DNA transformation, and the fact that Tzs is coregulated with the T-DNA translocation machinery is in accord with this notion (24, 37). However, so far there is no direct evidence for this role of Tzs during the *Agrobacterium*-plant interaction. As Tzs is not produced in all agrobacteria, it is not considered to be essential for virulence and it may be a host range factor that contributes to the infection of certain plants. In addition to the proteins that impact plant hormone homeostasis, the T-DNA encodes proteins that mediate the production of opines, a special family of conjugates between organic acids and amino acids (50, 54). These compounds serve as nutrients for *A. tumefaciens*, which, unlike most other bacteria, has the ability to metabolize them. The unique strategy of *A. tumefaciens* to exploit the resources of plants was named genetic colonization, and it relies on the ability to transfer genes from the bacteria into the host cell (45).

A type IV secretion system (T4SS) mediates the translocation of the single-stranded T-DNA covalently linked to the VirD2 protein into plant cells (6, 14, 34). The T4SS required for this translocation process consists of 12 components, the 11 VirB proteins (VirB1-VirB11) and VirD4. Three of the components of the T4SS, VirB4, VirB11, and VirD4, contain

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▽ Published ahead of print on 28 December 2007.

TABLE 1. Bacteria and yeast strains used in this study

Strain	Genotype and characteristics	Source/reference
C58	*A. tumefaciens* A136 pTiC58, virulent nopaline wild-type strain	46
CB1002	*A. tumefaciens* A136 pTiC58Δ*virB2*	This work
CB1005	*A. tumefaciens* A136 pTiC58Δ*virB5*	43
CB1008	*A. tumefaciens* A136 pTiC58Δ*virB8*	This work
A348	*A. tumefaciens* A136 pTiA6NC, virulent octopine wild-type strain	19
Ach5	*A. tumefaciens*, *Achillea millefolium* isolate, octopine-type Ti-plasmid	26
Chry 5	*A. tumefaciens*, *Chrysanthemum morifolium* isolate, succinamopine-type Ti-plasmid	26
A208	*A. tumefaciens* A136 pTiT37, nopaline-type Ti-plasmid	26
JM109	*E. coli*, [F′ *traD36 proAB*$^+$ *lacI*^{q}*lacZ*ΔM15] *recA1 endA1 gyrA96 thi-1 hsdR17 relA1 supE44* Δ(*lac-proAB*) λ$^-$	52
GJ1158	*E. coli*, *proU* promoter-controlled chromosomal RNA polymerase gene	12
Saccharomyces cerevisiae Y153	*MAT***a** *leu2-3,112 ura3-52 trp1-901 his3-Δ200 ade2-101 LYS2::GAL-HIS3 gal4Δgal80ΔURA3::GAL-lacZ*	15

Walker nucleotide binding and hydrolysis motifs; they interact and energize T4SS functions (5). VirB1, VirB3, VirB6, VirB7, VirB8, VirB9, and VirB10 assemble into a complex that spans the inner and the outer membrane and may form the substrate translocation channel (13, 25, 28, 49). The proteins VirB2 and VirB5 are components of the T4SS-determined T pilus, an extracellular structure that is believed to initiate cell-cell contact with plant cells prior to the initiation of T-complex transfer (16). VirB2 is the major T-pilus component that forms the main body of this extracellular structure (31). A yeast two-hybrid screen identified interaction partners in *Arabidopsis thaliana*, suggesting that VirB2 directly contacts the host cell during the substrate translocation process (23). VirB5 and its homologs TraC and TrbF were identified as minor components of T4SS-determined surface structures (42–44). VirB5 localizes at the T-pilus tip, and C-terminal variants were differentially affected in DNA transfer to different hosts, suggesting that VirB5 may also be involved in host-cell contact (3).

Details on the mechanism of T-pilus assembly are beginning to emerge, and based on work with purified components and analyses of membrane-bound T4SS complexes, a VirB4-VirB8-VirB2-VirB5 pilus assembly sequence was proposed (53). As an independent approach to gain insights into the mechanism of T-pilus incorporation of VirB5, we pursued a gel overlay approach to isolate interaction partners from *A. tumefaciens*. We detected a 26-kDa VirB5-binding protein from *A. tumefaciens*, and this protein was subsequently purified and identified as the *trans*-zeatin biosynthetic protein Tzs. The VirB5-Tzs interaction was confirmed, and an analysis of its subcellular localization showed that Tzs associates with discrete membrane protein complexes and that it is also exposed at the cell surface. A functional T4SS was required for efficient incorporation of Tzs into membrane complexes and for efficient translocation to the cell surface, suggesting that the secretion system and VirB5 may enable these processes by directly binding to Tzs.

MATERIALS AND METHODS

Cultivation of microorganisms. Cultures of *Escherichia coli* for cloning and protein overproduction were grown in LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) or in LBON medium (LB medium without NaCl) following standard procedures (12, 32). *A. tumefaciens* virulence genes were induced with acetosyringone (AS) in liquid or on solid AB minimal medium (1% glucose, 0.39% morpholineethanesulfonic acid [MES], 1 mM KNa-phosphate, 1× AB salts [20 g NH_4Cl, 6 g $MgSO_4 \cdot 7H_2O$, 3 g KCl, 0.2 g $CaCl_2$, 0.05 g $FeSO_4 \cdot 7H_2O$ per liter, pH 5.5], pH 5.5, 20× AB salts) as described previously (53). Yeast strains (*Saccharomyces cerevisiae*) for two-hybrid analysis were propagated in YEPD medium (2% tryptone, 1% yeast extract, 2% glucose, pH 7) for routine culture or in SD medium (6.7 g yeast nitrogen base without amino acid, 20 g glucose, and 0.87 g dropout mix [0.8 g adenine, 0.8 g Arg, 4 g Asp, 0.8 g His, 2.4 g Leu, 1.2 g Lys, 0.8 g Met, 2 g Phe, 8 g Thr, 0.8 g Trp, 1.2 g Tyr, 0.8 g uracil per liter] per liter, pH 7) for the analysis of interactions as described previously (15).

Plasmid and strain constructions. Standard molecular biology procedures were followed for the construction of strains (Table 1) and plasmids (Table 2) (32), and the oligonucleotides used are listed in Table 3. All PCR-amplified gene sequences were verified after cloning by DNA sequencing.

For the overexpression of fusions of VirB5 without the N-terminal signal peptide, the gene was PCR amplified with the oligonucleotides VirB5-5′ and VirB5-3′, followed by cleavage with Acc65I and PstI and ligation into similarly cut pT7-H_6TrxFus and pT7-7StrepII. To analyze the interactions of Tzs with the yeast two-hybrid system, the gene was PCR amplified with the oligonucleotides AS2-Tzs-5′ and AS2-Tzs-3′ and cleaved with BsphI and BamHI, followed by ligation into NcoI and BamHI-cleaved pAS2 and pACTII.

Strains CB1002 and CB1008, carrying in-frame deletions of *virB2* and *virB8*, respectively, on the Ti plasmid of strain C58, were constructed essentially as described previously (10, 43) using the oligonucleotides shown in Table 3. The *virB2* gene with 500 bp of upstream and downstream sequence was PCR amplified from intact *A. tumefaciens* cells by using oligonucleotides B2-5 and B2-3, and the fragment was cloned into the precleaved plasmid pTZ57R/T (InsTAclone kit; Fermentas), followed by deletion of the gene by inverse PCR with the oligonucleotides ΔB2-5 and ΔB2-3. The 1-kb fragment with the flanking regions was then excised with EcoRI and cloned into vector pK*mobsacB (41), and the gene deletion was introduced into the chromosome by double recombination (10, 43). A similar procedure was followed for the construction of CB1008 using oligonucleotides B8-5 and B8-3 for PCR amplification of *virB8* and of its flanking regions, followed by cloning and inverse PCR with the oligonucleotides ΔB8-5 and ΔB8-3. The fragment containing the flanking regions was then excised with XbaI and cloned into pK*mobsacB, and the gene deletion was introduced into the chromosome by double recombination as described above.

Overproduction and purification of VirB5 and of Tzs. Soluble fusion proteins (hexahistidyl- and StrepII-tagged VirB5) were overproduced in the NaCl-inducible T7 promoter expression strain GJ1158 as described previously (53) after expression for 90 min at 37°C (His_6TrxAVirB5) and for 48 h at 20°C (StrepIIVirB5). They were subsequently purified by StrepTactin-Sepharose or immobilized metal affinity chromatography (IMAC) and gel filtration chromatography as described previously (53). Native Tzs protein was overproduced and purified as described previously (27), and 500 μg was used for the generation of a specific antiserum after injection into rabbits (BioGenes, Germany).

Protein analytical methods, subcellular fractionation, and gel overlay assay. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (30), followed by Western blotting with specific antisera following standard procedures (21). Membrane protein complexes were isolated from *A. tumefaciens* by extraction with the mild detergent dodecyl-β-D-maltoside (DDM), followed by blue native electrophoresis as described previously (53). Proteins from other subcellular fractions were isolated after shearing and ultracentrifugation (T pili) and after precipitation of cell-free supernatants with acetone as described previously (7, 42).

A gel overlay assay based on the method described previously by Homann et al. (22) was applied for the detection of interaction partners of VirB5. Samples

TABLE 2. Plasmids used in this study

Plasmid	Genotype and characteristics	Source/reference
pPZP300	Str^r Spc^r; carries extra copies of the gene encoding the transcription factor VirG for increased virulence gene induction	28
pTreB2	Str^r Spc^r; pTre200 derivative for *lacI^q/trc* promoter-controlled expression of *virB2*	42
pTreB5	Str^r Spc^r; pTre200 derivative for *lacI^q/trc* promoter-controlled expression of *virB5*	43
pK*mobsacB	Kan^r *sacB*; mobilizable vector for the construction of in-frame deletion variants	41
pT7-H_6TrxFus	Carb^r; T7 promoter-controlled expression of hexahistidyl-thioredoxin (TrxA) fusion proteins	29
pT7-H_6TrxVirB5	Carb^r; for expression of hexahistidyl-thioredoxin-VirB5 fusion protein	This work
pT7-StrepII	Carb^r; T7 promoter-controlled expression of StrepII fusion proteins	53
pT7-7StrepIIVirB5	Carb^r; for expression of StrepII-VirB5 fusion protein	This work
pT7-7tzs	Carb^r; T7 promoter-controlled expression of Tzs	27
pAS2	Carb^r; for expression of fusions with the GAL4 DNA binding domain; selection in yeast in the absence of Trp	15
pACTII	Carb^r; for expression of fusions with the GAL4 activation domain; selection in yeast in the absence of Leu	15
pAS2-VirB5	Carb^r; *virB5* in pAS2	This work
pAS2-VirE2	Carb^r; *virE2* in pAS2	8
pAS2-Tzs	Carb^r; *tzs* in pAS2	This work
pACTII-VirB5	Carb^r; *virB5* in pACTII	This work
pACTII-VirE2	Carb^r; *virE2* in pACTII	8
pACTII-Tzs	Carb^r; *tzs* in pACTII	This work

were separated by SDS-PAGE, followed by Western blotting onto polyvinylidene difluoride (PVDF) membranes and incubation for 12 h at 4°C in renaturation buffer (10 mM HEPES, 10 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, pH 7.5) to remove the SDS and to enable refolding. The membranes were then washed three times with Tris-buffered saline containing Tween 20 (TBS-T) (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 8), and protein binding sites were blocked with 5% dry milk powder in TBS-T, followed by incubation with purified VirB5 fusion protein (1.5 μg/ml) in TBS-T with 5% dry milk powder for 5 h at 4°C. The membranes were then washed three times with TBS-T, and the bound VirB5 fusion protein was detected by Western blotting with a specific antiserum.

Interaction studies using affinity matrices (pulldown assays). Pulldown assays to detect the interaction of purified Tzs with hexahistidyl- or StrepII-tagged VirB5 fusion proteins bound to affinity matrices were conducted as described previously (53).

Purification of a VirB5-binding protein from *A. tumefaciens*. The VirB5-binding protein was purified from *A. tumefaciens*, and the gel overlay assay described above was applied to monitor the progress of individual purification steps. *A. tumefaciens* strain C58 carrying plasmid pPZP300 was cultivated under virulence gene-inducing conditions on eight AB minimal medium agar plates (15 cm diameter) for 4 days at 20°C in the presence of 200 μM AS for virulence gene induction. The bacteria were washed from the plates with 10 ml 50-mM Na-K-phosphate buffer per plate, sedimented, and resuspended in 20 ml 50-mM Na-K-phosphate buffer. Next, the bacteria were lysed by passage through a French press at 20,000 lb/in^2, followed by low-speed centrifugation (40 min at 12,000 rpm in an SS-34 rotor, Sorvall RC5B centrifuge) to remove cell debris and ultracentrifugation (2 h at 40,000 rpm in a Ti50.2 rotor, Sorvall OTD-50B ultracentrifuge) to remove membrane proteins. The VirB5-binding protein in the soluble fraction was further enriched by differential $(NH_4)_2SO_4$ precipitation (30 to 70% in steps of 10%), and the highest amount was detected in the 40% fraction. The precipitate was suspended in 1 ml 50 mM HEPES (pH 7), dialyzed in 2 liters of this buffer at 4°C for 12 h and then applied onto a Mono Q-Sepharose anion exchange column (Amersham Biosciences). The column was washed with 50 mM HEPES (pH 7), followed by elution with a linear gradient (50 mM to 1 M NaCl in 50 mM HEPES-puffer, pH 7) over 10 column volumes, and the fractions were precipitated by the addition of acetone. The samples were separated by electrophoresis on a 12% acrylamide gel and blotted onto a PVDF membrane, and the VirB5-binding protein localized by gel overlay assay was identified by N-terminal sequencing using standard protocols.

Yeast two-hybrid system analysis. Analyses of protein-protein interactions using the yeast two-hybrid system were conducted as described previously following standard procedures for the Matchmaker two-hybrid system (Clontech) (8, 15). The genes were cloned into pAS2 and pACTII, and plasmid-containing yeast cells were selected on SD medium in the absence of Leu and Trp. Six

TABLE 3. Oligonucleotides used in this study

Application	Name	Sequence and restriction site[a]
Construction of pT7-7 StrepIIVirB5 and pT7-7H_6TrxA-VirB5	VirB5-5′	5′-CAGGGTACCCAGTTCGTTGTCAGCGATCCGGCG-3′
	VirB5-3′	5′-GAGCTGCAGTCAGGGGACGGCCCCAAAGATG-3′
Construction of pAS2-Tzs and pACTII-Tzs	AS2-Tzs-5′	5′-GGAGGCTCATGATACTCCATCTCATCTACGGACC-3′
	AS2-Tzs-3′	5′-GACGGGGATCCTCACCGAATTCGCGTCAGCGTG-3′
Cloning of *virB2*	B2-5	5′-CCACACGAATTCCAAGTCGTGATGGACCGTCTCGA-3′
	B2-3	5′-CCACACGAATTCGACGGCAACGTGCATTGCGCATTT-3′
Deletion of *virB2*	ΔB2-5	5′-AGGAGGTCCGCAATAATGAATGATCGTCTGGAAGCAACCCTT-3′
	ΔB2-3	5′-TTATTGCGGACCTCCTTGATTTAAGTCGAACAAGAGTTGATCGTC-3′
Cloning of *virB8*	B8-5	5′-CGCAGTCTAGAGCAAAGTGGATCGGGCAACTTAT-3′
	B8-3	5′-CGCAGTCTAGACCTCTGCTCTCTGTTGATATTGCGCTT-3′
Deletion of *virB8*	ΔB8-5	5′-CCGTGCTCGAGTTATTCAGACCCCTTCATGGCGACCACCT-3′
	ΔB8-3	5′-CCGTGCTCGAGATGACGAAAAAAGCATTTCTCA-3′

[a] Restriction enzyme cleavage sites are underlined.

transformants from each plasmid combination were streaked on SD agar plates and lysed in liquid nitrogen, followed by soaking in Z buffer (16.1 g Na_2HPO_4, 5.5 g NaH_2PO_4, 0.75 g KCl, 0.25 g $MgCl_2$, 2.7 ml β-mercaptoethanol/liter) containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (1 mg/ml) to identify β-galactosidase production. The strength of the interaction was assessed based on the number of blue colonies among six transformants each from six independent transformation experiments.

Immunoelectron microscopy. Immunoelectron microscopy to detect cell-bound Tzs was conducted as described previously (3) using 1:250 diluted Tzs-specific primary antiserum and 1:10-diluted anti-rabbit 10-nm gold conjugates (Sigma-Aldrich).

RESULTS

Overlay assays detect a VirB5-binding virulence-induced protein in *A. tumefaciens*. In an attempt to identify *A. tumefaciens* proteins that contribute to the incorporation of the minor T-pilus component VirB5 into these extracellular structures, we overexpressed VirB5 as a fusion to hexahistidyl-thioredoxin. His_6TrxAVirB5 was purified by immobilized metal affinity chromatography, and a gel-overlay assay was conducted to identify binding partners in *A. tumefaciens* cell lysates. To this end, the lysates were separated by SDS-PAGE, followed by blotting onto a PVDF membrane and removal of the SDS to enable renaturation. The membranes were then incubated with purified His_6TrxAVirB5, and the bound fusion protein was detected after incubation with a VirB5-specific antiserum and a horseradish peroxidase-coupled secondary antiserum by chemiluminescence detection. This approach identified a 26-kDa protein only in lysates of virulence gene-induced strain C58, not in lysates from noninduced cells (Fig. 1A). This protein was slightly larger than the 25-kDa VirB5, which was detected by standard Western blotting as well as during the overlay assay procedure. The VirB5-binding protein was also present in lysates from the *virB5* gene deletion strain CB1005 (CB5) (Fig. 1A), excluding the possibility that it represents a modified VirB5 with a higher apparent molecular mass. In order to characterize its subcellular localization, we analyzed subcellular fractions and detected the VirB5-binding protein in the total cell lysate and in the soluble fraction (Fig. 1B). In contrast, VirB5 was detected in the total cell lysate and in the membrane fraction. Since all T4SS components localize either exclusively or primarily in the membranes, we concluded that the VirB5-binding protein is not likely a component of the transmembrane complex but that it may be an AS-induced soluble factor that aids in VirB5 assembly. To assess this possibility, the protein was purified and identified next.

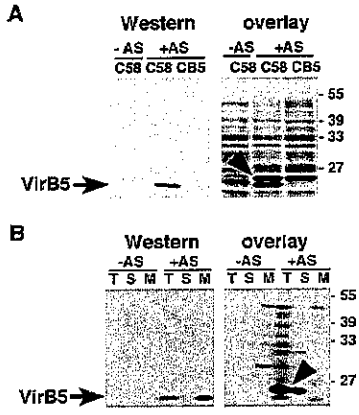

FIG. 1. Detection of a VirB5-binding protein using overlay assays. Wild-type C58 and the *virB5* deletion mutant CB1005 carrying pPZP300 were grown under virulence gene-inducing (+AS) or noninducing conditions (−AS). For subcellular fractionation, the cells were lysed in a French press, followed by separation of the total cell lysate (T) into soluble (S) and membrane fractions (M). (A) Cell lysates were separated by SDS-PAGE, followed by Western blotting with VirB5-specific antiserum (left panel). The overlay assay was conducted by incubating the PVDF membrane after electrotransfer of the proteins with purified His_6TrxAVirB5, followed by washing and Western blotting with VirB5-specific antiserum (right panel). (B) To analyze the subcellular localization of VirB5 and the interacting protein, samples of the T, S, and M fractions were separated by SDS-PAGE, followed by Western blotting (left panel) or the overlay assay was conducted, followed by Western blotting as described above (right panel). Arrows indicate VirB5, and arrowheads indicate the His_6TrxAVirB5-binding protein detected in the overlay assay; numbers on the right indicate the molecular masses of reference proteins.

Purification and identification of the VirB5-binding protein as Tzs. To purify the His_6TrxAVirB5-binding protein, strain C58 carrying the plasmid pPZP300 (for increased virulence gene expression) was grown in the presence of the virulence gene inducer AS and as controls, we also cultivated the negative controls CB1005 (with AS) and C58 (without AS). Cell lysis was conducted in a French press, the cell debris was removed by low-speed centrifugation, and the membranes were then separated from the soluble proteins by ultracentrifugation. To enrich the His_6TrxAVirB5-binding protein, we subjected the soluble fraction to stepwise fractionation with $(NH_4)_2SO_4$ and the highest amounts were detected in the 40% and 50% fractions in the case of strain C58 as well as that of CB1005 (Fig. 2A). VirB5 was also detected in the soluble fraction here, which is likely due to the more highly concentrated sample. This result is in accord with previous reports showing that a small portion of VirB5 is present in the soluble fraction (42, 53). The highest amount of VirB5 was precipitated with 30% $(NH_4)_2SO_4$, but the protein was also detected in the 40% and 50% fractions. These data are in accord with the possibility that VirB5 and the interacting protein form a complex in vivo.

We used Coomassie staining of SDS gels to analyze the differential salt solubility. This analysis revealed that the 40% $(NH_4)_2SO_4$ fraction contained a 26-kDa protein and the smallest amount of other proteins (data not shown), and it was therefore chosen for further purification by anion exchange chromatography. Only a portion of the VirB5-binding protein (revealed by overlay assay) and of soluble VirB5 (revealed by Western blotting) from the 40% $(NH_4)_2SO_4$ fraction bound to the anion exchange column, whereas most of both proteins eluted in the wash fractions (Fig. 2B). Interestingly, VirB5 and its interaction partner eluted in identical fractions during this procedure and this result is also consistent with the notion that they may form a complex. An analysis of the fractions eluted from the column by SDS-PAGE and Coomassie staining revealed that this procedure constituted an enrichment of the VirB5-binding protein, and we concluded that the purity was high enough for its identification. To this end, we blotted the

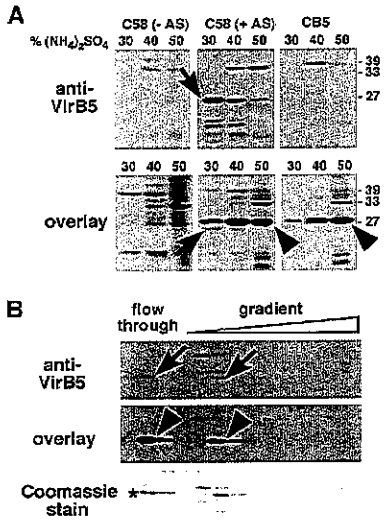

FIG. 2. Purification of the VirB5-binding protein from the soluble fraction of C58 cell lysates. Wild-type C58 and the *virB5* deletion variant CB1005 were grown under virulence gene-inducing (+AS) or noninducing conditions (−AS). (A) Cells were lysed in a French press and the soluble fractions were separated from the membranes by ultracentrifugation, followed by precipitation of proteins with increasing concentrations of $(NH_4)_2SO_4$ as indicated. The precipitates were analyzed by SDS-PAGE and Western blotting with VirB5-specific antiserum (upper panel) or by overlay assay with His_6TrxAVirB5, followed by the detection of bound VirB5 (lower panel). (B) Proteins precipitated with 40% $(NH_4)_2SO_4$ from C58 extracts were dialyzed and applied to a MonoQ anion exchange column, and the fractions eluted from the column (flowthrough and NaCl gradient) were analyzed by SDS-PAGE and Western blotting, overlay assay and detection of VirB5, or Coomassie staining. The protein indicated by the asterisk was electrotransferred to a PVDF membrane and subjected to Edman sequencing. Arrows show VirB5, and arrowheads indicate the His_6TrxAVirB5-binding protein detected using the overlay assay; numbers on the right indicate the molecular masses of reference proteins.

flowthrough fraction with the highest amount of the VirB5-binding protein onto a PVDF membrane and its identity was analyzed by N-terminal (Edman) sequencing. This analysis identified 11 of the 12 N-terminal amino acids counted from the N terminus as MLLHLIYGPTXS (X indicates an amino acid that could not be identified), which matches Tzs, the *trans*-zeatin biosynthetic protein from *A. tumefaciens* strain C58. The finding of Tzs as the VirB5 binding protein was unexpected, as there is no obvious connection between a protein involved in phytohormone biosynthesis in the cytoplasm and a T4SS component. However, the biological role of Tzs has not been firmly established and, in the following work, we verified the interaction with VirB5 and assessed its biological significance.

Pulldown and yeast two-hybrid analysis confirm the interaction between VirB5 and Tzs. To further characterize the VirB5-Tzs interaction, we cloned, overproduced, and purified Tzs as described previously (27) and we used the purified

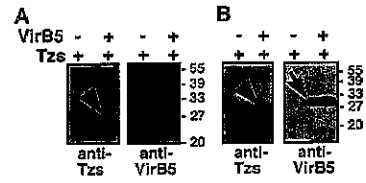

FIG. 3. Analysis of the VirB5-Tzs interaction by pulldown assays. Purified Tzs was incubated with affinity bead-bound StrepIIVirB5 and His_6TrxAVirB5 fusion proteins or with affinity beads alone, followed by sedimentation of the beads, washing, elution, and analysis of the bead-bound material by SDS-PAGE and Western blotting with specific antisera. (A) Analysis of proteins bound to Ni-nitrilotriacetic acid Sepharose after elution with imidazole. (B) Analysis of proteins bound to StrepTactin magnetic beads after elution with biotin. Arrows indicate VirB5 fusion proteins, and arrowheads indicate Tzs eluted from the affinity matrices. Molecular masses of reference proteins are shown on the right. −, absence of; +, presence of.

protein for the generation of a specific antiserum. We also overproduced and purified N-terminally StrepII-affinity-tagged VirB5 and used this fusion protein as well as His_6TrxAVirB5 to analyze the interaction with purified Tzs in pulldown assays. N-terminally affinity-tagged His_6TrxAVirB5 protein was attached to the IMAC affinity matrix and incubated with Tzs, followed by sedimentation of the matrix by centrifugation, washing, detection of the bound proteins by SDS-PAGE, and detection with specific antisera. This approach demonstrated that a VirB5 fusion protein bound Tzs to the IMAC matrix, and the use of a negative control (matrix without His_6TrxAVirB5) showed that this was not due to unspecific binding of Tzs (Fig. 3A). Similar results were obtained when the pulldown assays were conducted with StrepII affinity-tagged VirB5 and Strep-Tactin-Sepharose affinity matrix (Fig. 3B). These results also lead to the conclusion that Tzs binds to the VirB5 portion of the fusion proteins and not to the His_6TrxA domain.

As an independent assay to assess the VirB5-Tzs interaction, we used the yeast two-hybrid system. The genes encoding VirB5, Tzs, and (as a positive control) the VirE2 protein were fused to the DNA and the activation domains of the yeast GAL4 transcription factor. Interaction between two fusion partners resulted in the activation of the *lacZ* promoter and was monitored by the blue color of the yeast colonies. As in previous work, VirE2 was found to interact with itself (8) and we also detected a VirB5-VirB5 interaction, but this assay did not provide evidence for self-interaction of Tzs (Table 4). When pairwise combinations of the three proteins were tested, we noticed that VirB5 bound to the DNA binding domain led to *lacZ* gene activation when both Tzs and VirE2 were coexpressed as fusions to the activation domain, but the reciprocal experiments did not lead to gene activation. As the expression of VirB5 fused to the DNA binding domain alone did not activate the *lacZ* gene (data not shown), we conclude that the results of this assay lend additional support for the notion that Tzs and VirB5 interact. The evidence for a VirB5-VirE2 interaction presented here is novel, but we have not analyzed this question further in the context of this work. After confirming the VirB5-Tzs interaction with independent in vitro methods, we next analyzed whether Tzs associates with the T4SS complex in vivo.

TABLE 4. Analysis of VirB protein and Tzs interactions using the yeast two-hybrid system

Combination of vectors	Strength of interaction assessed by the no. of blue colonies[a]
pAS2-VirB5/pACTII-VirB5	++
pAS2-VirE2/pACTII-VirE2	+++
pAS2-Tzs/pACTII-Tzs	–
pAS2-VirB5/pACTII-Tzs	++
pAS2-Tzs/pACTII-VirB5	–
pAS2-VirB5/pACTII-E2	+++
pAS2-Tzs/pACTII-VirE2	–

[a] Relative numbers of β-galactosidase-producing colonies; six transformants each were tested from six independent transformation experiments.

Analysis of Tzs production and membrane association. Using the Tzs-specific antiserum, we first studied the conditions for production of Tzs in different agrobacteria. Tzs was detected exclusively in virulence gene-induced cells of *A. tumefaciens* strains C58 and A208 but not in strains A348, A281, Ach5, and Chry5 (data not shown). These results are in accord with its occurrence only on nopaline-type Ti-plasmids, such as in strains C58 and A208.

We next used the antiserum to determine the subcellular localization of Tzs in *A. tumefaciens* strain C58, and the T-pilus components VirB2 and VirB5 were used as controls. As described above, the cells were fractionated into total cell lysate (T), soluble (S), and membrane proteins (M) and Tzs was detected in all three fractions (data not shown). This result differs from that of the overlay assay, which detected the VirB5-binding protein exclusively in the soluble fraction (Fig. 1B). The fact that we analyzed more concentrated fractions of the membranes here and that detection with the Tzs-specific antiserum was more sensitive than the overlay assay likely explains this discrepancy.

To analyze the impact of VirB5 on the membrane association of Tzs in more detail, we next extracted membrane proteins with the mild detergent DDM, followed by separation under native conditions by blue native electrophoresis. This method was initially developed to assess the interactions between T4SS components, and it was applied here to determine the association of the translocation machinery with Tzs. As in previous work, both VirB2 and VirB5 were detected in the 100-kDa molecular mass range and, as expected, VirB2 was absent in CB1002 and VirB5 was absent in CB1005 (Fig. 4). An analysis with Tzs-specific antiserum revealed that the largest portion of Tzs was present in high-molecular-mass fractions larger than 140 kDa and in low-molecular-mass fractions smaller than 67 kDa (Fig. 4C). A minor fraction was also present in the 100-kDa molecular mass range, but the signal was not as well defined as in the cases of VirB2 and VirB5. These results were qualitatively similar in extracts from CB1002 and CB1008, but the amounts of Tzs were strongly reduced in CB1005. Tzs was not detected in the high- and low-molecular-mass fractions, but a relatively well-defined signal was present in the 100-kDa molecular mass range in CB1005 as well as in CB1008. Complementation of the *virB5* deletion in CB1005 pTrcB5 restored the wild-type pattern of Tzs fractionation, and these results demonstrate that VirB5 has a profound impact on the membrane association of Tzs. The fractionation of Tzs observed here is reminiscent of that of T4SS core components and of translocated substrates that were extracted in high-molecular-mass complexes with DDM. To assess whether Tzs is translocated from *Agrobacterium* by the T4SS, we analyzed next whether it is transferred to plant cells or to the cell exterior.

Extracellular localization of Tzs depends on T4SS function. The T4SS-dependent membrane association and the presence of positively charged residues at the C terminus of Tzs, which is a translocation signal of other T4SS substrates (48), opened the possibility that it may be translocated to plant cells. To test this possibility, the gene encoding Tzs was fused in frame to that encoding the Cre recombinase and its translocation to plant cells was tested following standard protocols (47). This analysis did not provide any evidence for a translocation of the fusion protein, indicating that Tzs is not a substrate translocated to plant cells (A. den Dulk-Raas and A. Vergunst, personal communication). Next, we tested whether Tzs is secreted to the cell exterior of strain C58 grown in liquid medium. As a positive control, we monitored the secretion of the C-terminal

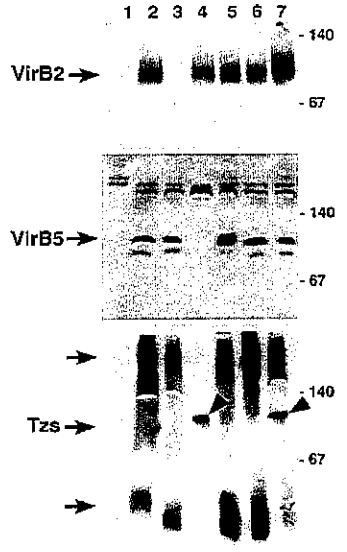

FIG. 4. Analysis of DDM-extracted membrane protein complexes by blue native electrophoresis. Strains C58, CB1002 (Δ*virB2*), CB1005 (Δ*virB5*), and CB1008 (Δ*virB8*) and complemented variants were cultivated on AB minimal medium in the absence (−AS, lane 1) or in the presence of AS (+AS, lanes 2 to 7) for virulence gene induction, followed by cell lysis, sedimentation of the membranes and extraction with 2% DDM. The samples were separated by blue native PAGE on a 15% gel, followed by Western blotting with VirB2-, VirB5-, and Tzs-specific antiserum. Lanes: 1, C58 without AS; 2, C58 with AS; 3, CB1002; 4, CB1005; 5, CB1002 pTrcB2; 6, CB1005 pTrcB5; 7, CB1008. Arrowheads point to Tzs in a 100-kDa complex in CB1005 and CB1008, and molecular masses of reference proteins are shown on the right (in kilodaltons). This experiment was conducted twice with qualitatively similar results.

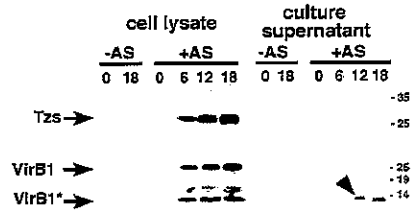

FIG. 5. Analysis of proteins secreted from *A. tumefaciens*. Strain C58 was cultivated in liquid AB minimal medium in the absence (−) or in the presence (+) of AS for virulence gene induction. The presence of VirB1, VirB1*, and Tzs was monitored in cell lysates as well as in culture supernatants at different time points after the induction of virulence gene expression (0 h, 6 h, 12 h, and 18 h). Samples were separated by SDS-PAGE, followed by Western blotting with VirB1 and Tzs-specific antiserum. The arrowhead points to secreted VirB1*, and molecular masses of reference proteins are shown on the right (in kilodaltons).

VirB1* fragment that is proteolytically processed from VirB1 (7). Tzs was not detected in the cell supernatant, and it is therefore not a secreted protein (Fig. 5).

T4SS-mediated translocation may alternatively lead to the incorporation or Tzs into T pili or to its display at the cell surface. To address these possibilities, we cultivated agrobacteria on solid agar medium, followed by the shearing of the cells to remove cell-bound T pili and flagella and ultracentrifugation to separate these high-molecular-mass extracellular structures in the ultracentrifugation pellet from low-molecular-mass structures in the ultracentrifugation supernatant. The T-pilus major component VirB2 and the minor component VirB5 as well as Tzs were detected in the ultracentrifugation pellet of samples from wild-type strain C58, suggesting that Tzs is incorporated into a high-molecular-mass structure that can be removed from the cells by shearing (Fig. 6). In accord with previous work, VirB2 and VirB5 were not detected in the

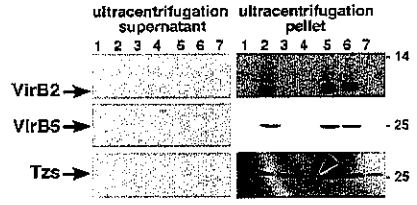

FIG. 6. Analysis of the composition of T-pilus fractions. Strains C58, CB1002 (ΔvirB2), CB1005 (ΔvirB5), and CB1008 (ΔvirB8) and complemented variants were cultivated on AB minimal medium in the absence (−) of AS (lane 1) or in the presence (+) of AS (lanes 2 to 7) for virulence gene induction, followed by shearing of cells and ultracentrifugation for separation of extracellular high-molecular-mass structures (ultracentrifugation pellet) and low-molecular-mass proteins released from the cells (ultracentrifugation supernatant). Samples were separated by SDS-PAGE, followed by Western blotting with VirB2-, VirB5-, and Tzs-specific antiserum. Lanes: 1, C58 without AS; 2, C58 with AS; 3, CB1002; 4, CB1005; 5, CB1002 pTrcB2; 6, CB1005 pTrcB5; 7, CB1008. The arrowhead points to the reduced amount of Tzs in the ultracentrifugation pellet from CB1005, and molecular masses of reference proteins are shown on the right (in kilodaltons).

ultracentrifugation pellets from strains CB1002, CB1005, and CB1008, but as expected, complementation of CB1002 and CB1005 restored pilus formation. In contrast, Tzs was detected in the ultracentrifugation pellets isolated from CB1002, in reduced amounts in those from CB1008, and in strongly reduced amounts in those from CB1005 (Fig. 6). The reduced association with the ultracentrifugation pellet fraction was complemented in CB1005 pTrcB5. These results demonstrated that Tzs can be sheared in a high-molecular-mass complex from the cell exterior, but since this association was not strictly dependent on VirB2 and VirB8, it is likely not a T-pilus component.

The results of the shearing experiments suggested that Tzs may be displayed on the cell surface, and we directly tested this possibility by immunoelectron microscopy. This approach has identified VirB5 on the cell surface and on the tips of T pili, whereas VirB2 was detected along the entire length of isolated T pili (3). When we conducted the immunoelectron microscopic analysis with Tzs-specific antiserum, the protein was detected evenly distributed across the entire surface of the wild-type strain C58, but it was not associated with T pili (Fig. 7). The number of gold grains indicating the presence of Tzs on the cell surface was modestly reduced on CB1002 and strongly reduced on CB1008, and the lowest amount was detected on CB1005. These reductions were complemented in CB1002 pTrcB2 and CB1005 pTrcB5 (Fig. 7). The results of the immunoelectron microscopic analysis correlated well with the detection of Tzs in pellets after shearing of the cells and ultracentrifugation (Fig. 6), showing that both assays detected a surface-exposed form of Tzs. Taken together, the work presented here demonstrates that Tzs is translocated to the cell surface of *A. tumefaciens* strain C58 and that the high efficiency of this process depends on VirB5 in the context of a functional T4SS.

DISCUSSION

The original goal of the work described here was to isolate proteins from *A. tumefaciens* that mediate the incorporation of VirB5 into T pili. To this end, we pursued an unbiased biochemical approach to isolate proteins from cell lysates that bind to purified VirB5 fusion protein and we envisaged that we may identify VirB proteins, other virulence-induced proteins, and possibly, non-virulence-induced proteins. The identification of Tzs, a protein that is believed to synthesize *trans*-zeatin ribotide phytohormones in the *A. tumefaciens* cytoplasm, came as a surprise, as there was no a priori reason to believe that such a protein would interact with the T4SS. In the course of this study, we have provided several lines of evidence supporting the notion that the Tzs-VirB5 interaction occurs in vivo and we gained insights into the mechanism of Tzs translocation across the cell envelope.

The first line of evidence suggesting a specific interaction was the finding that the VirB5 fusion protein bound only to one protein from *A. tumefaciens* cell lysate on PVDF membranes, the Tzs protein. Thus, VirB5 did not show features of a "sticky" protein that interacts with many partners. Under the conditions used, this interaction was apparently stronger than that with other known interaction partners of VirB5, such as VirB8 and VirB10 (53), that were not detected by overlay assay. This difference may be due to the fact that VirB8 and

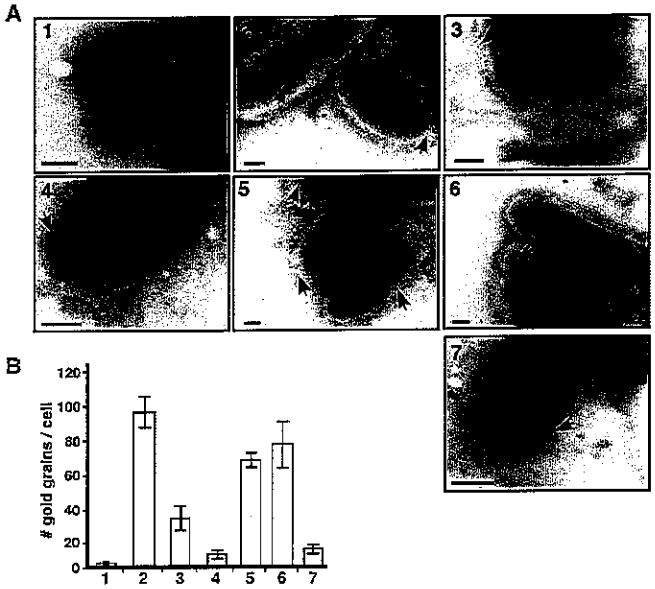

FIG. 7. Immunoelectron microscopy detects Tzs on the *A. tumefaciens* cell surface. Strains C58, CB1002 (Δ*virB2*), CB1005 (Δ*virB5*), and CB1008 (Δ*virB8*) and complemented variants were cultivated on AB minimal medium in the absence or in the presence of AS for virulence gene induction, followed by immunoelectron microscopy with Tzs-specific primary antibody and 10 nm gold-labeled secondary antibody. (A) Representative images of transmission electron micrographs; arrowheads point to gold grains on the cell surfaces of samples as follows: 1, C58 without AS; 2, C58 with AS; 3, CB1002; 4, CB1005; 5, CB1002 pTrcB2; 6, CB1005 pTrcB5; 7, CB1008. The contrast was increased to visualize the outline of cells for the purpose of presentation, but counting was conducted with reduced contrast settings that allowed the visualization of grains in even more heavily stained regions of the cells. Bars, 100 nm. (B) Quantification of results of the transmission electron microscopy analysis of Tzs on the cell surface; numbering of bars as for panel A. We counted 10 cells each from three independent induction experiments for each strain (total of 30 cells), and error bars show the standard deviations.

VirB10 do not refold on the membranes, but in any case, the limited number of interaction partners is consistent with a specific interaction. The second line of evidence stems from the apparent copurification of Tzs with a portion of soluble VirB5, which was observed during the initial purification of the VirB5-binding protein. Whereas this does not constitute a strict proof for an in vivo interaction, the results are consistent with the interaction detected with the overlay assay. The interaction between VirB5 and Tzs was subsequently confirmed using pulldown assays with purified proteins and the yeast two-hybrid system. The third line of evidence is based on the observation that a significant portion of Tzs associated with the membrane fraction. In the absence of VirB5, Tzs did not cofractionate with detergent-extracted high-molecular-mass complexes (larger than 140 kDa) and low-molecular-mass complexes (smaller than 67 kDa) and only small amounts of Tzs fractionated in the 100-kDa molecular mass range, similar to that of the VirB2-VirB5 pilus assembly complex. It is tempting to speculate that the detection of Tzs in the 100-kDa molecular mass range may reflect an interaction with VirB5 and VirB2, whereas high-molecular-mass Tzs may reflect its interaction with the core T4SS that was shown to fractionate in this molecular mass range (27, 53). The fourth line of evidence is based on the finding that the integrity of the T4SS is necessary for the efficient translocation of Tzs to the cell-surface that was monitored by the analysis of extracellular high-molecular-mass structures as well as by immunoelectron microscopy. Taken together, our data suggest a model for the contribution of the VirB5-Tzs interaction to the translocation of Tzs to the cell surface.

Surface-exposed Tzs was not released as a soluble protein into the supernatant, but it was removed from the cells by shearing and fractionated in a high-molecular-mass complex, together with T pili. This observation is reminiscent of findings made in the case of VirB7, which is a small lipoprotein and T4SS core complex component that translocates to the cell surface and is removed from the cells in a high-molecular-mass complex by shearing (38). The extracellular localization of VirB7 does not depend on T-pilus assembly, indicating that it is not an integral pilus component, but VirB7 may contribute to the assembly of this extracellular structure (38). Similar to VirB7, Tzs was detected on the surface of a *virB2* deletion strain, albeit at reduced levels. In contrast to VirB7, we observed that the amount of surface-exposed Tzs was strongly reduced in the absence of VirB8 and even more so in the absence of VirB5. Thus, an efficient translocation of Tzs to the surface depends on the integrity of the T4SS, and VirB5 is especially critical for this process. These observations differ

from those obtained in the case of VirB7, which translocates to the surface independently of individual T4SS components (38). The fact that VirB7 and Tzs are surface exposed in the absence of VirB2 and in immunoelectron microscopic analyses conducted here and elsewhere suggests that these proteins are not T-pilus components (3). The nature of the extracellular high-molecular-mass structure isolated by shearing remains elusive. One possibility is that shearing removes outer membrane vesicles (blebs) from the cells. Such structures are implicated in the translocation of virulence factors from bacteria (33, 35), and it may be interesting to assess in the future whether membrane vesicles contribute to the *Agrobacterium*-plant interaction.

The presented data suggest a mechanism for the translocation of Tzs to the cell surface. Tzs may bind to VirB5 in the inner membrane and it may subsequently interact with T4SS core complex components, followed by its translocation to the cell surface. This model is supported by the observation that a major portion of detergent-extracted Tzs fractionated in the molecular mass range of the T4SS core components. The absence of VirB5, but not of VirB2 and VirB8, reduced the amount of Tzs in these complexes, indicating that VirB5 is likely required to enable an early step of the interaction with the T4SS. Both VirB2 and VirB8 apparently facilitate a later step of the translocation, as the amount of surface-exposed Tzs was reduced in CB1002 and even more so in CB1008. The interaction partners among the T4SS core components are not known, but the data presented here suggest that in contrast to the case for VirB7, a functional T4SS and especially VirB5 are required for efficient translocation of Tzs to the cell surface.

Whereas this work demonstrates the surface localization of Tzs and suggests a key role of the VirB5 interaction for the translocation, it does not reveal the biological significance of this localization. The production of *trans*-zeatin ribotides in the cytoplasm is believed to be the primary function of Tzs (24, 37). However, the surface localization reported here opens up the possibility that Tzs may provide an additional or even an entirely different contribution to the *A. tumefaciens*-plant interaction. Different functions of surface-localized Tzs could be imagined, but further experimentation is required to assess these possibilities. First, the translocation of Tzs to the cell surface may down-regulate the cytoplasmic production of phytohormones. This metabolic pathway may be costly for the cells, and it may not contribute to the host cell interaction once the T-complex has been assembled and the *tmr* gene has been transferred. Thus, it may be advantageous to remove Tzs from the cytoplasm at this stage of the interaction. Second, surface-localized Tzs may convert metabolites from wounded and destroyed plant cells at the infection site to active phytohormones and thereby stimulate plant cell growth in the wound callus. Third, Tzs may be translocated to plant cells as a component of membrane vesicles and, in a manner similar to that of the Tmr protein, it may translocate to the chloroplasts and divert metabolites of the MEP pathway for the production of hydroxylated *trans*-zeatin ribotides (40). So far, there is no evidence for the formation of membrane blebs by agrobacteria, but blebs were detected in the closely related genus *Brucella* and it is therefore possible that they are also produced by *Agrobacterium* species (4, 17). The finding that Tzs as well as VirB7 associates with high-molecular-mass extracellular structures after shearing of cells is consistent with the possibility that membrane vesicles may be produced at the natural pathogen-host interface. Fourth, surface-localized Tzs may directly stimulate host-cell contact by binding to plant surface structures. The discovery of surface-localized GroEL, a protein that exerts an essential function in the bacterial cytoplasm, but contributes on the cell surface to pathogen-host adhesion in some bacteria, constitutes a precedent for this possibility (11, 18). The above-mentioned possibilities are speculative at this point, but the discovery of Tzs on the cell surface opens interesting avenues for the analysis of the *Agrobacterium*-plant interaction in the future.

ACKNOWLEDGMENTS

We are indebted to August Böck (Munich, Germany) and Meinhard Zenk (St. Louis, MO) for continued support and discussions and to Natalie Domke (Munich, Germany) for technical assistance. We are grateful to Annette Vergunst (Nîmes, France) and Amke den Dulk-Ras (Leiden, The Netherlands) for performing Cre recombinase reporter assays to determine whether Tzs is translocated into plant nuclei and to John Morala (McMaster) for help during the construction of strain CB1008.

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC grant 262104), the Canada Foundation for Innovation (CFI), the Ontario Innovation Trust (OIT), and the Deutsche Forschungsgemeinschaft (via SFB 369).

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Chapter 5

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

Preface

This chapter consists of the following article, in its originally published format: Carle, A., Höppner, C., **Ahmed Aly, K.**, Yuan, Q., den Dulk-Ras, A., Vergunst, A., O'Callaghan, D. and Baron, C. **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** (2006). Infection and Immunity 74(1):108-117. A copy of the original manuscript is included in my thesis with permission no. 2270920730245 from the publisher.

I performed the experiments shown in figures 2, 6 and 7, assembled the results of these figures and generated the figures. Dr. Christian Baron has helped improving the display of data in all figures so that they become easier for the reviewer to understand. I was not involved in the writing of the manuscript.

This article represents a collaboration between several laboratories and based on extensive trials to test the functionality of the *B. suis* T4SS when expressed in a heterologous host. There has been a long-term debate between several groups around the world on whether *Brucella* T4SS is a cryptic system or whether it is required for bacterial survival and virulence to mammalian hosts. Here, we show that the *B. suis* T4SS is functional when produced in the heterologous host *A. tumefaciens*. The functionality of that system was assessed by a recipient inter-bacterial conjugation assay. In addition, the absence of the T4SS structural protein VirB2 may result in the formation of a double membrane gap, which compromises the bacterial growth in the presence of toxic detergents.

The possibility that *virB2* deletion might create a membrane gap was also strengthened by the finding that many of the VirB structural proteins are secreted into the culture supernatant in the absence of VirB2. The results shown in this article are important because

they provide an *in-vivo* assay for the assessment of *Brucella* virulence and T4SS function. This work has been further utilized in future publications by other laboratory workers and will provide an essential tool for evaluating the function of *Brucella* T4SS upon introducing desired mutations in genes or gene deletions, following by detailed analysis of the effect of such deletions on T4SS function in a heterologous host.

In addition, with the recipient assay introduced in this article provides alternative research tools to conduct *Brucella* research if Biosafety level III requirements are not available.

INFECTION AND IMMUNITY, Jan. 2006, p. 108–117 Vol. 74, No. 1
0019-9567/06/$08.00+0 doi:10.1128/IAI.74.1.108–117.2006

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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Received 10 August 2005/Returned for modification 22 September 2005/Accepted 23 September 2005

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′-GGCAGAGCTCGACATAAGGAATAAAGATCATGAAAAC-3′] and virBsuis6-3 [5′-GAGGTCTAGAAAGGCCCTAATCCCTGTTGAACTG-3′]) and *virB7-12* (primers virBsuis7-5 [5′-GGCATCTAGAAGGAAATCATAATGAAAAAGGTAATCC-3′] and virBsuis12-3 [5′-GAGCCTGCAGGTTACTTGCGTAAAATTTCGATATC-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′-GCGCGAGCTCAGAAGGAGACGATCCTATGGTGCCA-3′] and virB1-3′ [5′-GCGCGACGCTCTTAGAAAACAACTACGCCGTCC-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′-CAGGGTACCCTCCAGCCCGCCGAAGCC-3′] and virB12-3 [5′-GAGCTGCAGTTACTTGCGTAAAATTTCGATATCCAC-3′]), cleaved with Acc65I and PstI (restriction sites are underlined), and ligated with similarly cleaved vector pT7H$_6$TrxFus (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).

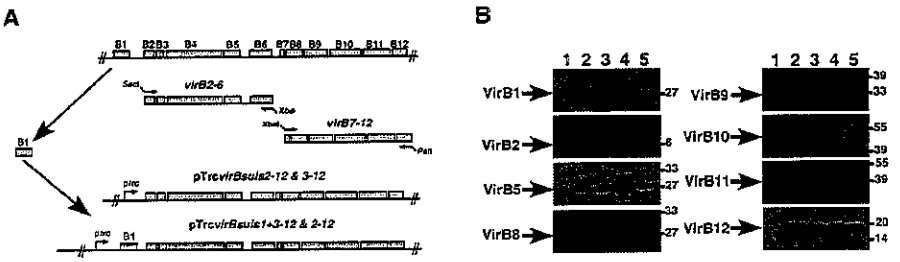

FIG. 1. Cloning of the *B. suis virB* operon and production of VirB proteins in *A. tumefaciens*. (A) Construction of different *virB* operon-containing pTrc300 derivatives for IPTG-induced expression. Portions of the *virB* operon containing *virB2-6* and *virB7-12* were PCR amplified and cloned separately, 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.

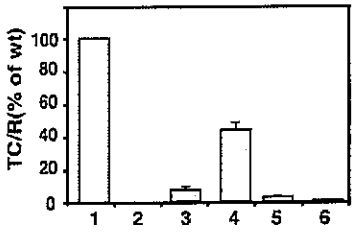

FIG. 2. Expression of the *B. suis virB* operon stimulates pLS1 transfer into *A. tumefaciens*. The recipient strain UIA143 carrying the Ti plasmid (bar 1), pTrc300 (bar 2), pTrcB3-12 (bar 3), pTrcB1+3-12 (bar 4), pTrcB2-12 (bar 5), and pTrcB1+2-12 (bar 6) were cultivated on AB minimal medium plates 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 (10^9)	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.

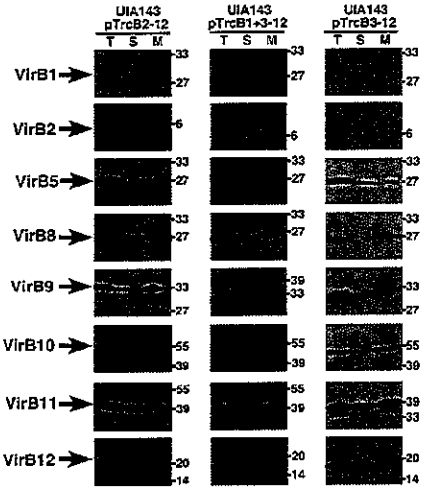

FIG. 3. Subcellular fractionation of *B. suis* VirB proteins produced in *A. tumefaciens*. Cells of UIA143 carrying pTrcB2-12, pTrcB1+3-12, and pTrcB3-12, respectively, were cultivated on AB minimal medium plates at 20°C for 3 days, followed cell lysis and membrane isolation. The protein content of subcellular 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.

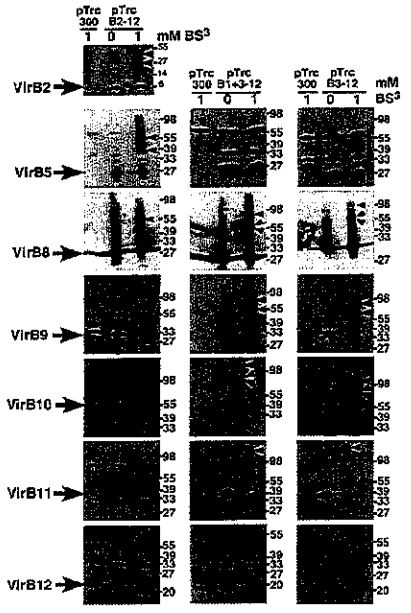

FIG. 4. Cross-linking monitors protein-protein interactions between *B. suis* VirB proteins in *A. tumefaciens*. Cells of UIA143 carrying pTrc300, pTrcB2-12, pTrcB1+3-12, and pTrcB3-12, respectively, were cultivated on AB minimal medium plates at 20°C for 3 days, followed by cross-linking with BS^3 (1 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

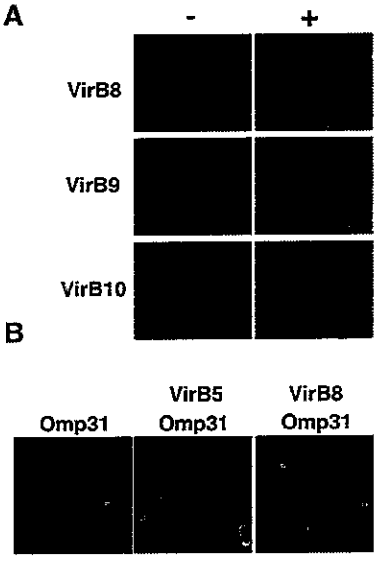

FIG. 5. Immunofluorescence analysis localizes *B. suis* VirB proteins in the cell envelope of *A. tumefaciens*. (A) *A. tumefaciens* strain C58 carrying pTrc300 (−) or pTrcB3-12 (+), respectively, was cultivated on AB minimal medium plates for 3 days in the presence of IPTG for *trc* promoter induction, 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 exoS* 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

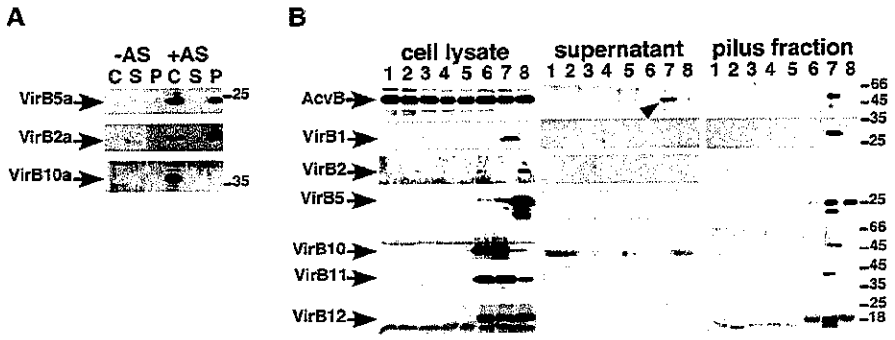

FIG. 6. Expression of the *B. suis virB1+3-12* operon induces periplasmic leakage in *A. tumefaciens*. Cells were cultivated on AB minimal medium plates at 20°C for 3 days, followed by shearing of the cells and analysis of protein content of subcellular fractions after SDS-PAGE and Western blotting: 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* VirBα 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

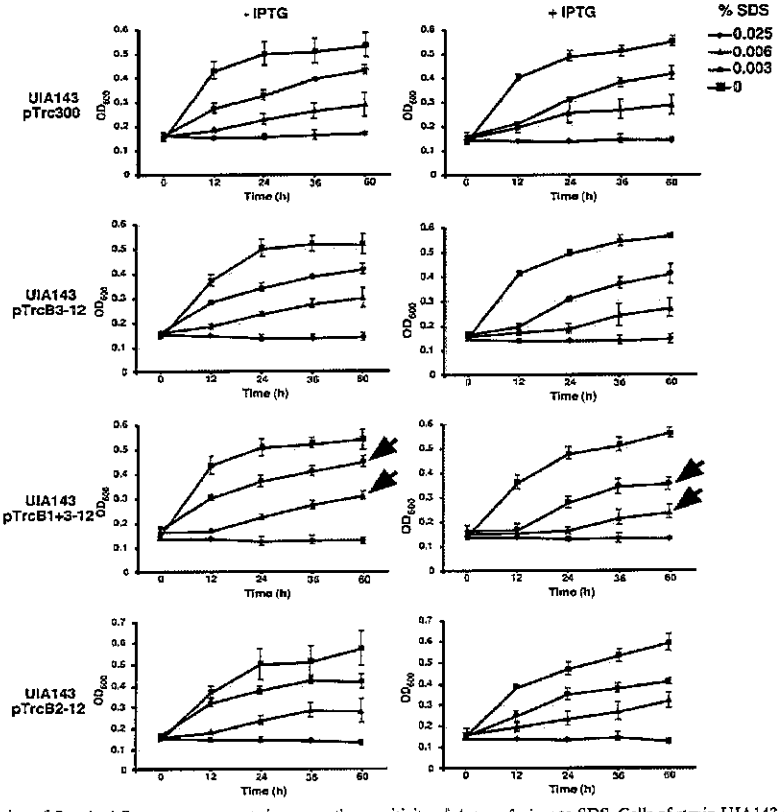

FIG. 7. Expression of *B. suis virB* operon constructs increases the sensitivity of *A. tumefaciens* to SDS. Cells of strain UIA143 carrying pTrc300 or different *virB* operon plasmids (pTrcB3-12, pTrcB1+3-12, or pTrcB2-12) were cultivated in liquid AB minimal medium at 20°C with or without IPTG for 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.

ACKNOWLEDGMENTS

We thank August Böck (Munich, Germany) for continued support and discussions.

This study was initiated with support from the EGIDE/DAAD French-German exchange program (PROCOPE) and the European Union (QLK2-CT-2001-01200). The Baron laboratory is supported by the Canadian Institutes of Health Research (CIHR grant MOP-64300), the Canada Foundation for Innovation, and the Ontario Innovation Trust. The O'Callaghan laboratory is supported by INSERM, the Université de Montpellier I (BQR), La Region Languedoc Roussillon, and La Ville de Nimes.

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Editor: D. L. Burns

Chapter 6

Identification of the VirB4-VirB8-VirB5-VirB2 pilus assembly sequence of type IV secretion systems

Preface

This chapter consists of the following article, in its originally published format: Yuan, Q., Carle, A., Gao, C., Sivanesan, D., **Aly, K.**, Höppner, C., Krall, L., Domke, N. and Baron, C. **Identification of the VirB4-VirB8-VirB5-VirB2 pilus assembly sequence of type IV secretion systems** (2005). Journal of Biological Chemistry 280(28):26349-26359. A copy of the original manuscript is included in my thesis with permission from the publisher.

I performed the experiments shown in figure 3 (A and B), assembled the results of these figures and generated the figures. Dr. Christian Baron has helped improving the display of data in all figures so that they become easier for the reviewer to understand. I was not involved in the writing of the manuscript.

This paper introduced an important procedure of extracting the VirB complex from Agrobacterial envelope using mild detergents. VirB4 was found to be required for VirB2-VirB5 integration into the T-pilus pre-assembly sub-complex, probably upon interaction with VirB8. This finding was also accompanied by finding that the *B. suis* VirB4 substitutes the *A. tumefaciens* VirB4 in T-pilus assembly and in operating a partially functional T4SS.

I conducted the EM portion of this article with quantification. My findings confirmed Dr. Yuan's findings that T-pili are indeed assembled when the *A. tumefaciens* VirB4 was substituted with the *B. suis* VirB4 as well as in the case of active site variants.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 280, No. 28, Issue of July 15, pp. 26349–26359, 2005
Printed in U.S.A.

Identification of the VirB4-VirB8-VirB5-VirB2 Pilus Assembly Sequence of Type IV Secretion Systems*

Received for publication, March 2, 2005, and in revised form, May 5, 2005
Published, JBC Papers in Press, May 18, 2005, DOI 10.1074/jbc.M502347200

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Type IV secretion systems mediate the translocation of virulence factors (proteins and/or DNA) from Gram-negative bacteria into eukaryotic cells. A complex of 11 conserved proteins (VirB1-VirB11) spans the inner and the outer membrane and assembles extracellular T-pili in *Agrobacterium tumefaciens*. Here we report a sequence of protein interactions required for the formation of complexes between VirB2 and VirB5, which precedes their incorporation into pili. The NTPase Walker A active site of the inner membrane protein VirB4 is required for virulence, but an active site VirB4 variant stabilized VirB3 and VirB8 and enabled T-pilus formation. Analysis of VirB protein complexes extracted from the membranes with mild detergent revealed that VirB2-VirB5 complex formation depended on VirB4, which identified a novel T-pilus assembly step. Bicistron expression demonstrated direct interaction of VirB4 with VirB8, and analyses with purified proteins showed that VirB5 bound to VirB8 and VirB10. VirB4 therefore localizes at the basis of a trans-envelope interaction sequence, and by stabilization of VirB8 it mediates the incorporation of VirB5 and VirB2 into extracellular pili.

Gram-negative bacteria use secretion systems to translocate macromolecules across their cell envelope of two membranes and the murein cell wall. The term type IV secretion system (T4SS)[1] was introduced for a group of protein machineries, which translocate proteins or protein-DNA complexes from donor to recipient cells. T4SSs are used by many bacterial pathogens for the translocation of virulence factors, *e.g.* by *Agrobacterium tumefaciens*, *Bartonella henselae*, *Bordetella pertussis*, *Brucella suis*, *Helicobacter pylori*, and *Legionella pneumophila* (1–3).

T4SSs from different bacteria translocate a wide variety of macromolecules to different types of recipients (bacteria, fungi, mammalian, and plant cells) (4). Nevertheless, the basic mechanism and structure of the translocation machinery are likely conserved (5). The best-characterized model is the plant pathogen *A. tumefaciens*. The T4SS of the closely related animal pathogen *B. suis* is encoded by an operon of similar organization, and it is essential for survival and multiplication inside mammalian cells (6). The T4SSs of these bacteria share 11 proteins, which can be divided into three groups. The first group comprises two inner membrane-associated NTPases (VirB4 and VirB11), which reside mainly in the cytoplasm but may traverse the inner membrane and contact periplasmic T4SS components (7–9). They contain Walker A nucleotide-binding motifs and are believed to energize T4SS assembly or substrate transfer. The second group consists of inner membrane VirB6 and periplasmic VirB7, VirB8, VirB9, and VirB10. They form the T4SS core and may constitute the translocation channel (10–13). The third group comprises the major T-pilus component VirB2, the minor component VirB5, and the pilus-associated protein VirB7 (14–17). VirB3 has not been firmly assigned, but its outer membrane localization and binding to VirB5 suggest that it is a pilus-associated protein (18, 19). Biochemical experiments based on extraction of VirB proteins with a mild detergent followed by separation under native conditions led to a model for T-pilus assembly (20). This model is refined here based on improved separation methods and the analysis of the contribution of VirB4.

VirB4 is the largest T4SS component (*A. tumefaciens*, 87 kDa; *B. suis*, 94 kDa) and exhibits the highest degree of conservation among T4SS components (31% identity and 52% similarity between *A. tumefaciens* and *B. suis*). It is homodimeric or homomultimeric and is essential for virulence (8, 21). An important feature is its Walker A nucleotide-binding site, which is essential for virulence and plasmid transfer (22, 23). ATPase activity of purified *A. tumefaciens* VirB4 was previously reported (24), but a more recent study argues against such an activity of the purified protein (25). Coordinated action of VirB4 with VirB11 and VirD4 was proposed to mediate the early DNA transfer reactions (26). In addition, VirB4 stabilized VirB3 and was required for its localization in the outer membrane (19). A study using the yeast two-hybrid system suggested that VirB4 binds to VirB1, VirB8, VirB10, and VirB11, but this was not substantiated with biochemical methods (27).

Interestingly, the VirB4 NTPase active site is essential for virulence, but it is not required for interactions with VirB11 and VirD4, for self-association, and for the stimulation of IncQ plasmid transfer into *A. tumefaciens* by the T4SS in the recipient (8). To unravel the role of VirB4, we constructed a *virB4* deletion mutant and assessed the complementation with *A. tumefaciens* and *B. suis* VirB4 and NTPase active site variants. We refined the protocol for the separation of detergent-extracted VirB proteins and identified a novel step in T-pilus assembly. Purified components were used to study interactions *in vitro*, and this revealed an interaction sequence from the

* This work was supported by Canadian Institutes of Health Research Grant MOP-64300, Natural Sciences and Engineering Research Council of Canada Grant 262104, and the European Union Frame Programme 5 Contract QLK2-CT-2001-01200 (to C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: T4SS, type IV secretion system; AS, acetosyringone; ARA, arabinose; DDM, dodecyl-β-D-maltoside; MES, 4-morpholineethanesulfonic acid.

VirB4-VirB8 complex to the pilus component VirB5 and the core component VirB10. This work gives fundamental insights into the contribution of VirB4 to T4SS complex stabilization and T-pilus assembly.

EXPERIMENTAL PROCEDURES

Cultivation of Bacteria—The strains and plasmids used in this study are given in Table I. Cultures of *Escherichia coli* JM109 for cloning experiments were grown at 37 °C in LB (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Antibiotics were added for plasmid propagation (50 μg/ml spectinomycin, 50 μg/ml streptomycin, 100 μg/ml carbenicillin). Overnight cultures of *A. tumefaciens* were grown in YEB (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 the propagation of pVS-BAD*Neo* and pVSBAD, followed by virulence gene induction in liquid AB glycerol minimal medium (0.5% glycerol, 0.4% morpholinoethansulfonic acid, 1 mM sodium potassium phosphate, 0.1% NH_4Cl, 0.03% $MgSO_4 \times 7H_2O$, 0.001% $CaCl_2$, 0.00025% $FeSO_4 \times 7H_2O$, pH 5.5) for 18 h or on AB agar plates for 3 days at 20 °C in the presence of acetosyringone (AS; 200 μM) and arabinose (ARA, 0.2%). For protein overproduction, *E. coli* strain GJ1158 was grown under aerobic conditions at 37 °C in LBON medium (1% tryptone, 0.5% yeast extract) to an A_{600} of 0.4–0.8, followed by the addition of NaCl at 0.3 M. Cultivation under aerobic conditions proceeded at different temperatures and times to assure maximal solubility and yield of the fusion proteins (VirB4s and its bicistron constructs, VirB5sp, VirB9sp, and VirB10sp: 16 h, 26 °C; VirB8sp: 4 h, 37 °C).

Plasmid and Strain Constructions and Mutagenesis—DNA manipulations followed standard procedures (28). *A. tumefaciens virB* genes were amplified from pGK217, and *B. suis virB4* genes were amplified from pUCvirB with oligonucleotides, cleaved with restriction sites, and cloned into vectors with compatible sites (Table II). The codons determining the ATP-binding site Lys residues in VirB4 and VirB4s were changed to Arg using the *in vitro* site-directed mutagenesis system (Promega). The sequences of PCR-amplified genes were confirmed by DNA sequencing.

Analysis of T4SS Functions: T-pilus Isolation, Conjugation, and Virulence Assays—Assays for T4SS functionality (T-pilus isolation, conjugation, and virulence assays) were performed as previously described (29).

Transmission Electron Microscopy—*A. tumefaciens* strains to be examined were cultivated on AB agar plates in the presence or absence of AS and ARA for gene induction. Cells were collected with 5 ml of 50 mM sodium potassium phosphate buffer, pH 5.5, and the cell density was adjusted to A_{600} of 1.5–2. 10 μl were applied onto UV-sterilized 200 mesh carbon-coated formvar copper electron microscopy grids and air dried in a laminar flow hood for 10 min. The grids were then stained with 2% phosphotungstic acid-0.01% glucose, pH 6, for 15 s prior to examination. Specimen images were taken with a JEOL 1200EX II transmission electron microscope. T-pili on 300 cells from three independent virulence induction experiments of each strain were counted (10 cells per visual field were analyzed).

Membrane Isolation, Detergent Extraction, Blue Native Electrophoresis, and Gel Filtration—Isolation of membranes and detergent extraction with 2% dodecyl-β-D-maltopyranoside (DDM) were performed as previously described (20). Blue native gradient gel electrophoresis (7–14% acrylamide, 16-cm-long gel) was performed for 20 h and 5 mA at 4 °C. Alternatively, for separation of small proteins, blue native gel electrophoresis was performed in a linear 15% polyacrylamide gel for 5–6 h and 5 mA at 4 °C in a mini-gel system (10-cm-long gel). Calibration was achieved by separation of reference proteins of known molecular masses: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (14 kDa, Amersham Biosciences). For gel filtration, 100 μl of 2% DDM-solubilized membrane proteins were applied to a Superdex 200 or Superdex 75 (XK 16/40; Amersham Biosciences), and chromatography was performed in 6-amino-caproic acid buffer (750 mM 6-amino-caproic acid, 50 mM BisTris, pH 7) containing 0.03% DDM performed at 4 °C with a flow rate of 0.5 ml/min in an Äkta fast protein liquid chromatography purifier (Amersham Biosciences). 100 μl of calibration kit proteins (ferritin, 1 mg/ml; all other proteins, 10 mg/ml) were separated in 6-amino-caproic acid buffer with 0.03% DDM.

SDS-PAGE and Western Blotting—Cells were incubated in Laemmli sample buffer for 5 min at 100 °C followed by SDS-PAGE. Chromatography samples were incubated in Laemmli sample buffer buffer for 30 min at 37 °C to avoid aggregate formation followed by SDS-PAGE using the Laemmli (for proteins larger than 20 kDa) or the Schägger and von Jagow system (for proteins smaller than 20 kDa) (30, 31). Western blotting was performed following standard protocols (32), with VirB protein-specific antisera.

Purification of Fusion Proteins—N-terminally hexahistidyl-thioredoxin (H_6TrxA)-tagged proteins were overproduced in GJ1158 using pT7-H_6TrxFus constructs (33). Cells from 400-ml cultures were suspended in 5 ml of lysis buffer (50 mM Hepes, 200 mM KCl, 5 mM $MgCl_2$, pH 7.5) with 0.5 mM phenylmethylsulfonyl fluoride and lysed by a French Pressure Cell (Aminco) at 18,000 p.s.i. The lysate was centrifuged twice (SS34 rotor, 30 min, 13,000 rpm at 4 °C), and the supernatant was applied to a high pressure liquid chromatography system (Äkta Purifier; Amersham Biosciences) with a Ni^{2+}-charged IMAC column (Talon™ Superflow; Clontech). Tagged proteins were eluted using a step gradient. At a flow rate of 0.5 ml/min, the column was first washed for 5 column volumes in buffer A (50 mM Hepes, 0.3–1 M NaCl, pH 7.5), followed by washing with buffer A with 20 mM imidazole (2.5 column volumes) and elution with buffer B (buffer A with 400 mM imidazole). The fractions were dialyzed overnight in H1B (50 mM Hepes, 200 mM NaCl, pH 7.5), followed by a second dialysis overnight in H2B (H1B with 50% glycerol and 2 mM dithiothreitol) and storage at −20 °C.

N-terminally StrepII-tagged proteins were overproduced in GJ1158 using pT7-7StrepII (34), and 400-ml cultures were lysed in 10 ml of S2B (0.1 M Tris-HCl, pH 8, 0.15 M NaCl, 1 mM EDTA, pH 8) with 0.5 mM phenylmethylsulfonyl fluoride. The cells were lysed, and the supernatant was collected as described above. Fusion proteins were purified with a 1-ml Strep-Tactin Superflow® column (IBA, Göttingen, Germany) following the instructions of the manufacturer using 2.5 mM desthiobiotin in the elution buffer. The fractions were subsequently purified by size exclusion chromatography using S2B at a flow rate of 0.5 ml/min. Superdex 75 or Superdex 200 (Amersham Biosciences) was used, depending on the molecular mass of the protein. The proteins were then dialyzed overnight in PSB (S2B buffer with 50% glycerol and 2 mM dithiothreitol) and stored at −20 °C.

Assays for Protein-Protein Interactions: Pull-down and Cross-linking Experiments—For pull-down experiments, 10 μl each of purified StrepII- and H_6TrxA-tagged proteins concentrated at 5 pmol/μl in PSB were mixed, followed by the addition of 80 μl of S2B and incubation for 30 min at 22 °C. Next, 20 μl of Strep-Tactin Sepharose beads (50% suspension in S2B; IBA) were added, followed by a 15-min incubation at 22 °C. The Sepharose beads were subsequently sedimented by centrifugation and washed three times with 200 μl of S2B. Bound proteins were eluted with 50 μl of S2B with 1 mM biotin, mixed with 1 volume of Laemmli sample buffer, and analyzed by SDS-PAGE and Western blotting.

For chemical cross-linking, 5 μl each of 10 pmol/μl stock solutions of purified StrepII-tagged proteins in PSB (or 5 μl of PSB as negative control) were mixed for 5 min at 22 °C, 90 μl of CLB (50 mM MES-KOH, 150 mM NaCl, 1 mM EDTA, pH 6.5) were added, and the mixture was incubated for 30 min. The cross-linking agent disuccinimidyl suberate (10 mM stock in Me_2SO; Pierce) was added in different concentrations (0.05 and 0.1 μM), and the samples were incubated for 1 h at 22 °C, followed by the addition of 1 volume of Laemmli sample buffer and analysis by SDS-PAGE and Western blotting.

Yeast Two-hybrid Assay—The Matchmaker 3 system (Clontech) was used for the analysis of protein-protein interactions with the yeast two-hybrid system following the manufacturer's protocols. The genes encoding the periplasmic domains VirB5sp, VirB8sp, VirB9sp, and VirB10sp were cloned into pGADT7 (GAL4 activation domain fusion) and pGBKT7 (DNA binding domain fusion) as described above. The plasmids were transformed into *Saccharomyces cerevisiae* AH109 using the lithium acetate method (Clontech Manual; Clontech), and plasmid-carrying cells were selected on minimal medium without leucine and tryptophan. Interactions between VirB proteins, which tethered both domains of GAL4 together, were identified by growth of plasmid-carrying cells on minimal medium without histidine and adenine and by β-galactosidase activity.

RESULTS

VirB4 Stabilizes VirB3 and VirB8—To study the role of VirB4, we deleted *virB4* in the Ti plasmid of wild-type C58, resulting in strain CB1004 (Δ*virB4*). Next, virulence genes were induced by cultivation on acidic minimal medium with acetosyringone followed by SDS-PAGE and Western blotting. Specific antisera were applied to compare the VirB protein

TABLE I
Bacterial strains and plasmids

	Genotype or description	Source/ref. no.
Strains		
E. coli JM109	*endA1 gyr96 thi hsdR71 supE44 recA1 relA1* (Δ*lac-proAB*) (F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*)	45
E. coli GJ1158	*ompT hsdS gal dcm* Δ*malAp510 malP::*(*proUp*-T7 RNAP) *malQ::lacZhyb11* Δ*izhf-900::*Tn*10*ΔTet)	46
A. tumefaciens C58	Wild type, pTiC58	47
A. tumefaciens A348	Wild type, pTiA6NC	47
A. tumefaciens CB1004	pTiC58 carrying an in-frame deletion of *virB4*	This work
A. tumefaciens PC1004	pTiA6NC carrying an in-frame deletion of *virB4*	48
A. tumefaciens UIA143(pTiA6)	A348, ery^r, *recA ery140*	35
S. cerevisiae AH109	MATa *trp1–901 leu2–3 ura3–52 his3–200 gal4*Δ *gal80*Δ LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3 GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2 URA3::MEL1$_{UAS}$-MEL1$_{TATA}$-*lacZ*	Clontech
Plasmids		
pVSBAD*Nco*	str^r, spc^r, pVS1 origin, AraC-controlled promoter expression vector	49
pVSBAD	str^r, spc^r, pVS1 origin, AraC-controlled promoter expression vector	49
pT7-7StrepII	car^r, T7 promoter expression vector for N-terminal StrepII affinity peptide fusions	34
pT7-H$_6$TrxFus	car^r, T7 promoter expression vector, for N-terminal H$_6$TrxA affinity peptide fusions	33
pLS1	car^r, IncQ plasmid for VirB/D4-mediated conjugative transfer experiments	50
pGK217	car^r, pUC18 with 11-kb *virB* fragment from *A. tumefaciens*	51
pUC*virB*	car^r, pUC18 with 12-kb *virB* fragment from *B. suis*	52
pVSBADVirB4	pVSBAD carrying a 2.4-kb Acc65I/PstI *virB4* fragment from *A. tumefaciens* C58	This work
pVSBADVirB4^{K439R}	pVSBADVirB4, mutated *A. tumefaciens virB4* gene, encoding Walker A site K439R variant	This work
pVSBAD*Nco*VirB4s	pVSBAD*Nco* carrying 2.5-kb NcoI/XbaI *virB4* fragment from *B. suis*	This work
pVSBAD*Nco*VirB4s^{K464R}	pVSBAD*Nco*VirB4s, mutated *B. suis virB4* gene, encoding Walker A site K464R variant	This work
pT7-7StrepIIVirB4s	pT7-7StrepII carrying 2.5-kb Acc65I/EcoRI *virB4* fragment from *B. suis*	This work
pT7-7StrepIIVirB4s-VirB8sp	pT7-7StrepIIVirB4s bicistron with 492-bp EcoRI/PstI *virB8* fragment from *B. suis* (encoding 163-amino acid periplasmic domain)	This work
pT7-7StrepIIVirB4s-VirB8s	pT7-7StrepIIVirB4s bicistron with 720-bp EcoRI/PstI *virB8* fragment from *B. suis* (encoding 239-amino acid, full-length protein)	This work
pT7-7StrepIIVirB5sp	pT7-7StrepII carrying 666-bp Acc65I/PstI *virB5* fragment from *B. suis* (encoding 221-amino acid periplasmic domain)	This work
pT7-7StrepIIVirB8sp	pT7-7StrepII carrying 492-bp Acc65I/PstI *virB8* fragment from *B. suis* (encoding 163-amino acid periplasmic domain)	53
pT7-7StrepIIVirB9sp	pT7-7StrepII carrying 813-bp Acc65I/PstI *virB9* fragment from *B. suis* (encoding 271-amino acid periplasmic domain)	This work
pT7-7StrepIIVirB10sp	pT7-7StrepII carrying 1020-bp Acc65I/PstI *virB10* fragment from *B. suis* (encoding 339-amino acid periplasmic domain)	This work
pT7-H$_6$TrxVirB5sp	pT7-H$_6$TrxFus carrying 666-bp Acc65I/PstI *virB5* fragment from *B. suis* (encoding 221-amino acid periplasmic domain)	54
pT7-H$_6$TrxVirB8sp	pT7-H$_6$TrxFus carrying 492-bp Acc65I/PstI *virB8* fragment from *B. suis* (encoding 163-amino acid periplasmic domain)	54
pT7-H$_6$TrxVirB9sp	pT7-H$_6$TrxFus carrying 813-bp Acc65I/PstI *virB9* fragment from *B. suis* (encoding 271-amino acid periplasmic domain)	This work
pT7-H$_6$TrxVirB10sp	pT7-H$_6$TrxFus carrying 1020-bp Acc65I/PstI *virB10* fragment from *B. suis* (encoding 339-amino acid periplasmic domain)	This work
pGADT7	car^r, GAL4 AD fusion vector, LEU2 marker for selection in yeast	Clontech
pGBKT7	kan^r, GAL4 DNA-BD fusion vector, TRP1 marker for selection in yeast	Clontech
pGADT7-T	pGADT7 construct; encodes fusion of GAL4 AD and SV40 T-antigen; positive control	Clontech
pGBKT7–53	pGBKT7 construct; encodes fusion of GAL4 DNA-BD and mouse-p53, positive control	Clontech
pGADT7-VirB5sp	pGADT7 carrying 657-bp NdeI/BamHI *virB5* fragment from *B. suis* (encoding 218-amino acid periplasmic domain)	This work
pGADT7-VirB8sp	pGADT7 carrying 510-bp NdeI/BamHI *virB8* fragment from *B. suis* (encoding 170-amino acid periplasmic domain)	This work
pGADT7-VirB10sp	pGADT7 carrying 1020-bp NdeI/BamHI *virB10* fragment from *B. suis* (encoding 339-amino acid periplasmic domain)	This work
pGBKT7-VirB5sp	pGBKT7 carrying 657-bp NdeI/BamHI *virB5* fragment from *B. suis* (encoding 218-amino acid periplasmic domain)	This work
pGBKT7-VirB8sp	pGBKT7 pGADT7 carrying 510-bp EcoRI/PstI *virB8* fragment from *B. suis* (encoding 170-amino acid periplasmic domain)	This work
pGBKT7-VirB9sp	pGBKT7 carrying 816-bp NcoI/PstI *virB9* fragment from *B. suis* (encoding 272-amino acid periplasmic domain)	This work
pGBKT7-VirB10sp	pGBKT7 carrying 1020-bp EcoRI/PstI *virB10* fragment from *B. suis* (encoding 339-amino acid periplasmic domain)	This work

levels in cells of non-induced and virulence gene-induced C58 (controls) and in CB1004. The levels of most VirB proteins were comparable in C58 and CB1004 (Fig. 1*B*), but VirB3, VirB8, and VirB4 were not detected or were detected only in very small quantities in CB1004 (Fig. 1*A*). Whereas the absence of VirB4 was expected, and reduced levels of VirB3 had been

TABLE II
Oligonucleotide sequences

Restriction sites used for cloning are underlined.

Name/restriction	Sequence	Constructed plasmid
virB4 expression		
VirB4–5′/Acc65I	5′-CGGGGTACCCACAGGAAACAGACCATGCTCGGAGCA-3′	pVSBADVirB4
VirB4–3′/PstI	5-GCGGCTGCAGTCAATCTTTTGCCTCGTGGTAA-3′	
VirB4s-5′/NcoI	5′-GCGGCCATGGTGGGCGCTCAATCCAAATACGCGCAA-3′	pVSBAD*Nco*VirB4s
VirB4s-3′/XbaI	5′-GGCGTCTAGATCACCTTCCTGTTGATTTGGACGACG-3′	
VirB4M5	5′-CCCATCGGGAGGGGTAGAACGACGCTCATGACCTTT-3′	pVSBADVirB4^{K439R}
VirB4sM5	5′-CCAGTCCGGCGCTGGTAGAACTGTCCTGATGAACTTCTGC-3′	pVSBAD*Nco*VirB4s^{K464R}
VirB protein overproduction		
T7B4suis5′/Acc65I	5′-GCGCGCGGTACCCATGATGGGCGCTCAATC-3′	pT7-7StrepIIVirB4s
T7B4suis3′/EcoRI	5′-GCGCGAATTCTCATTGCAGGTTCTCCCCGGGC-3′	
T7B8suisBCsp-5′/EcoRI	5′-CCAGAATTCGAAGGAGATATATATATGCGCGTCAACGCACAGACAGGTGCGCCT-3′	
T7B8suisBCsp-3′/PstI	5′-AAAACTGCAGTCATTGCACCACTCCCATTTCTGGATCAAC-3′	pT7-7StrepIIVirB4s-VirB8sp
T7B8suisBCs-5′/EcoRI	5′-CCAGAATTCGAAGGAGATCTAATCATGTTTGGACGCAAACAATC-3′	
T7B8suisBCs-3′/PstI	5′-AAAACTGCAGTCATTGCACCACTCCCATTTCTGGATCAAC-3′	pT7-7StrepIIVirB4s-VirB8s
T7B5suis5′/Acc65I	5′-CAGGGTACCCGCGCACGCGCAGCTCC-3′ (54)	pT7-7StrepIIVirB5sp and
T7B5suis3′/PstI	5′- GAGCTGCAGCTAATAGGCGGCTTCCAGTGC-3′ (54)	pT7H$_6$TrxFusVirB5sp
T7B8suis5′/Acc65I	5′-CAGGGTACCCCGCGTCAACGCACAGAC-3′ (54)	pT7-7StrepIIVirB8sp and
T7B8suis3′/PstI	5′- GAGCTGCAGCTATTGCACCACTCCCATTTCTGG-3′ (54)	pT7H$_6$TrxFusVirB8sp
T7B9suis5′/Acc65I	5′-CAGGGTACCCCCGTCCGGCTCAAAATACG-3′	pT7-7StrepIIVirB9sp and
T7B9suis3′/PstI	5′-GAGCTGCAGCTATTGCAGGTTCTCCCCGGGC-3′	pT7H$_6$TrxFusVirB9sp
T7B10suis5′/Acc65I	5′-CAGGGTACCCCACATGAGGGGCAATGCAG-3′	pT7-7StrepIIVirB10sp and
T7B10suis3′/PstI	5′-GAGCTGCAGCTACTTCGGTTGGACATCATACAC-3′	pT7H$_6$TrxFusVirB10sp
Yeast two-hybrid analysis		
BKT7-B5sp-5/NdeI	5′-CGCGCGCATATGCAGCTCCCGGTGACAGATG-3′	pGBKT7-VirB5sp and
BKT7-B5sp-3/BamHI	5′-GCGCGCGGATCCATAGGCGGCTTCCAGTGCTTT-3′	pGADT7-VirB5sp
BKT7-B8sp-5/EcoRI	5′-GCGCGCGAATTCCAACATGTGCCCTACCTGGTG-3′	pGBKT7-VirB8sp
BKT7-B8sp-3/PstI	5′-CGCGCGCTGCAGTTGCACCACTCCCATTTCTGGATG-3′	
BKT7-B9sp-5′/NcoI	5′-CGCGCGCCATGGACCAAAATCCCGTCCGGCTCA-3′	pGBKT7-VirB9sp
BKT7-B9sp-3/PstI	5′-CGCGCGCTGCAGTTGCAGTTGCAGGTTCTCCCCGGGC-3′	
BKT7-B10sp-5/EcoRI	5′-GCGCGCGAATTCCACATGAGGGGCAATGCAGAG-3′	pGBKT7-VirB10sp
BKT7-B10sp-3/PstI	5′-CGCGCGCTGCAGCTTCGGTTGGACATCATACACACT-3′	
ADT7-B8sp-5/NdeI	5′-CGCGCGCATATGCAACATGTGCCCTACCTGGTG-3′	pGADT7-VirB8sp
ADT7-B8sp-3/BamHI	5′-GCGCGCGGATCCTTGCACCACTCCCATTTCTGGATG-3′	
ADT7-B10sp-5/NdeI	5′-CGCGCGCATATGCACATGAGGGGCAATGCAGAG-3′	pGADT7-VirB10sp
ADT7-B10sp 3/BamHI	5′-GCGCGCGGATCCCTTCGGTTGGACATCATACACACT-3′	

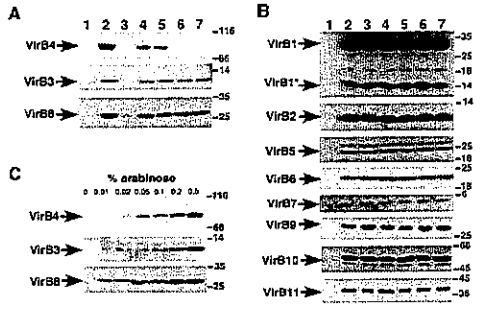

FIG. 1. **VirB protein levels in C58 and CB1004 and after complementation with VirB4 and VirB4s and their active site mutants.** *A. tumefaciens* wild-type C58 and *virB4* deletion CB1004 complemented with different constructs were grown on AB medium in the presence or absence of virulence gene inducer AS and pBAD inducer ARA, followed by SDS-PAGE and analysis with specific antisera. *A*, VirB protein levels affected in the absence of VirB4. *B*, VirB protein levels not affected in the absence of VirB4. *C*, modulation of VirB4, VirB3, and VirB8 by levels of arabinose inducer in CB1004 pVSBAD-VirB4 plus AS. *A* and *B*: *lane 1*, C58 −AS +ARA: *lane 2*, C58 +AS +ARA; *lane 3*, CB1004 pVSBAD*Nco* +AS +ARA; *lane 4*, CB1004 pVSBADVirB4 +AS +ARA; *lane 5*, CB1004 pVSBADVirB4^{K439R} +AS +ARA; *lane 6*, CB1004 pVSBAD*Nco*VirB4s +AS +ARA; *lane 7*, CB1004 pVSBAD*Nco*VirB4s^{K464R} +AS +ARA. Molecular masses of reference proteins are shown on the *right* (in kDa).

reported previously (19), the reduced levels of VirB8 showed that VirB4 plays a role for the accumulation of this protein.

A previous study in a different *A. tumefaciens* strain suggested that the structure of VirB4, but not its NTPase activity, is required for VirB protein stabilization (8). To assess this possibility, *A. tumefaciens virB4* was cloned behind the tightly controlled *E. coli* arabinose (BAD) promoter of broad host range vector pVSBAD. An active site change was engineered, and the cloning vector pVSBAD and constructs encoding VirB4 and its Walker A derivative (VirB4^{K439R}) were introduced into CB1004. Analysis of the resulting strains demonstrated that production of VirB4 as well as production of VirB4^{K439R} restored the levels of VirB3 and VirB8 (Fig. 1*A*). To further study the requirement of VirB4 sequence and structure, we cloned the gene encoding the *B. suis* VirB4 homolog (VirB4s) into pVSBAD*Nco* and engineered an active site change (VirB4s^{K464R}). The production of both VirB4s and VirB4s^{K464R} fully restored levels of VirB3 and VirB8 (Fig. 1*A*). When CB1004 complemented with plasmids encoding VirB4 or VirB4s (data not shown) was cultivated with gradually increasing arabinose inducer levels (0.01–0.5%), a gradual increase of VirB4 and concomitant increases of VirB3 and VirB8 levels were observed (Fig. 1*C*). These results showed that the NTPase activity is not important for the stabilization of VirB3 and of VirB8 and that the heterologous VirB4s provided the required structural information.

The Active Site of VirB4 Is Required for Substrate Transfer but Not for T-pilus Formation and pLS1 Recipient Activity—We next determined the functionality of *A. tumefaciens* and *B. suis* VirB4 and of their active site variants using four different complementation assays. First, the virulence was tested on wounded *Kalanchoë diagremontiana* leaves. Tumor formation was observed only at sites inoculated with C58 and CB1004 producing wild-type VirB4 and VirB4s, respectively (Fig. 2*A*). The requirement for the NTPase active site(s) is in accord with previous reports, but complementation by *B. suis*

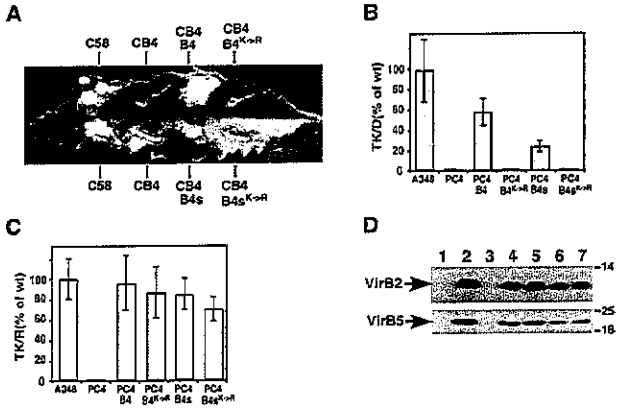

FIG. 2. **Complementation of T4SS functions in *A. tumefaciens virB4* deletions.** *A. tumefaciens* wild-type strains C58 and A348 and *virB4* deletion mutants CB1004 (*CB4*) and PC1004 (*PC4*) complemented with different constructs (pVSBADVirB4 (*B4*), pVSBADVirB4^{K439R} (*B4*$^{K\rightarrow R}$), pVSBAD*Nco*VirB4s (*B4s*), and pVSBAD*Nco*VirB4s^{K464R} (*B4s*$^{K\rightarrow R}$)) were assayed for different T4SS functions. *A*, analysis of tumor formation after infection of *K. diagremontiana*. *B*, pLS1 donor activity; donor strains A348 and complemented PC1004 carrying pLS1 were co-cultivated with recipient *A. tumefaciens* UIA143 (pTiA6) for 3 days in the presence of arabinose under virulence gene-inducing conditions, followed by the quantitation of pLS1-carrying recipients (*TK/D*, transconjugants/donor). *Error bars* indicate S.D. values derived from three independent experiments. *C*, pLS1 recipient activity; donor strain A348 pLS1 was co-cultivated with recipient strains A348 and complemented PC1004 for 3 days in the presence of arabinose under virulence gene-inducing conditions, followed by the quantitation of pLS1-carrying recipients (*TK/R*, transconjugants/recipient). *D*, T-pilus formation; T-pili were isolated after growth of the bacteria on AB minimal media by shearing and ultracentrifugation, and pilus components VirB2 and VirB5 were detected by SDS-PAGE and Western blotting. *Lane 1*, C58 −AS +ARA; *lane 2*, C58 +AS +ARA; *lane 3*, CB1004 pVSBAD*Nco* +AS +ARA; *lane 4*, CB1004 pVSBADVirB4 +AS +ARA; *lane 5*, CB1004 pVSBADVirB4^{K439R} +AS +ARA; *lane 6*, CB1004 pVSBAD*Nco*VirB4s +AS +ARA; *lane 7*, CB1004 pVSBAD*Nco*VirB4s^{K464R} +AS +ARA. Molecular masses of reference proteins are shown on the *right* (in kDa).

TABLE III
Conjugative transfer of pLS1 from A. tumefaciens donors A348, virB4 mutant PC1004, and its complemented derivatives into A. tumefaciens UIA143 pTiA6 as recipient

Donors[a] pLS1	Recipient[a]	Donors	TK[b]	TK/donor	TK/donor
		$\times 10^6$			%
A348 pLS1	UIA143 pTiA6	79	63,000	7.97×10^{-5}	100
PC1004 pVSBAD*Nco*	UIA143 pTiA6	82	2	2.4×10^{-8}	0.03
PC1004 pVSBADVirB4	UIA143 pTiA6	68	3,200	4.7×10^{-5}	56
PC1004 pVSBADVirB4^{K439R}	UIA143 pTiA6	75	3	4.0×10^{-8}	0.05
PC1004 pVSBAD*Nco*VirB4s	UIA143 pTiA6	73	1,500	2.05×10^{-5}	26
PC1004 pVSBAD*Nco*VirB4s^{K464R}	UIA143 pTiA6	59	2	3.38×10^{-8}	0.04

[a] Ratio of donors to recipients mixed for conjugation was 1:5 (29, 35).
[b] TK, transconjugants.

VirB4s shows that despite low sequence identity, the heterologous protein exerts all VirB4 function(s). Tumors induced by VirB4s-complemented CB1004 were smaller than those induced by the VirB4-complemented strain. We have not attempted to measure tumor formation and used the more readily quantifiable plasmid conjugation systems for this purpose. To this end, when T4SS-mediated IncQ group plasmid pLS1 transfer between Agrobacteria was employed as second assay, only *A. tumefaciens* and *B. suis* wild-type VirB4 complemented the *virB4* defect (Fig. 2*B* and Table III). As a third assay, we exploited the ability of T4SS to stimulate conjugative pLS1 transfer upon production in the recipient (35). The ability of a *virB4* deletion strain to serve as pLS1 recipient was restored by VirB4 as well as VirB4^{K439R}, which is in accord with previous work, and production of the *B. suis* proteins had similar effects (Fig. 2*C* and Table IV). As a fourth assay, T-pili were isolated from the cells by shearing and ultracentrifugation, followed by detection of major T-pilus component VirB2 and minor component VirB5, respectively. Both proteins were not detected in the pilus fractions from CB1004, but production of VirB4, VirB4s, and their active site variants fully restored T-pilus formation (Fig. 2*D*). The active site of VirB4 was obviously not required for T-pilus assembly, which constitutes a remarkable difference to previous reports (22). To directly assess T-pilus formation, we analyzed the cells from three independent induction experiments (300 cells of each strain were counted) by transmission electron microscopy. Analysis of wild-type strain C58 under virulence gene-inducing conditions revealed T-pili on 77% of the cells (Fig. 3), whereas no T-pili were observed on C58 grown under non-inducing conditions or CB1004 pVSBAD*Nco*. T-pilus formation in CB1004 was at least partly restored by production of VirB4 (61%), VirB4s (56%), VirB4^{K439R} (38%), and VirB4s^{K464R} (36%). Whereas the formation of T-pili in CB1004 carrying the active site variants

TABLE IV
Conjugative transfer of pLS1 from A. tumefaciens donor A348 into A. tumefaciens wild type, virB4 mutant PC1004, and its complemented derivatives as recipients

Donor[a]	Recipients[a]	Recipients	TK[b]	TK/recipient	TK recipient
		$\times 10^7$			%
A348	A348	42	55,000	1.31×10^{-4}	100
pLS1	pVSBAD*Nco*				
A348	PC1004	31	66	2.13×10^{-7}	0.16
pLS1	pVSBAD*Nco*				
A348	PC1004	39	49,000	1.26×10^{-4}	96
pLS1	pVSBADVirB4				
A348	PC1004	51	58,000	1.14×10^{-4}	87
pLS1	pVSBADVirB4^{K459R}				
A348	PC1004	26	29,000	1.12×10^{-4}	85
pLS1	pVSBAD*Nco*VirB4s				
A348	PC1004	28	26,000	9.29×10^{-5}	71
pLS1	pVSBAD*Nco*VirB4s^{K464R}				

[a] Ratio of donors to recipients mixed for conjugation was 5:1 (29, 35).
[b] TK, transconjugants.

was reduced as compared with the wild-type, the difference from the negative controls was very clear, showing that the intact active site was not required for the formation of this structure (Fig. 3*B*). The above results suggest that VirB4 contributes only structural information to T4SS assembly and T-pilus formation. In contrast, T-DNA translocation required a wild-type Walker A box, but heterologous VirB4s fully complemented those functions. Biochemical methods were used next to analyze the molecular basis of the stabilization phenomenon.

VirB4 Is Required for the Association of Pilus-associated Proteins with VirB3, VirB6, and VirB8—Extraction of membrane proteins with mild detergents greatly contributed to the understanding of protein uptake machineries in mitochondria (36, 37), and we have also found applications for the analysis of bacterial protein translocation systems (38). We have previously adapted this method to the extraction of VirB protein subcomplexes from the membranes of *A. tumefaciens* (20). Further analyses revealed two subassemblies, a low molecular mass complex of pilus-associated proteins and a high molecular mass complex containing the T4SS core components. This method was applied here to assess the contribution of VirB4. Membranes were isolated from C58, CB1004, and CB1004 producing VirB4, VirB4s, and their active site variants, followed by the extraction with DDM (2%, w/v) and analysis of subcellular fractions by Western blotting. Similar to previous reports, VirB2 was detected in the total cell lysate (Fig. 4*A*, *T*) and in the membranes (*M*). VirB5 was predominantly detected in the membranes, but a significant portion was present in the soluble fraction (Fig. 4*A*, *S* = cytoplasmic and periplasmic). Differences were not apparent, showing that the absence of VirB4 did not impact the membrane association of VirB2 and VirB5 (Fig. 4*A*).

To characterize the formation of the characteristic low molecular mass VirB2-VirB5 complex, the DDM-solubilized proteins were separated by blue native electrophoresis in a 7–14% polyacrylamide gel. Similar to previous findings, VirB2 and VirB5 co-localized in complexes of 140 kDa in samples from C58, but only very low amounts of both proteins were detected in samples from CB1004 (Fig. 4*B*). Production of VirB4, VirB4s, and their active site variants restored the levels of VirB2 and VirB5 in these complexes (Fig. 4*B*). These results suggest that whereas the membrane association is not affected in CB1004, VirB4 is necessary for the incorporation of VirB2 and VirB5 into complexes of 140 kDa.

Because blue native PAGE separation has limited resolution, we separated DDM extracts by gel filtration next. In contrast to the previously described procedure, gel filtration was conducted in the presence of 0.03% DDM, which is higher than the critical micellar concentration (0.006–0.007%), to avoid detergent dilution. Similar to previous observations in C58, a high molecular mass complex with the core components VirB4, VirB6, VirB7, VirB8, VirB9, VirB10, and the substrate VirE2 was detected (Fig. 5*A*). VirB3, VirB6, and VirB8 co-fractionated with VirB2, VirB5, and VirB7 in the low molecular mass complex of pilus-associated proteins (Fig. 5*A*). Analysis of samples from CB1004 revealed a drastically different distribution. Similar to the wild-type, VirB9, VirB10, and VirE2 were detected in the high molecular mass complex. In contrast, VirB6 and VirB7 eluted exclusively in the high molecular mass fractions (Fig. 5*B*). As expected, VirB3 and VirB8 were not detected. In addition, we did not detect VirB2 and VirB5 in the fractions eluting from the column. Analysis of samples from CB1004 producing VirB4, VirB4s, and their active site variants revealed VirB protein complexes as in the wild-type (data not shown). Thus, the NTPase activity of VirB4 was not required for the formation of the wild-type VirB protein complexes.

VirB2 and VirB5 Do Not Co-fractionate with Each Other and Other T4SS Components in CB1004—The observation that VirB2 and VirB5 were not detected in the gel filtration fractions from CB1004 was unexpected because both pilus components associated with the membranes and were extracted by DDM similar to the wild-type (Fig. 4*A*). One explanation was that VirB2 and VirB5 do not interact with other VirB proteins in CB1004 and are therefore present in low molecular mass complexes. To assess this possibility, we adapted the fractionation techniques to separate low molecular mass proteins. First, blue native electrophoresis was conducted in linear 15% acrylamide gels, which efficiently separated only small proteins. Using this technique, both VirB2 and VirB5 were detected in low molecular mass complexes in extracts from CB1004 (Fig. 6). VirB2 had an apparent molecular mass of 13 kDa, and VirB5 had an apparent molecular mass of 30 kDa. In strains C58 and complemented CB1004, VirB2 and VirB5 were detected in complexes similar to the wild-type.

As a second approach to identify VirB2 and VirB5 in CB1004, we separated the DDM extracts on a Superdex 75 gel filtration column, which resolved proteins smaller than 100 kDa. In extracts from C58, VirB2 and VirB5 co-eluted in fractions of high molecular masses (>150 kDa), which could not be resolved (Fig. 7*A*). In extracts from CB1004, VirB2 and VirB5 eluted in separate fractions of low molecular masses of 13 and 30 kDa, respectively (Fig. 7*B*), and complementation of CB1004 restored the wild-type situation (data not shown). The results show that in the absence of VirB4, the interactions of VirB2 and VirB5 with each other and with other T4SS components are weakened or abolished.

VirB4s Binds to the Periplasmic Domain of VirB8s—The absence of VirB4 strongly impacts the formation of VirB2-

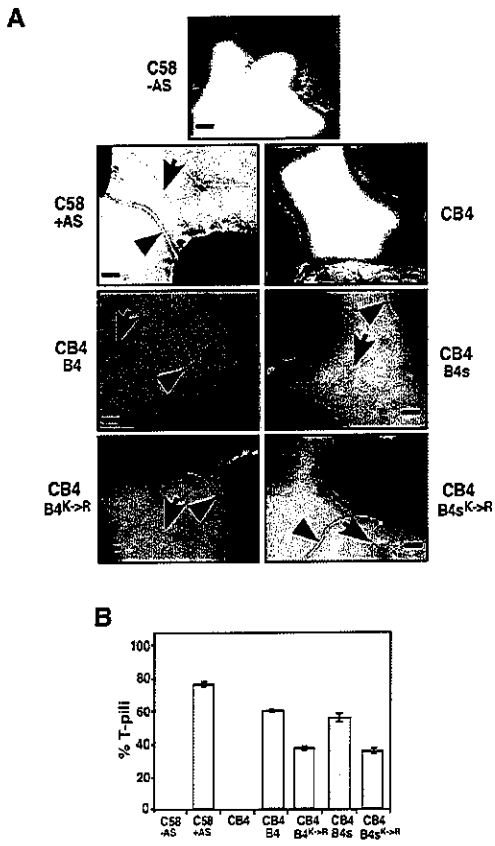

FIG. 3. **Transmission electron microscopy to visualize T-pili on *A. tumefaciens* C58 and the complemented *virB4* deletion mutant.** *A. tumefaciens* wild-type strain C58 (−AS +ARA and +AS +ARA) and *virB4* deletion mutant (all grown +AS +ARA) CB1004 pVSBAD*Nco* (*CB4*), complemented with different constructs pVSBADVirB4 (*B4*), pVSBADVirB4^{K430R} (*B4$^{K\rightarrow R}$*), pVSBAD*Nco*VirB4s (*B4s*), and pVSBAD*Nco*VirB4s^{K446R} (*B4s$^{K\rightarrow R}$*), were cultivated on AB minimal medium followed by analysis of T-pilus formation by negative staining and transmission electron microscopy (*A*). Results of counts of 300 cells from each strain from three independent experiments are shown in *B*. *Scale bars* correspond to 100 nm, *arrows* indicate T-pili, and *arrowheads* indicate flagella. *Error bars* indicate S.D. values derived from three independent experiments.

VirB5 complexes, but it was not obvious whether this effect was direct or indirect via stabilization of VirB3 and/or VirB8. Interaction between VirB4 and VirB8 had been predicted, and our data are consistent with this finding (27). We directly tested this possibility using bicistron expression (39). Because biochemical work with *A. tumefaciens* VirB proteins proved to be very difficult (low solubility and yield upon overproduction),[2] we performed the following experiments with *B. suis* VirB homologs (named VirBs). The *virB4s* gene was cloned into pT7-7StrepII to produce an N-terminal fusion with the StrepII peptide for affinity purification and detection. Next, two fragments of the VirB8s-encoding gene (full-length and one encoding the periplasmic domain VirB8sp) were cloned downstream of *virB4s*. The resulting constructs pT7-7StrepIIVirB4s, pT7-7StrepIIVirB4sVirB8s, and pT7-7StrepIIVirB4sVirB8sp were transformed into *E. coli* strain GJ1158, followed by expression and detection of StrepII-VirB4s with a StrepII-specific mono-

[2] Q. Yuan, A. Carle, C. Gao, D. Sivanesan, K. A. Aly, C. Höppner, L. Krall, N. Domke, and C. Baron, unpublished observations.

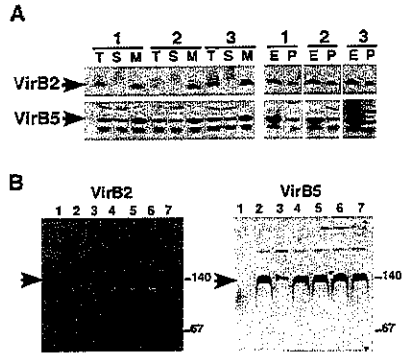

FIG. 4. **Analysis of DDM extraction of VirB2 and VirB5 and separation by blue native PAGE.** *A. tumefaciens* wild-type C58 and *virB4* deletion CB1004 complemented with different constructs were grown on AB minimal medium in the presence of AS and pBAD inducer ARA. *A*, cells were lysed, followed by fractionation of the cell lysate (*T*) into soluble (*S*) and membrane fractions (*M*) (*1*, C58 +AS +ARA; *2*, CB1004 pVSBAD*Nco* +AS +ARA; *3*, CB1004 pVSBADVirB4 +AS +ARA). Membranes were incubated with 2% DDM, and soluble proteins (*E*) were separated by ultracentrifugation from insoluble proteins (*P*). Fractions were analyzed by SDS-PAGE and Western blotting. *B*, DDM-solubilized membrane proteins were mixed with Coomassie Blue G-250 followed by separation in blue native 7–14% acrylamide gradient gels, followed by Western blotting. *Lane 1*, C58 −AS +ARA; *lane 2*, C58 +AS +ARA; *lane 3*, CB1004 pVSBAD*Nco* +AS +ARA; *lane 4*, CB1004 pVSBADVirB4 +AS +ARA; *lane 5*, CB1004 pVSBADVirB4^{K430R} +AS +ARA; *lane 6*, CB1004 pVSBAD*Nco*VirB4s +AS +ARA; *lane 7*, CB1004 pVSBAD*Nco*VirB4s^{K446R} +AS +ARA. Molecular masses of reference proteins are shown on the *right* (in kDa).

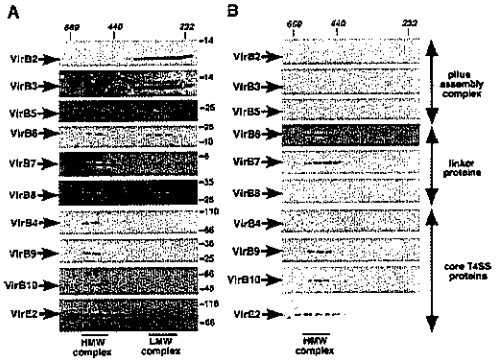

FIG. 5. **Analysis of DDM-extracted virulence protein complexes from C58 and CB1004 by Superdex 200 gel filtration.** *A. tumefaciens* wild-type C58 and *virB4* deletion strain CB1004 were grown on AB minimal medium in the presence of AS and pBAD inducer arabinose. Cells were lysed, and membranes were isolated and incubated with 2% DDM, followed by gel filtration in the presence of 0.03% DDM and analysis of the fractions by SDS-PAGE and Western blotting. Low molecular weight (*LMW*) fractions contain the pilus assembly complex, and high molecular weight (*HMW*) fractions contain the core T4SS proteins; linker proteins are part of both complexes. *A*, analysis of extracts from C58. *B*, analysis of extracts from CB1004. Molecular masses of reference proteins are shown on the *right* (in kDa).

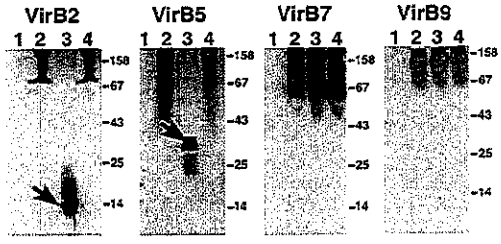

FIG. 6. **DDM extraction and analysis of low molecular mass VirB complexes by blue native PAGE.** *A. tumefaciens* wild-type C58 and *virB4* deletion CB1004 complemented with different constructs were grown on AB minimal medium in the presence or the absence of AS in the presence of pBAD inducer ARA. Cells were lysed, membranes were isolated and incubated with 2% DDM, and solubilized membrane proteins were mixed with Coomassie Blue G-250 followed by separation in a blue native 15% acrylamide gel and Western blotting. *Lane 1*, C58 −AS +ARA; *lane 2*, C58 +AS +ARA; *lane 3*, CB1004 pVSBAD +AS +ARA; *lane 4*, CB1004 pVSBADVirB4 +AS +ARA. *Arrows* indicate low molecular mass forms of VirB5 and VirB2. Molecular masses of reference proteins are shown on the *right* (in kDa).

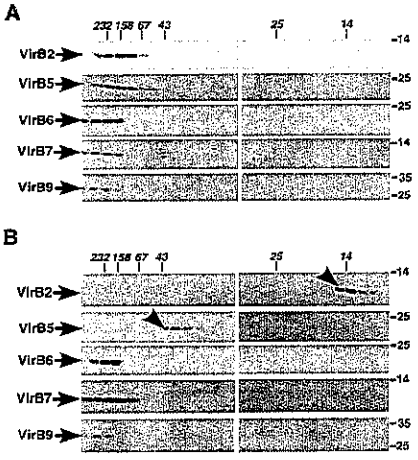

FIG. 7. **Analysis of DDM-extracted virulence protein complexes from C58 and CB1004 by Superdex 75 gel filtration.** *A. tumefaciens* wild-type C58 and *virB4* deletion CB1004 were grown on AB minimal medium in the presence of AS and pBAD inducer arabinose. Cells were lysed, and membranes were isolated and extracted with 2% DDM, followed by gel filtration and analysis of the fractions by SDS-PAGE and Western blotting. *A*, analysis of extracts from C58. *B*, analysis of extracts from CB1004. *Arrowheads* indicate low molecular mass forms of VirB5 and VirB2. Molecular masses of reference proteins are shown on the *right* (in kDa).

clonal antibody and the VirB8s variants with a specific antiserum (data not shown). To study interactions, cell lysates were applied to a streptavidin affinity matrix, which enriched StrepIIVirB4s. VirB8s and VirB8sp co-eluted with StrepIIVirB4s, and controls showed that the column had very weak affinity for non-tagged VirB8sp (data not shown). To assess whether the co-elution reflects a stable interaction, we subjected samples to two separation procedures. First, the proteins were separated by blue native PAGE, and VirB4s was detected in complexes slightly larger than 440 kDa in all cases (Fig. 8*A*). A minor portion was detected in larger complexes in samples from strains carrying the bicistron constructs. VirB8s and VirB8sp were mainly detected in complexes of similar size such as VirB4s, but minor fractions were present in higher and lower molecular mass forms (Fig. 8*A*). As a control, we separated StrepII-VirB8sp, and it was detected as monomer of 20 kDa. The results suggest that VirB4s formed multimers and that VirB8s and VirB8sp bound to this complex when expressed from bicistron constructs. As a second approach, the affinity column-enriched samples were subjected to gel filtration over a Superdex 200 column. StrepIIVirB4s co-eluted with VirB8s and VirB8sp in fractions 8 (corresponding to 440 kDa) and 9, suggesting that they form a complex (Fig. 8*B*). StrepIIVirB4s enriched from pT7-7StrepIIVirB4s-carrying cells eluted exclusively in fraction 9, showing that the StrepIIVirB4s complex was smaller than the StrepIIVirB4s-VirB8s and StrepIIVirB4s-VirB8sp complexes (Fig. 8*C*). As a control, StrepIIVirB8sp was analyzed and proved to be much smaller than StrepIIVirB4s complexes (Fig. 8*C*). Finally, to assess the stability of the StrepIIVirB4s-VirB8s complex, it was applied to a Superdex 75 column, which would permit the detection of VirB8s, if it dissociated from StrepIIVirB4s. Both StrepIIVirB4s and VirB8s were exclusively detected in the void elution volume, indicating a large and stable complex (Fig. 8*D*). Taken together, our experiments show that StrepIIVirB4s forms multimers, which constitutes direct evidence for the formation of the hexameric complex recently predicted based on a bioinformatics and modeling approach (40). In addition, it forms stable complexes with full-length VirB8s as well as with its periplasmic domain VirB8sp.

VirB5s Binds to T4SS Core Components VirB8s and VirB10s—After demonstrating the interaction between VirB4s and VirB8s, we next assessed the possibility that VirB8s may bind to VirB5s and thereby impact its incorporation into pili. We also analyzed the core components VirB9s and VirB10s because their *A. tumefaciens* homologs were previously shown to interact with VirB8 (13). First, we performed pull-down assays with differentially tagged VirBs proteins. To this end, the genes encoding the periplasmic domains of VirB5s, VirB8s, VirB9s, and VirB10s were cloned into pT7-7StrepII and pT7-H_6TrxFus, respectively, for overexpression and purification of StrepII- and H_6TrxA-tagged proteins. The different sizes of the tags enabled identification based on their molecular masses and recognition by specific antisera. StrepIIVirB5sp was mixed with H_6TrxAVirB5sp, H_6TrxAVirB8sp, H_6TrxAVirB9sp, and H_6TrxAVirB10sp, respectively, and streptactin-Sepharose beads were added to isolate the StrepII-tagged bait and the H_6TrxA-tagged preys. StrepIIVirB5sp pulled down H_6TrxAVirB8sp and H_6TrxAVirB10sp, but not H_6TrxAVirB9sp (Fig. 9*A*), suggesting interactions with these two core T4SS components. H_6TrxAVirB5sp bound non-specifically to the affinity matrix so that this assay could not be used to assess the self-interaction of this protein (data not shown).

As an alternative method, we applied the cross-linking agent disuccinimidyl suberate to StrepII-tagged VirBsp proteins and mixtures thereof, followed by SDS-PAGE and Western blot analysis. Application of disuccinimidyl suberate led to the formation of higher molecular mass complexes, indicating homomultimer formation (Fig. 9*B*). When mixtures with StrepIIVirB5sp were subjected to cross-linking, we observed changes of the cross-linking patterns. Novel complexes appeared when StrepIIVirB5sp was cross-linked in the presence of StrepIIVirB8sp and StrepIIVirB10sp (Fig. 9*B*). Similar to the results of the pull-down experiments, this indicated interactions with StrepIIVirB5sp (Fig. 9*B*).

Third, the regions encoding the periplasmic domains were cloned into pGADT7 (fusion to GAL4 activation domain) and pGBKT7 (fusion to GAL4 DNA binding domain), and their

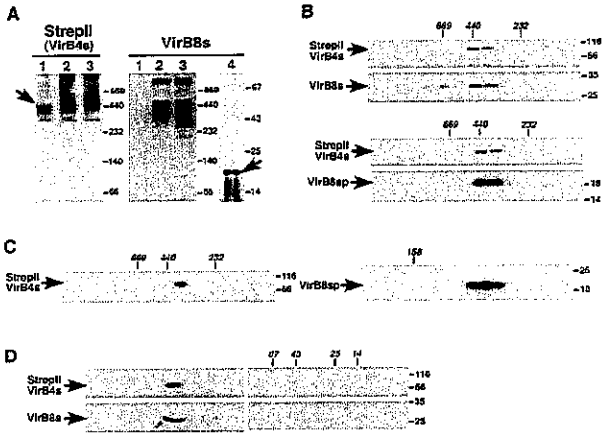

FIG. 8. **Blue native PAGE and gel filtration analysis of VirB4s oligomerization and complex formation with VirB8s.** T7 expression in *E. coli* GJ1158 carrying pT7-7StrepIIVirB4s (*lanes 1*), pT7-7StrepIIVirB4sVirB8s (*lanes 2*), pT7-7StrepIIVirB4sVirB8sp (*lanes 3*), and pT7-7StrepIIVirB8sp (*lane 4*), respectively. Tagged proteins were enriched via streptavidin-Sepharose followed by different fractionation techniques, SDS-PAGE, and detection with StrepII (tag)-specific or VirB8s-specific antisera. *A*, blue native PAGE analysis. *Arrows* indicate VirB4s and VirB8sp. *B*, Superdex 200 gel filtration of extracts from bicistron expressions (StrepIIVirB4s-VirB8s and StrepIIVirB4s-VirB8sp). *C*, Superdex 200 gel filtration of extracts from monocistron expressions (StrepIIVirB4s and StrepIIVirB8sp). *D*, Superdex 75 gel filtration of extracts from bicistron expression (StrepIIVirB4s-VirB8s). Molecular masses of reference proteins are shown on the *right* (in kDa).

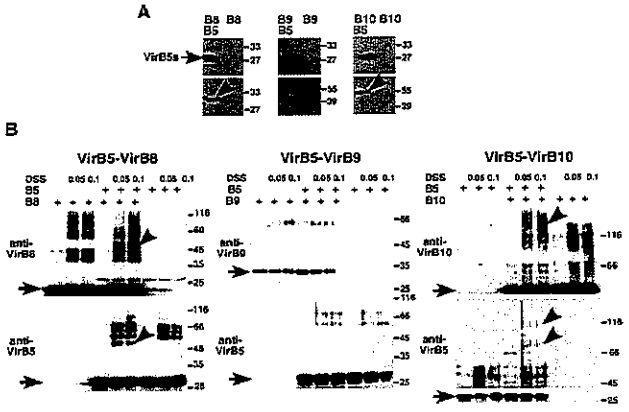

FIG. 9. **Biochemical experiments show interactions between periplasmic T4SS components.** Interactions between StrepII- and H_6TrxA-tagged *B. suis* VirBs proteins studied by pull-down and cross-linking; analyses were performed by SDS-PAGE followed by Western blotting. *A*, equimolar mixtures of StrepIIVirB5sp with H_6TrxAVirB8sp, H_6TrxAVirB9sp, and H_6TrxAVirB10sp were precipitated with streptavidin-Sepharose beads, followed by washing and analysis of bound proteins; *arrowheads* indicate H_6TrxAVirB8sp and H_6TrxAVirB10sp co-precipitated with StrepIIVirB5sp. *B*, StrepIIVirB5sp, StrepIIVirB8sp, StrepIIVirB9sp, and StrepIIVirB10sp were incubated alone and in equimolar mixtures with disuccinimidyl suberate (0.05 and 0.1 mM) prior to SDS-PAGE. *Arrows* indicate monomeric proteins; *arrowheads* indicate cross-linking products detected with one or both of the antisera. Molecular masses of reference proteins are shown on the *right* (in kDa).

interactions were tested using the yeast two-hybrid system. An interaction of the prey VirB5sp with the bait VirB8sp was shown by restoration of growth of the yeast strain AH109 on medium without adenine and on medium without histidine and by β-galactosidase activity (Table V). This approach also suggested self-interaction of VirB5sp. However, it did not show interactions with the baits VirB9sp and VirB10sp, and assays in the reverse order with VirB5sp as bait did not indicate interactions (data not shown). Nevertheless, we demonstrated the not previously reported VirB5sp-VirB8sp interaction with three independent methods, and two of those suggested that VirB5sp also interacts with VirB10sp.

DISCUSSION

In this study, we define the contribution of VirB4 to T4SS stabilization and pilus assembly via a VirB4-VirB8-VirB5-VirB2 interaction sequence. There is compelling evidence for the requirement of the Walker A nucleotide-binding site for virulence, but the enzymatic activity (presumably ATPase) has not been demonstrated. We show here that it is dispensable for T4SS stabilization and pilus assembly, suggesting that its role is to energize T-complex translocation. Similar to previous findings, we showed that VirB4 stabilizes VirB3, and here we reveal that it also stabilizes VirB8. The Walker A active site was not required for stabilization, and the *B. suis* homolog VirB4s fully complemented the *virB4* deletion CB1004. In accord with previous work, VirB4 was required for T-pilus formation, and VirB4 and VirB4s restored T-pilus formation in CB1004. In contrast to previous findings, however, the active site variants VirB4^{K439R} and VirB4s^{K464R} fully complemented T-pilus formation (22). This difference may be explained by the active site change we introduced, which differs from those in previous studies. The active site Lys was here changed to Arg, a conservative change, which is known to abolish the NTPase function of Walker sites but preserves active site structure and NTP binding (41, 42). In contrast, in most previous studies, the Lys residue was changed to Glu, Gln, or Met, or small deletions were introduced (8, 9, 22). These changes may cause substantial alterations of the conformation, and the amounts of some VirB4 variants were reduced, which may explain the effects on T-pilus formation. Because the change introduced here abolished virulence but not pilus assembly, we conclude that the NTPase activity is exclusively required to energize T-complex translocation, which is in accord with recent studies using a transfer DNA immunoprecipitation assay (26).

The fact that T-pilus assembly depends on VirB4 but not on its NTPase activity was surprising at first, and we further

TABLE V
Results of yeast two-hybrid assays

Prey vector	pGADT7-VirB5sp					pGADT7-VirB8sp					pGADT7-virB10sp	
Bait vector	pGBKT7-					pGBKT7-					pGBKT7-	
	-	virB5	virB8	virB9	virB10	-	virB5	virB8	virB9	virB10	-	virB5
–Ade[a]	–	+	+	–	–	–	–	+	+	+	–	–
–His[a]	–	+	+	–	–	–	–	+	–	+	–	–
lacZ assay[b]	–	+	+	–	–	–	–	+	–	+	–	–

[a] Growth on S.D.-media –Leu/–Trp without given supplements.
[b] Positive result, β-galactosidase activity indicated by blue colony color on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates.

dissected this process with biochemical methods. T-pili were not formed in CB1004, but the levels of VirB2 and VirB5 did not differ from C58, and they were extracted with similar efficiency from the membranes. Therefore, in contrast to VirB3 and VirB8, their stability is not reduced, and their membrane association is not affected in the absence of VirB4. We then analyzed the VirB protein subcomplexes. First, DDM extracts were separated by gel filtration. In our previous work, the separation had been conducted without detergent in the column buffer, which raised concerns about protein solubility upon dilution of the detergent (20). Here we conducted the separation in the presence of 0.03% DDM, which is higher than the critical micellar composition. Use of the modified procedure led to changes of VirB protein fractionation as compared with previous work. In the absence of DDM, VirB3, VirB6, and VirB8 had eluted exclusively with the high molecular mass core T4SS components. In the presence of 0.03% DDM, however, VirB3 eluted exclusively with VirB2 and VirB5 in the low molecular mass fraction, whereas VirB6 and VirB8 were equally distributed between the high molecular mass core complex and the low molecular mass complex with VirB2, VirB3, and VirB5. These results reflect the association between VirB proteins more appropriately because the inclusion of DDM prevents artifacts due to detergent dilution. Thus, VirB6 and VirB8 may link the core T4SS proteins to the pilus assembly complex of VirB2, VirB5, VirB7, and VirB3. VirB3 had previously been shown to localize to the outer membrane and to interact with VirB5, suggesting that it may indeed be part of the pilus assembly subcomplex (18, 19).

Analysis of extracts from the *virB4* deletion mutant further supported the notion that VirB6 and VirB7 link the core components and the pilus assembly subcomplex. In extracts from CB1004, VirB6 and VirB7 were exclusively detected in the high molecular mass fraction with VirB9 and VirB10. To our surprise, VirB2 and VirB5 were not detected in any of the fractions from CB1004 eluting from the Superdex 200 column. However, fractionation over a Superdex 75 column, which separates small proteins, showed that VirB2 and VirB5 eluted as dimers and monomers, respectively. Similarly, after separation of DDM extracts from CB1004 by blue native PAGE in gels of high acrylamide concentration, the pilus components were detected in small complexes. Complementation of CB1004 with plasmids expressing VirB4, VirB4s, or its active site variants restored the wild-type situation, showing that the NTPase activity is not required for the formation of VirB protein subcomplexes. Taken together, in CB1004, VirB2 and VirB5 did not co-fractionate with each other and with other VirB proteins. This may reflect a loss of interaction or a weakened interaction, which could be dissociated by native separation techniques. Our results show that VirB4, but not its NTPase activity, is essential for the formation of interactions between VirB2 and VirB5 and with VirB3, VirB6, VirB7, and VirB8.

The membrane fractionations did not show whether the effects of VirB4 on the stabilization of VirB3/VirB8 and on the VirB5-VirB2 interaction were direct or indirect. To address this question, we conducted *in vitro* experiments. When the genes encoding VirB4s and VirB8s were expressed as a bicistron in *E. coli*, the gene products formed a stable complex, and the periplasmic domain VirB8sp was sufficient. VirB4s therefore binds to and stabilizes VirB8s, which is in line with predictions by yeast two-hybrid analysis. In addition, analyses by gel filtration and blue native PAGE revealed that VirB4s forms multimers, and their size is in accord with the hexamers recently predicted based on a bioinformatics approach (40). Our results therefore support the notion that VirB4 proteins function as homo-hexameric complexes much like VirB11 and VirD4 (43, 44). We next analyzed whether VirB8sp or other core T4SS components interact with VirB5sp. Using pull-down and cross-linking experiments and yeast two-hybrid analysis, we showed that VirB5sp binds to VirB8sp as well as to VirB10sp. This suggests that VirB4-stabilized VirB8 impacts VirB5-VirB2 complex formation via its direct interaction with VirB5 and perhaps in concert with VirB10. Based on the data in this study, we propose a refined model of T-pilus assembly, which takes the contribution of VirB4 into account (Fig. 10).

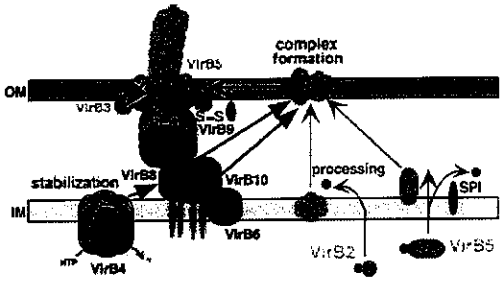

FIG. 10. **A model for T-pilus assembly in *A. tumefaciens* showing the impact of VirB4.** Signal peptidase I (*SPI*) removes signal peptides from precursors of the main pilus component VirB2 and the minor pilus component VirB5, followed by cyclization of VirB2 by an unknown cellular protein. Processed VirB2 and VirB5 subsequently associate with the membranes. VirB4 stabilizes VirB8, which binds to VirB5, possibly in concert with VirB10. Stabilized and properly oriented VirB5 forms a complex with VirB2, which is a key step in the formation of the pilus assembly subcomplex. We suggest that the effect of VirB4 on the localization and stability of VirB3 is mediated via VirB5 properly localized in the pilus assembly subcomplex.

VirB4 resides at the inner face of the cytoplasmic membrane, its short N-terminal domain is exposed to the periplasm (9), and it binds to and stabilizes VirB8 via its periplasmic domain. VirB8 binds to VirB5, which is stabilized by VirB6 (12). Stabilized and properly oriented VirB5 forms a complex with VirB2, which is a key step in the formation of the pilus assembly subcomplex. We have not directly assessed whether VirB4 or VirB8 binds to and stabilizes VirB3, which may also be a component of the pilus assembly subcomplex. Nevertheless, *B. henselae* VirB5 was shown to directly bind VirB3 (18), and we suggest that the effect of VirB4 on the localization and

stability of VirB3 is mediated via VirB5 properly localized in the pilus assembly subcomplex. The above model is supported by the results of the gel filtration experiments, which showed that in C58, VirB6 and VirB8 partly co-fractionated with the pilus assembly subcomplex. VirB6 and VirB8 therefore link the core components to the pilus assembly subcomplex. In the absence of VirB4, the key component VirB8 is not stabile, which leads to a loss of VirB5-VirB2 and VirB5-VirB3 interactions, the pilus assembly subcomplex does not form, and VirB6 and VirB7 re-distribute to the core complex. Taken together, the experiments presented here reveal several novel features of the T-pilus assembly process in *A. tumefaciens*, which are likely conserved in other T4SSs. They provide a concise explanation for the observation that VirB4 stabilizes the T4SS but that its presumptive ATPase activity is not necessary.

In addition, this work constitutes the basis for future experiments to study the activation of NTPase activity of VirB4. Despite efforts in different laboratories, nucleotide hydrolysis by this protein has not been conclusively demonstrated. A detailed biochemical analyses of purified VirB4 homologs TrbE from RP4 and TrwK from R388 showed that the purified proteins do not hydrolyze ATP or GTP (25). It is thus likely that hydrolysis depends on interaction(s) with other T4SS components or substrates. The bicistron approach we pursued here showed the interaction of VirB4s with VirB8s, and it will be interesting to assess whether VirB4s hydrolyzes nucleotides under these conditions. Similar approaches could be pursued to systematically study other putative VirB4 interaction partners, such as VirB11 and translocated substrates. This work may reveal in the future which of these interactions triggers the NTPase activity of VirB4.

Acknowledgments—We are indebted to David O'Callaghan (Nîmes, France) and August Böck (Munich, Germany) for continued support and discussions and to Patricia C. Zambryski (University of California, Berkeley, CA) for the communication of results prior to publication. We thank Klaus Schultes (McMaster University) for assistance with electron microscopic analyses.

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Chapter 7

Indirect forced localization of the *Agrobacterium tumefaciens* VirB5 to transient envelope destinations abolishes T-pilus elongation and type IV apparatus function

(Manuscript for submission as a short note)

Preface

This chapter consists of the following article to be submitted for publication:

Aly, K. A. and Baron, C. **Indirect forced localization of the *Agrobacterium tumefaciens* VirB5 to transient envelope destinations abolishes T-pilus elongation and type IV apparatus function**

I performed the experiments in this article. I generated and assembled the figures. Dr. Christian Baron has helped improving the display of data in all figures so that they become easier for the reviewer to understand. I wrote the manuscript and sent it to Dr. Baron who will provide his intellectual input and suggestions of manuscript changes in future.

This article describes a VirB5 variant that incorporates into the bacterial surface but without permitting T-pilus elongation. In this variant-producing strain, T4SS was nonfunctional as assessed by two different assays. It has been previously speculated that the T-pilus function might be limited to the T-pilus tips since these tips might be the point of interaction with both, prokaryotic and eukaryotic recipients. This study provides strong but indirect evidence that the filament portion of the pilus is also essential for the apparatus function. This is the first study to provide such evidence regarding the importance of the filament portion of the T-pilus in T4SS function and will contribute towards better understanding the T-pilus role during bacterial pathogenesis.

Title: Indirect forced localization of the *Agrobacterium tumefaciens* VirB5 to transient envelope destinations abolishes T-pilus elongation and type IV apparatus function

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Running title: Indirect forced localization of VirB5

Key words: *Agrobacterium*, type IV secretion, VirB proteins, immuno-EM.

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ABSTRACT

VirB2 and VirB5 are the major and minor components of the *Agrobacterium tumefaciens*-determined T-pilus. The T-pilus is a filamentous structure that facilitates the transfer of macromolecular effectors into recipient bacteria/eukaryotic hosts and thereby significantly contributes to Agrobacterial pathogenesis. To gain insights into the T-pilus elongation process and its effect on T4SS function, we engineered and overproduced several variants of the *Agrobacterium tumefaciens* T-pilus tip protein VirB5 to localize to different apparatus destinations. VirB5 with an altered signal sequence-processing site was specifically designed to associate with the outer membrane. In this variant, the sequence of the VirB5 leader peptide was specifically designed for recognition and hypothetical cleavage by signal peptidase II enzyme instead of signal peptidase I as in the case of wild-type VirB5. In the *virB5* deletion mutant CB1005 producing this variant (VirB5SP), gold grains detected VirB5SP on two cell surface locations on average versus nine surface locations in the case of VirB5 produced from the wild-type strain C58, as concluded upon the quantification of immuno-EM data. VirB5SP was also detected by Western blot analysis of Agrobacterial appendages sheared and sedimented by high-speed centrifugation. Alternatively, an inner membrane bound VirB5 through its amino- or carboxyl-terminus was engineered to localize to the periplasmic space. Under all conditions, T-pilus elongation was abolished and T4SS was non-functional as assessed by donor inter-bacterial conjugation and tumor formation on plants. Aside from the hypothetical importance of the T-pilus tips, this study provides documentation of the necessity of the filament portion of the T-pilus for T4SS function.

INTRODUCTION

Type IV secretion systems (T4SS) are conserved conjugation machineries often utilized to "share DNA information" between Gram-negative bacteria, a process regarded as a major contributor to microbial resistance to all families of antibiotics (Baron 2005; Juhas, Crook et al. 2007). In addition, T4SS are also coined as virulence tools employed by many pathogens to interfere with the physiological norm of their eukaryotic hosts and cause mild to severe infections (Rieder, Merchant et al. 2005; Pei, Wu et al. 2008). Due to the multifunctional nature of these machineries, they gained interest as potential drug targets (Savvides, Yeo et al. 2003; Baron 2006). In the model plant pathogen *Agrobacterium tumefaciens*, a trans-envelope T4SS apparatus assembles from 11 VirB proteins and VirD4. The VirB apparatus is composed of two distinct sub-complexes that do not co-fractionate as analyzed by Gel filtration of membrane proteins extracted with mild detergents (Krall, Wiedemann et al. 2002). The first sub-complex is of a high molecular mass of around 260 kDa, which constitutes the innermost compartment of the apparatus. It is believed that the functional forms of 6 proteins: VirB4, VirB6, VirB8, VirB10, VirB11 and VirD4 mainly reside at or associate with the inner membrane (Ward, Draper et al. 2002; Atmakuri, Cascales et al. 2004; Cascales and Christie 2004; Terradot, Bayliss et al. 2005; Draper, Middleton et al. 2006; Paschos, Patey et al. 2006). The second sub-complex is of a smaller mass of approximately 140 kDa and is predominantly composed of the outermost T-pilus proteins VirB2 and VirB5. Whereas our knowledge regarding the proteins involved in the T-pilus assembly process has been certainly advanced, the nature of the T-pilus elongation process, the theory around its initiation and its effect on T4SS function remain significantly elusive.

Individual VirB proteins may localize to multiple apparatus destinations as shown by an array of previous publications. For example, VirB1 is a lytic transglycosylase that shares significant sequence similarity to goose-egg white lysozyme (Llosa, Zupan et al. 2000). The *A. tumefaciens* VirB1 interacts with VirB4, VirB8, VirB10 and VirB11 (Ward, Draper et al. 2002). In addition, VirB1 from the mammalian pathogen *Brucella suis* has been shown to interact with core complex proteins VirB8, VirB9 and VirB11 (Hoppner, Carle et al. 2005). Collectively, research findings have substantiated the role of VirB1 as a muramidase that cleaves β-1,4-glycosidic bonds in the peptidoglycan cell wall polymer, resulting in the

formation of 1,6-anhydromuropeptides. This type of cleavage generated by VirB1 is believed to create openings in the bacterial cell wall (Mushegian, Fullner et al. 1996; Blackburn and Clarke 2001). VirB1 function is likely substantiated at the periplasmic section of the bacterial envelope. Contrary to the classical perception of VirB1 as a periplasmic transglycosylase, a C-terminal segment of VirB1 was found to be secreted into the extracellular environment (Baron, Llosa et al. 1997; Aly, Krall et al. 2008). VirB1 also co-fractionates with the outermost section of the VirB complex and may contribute to the T-pilus assembly process (Zupan, Hackworth et al. 2007). These results shed light on the presence of a periplasmic, outer membrane/T-pilus associated and externally secreted forms of VirB1. VirB1 may be assigned multiple functions that require its incorporation into different apparatus destinations, and this hypothesis likely applies to other VirB proteins such as VirB5. It has been established that VirB2 and VirB5 are the major and minor components of the Agrobacterial T-pilus. Recently, the association of VirB5 with the T-pilus tips has been directly confirmed (Aly and Baron 2007). VirB5 was additionally detected on the Agrobacterial cell surface. The tips of the T4SS appendage from the human pathogen *Helicobacter pylori* were also found to be enriched with the VirB5-like protein CagL (Kwok, Zabler et al. 2007; Backert, Fronzes et al. 2008). Moreover, a periplasmic form of VirB5 may also exist and likely contributes to the translocation of the Agrobacterial effectors into the external compartment of the VirB system (Cascales and Christie 2004). Taken together, these data assert that individual VirB proteins may localize, whether through independently functional forms or in a stepwise fashion, at multiple apparatus destinations presumably to fulfill several structural and/or functional activities.

Among the dynamics of the VirB system, the nature of the T-pilus elongation process and its effect on T4SS function have not been previously studied. It is not clear which form of VirB5 is required for T-pilus elongation or whether the T-pilus assembly is initiated at the inner or outer membrane. The T-pilus tip VirB5 may be required for host cell recognition and/or adherence but not necessarily for T-pilus elongation. Aside from the hypothetical importance of the T-pilus tip in host cell recognition and/or attachment, the remaining filament itself may not be required for T4SS function unless otherwise investigated. To gain further insights into these questions and to the importance of the T-pilus filament for T4SS function, we engineered and overproduced several variants of VirB5 that pause at different

VirB complex destinations, followed by detailed monitoring of such forced localization on both, T-pilus elongation as well as T4SS function.

MATERIALS AND METHODS

Cultivation of bacteria, strain and plasmid constructions

Cultures of *E. coli* JM109 for cloning experiments and *A. tumefaciens* for virulence gene inductions on AB minimal medium with acetosyringone (AS) and isopropyl-β-thiogalactopyranoside (IPTG) ad 0.5 mM for induction of plasmid-encoded genes were cultivated as described (Yuan, Carle et al. 2005). DNA manipulations followed standard procedures (Maniatis 1982.). *A. tumefaciens virB5* in pTrcB5 (Schmidt-Eisenlohr, Domke et al. 1999) were amplified by polymerase chain reaction with oligonucleotides (Tab. 1) and cloned downstream to an IPTG-inducible *trc* promoter carried on the expression vector pTrc200. The regions encoding N- and C-terminal membrane anchors of *B. suis* VirB10 were created by annealing and ligation of oligonucleotides into pTrc200, followed by PCR-amplification and cloning of the gene encoding the periplasmic domain of VirB5 resulting in pTrcB5N and pTrcB5C. The sequences of PCR-amplified genes were confirmed by DNA sequencing.

Analysis of T4SS functions: T-pilus isolation, conjugation and virulence assays

Assays for T4SS functionality (T-pilus isolation, conjugation and virulence assays) were performed as previously described (Höppner, Liu et al. 2004; Yuan, Carle et al. 2005).

SDS/PAGE and Western blotting

SDS-PAGE was conducted according to Laemmli (for proteins larger 20 kDa) or Schägger and v. Jagow (for proteins smaller 20 kDa) (Laemmli 1970; Schägger and von Jagow 1987). Western blotting was performed following standard protocols with VirB protein-specific antisera as described (Harlow and Lane 1988; Yuan, Carle et al. 2005).

Transmission electron microscopy (TEM) and immuno-EM

Negative staining for visualization of T-pili was conducted essentially as reported (Yuan, Carle et al. 2005). Virulence-induced *A. tumefaciens* were collected with 5 ml of 50 mM sodium potassium phosphate buffer, pH 5.5, and the optical density (OD_{600}) adjusted to 1.5–2. 10 µl samples were applied onto UV-sterilized 200 mesh carbon-coated formvar copper grids and air-dried. The grids were then stained with 1% phosphotungstic acid-0.01% glucose, pH 6, for 15 s prior to examination.

For immuno-EM, strains were cultivated on AB minimal medium agar plates as above, collected with liquid AB minimal medium supplemented with AS and the cell density was adjusted to OD_{600} of 0.25. 15 µl were applied onto UV-sterilized 200 or 300 mesh carbon-coated formvar nickel electron microscopy grids and cultivated for 10-12 h at 20°C in a humid chamber. The grids were then fixed for 1 h with 2% formaldehyde and 0.5% glutaraldehyde in 50 mM sodium cacodylate buffer at pH 5.5 as described (Jin, Hu et al. 2001). After fixation, the grids were subjected to immuno-gold labeling largely as described (Quintero, Busch et al. 1998). Grids were blocked with 5% skim milk in TBST (20 mM Tris/HCl, 137 mM NaCl, 0.1% Tween-20, pH 8) for 20 min, followed by incubation an a drop of 1:250 diluted anti-VirB5 in 5% skim milk and TBST for 45 min at room temperature. Grids were then washed 3x5 min, incubated with the secondary antibodies for 45 min at room temperature (1:10 diluted anti-rabbit 10 nm. gold conjugate, Sigma-Aldrich) in TBST with 5% skim milk, 0.1% BSA and 5% fetal bovine serum. Finally, the grids were washed and negative stained with 1% of phosphotungstic acid in 0.01% glucose (pH 6) for 15 s prior to examination. Images were recorded with a JEOL JEM-1200EX or JEOL 1200EX II transmission electron microscopes.

RESULTS

Forced periplasmic and outer membrane localization of VirB5 inhibits pilus elongation

Analysis of the amino acid sequence shows that VirB5-like proteins are overall hydrophilic. However, both VirB5 and its homolog TraC were shown to associate with the membranes and this is likely mediated by protein-protein interactions (Schmidt-Eisenlohr, Domke et al. 1999; Yeo, Yuan et al. 2003). To shed light on the correlation between membrane association and incorporation of VirB5 into T-pilus, we modified the *virB5* gene to direct production of fusion proteins with membrane-standing regions. The *Agrobacterium tumefaciens* VirB5N carried 58 amino acids of the *Brucella suis* VirB10 inner membrane anchor, which encodes one transmembrane helix and a short cytoplasmic domain. This anchor was fused to the N-terminus of the processed VirB5 form, which naturally incorporates into the outermost section of the VirB complex. VirB5C carried a similar membrane-standing region at its C-terminus. These modifications localized both proteins to the periplasmic space with a small inner membrane-anchoring domain, but their orientation was different. In contrast, VirB5SP carried the signal peptide from *A. tumefaciens* VirB7 directing lipoprotein modification by signal peptidase II and localization to the outer membrane (Fig. 1A) (Spudich, Fernandez et al. 1996; Baron, Thorstenson et al. 1997). Different constructs were subcloned into the expression vector pTrc200 and independently transformed into the *virB5* deletion mutant CB1005, followed by growth and induction of the *trc* promoter using 0.5 mM isopropyl-ß-D-thiogalactopyranoside (IPTG). Western blot analysis of cell lysates showed that the three different VirB5 variants were produced at levels comparable to the wild type VirB5 produced from strain C58, but VirB2 was not incorporated into T-pili (Fig. 1B). In accord with these findings, VirB5N and VirB5C were not detected in fractions containing extracellular high molecular mass structures. In contrast, VirB5SP was detected in these fractions, but the absence of VirB2 showed that T-pili were likely not formed (Fig. 1B).

The ability of the chimeric VirB5 proteins to function in T4SS was assessed by plant infection and donor bacterial conjugation assays

The T4SSs of strains producing the forced localization variants were subject to donor bacterial conjugation and plant infection assays largely as described (Höppner, Liu et al.

2004). Briefly and for the analysis of pLS1 donor activity, A348 and PC1005 carrying pTrc200 with and without VirB5 variant-encoding genes were co-cultivated with UIA143(pTiA6) recipient cells in a 1:5 ratio for 3 days on AB minimal medium agar containing 500 µM AS and 500 µM IPTG followed by plating on selective agar media (CAR, 150 µg/ml; ERY, 150 µg/ml) and quantitation of the transconjugant and donor cells. For plant infection assays, leaves of the *Kalanchöe diagremontiana* plant were wounded using 26.5 gauge needles followed by inoculation with bacterial cultures grown in AB minimal media supplemented with 0.5 mM IPTG when necessary. The T4SSs of strains producing the forced localization variants were non-functional in both assays as demonstrated by avirulence in tumor assay and their inability to transfer pLS1 (Fig. 2, Tab. 2 and Tab. 3). The impact of the forced membrane localization on pilus elongation into the cell exterior was analyzed next by TEM.

To investigate the location of VirB5 in A. tumefaciens virB5 deletion strain CB1005 producing the VirB5SP variant, cells were examined by negative staining and immuno-EM

VirB2 was not detected in fractions of extracellular high molecular mass structures and in accord with these results, we have not visualized T-pili assembled on CB1005 strain producing VirB5N, VirB5C or VirB5SP variants (Fig. 3A) upon TEM examination. To assess whether any of these VirB5 variants localize to the cell surface, we performed immuno-electron microscopy without cells permeation using VirB5-specific antiserum as primary antibody and goat anti-rabbit as secondary antibody conjugated to 10 nm gold grains (Sigma Aldrich). VirB5N and VirB5C were not detected on cell surface. In contrast, VirB5SP was detected on the surface, but in reduced amounts to 20% (approximately 2 gold grains per cell surface as compared to 9 in case of the wild-type VirB5 produced from virulence induced strain C58) (Fig. 3A and B). The localization of VirB5SP on the surface is in accord with the fact that it can be removed from the cells be shearing and sedimentation as part of a high molecular mass structure. The N-terminal lipoprotein modification perhaps anchors VirB5SP to the outer membrane and thereby preventing T-pilus elongation into the external environment.

DISCUSSION

The purpose of this study is to investigate the necessity of the T-pilus filament for T4SS function. It is currently thought that the T-pilus is composed of VirB2 filament and VirB5 tip. The T-pilus extends into the extracellular environment of Agrobacteria and may initiate contact with host cells. The significant advancement provided by this study was the ability to design and produce a VirB5 variant (VirB5SP) that can incorporate into the cell surface but without permitting T-pilus elongation (Fig. 3B). In this variant producing strain, VirB5 was labeled by gold grains on the cell surface. Thus, the tip portion of the T-pilus was surface exposed but the elongation of T-pili was not visualized by EM investigation. Futher characterization of this variant has allowed us to investigate the importance of the T-pilus filament for T4SS function. Strain CB1005 producing VirB5SP failed to conjugate with recipient bacteria or to incite tumors on the surface of *Kalanchöe diagremontiana* leaves. If the T-pilus function is limited to an exclusive role of its tip, then VirB5SP variant should be partially functional, which was not found to be the case upon conducting two different functional assays (Fig. 2). In these assays, *virB5* deletion strain producing VirB5N, VirB5C and VirB5SP variants failed to incite tumors on *Kalanchöe diagremontiana* leaves and did not mediate donor inter-bacterial conjugation. The VirB5SP variant has incorporated into the outermost section of the apparatus as indicated by immuno-EM analysis. However and throughout the translocation process into the cell surface, TEM visualization have not provided any evidence for T-pilus elongation. This may be attributed to the hypothesis that T-pilus elongation may initiate at the cell surface. Since VirB5SP was designed to anchor to outer membrane lipids, this interaction may have likely prevented T-pilus elongation into the cell exterior. In accord with this hypothesis and if the initiation of the T-pilus elongation normally takes place anywhere between the inner and outer membranes, this would have been recorded in the form of the assembly of any -even short pili- which was not found to be the case. Western blot analysis of high molecular mass structures sheared and sedimented by ultracentrifugation from all variant-producing strains did not indicate the presence of signals of the major T-pilus protein, VirB2.

In addition, TEM analysis did not provide evidence for T-pilus assembly on the surface of CB1005 strain producing the VirB5N, VirB5C and VirB5SP variants. This

interpretation also matches with the fact that VirB5N and VirB5C variants anchored to the inner membrane through their N- or C-terminus did not lead to T-pilus elongation.

In wild-type T-pilus, VirB5 represents the cap that is directly/indirectly linked to the VirB2 filament. However and in case of VirB5SP, VirB2 was not incorporated into high molecular mass structures (Fig. 1). We do not anticipate that the minor modification of VirB5 N-terminal sequence of this variant has contributed to such result. This is because of two reasons: first, the VirB5 leader sequence processing site in this variant identically resembles that found in VirB7. VirB7 has been previously shown to co-fractionate with T-pilus protein components regardless of its association with outer membrane lipids. Thus, similar modification in VirB5 would not likely cause drastic changes in the assigned role or VirB2 incorporation into high molecular mass structures. Second, VirB5SP level of stability in cell lysates was largely comparable to wild-type VirB5 level of stability (Fig. 1). Thus, it is unlikely that VirB5SP has undergone a conformational change that may have affected its stability in cells or prevented VirB2 from integration into the outer leaflet of the bacterial envelope. VirB2 failure to incorporate into these structures can be explained in the context that VirB2/VirB5 interaction may take effect directly before the initiation of the T-pilus elongation at the bacterial cell surface. VirB5SP was anchored to the outer membrane, leading to the blockage of T-pilus elongation, the early stages of which may require direct interaction between VirB2 and VirB5.

In a previous publication, we found that VirB4, the largest T4SS component is required for VirB2/VirB5 co-fractionation into the T-pilus pre-assembly sub-complex (Yuan, Carle et al. 2005). Taken together and based on the findings presented here, we hypothesize a model of T-pilus elongation (Fig. 4). VirB4 is required to mediate VirB2/VirB5 co-migration into the cell surface. This co-migration hypothesis is suggested based on VirB4 requirement for VirB2/VirB5 co-fractionation as previously shown by Gel filtration analysis (Yuan, Carle et al. 2005). However, co-migration or co-fractionation of both proteins does not dictate direct interaction. VirB5 becomes surface exposed and this has been concluded from previous studies where VirB5 was labeled by gold grains on both, T-pilus tips as well as on the bacterial cell surface (Aly and Baron 2007). Upon surface exposure, VirB2 and VirB5 may initiate a state of direct interaction, followed by T-pilus elongation into the cell exterior where

VirB5 remains incorporated into T-pilus tips. This condition may subsequently recruit more units of VirB2 to the base of the T-pilus in a fashion that adds to the T-pilus length from its base until the elongation process becomes terminated under largely unknown circumstances.

Whereas we understand that this model may be speculative, this study has certainly advanced our knowledge regarding the importance of the T-pilus filament for T4SS function and also provided important insights that led to hypothesizing a model of the initial stage of T-pilus elongation. Future studies will directly address the nature of VirB2/VirB5 interaction and provide more details regarding the biochemical and biophysical basis of T-pilus elongation.

Acknowledgements

This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC grant: 262104) the Canada Foundation for Innovation (CFI) and the Ontario Innovation Trust (OIT) to C.B.

Figure Legends

Fig. 1: Analysis of VirB5 forced membrane localization variants.
(A) Schematic presentation of the designed VirB5 variants. **(B)** Wild type strain C58, CB1005 (Δ*virB5*) and CB1005 complemented with pTrc200 expressing VirB5 membrane localization variants were grown on AB minimal medium under virulence-inducing (+AS) and non-inducing (-AS) conditions. IPTG (0.5 mM) was added to induce transcription of pTrc200-encoded *virB5* genes. Samples from subcellular fractions were separated by SDS-PAGE, followed by Western blot analysis with VirB2- and VirB5-specific antisera. C58 -AS (1), C58 +AS (2), CB1005 (3), and CB1005 carrying pTrc200 (4), pTrcB5 (5), pTrcB5N (6), pTrcB5C (7), pTrcB5SP (8). Arrowhead indicates localization of VirB5SP in extracellular high molecular mass fraction. Numbers on the right indicate molecular masses of reference proteins.

Fig. 2: (A) Analysis of tumor formation after wounding and infection of *K. diagremontiana* plants. **(B)** Quantitation of plasmid transfer pLS1 donor activity. Donor strains A348 and complemented PC1005 carrying pLS1 were co-cultivated with recipient *A. tumefaciens* UIA143 (pTiA6) for three days in the presence of IPTG under virulence gene-inducing conditions, followed by the quantitation of pLS1-carrying recipients (TC/D = transconjugants per donor). Error bars indicate standard deviations derived from three independent experiments.

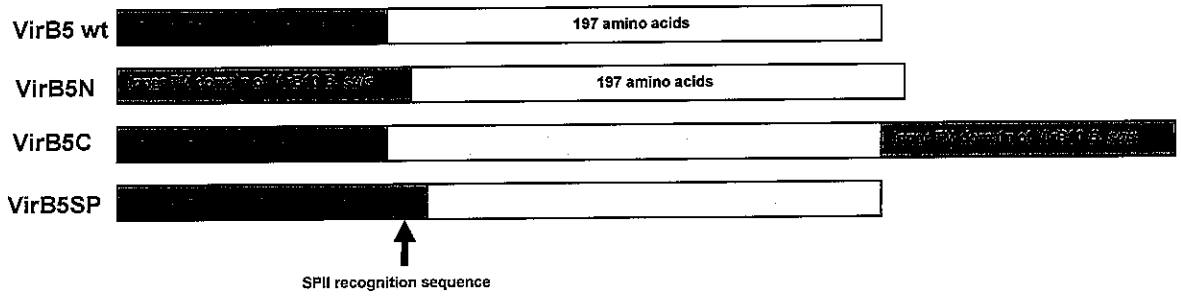
Fig. 3: Electron microscopic analysis of the localization of VirB5N, VirB5C and VirB5SP on the surface of CB1005 cells. Strains CB1005 carrying pTrcB5N (N), pTrcB5C (C) or pTrcB5SP (SP) were cultivated on AB minimal medium plates at 20°C for three days under virulence-inducing conditions in the presence of 0.5 mM IPTG. **(A)** Electron microscopic analysis of cells by negative staining or immuno-detection with VirB5-specific primary and 10 nm gold-coupled secondary antiserum. Arrows indicate gold grains on the cell surface and size bars indicate 100 nm. **(B)** Quantitation of gold grains on the cell surface as compared to the wild type. The average number of gold grains on 60 cells from three different virulence induction experiments per strain was determined.

Fig. 4: Model of T-pilus elongation. VirB4 is required to mediate VirB2/VirB5 co-migration into the cell surface. Upon cell surface exposure, VirB2 and VirB5 may initiate a state of direct interaction, followed by T-pilus elongation into the cell exterior where VirB5 remains incorporated on the T-pilus tips. This condition may subsequently recruit more units of VirB2 to the base of the T-pilus in a fashion that adds to the T-pilus length from its base.

FIGURES

Fig. 1

A



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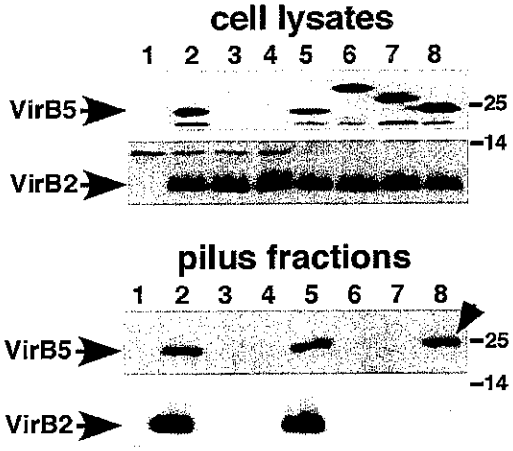
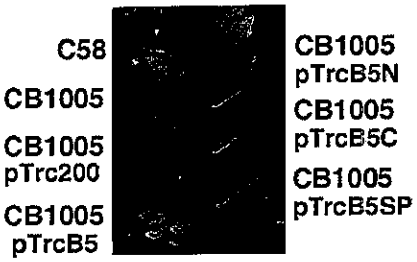


Fig. 2

A



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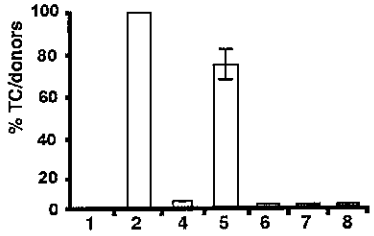
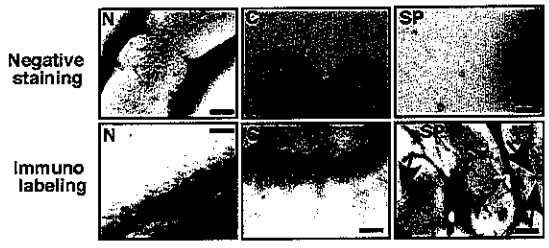


Fig. 3

A



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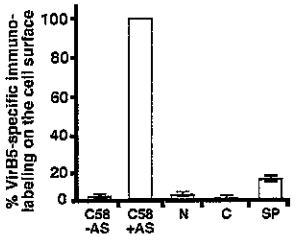
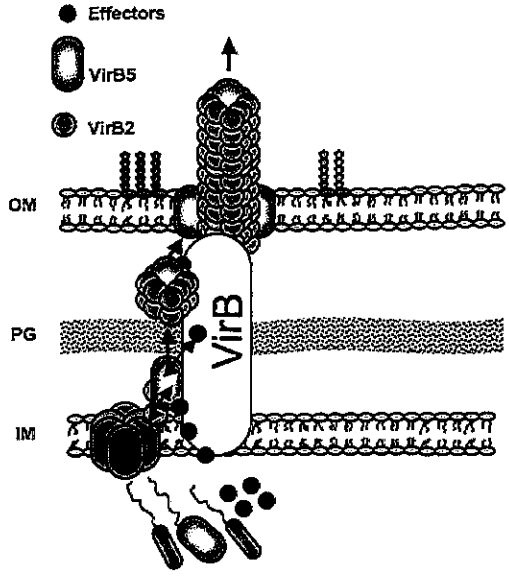


Fig. 4



TABLES

Table 1: Oligonucleotide sequences and constructed plasmids

Name/ restriction	Sequence*	Constructed plasmid
VirB5-5'/AflIII	5'-CCACATGTCGATCATGCAACTTGTTGC- 3'	pTrcB5
VirB5-3'/ScaI	5'-GAAAGTACTCAGGGGACGGCCC- 3'	
VirB10sN-5'/AflIII	5'-CCACATGTCGCAGGAAAACATTCCGGTGCAGC- 3'	pTrcB5N
VirB10sN-3'/EcoRI	5'-GCGAATTCATTGCCCCTCATGTGAAACACG- 3'	
VirB5N-5'/EcoRI	5'-CCGAATTCCAGTTCGTTGTCAGCGATCCG- 3'	
VirB5N-3'/HindIII	5'-CCAAGCTTTCAGGGGACGGCCC- 3'	
VirB5C-5'/AflIII	5'-CCACATGTCGATCATGCAACTTGTTGC- 3'	pTrcB5C
VirB5C-3'/PstI	5'-GGCCTGCAGGGGGACGGCCCCAAAG- 3'	

VirB10sC-5'/PstI	5'-CACTGCAGAGGATGCACGTGTTGCTCTTTCTCTTTGTC GTGGGCTTCAT- 3'	
VirB10sC-3'/HindIII	5'-CCAAGCTTAGCGGGTGCGCTTAAACACGAGCAGCCA CAGCAGCAC-3'	
VirB5SP-5'/AflII	5'CCACATGTCGATCATGCAACTTGTTGCTGCGGCCATGGCC GTCAGCCTTCTTGGGGGGTGCCAGTTCGTTGTCAGCGATCC GGCGAC- 3'	pTrcB5SP
VirB5SP-3'/HindIII	5'-CGAAGCTTTCAGGGGACGGCCCCAAAGATGACCGCA GTCTTTTGATGCTCCTTACGTTGAGCGGCC-3'	

*Restriction sites used for cloning are underlined

Table 2: Summary of results

Variant	Stability	Pilus incorporation	Immuno EM	pLS1 transfer	Virulence
VirB5 wt	+++	+++	tip	+++	+++
VirB5N	+++	-	-	-	-
VirB5C	+++	-	-.	-	-
VirB5SP	+++	-	-	-	-

Table 3: Analysis of pLS1 transfer from *A. tumefaciens* donors A348, PC1005 and complemented derivatives into *A. tumefaciens* UIA143 pTiA6

Donors pLS1	Recipient	Donors ($x10^6$)	TC	TC/donor	TC/donor (%)	*SD (+/-)
A348	UIA pTiA6	48	18900	$3.93x10^{-4}$	100	0
PC1005 pTrc200	UIA pTiA6	52	300	$5.7x10^{-6}$	1.45	0.69
PC1005 pTrcB5	UIA pTiA6	57	16800	$2.95x10^{-4}$	75.06	4.28
PC1005 pTrcB5N	UIA pTiA6	62	100	$1.6x10^{-6}$	0.41	0.04
PC1005 pTrcB5C	UIA pTiA6	65	300	$4.6x10^{-6}$	1.17	0.32
PC1005 pTrcB5SP	UIA pTiA6	57	100	$1.7x10^{-6}$	0.43	0.01

* SD = standard deviation from 3 repetitions

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Chapter 8
DISCUSSION

The *A. tumefaciens* VirB5 protein is required for the assembly of the T-pilus. The T-pilus is a filamentous structure which extends into the extracellular environment. It is believed that the T-pilus contributes to bacterial pathogenesis by mediating the initial stages of host cell contact. The objective of my thesis was to understand the role of VirB5 during the T4SS process. The location of VirB5 in the T-pilus and its role in agrobacterial pathogenesis were long-standing questions in T4SS research. Previous findings have systematically established the hypothesis that VirB2 is the major component of the T-pilus and that VirB5 may be a minor T-pilus protein. This hypothesis lacked direct evidence such as immuno-EM labeling of both proteins, followed by direct visualization. To this end, I have performed a collection of experiments to investigate the location of VirB5 in the T-pilus and further study its role in the virulence of *A. tumefaciens*.

8.1 VirB5 is exposed on the bacterial surface

We attempted to determine the localization of VirB5 in the T4SS complex from the plant pathogen *A. tumefaciens*. We first artificially forced VirB5 to localize to various subcellular compartments by implementing a systematic method of VirB5 fusion to an inner trans-membrane segment from the *B. suis* VirB10 protein (**Chapter 7**). Fusion of that domain to the N- or the C-terminus of VirB5 was followed by detailed analysis of T4SS function. Inner membrane-bound VirB5 failed to contribute to T-pilus assembly, tumor formation on plants or inter-bacterial conjugation. We concluded that the location of VirB5 in the T4SS complex may not be associated with the inner membrane. Next, we attempted to localize VirB5 to the bacterial outer membrane by modifying its signal sequence processing site (VirB5SP). In the VirB5SP variant, the signal peptide cleavage site was designed for recognition by the bacterial signal peptidase II enzyme instead of signal peptidase I. The resulting VirB5 variant was likely cleaved by signal peptidase II enzyme which is specializes in cleaving outer membrane associated lipoproteins (**Chapter 7**). Whereas this variant served as an excellent tool to understand the biological significance of the filament portion of the T-pilus during the T4SS process, it revealed that this form of VirB5, which is forced to localize to the bacterial surface remains incompetent in terms of its failure to contribute to T-pilus

assembly or T4SS virulence. We concluded that VirB5 may be very likely associated with the T-pilus itself and that a T-pilus associated form is required for a functional T4SS.

Our next systematic step was to use an immuno-EM approach to localize VirB5 in the T-pilus. My results confirmed that VirB5 localizes at the end of broken pili, T-pilus tips as well as on the bacterial cell surface (**Chapter 3**). The cell surface associated form of VirB5 may be explained as an early growing T-pilus. These results provide strong evidence which supports previous hypotheses of VirB5 surface exposure and association with conjugative and pathogenic pili (Yeo, Yuan et al. 2003) (Schmidt-Eisenlohr, Domke et al. 1999).

8.2 VirB2 is detected along the entire T-pilus length

Immuno-EM analysis confirmed that VirB2 is the major component of the T-pilus. Gold labeled antibodies detected VirB2 along the entire length of detached T-pili but not on cell bound ones (**Chapter 3**). Lack of VirB2 detection by gold lebeled antibodies in cell bound T-pili represents an interesting finding that might be followed upon in future studies. One possible explanation is that unlike detached pili, cell-bound pili may be tightly compressed in a fashion which renders the VirB2 epitope inaccessible. Cryo-EM reconstruction studies in future should reveal major structure variations between cell-bound T-pili and detached ones. Nevertheless, this finding provided the first direct documentation of the appendage composition in any T4SS.

8.3 Possible models of VirB2/VirB5 interaction in the T-pilus

VirB4 is required for VirB2/VirB5 incorporation into the T-pilus pre-assembly sub-complex (Yuan, Carle et al. 2005). This finding together with the direct evidence of my immuno-EM results revealing that VirB2 and VirB5 are the major and minor T-pilus proteins, led to hypothesizing two different models of VirB2/VirB5 incorporation into T-pili. In the first model (Fig. 1), VirB2-VirB5 interaction is mediated by VirB4 in the bacterial inner membrane. Several units of VirB2 may interact with one or more units of VirB5 leading to the assembly of one T-pilus "block". Many blocks migrate to the bacterial surface where they interact under certain condition(s) prior to the T-pilus elongation process into the cell exterior.

Based on this model, the T-pilus must be composed of many of VirB2/VirB5 building blocks. We do not anticipate this model to represent the most accurate description of the T-pilus assembly process for two reasons. First, this model implies the presence of large amounts of VirB5 in pili, which is not in accord with our observations that VirB5 is a minor T-pilus protein. Second, VirB5 likely plays a role as an adhesin through one of 3 different alternatives: 1- As an adhesin required for the linkage of T-pilus blocks during the T-pilus assembly process, 2- As an adhesin required for binding several units of the major T-pilus component VirB2 and 3- As an adhesin required for the detection of receptors on the host surface. It is unlikely that VirB5 carries out all these functions, especially due to its relatively short amino acid sequence number (220 amino acids), simple structure, no reports regarding its post-translational modification, and due to the absence of domains that belong to any families of adhesion proteins.

The fact that VirB5 was found to associate with the T-pili suggests its role as an adhesin likely required for the detection of the recipient during T4SS function. This hypothesis is in accord with the second possible role of VirB2/VirB5 in T-pilus assembly. In the second model (Fig. 2), several units of VirB2 may interact with one or more units of VirB5 leading to the formation of a T-pilus building block. This block migrates to the cell surface and VirB5 contributes to the process of T-pilus elongation into the extracellular environment. The T-pilus elongation process recruits more VirB2 subunits to the pilus base where the elongation process remains constantly activated. The T-pilus elongation process eventually terminates by an unknown mechanism. Factors contributing to T-pilus elongation or determining the termination of this process remain largely unknown and consequently, it is not known if the T-pilus elongation rate becomes significantly lower near the end of the elongation process.

Figure 1. First possible model of VirB2/VirB5 interaction. VirB4 mediates VirB2/VirB5 interaction near or at the inner membrane. Upon interaction, VirB2 and VirB5 assemble a T-pilus building block. Many of these blocks polymerize leading the elongation of a filamentous T-pilus structure.

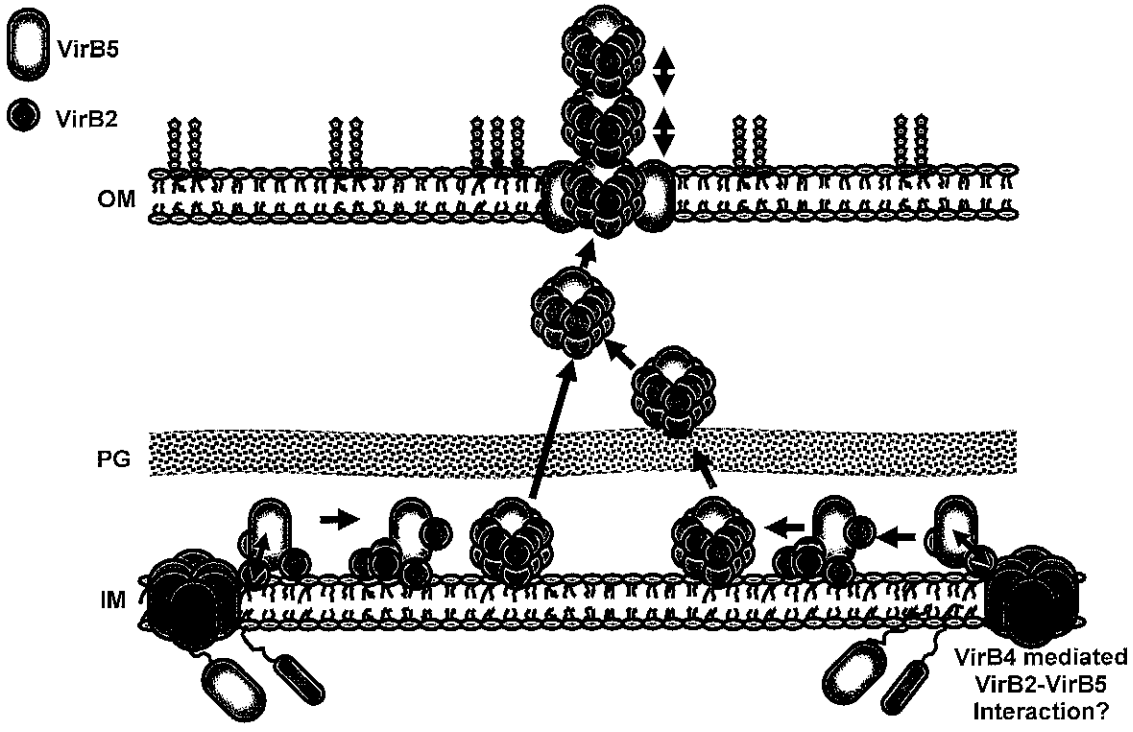
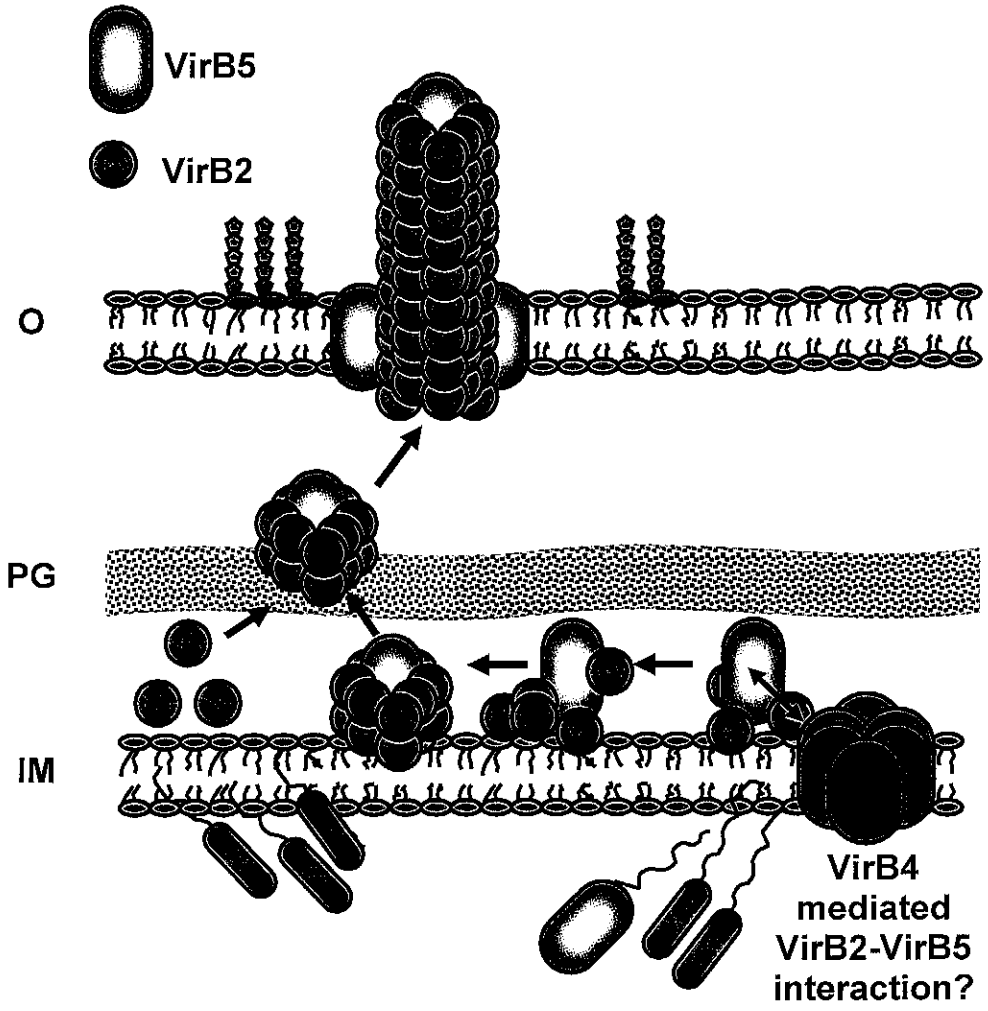

VirB5
VirB2
OM
PG
IM
VirB4 mediated
VirB2-VirB5
Interaction?

Figure 2. Second possible model of VirB2/VirB5 interaction. VirB4 mediates VirB2/VirB5 interaction near or at the inner membrane. Upon interaction, VirB2 and VirB5 assemble a T-pilus leading block. Upon reaching the bacterial outer membrane, VirB5 initiates the process of T-pilus elongation into the extracellular environment followed by recruiting more VirB2 units to the T-pilus base.


VirB5
VirB2
O
PG
IM
VirB4
mediated
VirB2-VirB5
interaction?

8.4 VirB5 significantly contributes to Agrobacterial pathogenesis

A different area of preliminary research employed a gel overlay assay together with gradient ammonium sulfate precipitation to detect a VirB5 interaction partner from *A. tumefaciens*. Mass Spectrometry analysis identified that this protein is the *trans*-zeatin synthesizing enzyme (Tzs). Research on cytokinins in plants suggests that Tzs is a host range factor that might be required for infecting specific plant hosts. In accordance with this hypothesis, some genome sequences such as the sequence of the octopine *A. tumefaciens* strain A348 do not encode *tzs* genes. However, the nopaline strain C58 encodes *tzs* on its genome. Tzs catalyzes the last step of the biosynthesis of the *trans*-zeatin ribotides inside *A. tumefaciens*. The ribotide products may contribute to pathogenesis by increasing the efficiency of T-DNA translocation into plant hosts. Following up on these preliminary findings (**Chapter 4**), I found that similar to T4SS induction conditions, Tzs production also requires the presence of acetosyringone in bacterial minimal media. This provided a clue that Tzs function might be linked to that of T4SS. Next, I decided to analyze the possibility that Tzs can be sedimented together with sheared agrobacterial appendages (**Chapter 4**). I unexpectedly detected significant amounts of Tzs in sheared and sedimented T-pili. This finding switched our focus to the possibility that Tzs might localize to the bacterial surface. This was an unexpected finding due to the fact that Tzs has been regarded for a long time as a soluble protein that might exclusively reside in the bacterial cytosol.

Immuno-EM analysis confirmed my previous observations and results revealed that Tzs localizes on the bacterial surface. Tzs is detected over the entire bacterial surface and may contribute to bacterial recognition of specific hosts. Whereas *virB5* deletion leads to extremely attenuated Tzs surface exposure, this process likely depends on other T4SS proteins. In case of *virB2* and *virB8* deletion mutants, I also found significant alterations in Tzs surface exposure. We concluded that whereas VirB5 may be required for the extracellular localization of Tzs, this process is likely T4SS-dependent.

Blue Native Electrophoresis analysis of Tzs co-fractionation with various membrane proteins revealed remarkable alterations in the case of *virB5* deletion mutant when compared to the wild-type pattern of association with membrane protein complexes. Taken together, my results published in that article have enriched our understanding regarding the role of VirB5

during the T4SS process and that it might be involved in multiple processes other than T-pilus assembly and/or elongation.

8.5 Recipient assay for the evaluation of *B. suis* T4SS

B. suis is a mammalian pathogen which employs T4SS for intracellular survival in mammalian hosts. The role of T4SS during *Brucella* virulence was debatable for an extended period of time. This is because many Gram-negative pathogens carry sets of T4SS in a cryptic form on their genomes. Some convincing studies have shown that in the absence of T4SS, the intracellular survival of *Brucella* species in mammalian hosts is severely attenuated (Boschiroli, Ouahrani-Bettache et al. 2002). Subsequently, other studies have confirmed that replacing functional VirB proteins from some pathogens with their orthologs from the *Brucella* T4SS did not remarkably affect the intrinsic T4SS function. However, a functional assay for the *Brucella* T4SS has been long waited for in T4SS research.

An assay was developed by former students in the Baron laboratory to characterize the *B. suis* T4SS function by testing its contribution to recipient competence of plasmid transfer in *A. tumefaciens* (**Chapter 5**). To this end, a recipient *A. tumefaciens* strain expressing the *B. suis* T4SS was incubated with a donor *A. tumefaciens* strain expressing wild-type *A. tumefaciens* T4SS. I found that the recipient strain was partially functional in acquiring an IncQ plasmid from the donor strain. This finding strengthens the notion that the *B. suis* T4SS is not cryptic and that its function can be studied upon expression in heterologous hosts (**Chapter 5**). I also found that recipient competence can be maximized when *virB2* was deleted from the *B. suis virB* operon. One possible interpretation of such a result can be understood in the context that in the absence of VirB2, a lack of membrane protection allows conjugative plasmids to translocate from recipient into donor cells in higher efficiency (**Chapter 5**).

To test this hypothesis, cells were grown in the presence of a toxic detergent (sodium lauryl sulphate) (SDS). Growth of agrobacterial cells expressing the *B. suis* T4SS in low concentrations of SDS was negatively affected when the *virB2* gene product was absent. This might also explain that many of the essentially structural *B. suis* VirB proteins are secreted

into the supernatant. This shows the lack of tight control over the integrity of bacterial membranes when VirB2 is not produced (**Chapter 5**).

These data hypothesize that VirB2 spans both bacterial membranes and will open future area of research by trying to investigate the depth of the T-pilus (VirB2) inside agrobacterial cells. This is also considered a long-standing question in T4SS research.

8.6 Concluding remarks and outlook

In summary, this thesis provided significant advancement in the area of T-pilus assembly and the role of VirB5 during the T4SS process. I was able to document the appendage structure for the first time in any T4SS by immuno-EM analysis. The location of VirB5 in the apparatus has been a long-standing question in the field. By identifying various locations of VirB5 in the apparatus, I have advanced that area of research and possible applications of my thesis findings are numerous. Aside from the location of VirB5 in the T4SS apparatus, I also found that VirB5 contributes to Agrobacterial pathogenesis by interacting with the host range factor Tzs. In *virB5* deletion mutant CB1005, Tzs integration into the cell surface was strongly reduced. This finding may provide an explanation behind the avirulence of the T4SS *virB* deletion mutants, since factors other than the VirB system and its effectors might be involved in bacterial pathogenesis.

Taken together, my research achievements have hopefully met with the expectations from a Ph.D. student. Novel results introduced in my thesis will hopefully inspire future Graduate students to continue the path of critical thinking to follow upon these findings.

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