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.

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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 cofractionates 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 μ l 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 μ l 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 OD₆₀₀ 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 ($\Delta virB5$) 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.

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

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.

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 staining 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 diagraphmontiana* 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. diagraphmontiana* 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. diagraphmontiana* 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

Table 1. Analysis of pLS1 transfer from *A. tumefaciens* donors A348, *virB5* deletion strain PC1005 and complemented derivatives into *A. tumefaciens* UIA143 pTiA6

The SD was based on three repetitions; TC, transconjugants.

Donors pLS1	Recipient	Donors ($\times 10^6$)	TC	TC/donor	TC/donor (%)	SD
A348	UIA pTiA6	73	18 400	2.52×10^{-4}	100	0
PC1005 pTrc200	UIA pTiA6	53	1400	2.64×10^{-5}	10.5	2.7
PC1005 pTrcB5	UIA pTiA6	65	8870	1.36×10^{-4}	54	10.1
PC1005 pTrcB5Δ1	UIA pTiA6	35	1070	3.1×10^{-5}	12.3	2.1
PC1005 pTrcB5Δ2	UIA pTiA6	57	1000	1.75×10^{-5}	7	2.4
PC1005 pTrcB5Δ3	UIA pTiA6	48	730	1.52×10^{-5}	6	0.5
PC1005 pTrcB5Δ4	UIA pTiA6	42	12 000	2.86×10^{-4}	113.5	20.5
PC1005 pTrcB5Δ5	UIA pTiA6	47	600	1.28×10^{-5}	5.1	1.1

procedure. This observation opened the possibility that the epitope recognized by the antibodies may be accessible in detached T-pili. To assess this possibility, we analysed T-pili isolated from the cells by shearing and ultracentrifugation. In isolated T-pili, we detected gold grain label along the entire length (Fig. 5c, d) of 96 % of the T-pili isolated from strain C58, and at slightly lower levels on T-pili from CB1005 expressing the VirB5Δ3 and VirB5Δ4 variants (Supplementary Table S1). Gold grains were exclusively detected on detached (sheared) T-pili and not on cell-bound pili or flagella. The results were similar in the wild-type strain C58 (Fig. 5a–d) and in the *virD4* deletion variant CB2004 (Fig. 5e). These results indicate that the accessibility of the epitope recognized by the VirB2-specific antiserum differs between cell-bound and detached T-pili, and that the coupling of substrates mediated by VirD4 does not impact this phenomenon.

Immuno-EM detects VirB5 on the cell surface and on the T-pilus tips

We next performed immuno-EM analysis with a VirB5-specific antiserum and 10 nm gold-labelled secondary antiserum, and detected the label in three different locations: on the cell surface, on the tips of T-pili, and on the ends of T-pili isolated by shearing and ultracentrifugation. Quantification of our observations revealed that an average of nine gold grains per cell was detected on

the surface, showing that in contrast to VirB2, cell-bound VirB5 was readily accessible to antibodies (Fig. 6a). Gold labels on the T-pilus tips were detected on 26 % of the T-pilus-carrying wild-type cells (Fig. 6b, c) and they were also detected at a similar percentage on the ends of T-pili isolated by shearing and ultracentrifugation (Fig. 6f, Supplementary Table S1), but not along the length of T-pili. Similar observations were made in the *virD4* deletion strain CB2004 (Fig. 6g), showing that coupling of the substrate to the T4SS does not impact the localization of VirB5. Controls with non-induced or virulence-induced cells incubated without primary or secondary antibodies showed that the detection of VirB5 was specific (Supplementary Fig. S3A–D). Also, using specific antisera and immuno-EM analysis as above, we did not detect VirB1 or VirB7 on the cell surface and on the T-pili (results not shown). In the case of these two proteins, partial exterior localization has previously been demonstrated (Baron *et al.*, 1997; Sagulenko *et al.*, 2001), and we concluded either that they are not present on the surface and in the T-pili or that the epitopes were not accessible to the antisera under the conditions used here.

In the experiments described above, VirB5 variants with shortened C termini proved to be particularly interesting, as these changes impacted pilus length as well as gene transfer to different hosts. To assess whether VirB5 also localized on the tips of these pili, we analysed CB1005

Table 2. Analysis of pLS1 transfer from *A. tumefaciens* donors A348, *virB5* deletion strain PC1005 and complemented derivatives into *A. tumefaciens* UIA143 pTiA6

The SD was based on three repetitions; TC, transconjugants.

Donors pLS1	Recipient	Donors ($\times 10^6$)	TC	TC/donor	TC/donor (%)	SD
A348	UIA pTiA6	48	18 900	3.93×10^{-4}	100	0
PC1005 pTrc200	UIA pTiA6	52	300	5.7×10^{-6}	1.5	0.7
PC1005 pTrcB5	UIA pTiA6	57	16 800	2.95×10^{-4}	75.1	4.3
PC1005 pTrcB5AAA	UIA pTiA6	47	200	4.2×10^{-6}	1.1	0.3
PC1005 pTrcB5AVA	UIA pTiA6	52	100	1.9×10^{-6}	0.5	0.04
PC1005 pTrcB5AAP	UIA pTiA6	56	15 300	2.72×10^{-4}	69.2	11.1

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.

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

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).

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 pilus 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 (VirB5 Δ 4 and VirB5AAP) did complement the defect of a *virB5* deletion strain in transfer of the IncQ plasmid pLS1 to recipient agrobacteria. The fact that VirB5 Δ 4 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.

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