Molecular characterization of the *Agrobacterium tumefaciens* DNA transfer protein VirB6

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The VirB proteins of *Agrobacterium tumefaciens* assemble a T-pilus and a type IV secretion (T4S) apparatus for the transfer of DNA and proteins to plant cells. VirB6 is essential for DNA transfer and is a polytopic integral membrane protein with at least four membrane-spanning domains. VirB6 is postulated to function in T-pilus biogenesis and to be a component of the T4S apparatus. To identify amino acids required for VirB6 function, random mutations were introduced into *virB6*, and mutants that failed to complement a deletion in *virB6* in tumour formation assays were isolated. Twenty-one non-functional mutants were identified, eleven of which had a point mutation that led to a substitution in a single amino acid. Characterization of the mutants indicated that the N-terminal large periplasmic domain and the transmembrane domain TM3 are required for VirB6 function. TM3 has an unusual sequence feature in that it is rich in bulky hydrophobic amino acids. This feature is found conserved in the VirB6 family of proteins. Studies on the effect of VirB6 on other VirB proteins showed that the octopine Ti-plasmid VirB6, unlike its nopaline Ti-plasmid counterpart, does not affect accumulation of VirB3 and VirB5, but has a strong negative effect on the accumulation of the VirB7–VirB7 dimer. Using indirect immunofluorescence microscopy the authors recently demonstrated that VirB6 localizes to a cell pole in a VirB-dependent manner. Mutations identified in the present study did not affect polar localization of the protein or the formation of the VirB7–VirB7 dimer. A VirB6–GFP fusion that contained the entire VirB6 ORF did not localize to a cell pole in either the presence or the absence of the other VirB proteins. IMF studies using dual labelling demonstrated that VirB6 colocalizes with VirB3 and VirB9, and not with VirB4, VirB5 and VirB11. These results support the conclusion that VirB6 is a structural component of the T4S apparatus.

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INTRODUCTION

Agrobacterium tumefaciens, a Gram-negative phytopathogenic soil bacterium, uses type IV secretion (T4S) to deliver DNA and proteins to plant cells, causing the neoplastic crown gall tumour disease (Zupan *et al.*, 2000). Tumour formation results from the transfer and stable integration of a segment of the tumour-inducing Ti-plasmid DNA into the plant nuclear genome. The virulence (*vir*) region of the Ti-plasmid encodes functions essential for the processing of the Ti-plasmid and substrate transfer. Eleven proteins encoded in the *virB* operon and VirD4 are required for substrate transfer (Berger & Christie, 1994; Vergunst *et al.*, 2000). The VirB proteins presumably assemble a transport apparatus to facilitate substrate transfer. VirD4 functions as a coupling protein facilitating communication between the substrates and the apparatus (Cabezón *et al.*, 1997). The VirD4/VirB proteins are conserved in animal and human pathogens that include *Bordetella pertussis*, *Helicobacter pylori*, *Legionella*

pneumophila, *Brucella suis* and *Rickettsia prowazekii* (Covacci *et al.*, 1999; Cascales & Christie, 2003). These pathogens, members of the T4S family, use the VirD4/VirB system to deliver macromolecules to the host cell. The VirD4/VirB proteins are also found conserved in conjugative bacterial plasmids. Like *A. tumefaciens*, conjugative plasmids use the VirD4/VirB system to transfer plasmid DNA between bacteria.

Functional analysis of the VirB proteins showed that VirB1, a putative transglycosylase, is the only non-essential VirB protein but is required for a high efficiency of DNA transfer (Berger & Christie, 1994; Mushegian *et al.*, 1996). VirB2 is a major component of the T-pilus, a structure essential for DNA transfer (Fullner *et al.*, 1996; Lai & Kado, 1998). VirB5 is a minor component of the T-pilus (Schmidt-Eisenlohr *et al.*, 1999). VirB4 and VirB11 are two ATP-binding proteins that provide energy essential for the function of the transport apparatus (Atmakuri *et al.*, 2004; Judd *et al.*, 2005b). VirB6, VirB7, VirB8, VirB9 and VirB10 are postulated to assemble a T4S apparatus for substrate delivery (Das & Xie, 2000; Judd *et al.*, 2005a, b). Many interactions likely

Abbreviations: IMF, immunofluorescence microscopy; T4S, type IV secretion; TM, transmembrane.

to be required for the assembly of the T4S apparatus have been identified (Finberg *et al.*, 1995; Anderson *et al.*, 1996; Beaupre *et al.*, 1997; Das & Xie, 2000; Ward *et al.*, 2002; Krall *et al.*, 2002; Jakubowski *et al.*, 2004; Atmakuri *et al.*, 2004; Judd *et al.*, 2005a, b). VirB7 forms a disulfide-linked VirB7–VirB7 homodimer and a disulfide linked VirB7–VirB9 heterodimer. The VirB7 homodimer is postulated to function in T-pilus biogenesis and the VirB7–VirB9 heterodimer in the assembly of the T4S apparatus (Hapfelmeier *et al.*, 2000; Das & Xie, 2000). VirB7–VirB10 form a protein complex that localizes to the bacterial cell pole (Judd *et al.*, 2005b).

VirB6 is a polytopic integral membrane protein with four or five transmembrane (TM) segments (Jakubowski *et al.*, 2004; Judd *et al.*, 2005a). VirB6 affects stability of two VirB proteins, viz. VirB3 and VirB5, and the formation of the VirB7–VirB7 dimer (Hapfelmeier *et al.*, 2000). Since these proteins and probably the VirB7 homodimer are involved in T-pilus biogenesis, VirB6 was postulated to regulate T-pilus formation. Several recent studies suggest an essential role of VirB6 in T4S apparatus assembly and function as well. VirB6 forms complexes with the core T4S apparatus components, and small insertions at random sites within the coding region blocked various steps in the T-DNA translocation process (Cascales & Christie, 2004; Jakubowski *et al.*, 2004). VirB6, like the coupling protein VirD4, localized to the cell pole, and five VirB proteins are required for its polar localization (Judd *et al.*, 2005a). A conserved tryptophan at position 197 and the C-terminal end of VirB6 are required for polar localization of the protein (Judd *et al.*, 2005a). Mutants defective in polar targeting failed to transfer DNA to plant cells. In a separate study a VirB6–GFP fusion was reported to localize to a cell pole in the absence of the other VirB proteins (Jakubowski *et al.*, 2004).

In the present study we report the identification of individual amino acids that are essential for VirB6 function. We demonstrate that the primary sequence of one of the TM segments plays an important role in VirB6 function and the sequence of this domain is conserved in the VirB6 family of proteins. VirB6 colocalizes with two core constituents of the T4S apparatus and VirB6–GFP does not localize to a cell pole.

METHODS

Strains. *A. tumefaciens* A348 contains octopine Ti-plasmid pTiA6 in A136. *A. tumefaciens* A136 is a strain cured of the nopaline Ti-plasmid pTiC58. PC1006 is a derivative of *A. tumefaciens* A348 with a nonpolar deletion in pTiA6 *virB6* (Berger & Christie, 1994). *A. tumefaciens* AD886 and AD1068 are derivatives of PC1006 harbouring plasmid pAD1482 and phisB6, respectively (Judd *et al.*, 2005a). Plasmids pAD1482 and phisB6 are tetracycline-resistant, wide-host-range IncP plasmids that contain a chimeric *virD_p–virB6* gene or its derivative encoding a hexa-histidine tag at the VirB6 C-terminus (Judd *et al.*, 2005a).

VirB6–GFP fusion. A *virB6–gfp* (green fluorescent protein) fusion gene where the last codon of *virB6* was fused to codon 3 of *gfp* mut3

(Cormack *et al.*, 1996) was constructed as follows. Plasmid pGFP2X is a pUC118 derivative with the mutant *gfp* mut3 gene. A unique *Xho*I site at codon 3 of *gfp* was introduced during PCR-mediated cloning of the structural gene. A 1.4 kb DNA fragment containing the *virD_p–virB6* gene lacking the translation stop codon was obtained by PCR and cloned as an *Acc65I–Xho*I fragment into plasmid pGFP2X to construct plasmid pAD1759. Plasmid pAD1759 containing the *virD_p–virB6–gfp* chimeric gene was fused to plasmid pAD1412 to construct the wide-host-range plasmid pB6GFP.

Mutagenesis of *virB6*. Random mutations in *virB6* were introduced by error-prone PCR using *Taq* DNA polymerase (Vogel & Das, 1994). The regions encoding amino acids 1–168 and 168–295 were amplified by PCR and purified with the QIAquick PCR purification kit (Qiagen). The PCR fragments were cloned as a *Sph*I–*Eco*RI or *Eco*RI–*Bgl*II (encoding residues 1–168 and 168–295, respectively) fragment into pAD1482 DNA to create a chimeric *virB6* gene encoding a mutagenized N- or C-terminal region. Other mutations were introduced by deoxyoligonucleotide-directed site-specific mutagenesis using uracil-containing single-stranded pAD1426 as a template (Judd *et al.*, 2005a). All mutations were identified by DNA sequence analysis. All plasmids were fused to the wide-host-range plasmid pAD1412 for analysis in *A. tumefaciens*.

Anti-VirB antibodies. Polyclonal antibodies against a His-tagged TrpE–VirB6 fusion protein were raised in rabbits and purified by affinity chromatography (Judd *et al.*, 2005a). Other VirB antibodies used in this study have been described previously (Kumar *et al.*, 2000; Judd *et al.*, 2005b). For analysis by Western blotting, proteins were denatured for 30 min at 37 °C, separated by electrophoresis on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Judd *et al.*, 2005a). Antigens were detected by the chemiluminescence method using peroxidase-conjugated secondary antibodies (Amersham).

Immunofluorescence microscopy (IFM). The procedure for IFM was as described previously (Kumar & Das, 2002; Judd *et al.*, 2005a). Briefly, *A. tumefaciens* was grown overnight in induction medium or uninduced conditions to mid-exponential phase (OD₆₀₀ ~0.5) at 20 °C. At this growth temperature the VirB proteins accumulate at a similar level and no defect in either the assembly of the T-pilus or virulence is observed (Baron *et al.*, 2001). Cells were collected by low-speed centrifugation, washed in PBS, fixed in a 4% paraformaldehyde solution and permeabilized by treatment with lysozyme (5 mg ml⁻¹, final concentration) for 10 min at 4 °C. Samples were placed on a polylysine-coated multi-well slide, washed and treated overnight with affinity-purified anti-VirB6 antibodies (1:600 dilution) at 4 °C followed by Alexa-fluor 488 (green) conjugated secondary antibodies (Molecular Probes). Samples were viewed in a Nikon E800 fluorescence microscope equipped with a CoolCam 2000, liquid-cooled, three-chip colour video camera. Images were processed with Adobe Photoshop version 6.0 for presentation.

A. tumefaciens AD1068, which expresses a His-tagged VirB6 protein, was used for colocalization experiments. AD1068 is a derivative of PC1006 containing plasmid phisB6 (Judd *et al.*, 2005a). For colocalization analysis, the slides were first probed with anti-VirBn antibodies followed by the cognate secondary antibodies, and then with monoclonal anti-His antibodies and the cognate secondary antibodies. Changing the order of addition of the two primary antibodies had no effect on final results. Only those bacteria that were labelled with both probes were used for quantitative analysis. Foci were considered coincident when at least one half of each focus overlapped with the other (Lachmanovich *et al.*, 2003).

Other methods. Plasmid DNA was introduced into *A. tumefaciens* by electroporation (Mersereau *et al.*, 1990). Bacteria were grown at 30 °C in AB minimal medium (Chilton *et al.*, 1974) supplemented

with the appropriate antibiotic. For induction, cells were grown in AB Mes pH 5.8 medium at 20 °C in the absence or presence of the inducer acetosyringone (100 µM, final concentration) where appropriate. Virulence was monitored by tumour formation assays on *Kalanchoe daigremontiana* leaves (Judd *et al.*, 2005a). All infections were performed a minimum of four times and tumours were scored 2–3 weeks post-infection.

RESULTS

Mutagenesis and isolation of *virB6* mutants

To gain insight into the domains required for VirB6 function, we isolated and characterized mutants that were severely impaired in DNA transfer. Random mutations were introduced in *virB6* by error-prone PCR with *Taq* DNA polymerase (Vogel & Das, 1994). The inherent low fidelity of *Taq* DNA polymerase introduces errors in the amplified DNA. The error rate was further increased by the addition of the divalent metal ion Mn^{2+} , and lowering the concentration of one deoxynucleotide (0.2 mM dCTP vs 1 mM dGTP, dATP and dTTP). To ensure that the mutations were distributed throughout the protein, regions encoding the N-terminal and the C-terminal halves of the protein were mutagenized separately. The *virB6* gene in pAD1482 is flanked by unique *SphI* and *BglII* sites, and a unique *EcoRI*

site is present within the *virB6* coding region (amino acid 168). Following PCR amplification, either the *SphI*–*EcoRI* or the *EcoRI*–*BglII* fragment of pAD1482 was replaced with a mutant *virB6* fragment. Two *virB6* mutant libraries were constructed by isolating plasmid DNA from ~3000 independent transformants in *E. coli*. The libraries were introduced into the nonpolar pTiA6 *virB6* deletion strain *A. tumefaciens* PC1006. Transformants were tested for *virB6* function by monitoring their ability to complement the *virB6* deletion mutant in tumour formation assays on *Kalanchoe* leaves.

Screening of several hundred transformants led to the isolation of 21 mutants with an avirulent phenotype (Fig. 1). DNA sequence analysis of the putative *virB6* mutant genes identified thirteen unique mutants (Table 1). Eleven mutants had a single nucleotide change that led to a change in the amino acid sequence, one was a double mutant with changes in two amino acids, and the remaining one was a triple mutant with mutations in three amino acids. Several mutations mapped to two regions of the protein: four to the large periplasmic loop P1 (aa 56–165) including two at adjacent residues (D118G, S133P, E134G, Q150A) and three to the transmembrane domain TM3 (L210P, L213P, I217T). Of the remaining four, two mapped to the second periplasmic domain (T224I, L249R), one to the TM4 domain (A267T), and one near the N-terminal end of the protein (F9S). The double and triple mutants had one mutation at the N-terminal end (F9S, A7V). Since the F9S mutant is avirulent (mutant 2, Table 1), the phenotype of these two mutants may be due to the defect near the N-terminus of the protein. In protein transfer assays, none of the mutants supported VirE2 transfer in complementation *in planta* (Otten *et al.*, 1984).

Fig. 1. Phenotype of the *virB6* mutants. The effect of a mutation on DNA transfer was monitored by complementation using tumour formation assays. All mutants shown here have an avirulent phenotype. The number identifies a mutation listed in Table 1. A348, wild-type; pB6/ΔB6, PC1006 complemented with *virB6* on the plasmid pAD1482. The line drawing indicates the relative position of the mutations (down arrowhead) in the linear map of the 295 aa protein. TM1–4, the four transmembrane domains as defined by Judd *et al.* (2005a).

Table 1. Identification of the VirB6 mutants

Mutant	Base change(s)*	Amino acid change(s)
2	T5335→C	Phe-9→Ser
6	A5662→G	Asp-118→Gly
18	T5707→C	Ser-133→Pro
7	A5709→G	Glu-134→Gly
1	A5757→G	Gln-150→Ala
14	T5938→C	Leu-210→Pro
12	T5947→C	Leu-213→Pro
13	T5958→C	Ile-217→Thr
15	C5980→T	Thr-224→Ile
16	T6054→G	Leu-249→Arg
17	G6107→A	Ala-267→Thr
5	T5335→C	Phe-9→Ser
	A5697→G	Lys-130→Arg
3	C5328→T	Ala-7→Val
	G5714→A	Gly-136→Ser
	T5778→A	Leu-157→His

*Residue number according to Ward *et al.* (1988).

The primary sequence of TM3 is important for VirB6 function

Three mutations mapped to the transmembrane domain TM3, while only one (A267T in TM4) mapped to the other three TM domains combined. If the sole function of a TM domain is to span the inner membrane, mutations in each TM domain are expected to appear at a low and similar frequency. A high frequency of mutation in TM3 suggests either that this region is a mutational hotspot or that the domain has a second function. We investigated the possibility that both hydrophobicity and the primary amino acid sequence of TM3 play an important role in VirB6 function. If this hypothesis is correct, we expect that the sequence of TM3 will be conserved in the VirB6 family of proteins.

The pTiA6 VirB6 TM3 has an unusual primary amino acid sequence in that it is rich in leucine (Fig. 2). Within an eight-residue segment (aa 206–213), seven residues are leucine and six of these are in tandem. This region is preceded by an essential tryptophan at position 197 that is invariant in the VirB6 family of proteins (Judd *et al.*, 2005a). A comparison of sequences among the VirB6 homologues showed a high degree of sequence conservation in this region. The TM3 homologous domain is rich in the three bulky, nonpolar amino acids, leucine, isoleucine and valine, which occupy seven positions within a thirteen-residue segment. The conservation of L/I/V sequences and the mapping of several mutations to the TM3 domain suggest that the primary sequence of TM3 plays an important role in VirB6 function.

Fig. 2. Sequence conservation in the TM3 region of the VirB6 proteins. Amino acid sequences (single-letter code) of pTiA6 VirB6 (B6AGA6) and a subset of its homologues, the *Bartonella henselae* (accession no. AAF00944), *Bordetella pertussis* (C47301), *Brucella suis* (NP_699271), *Caulobacter crescentus* (NP_421223), *E. coli* pKM101 (I79270), *Legionella pneumophila* (AAM08244), *Rickettsia prowazekii* (NP_220499), *Sinorhizobium meliloti* (NP_439960), and *Xylella fastidiosa* (AAF85580) homologues, are shown. The numbers identify the location of the first and the last residue. The TM3 domain is underlined and the location of the three avirulent mutants (m12–14) identified in this study is shown by a down arrow. The invariant tryptophan is in bold. z, leucine/isoleucine/valine; +, positively charged residue; –, negatively charged residue.

The L/I/V content of the TM3 equivalent domains (pTi VirB6 residues 201–221) of six VirB6 homologues shown in Fig. 3 ranged between 48 % and 75 %. For comparison, the L/I/V content of the TM1, TM2 and TM4 domains were 28–61 %, 26–52 % and 16–28 %, respectively. Also note the high conservation of TM1, TM2, TM3 and the TM2–TM3 intervening region sequences in this family of proteins (Fig. 3). The latter region also contains the only three

Fig. 3. Sequence similarity in the VirB6 family of proteins. The *A. tumefaciens* VirB6 protein sequence was aligned with a subset of its homologues using Multalin (Corpet, 1988); see the legend to Fig. 2 for accession numbers. The four transmembrane domains of the *A. tumefaciens* protein are underlined and identified as TM1–TM4 above the sequences. In the consensus sequence the invariant residues are indicated in bold capitals. The residue numbers at the top do not match that of the *A. tumefaciens* protein because of the gaps introduced for optimal alignment.

invariant residues, proline 179, threonine 190 and tryptophan 197, found in this protein family (the COG3704.1 family in the conserved domain database, NCBI). The assignments of three of the TM domains of the *A. tumefaciens* protein, TMS2 and TMS5 (Jakubowski *et al.*, 2004) and TM4 (Judd *et al.*, 2005a), differed in the two published studies. There is very little sequence conservation within these domains in the VirB6 family of proteins (Fig. 3). The sequence divergence is quite striking in the TMS2 (residues 65–83) domain. This domain is significantly rich in hydrophilic residues, and in several homologues includes two or more charged residues interspersed throughout the domain. These features make it less likely that the TMS2 equivalent domains function in membrane spanning.

Two of the mutations that mapped to TM3 are leucine to proline changes in amino acids 210 and 213. As a proline residue can function as a helix-breaker, the mutation may affect the formation of the TM α -helix, which may lead to an aberrant topology of the mutant protein. However, proline is not uncommon within TM domains (the VirB6 TM2, and the TM3 homologues of both *Caulobacter* and *Rickettsia* have one; see Fig. 2). To investigate the role of proline at positions 210 and 213, we determined whether another amino acid is tolerated at these positions. A leucine to alanine change in the respective codon was introduced by site-specific mutagenesis. The primary reason for introducing such a conservative change was to minimize the effect of the mutation on hydrophobicity, and hence the membrane-spanning property, of the TM domain. This, in turn, allowed probing of the importance of the TM3 primary sequence on protein function. In complementation assays, the VirB6 L210A mutation abolished DNA transfer and the L213A mutant was fully functional. The disruption of the tandem hexa-leucine motif in the L210A mutant probably led to the loss of protein function.

In mutational studies, impairment of protein function can result from an undesired effect of the mutation on protein stability. Unfortunately, quantitative analysis on the VirB6 mutants could not be performed using Western blot assays because we were unable to detect the wild-type protein and the mutant proteins in such an assay (Judd *et al.*, 2005a and data not shown). The anti-VirB6 antibodies used in these studies, however, did recognize a His-tagged VirB6, four other VirB6 mutants, and VirB6-PhoA fusions in Western blot assays, and wild-type VirB6 and its mutants in IFM (Judd *et al.*, 2005a). The failure to detect VirB6 and most of its mutants in Western blot assays is probably due to the formation of SDS-resistant oligomer that fails to enter the gel. IFM was used to examine whether the point mutants reported here have a major effect on the accumulation of the mutant proteins. All mutants did express VirB6 at a level comparable to that of the wild-type strain, suggesting that the null phenotype is not due to instability of a mutant protein, but due to an effect on protein function (see Fig. 5).

The VirB6 mutants do not affect stability of other VirB proteins

VirB6 from the nopaline Ti-plasmid pTiC58 stabilizes several VirB proteins and their complexes (Hapfelmeier *et al.*, 2000). In a *virB6* deletion mutant, both VirB3 and VirB5 accumulated to a lower level than that in the wild-type strain, and the formation of the VirB7 homodimer was abolished. To determine whether the octopine-type plasmid pTiA6 VirB6 acts in a similar manner, we characterized *A. tumefaciens* PC1006, the strain with a non-polar deletion in *virB6*. Analysis of total proteins from wild-type A348, PC1006 and AD886 (PC1006/pAD1482) by Western blot assays using VirB-specific antisera showed that a deletion in the pTiA6 *virB6* gene had no appreciable effect on the accumulation of VirB3, VirB5, VirB8, VirB9 and VirB10 (Fig. 4). In control experiments, the level of VirE2 was not affected by the deletion in *virB6*. In agreement with a

Fig. 4. Effect of VirB6 on the accumulation of other VirB proteins, monitored by Western blot assays using purified anti-VirBn antibodies. Total protein of *A. tumefaciens* grown in induction medium in the presence of acetosyringone was separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with the appropriate antibodies. All antibodies except anti-VirB6 antibodies were used at 1:2000 dilution. Anti-VirB6 antibodies were used at a 1:1000 dilution. Accumulation of VirE2 was monitored as a control. For the analysis of VirB7-VirB7 and VirB7-VirB9 dimers, samples were denatured under non-reducing conditions in the absence of β -mercaptoethanol and the blot was probed with anti-VirB7 antibodies. SDS-12.5% PA gels were used for the analysis of VirB5, VirB8-VirB10 and VirE2. VirB3 and VirB7 and its complexes were analysed in a SDS-15% PA gel run in Tris/Tricine buffer (Schägger & von Jagow, 1987). -Ti, A136; Δ B6, PC1006; Δ B6/pB6, PC1006/pAD1482; WT, A348.

Fig. 5. Effect of mutation in *virB6* on VirB7 accumulation. Vir protein accumulation was analysed as described in the legend of Fig. 4. Samples analysed: –, A136; +, A348; $\Delta B6/pB6$, PC1006/pAD1482; $\Delta B6$, PC1006; lanes 5–17, *virB6* mutants (the number identifies the mutant listed in Table 1).

previous study (Jakubowski *et al.*, 2003), we observed that the deletion of pTiA6 *virB6*, like that of the pTiC58 *virB6*, had a negative effect on the accumulation of VirB7 monomer and abolished the formation of VirB7–VirB7 dimer. Expression of *virB6 in trans* restored the level of the VirB7 homodimer to near wild-type level. These results suggest that VirB6 stabilizes VirB7, and may play a role in the assembly and/or stability of the VirB7 homodimer. However, VirB6 is not required for the formation of the VirB7–VirB9 heterodimer. To determine if a point mutation in VirB6 affects the other VirB proteins we studied the effect of each mutation on the accumulation of VirB3, VirB5, VirB7, VirB9, VirB7–VirB7 dimer and VirB7–VirB9 dimer (Fig. 5 and data not shown). None of the mutations had an effect on these proteins or the protein complexes.

All VirB6 mutants localize to the cell pole

In a recent study we demonstrated that VirB6 localizes to the cell pole (Judd *et al.*, 2005a). In order to determine if a point mutation affects polar localization of VirB6, we determined the subcellular location of all the VirB6 mutants by IFM. All the mutant proteins, like the wild-type protein, showed fluorescent foci at a cell pole, indicating that none of the mutations affected the subcellular location of VirB6 (Fig. 6a and data not shown). The presence of VirB6-specific fluorescent foci in all mutants confirmed that the mutant protein was expressed in the bacterium. As we were unable to detect the mutant proteins in Western blot assays, IFM analysis demonstrated that a mutation did not significantly destabilize the protein. While none of the mutants described here showed abnormal subcellular localization of the protein, we have previously identified two determinants of polar localization of VirB6, the conserved tryptophan at position 197 and the C-terminal end (Judd *et al.*, 2005a). To study whether a mutation in *virB6* affects the subcellular localization of another VirB protein, we determined the subcellular location of VirB8, VirB9 and VirB10 in the VirB6 mutants. No change in the subcellular location of any of the proteins was observed (data not shown), suggesting that the VirB6 mutants are unlikely to be defective in the assembly of the T4S apparatus.

A VirB6–GFP fusion fails to localize to the cell pole

Our recent studies demonstrated that polar localization of VirB6 required five VirB proteins, VirB7–VirB11 (Judd *et al.*, 2005a). However, another report showed that a VirB6–GFP fusion protein could localize to the cell pole in the absence of the Ti-plasmid (Jakubowski *et al.*, 2004). To address this discrepancy, we constructed a chimeric *pvirD_p-virB6-gfp* that expresses a fusion protein in which GFP is fused in-frame to the last amino acid of VirB6. When expressed in either the Ti-plasmidless *A. tumefaciens* A136 or the non-polar *virB6* deletion mutant PC1006 the fusion protein failed to localize to the cell pole (Fig. 6b). The protein was distributed throughout the cell periphery in both strains. In contrast, a GFP–VirD4 fusion, like the wild-type protein, localized to the cell pole. In Western blot assays, no difference in the level of the VirB6–GFP fusion in the two strains was observed (data not shown). With bacteria grown for an extended period on solid induction medium at room temperature, analysis over a 7 day period showed only a slight increase in bacteria containing polar fluorescence, but these constituted ≤ 5 –10 % of the total bacterial population with observable fluorescence. It is of interest to note that VirB6–GFP failed to localize to the cell pole even in the presence of the other VirB proteins, indicating that addition of GFP to the C-terminal end of VirB6 abrogated targeting to the cell pole. Consistent with these results is the observation that the *virB6-gfp* fusion was non-functional in T-DNA transfer assays (data not shown). This result is not unexpected because the C-terminal end of the protein is essential for both DNA transfer and polar targeting (Judd *et al.*, 2005a).

VirB6 colocalizes with a subset of VirB proteins

We recently reported that VirB6 colocalized with two components essential for T4S, VirD4 and VirB8 (Judd *et al.*, 2005a, b). Those studies and others suggested that VirB6 is a core component of the T4S apparatus (Jakubowski *et al.*, 2003). To gain additional support for this hypothesis, we studied colocalization of VirB6 and other VirB proteins. It is expected that proteins that are components of a protein complex will colocalize with one another. In another study we observed that VirB8 colocalized with a subset of the VirB

Fig. 6. Subcellular localization of VirB6 mutants and VirB6-GFP. (a) Subcellular location of VirB6 and its mutants was determined by indirect IFM. Location of a subset of the VirB6 mutants is shown. Panel A, negative control A136; B, positive control wild-type VirB6 (A348); C–F, VirB6 mutants 7, 18, 14 and 16 (see Table 1), respectively. Arrowheads identify polar foci. (b) Subcellular location of VirB6-GFP in the absence or presence of the other VirB proteins, and of a GFP-VirD4 fusion, monitored by fluorescence microscopy. A small amount of bacteria grown on solid induction medium at room temperature was resuspended in acidic medium and a 2–5 μ l aliquot was placed on a polylysine-coated slide for viewing. The VirB6-GFP fusion was found distributed throughout the cell surface while the GFP-VirD4 fusion localized exclusively to the cell pole. All bacteria were GFP positive. With the VirB6-GFP fusion a few areas of higher protein concentration forming foci were evident; however, in addition to these foci the bacteria exhibited fluorescence throughout the cell surface.

proteins and postulated that these proteins are the principal components that function in the assembly of the T4S apparatus (Judd *et al.*, 2005b). VirB8 did not colocalize with three of the VirB proteins, VirB4, VirB5 and VirB11. To determine whether VirB6 exhibits a similar behaviour, colocalization analysis of a His-tagged VirB6 and other VirB proteins was performed. Since colocalization analysis requires the use of two primary antibodies from different sources and all of our antibodies were raised in rabbits, the wild-type strain could not be used for this analysis. The His-tagged VirB6 was used instead because it is fully functional in DNA transfer and complemented the *virB6* deletion mutant (Judd *et al.*, 2005a), and monoclonal antibodies against the tag are commercially available. His-tagged VirB6 probed with monoclonal anti-His antibodies formed red foci (Fig. 7). The other VirB proteins probed with the cognate rabbit anti-VirB antibodies formed green foci. In a superimposed image, foci of proteins that colocalize had a

yellow colour resulting from the combination of red and green. VirB6, like VirB8, colocalized with VirB3 and VirB9, and did not colocalize with VirB4, VirB5 and VirB11 (Fig. 7, Table 2).

DISCUSSION

T4S is used for horizontal gene transfer in bacteria and for the delivery of detrimental macromolecules to plants, humans and animals. To facilitate substrate transfer, bacteria assemble a macromolecular structure that spans both membranes. The present study addresses the role of VirB6 in the assembly and function of the T4S apparatus. Mutational analysis reported here identified two regions, the first periplasmic loop (loop P1) and the transmembrane domain TM3, that are important for function. Another study found that small insertions within this periplasmic loop affected an early step in T-DNA transfer, the sequential delivery of DNA from

Fig. 7. Colocalization of His-tagged VirB6 and other VirB proteins expressed in *A. tumefaciens* AD1068, monitored by IFM. Fixed bacteria were probed successively with rabbit anti-VirBn antibodies, Alexa-fluor 488 (green) conjugated goat anti-rabbit antibodies, monoclonal anti-His antibodies and Alexa-fluor 594 (red) conjugated goat anti-mouse antibodies. Merged images of localization of His-VirB6 and the indicated VirB protein are shown. Arrowhead, yellow coincident foci; black arrow, red VirB6 foci; white arrow, green VirBn foci. Panel A, control experiment where bacteria were probed with monoclonal anti-His antibodies followed by Alexa-fluor 488 conjugated anti-rabbit secondary antibodies.

VirB11 to VirB6 (class I mutants of Jakubowski *et al.*, 2004). The insertion mutants gave rise to high-molecular-mass VirB9 complexes in non-reducing gels. None of the point mutants studied here exhibited a similar phenotype (data not shown).

Three mutants mapped to an 8 aa segment in the TM3 domain while only one mutant was found within the remaining TM domains. The richness of three bulky hydrophobic residues, leucine, isoleucine and valine, in this domain and the conservation of this feature in the VirB6 homologues suggest that both hydrophobicity and the primary sequence of TM3 are important for VirB6 function. This domain is preceded by another conserved region that includes an invariant tryptophan at position 197, and a conserved threonine at 189, a basic residue at 190 and an acidic residue at 195 (Fig. 2). High sequence conservation in

this region suggests functional importance. Tryptophan 197 is required for polar localization of VirB6 (Judd *et al.*, 2005a). Insertion mutations around this region, the class II mutants of Jakubowski *et al.* (2004), failed in a subsequent step in T-DNA delivery, the transfer of DNA from VirB6 to VirB8.

Deletion of the pTiA6 *virB6* gene had an effect on VirB7 and its complexes. Contrary to the observations with the pTiC58 VirB6 (Hapfelmeier *et al.*, 2000), we did not observe a destabilizing effect of pTiA6 VirB6 on the T-pilus-associated proteins VirB3 and VirB5. In agreement with earlier reports, we observed that a deletion in *virB6* abolished the formation of the VirB7 homodimer, but not that of the VirB7-VirB9 heterodimer (Hapfelmeier *et al.*, 2000; Jakubowski *et al.*, 2003). The later is in agreement with our previous report that demonstrated that VirB7 and VirB9 are sufficient to form the VirB7-VirB9 dimer (Anderson *et al.*, 1996). The absence of the VirB7 homodimer, and not that of the VirB7-VirB9 heterodimer, in the *virB6* deletion mutant confirms an earlier conclusion that the two dimers form through independent pathways.

The effect of VirB6 on VirB7-VirB7 dimer formation may also be an indirect one. The VirB7-VirB7 dimer may be a dead-end complex to sequester excess VirB7 that could not participate in the formation of the preferred VirB7-VirB9 complex. Since VirB6 stabilizes VirB7, the absence of VirB6 significantly reduces the amount of VirB7 in the cell. All of the VirB7 interacts with the preferred partner VirB9 to form the VirB7-VirB9 complex, leaving no excess protein to form the VirB7 homodimer. The undetectable level of the VirB7 homodimer in the *virB6* deletion mutant indicates that that VirB7 has a much higher affinity for VirB9 than for itself. In the presence of VirB6, the bacterium accumulates more VirB7. After all available VirB9 is used up in the formation of the VirB7-VirB9 complex, the excess VirB7 forms homodimer to sequester its reactive cysteine. This property was noted previously in the VirB9 protein (Anderson *et al.*, 1996). In the absence of VirB7, VirB9 forms a disulfide-linked homodimer as well as other non-specific disulfide-linked complexes with cellular proteins. When VirB7 was co-expressed with VirB9, formation of only the preferred VirB7-VirB9 complex was observed.

Subcellular localization studies presented here demonstrate that VirB6 colocalized with at least two core components of the T4S apparatus. It colocalized with VirB8 and VirB9, and not with VirB4, VirB5 and VirB11 (Fig. 6 and Judd *et al.*, 2005b). These results, in conjunction with colocalization studies with VirB8 presented elsewhere (Judd *et al.*, 2005b), support the conclusion that VirB6 is a core component of the T4S apparatus. Localization of VirB6 to a cell pole required the presence of VirB7-VirB11, suggesting a requirement for an interaction of VirB6 with a polar VirB7-VirB10 complex (Judd *et al.*, 2005a, b). While VirB11 is required for this process, the VirB6 complex and VirB11 occupy different regions of the cell pole, as neither VirB6 nor VirB8 colocalized with VirB11. This situation is analogous

Table 2. Colocalization of VirB6 and other VirB proteins

VirB protein	No. of cells	Total pairs of foci	Percentage coincident
VirB3	50	51	59
VirB4	62	64	17
VirB5	86	90	28
VirB9	55	55	78
VirB11	66	66	15

to the polar location of replicons in *A. tumefaciens* in which four replicons (those of linear chromosome, circular chromosome, pTiC58 and pAtC58) localized to the cell pole but neither plasmid replicon colocalized with another replicon (Kahng & Shapiro, 2003). Compatible plasmids in bacteria are also found to occupy non-overlapping sites within the same subregion of a cell, e.g. mid-cell, quarter-cell and cell pole (Ho *et al.*, 2002). Occupation of non-overlapping sites by the VirB6–VirB10 complex and VirB4/VirB5/VirB11 suggests that the latter group of VirB proteins is positioned at the cell pole for the proper functioning of the T4S apparatus.

According to the current model of DNA transfer, the *A. tumefaciens* T-DNA is delivered sequentially from VirD4 to VirB11, VirB6, VirB8 and VirB2/VirB9 (Cascales & Christie, 2004). Our colocalization results are in apparent disagreement with this model, which predicts VirB11 to colocalize with the other VirB proteins. A likely explanation for the difference is that subcellular localization of VirB11 (and VirB4) is dynamic in nature. Dynamic localization of bacterial proteins and origins of replications is well documented (Gitai *et al.*, 2004). We postulate that under specific conditions, e.g. in the presence of a substrate, recipient or both, VirB4 and VirB11 (transiently) associate with the VirB6–VirB10 complex to form an export-competent complex. This association may represent the last step in the assembly of the T4S apparatus or the conversion of an export-incompetent fully assembled apparatus into an export-competent apparatus.

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