

Biogenesis of T Pili in *Agrobacterium tumefaciens* Requires Precise VirB2 Propilin Cleavage and Cyclization

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VirB2 propilin is processed by the removal of a 47-amino-acid signal peptide to generate a 74-amino-acid peptide product in both *Escherichia coli* and *Agrobacterium tumefaciens*. The cleaved VirB2 protein is further cyclized to form the T pilin in *A. tumefaciens* but not in *E. coli*. Mutations in the signal peptidase cleavage sequence of VirB2 propilin cause the formation of aberrant T pilin and also severely attenuate virulence. No T pilus was observed in these mutants. The potential role of the exact VirB2 propilin cleavage and cyclization in T pilus biogenesis and virulence is discussed.

T pili are generated when *Agrobacterium tumefaciens* cells are induced by plant phenolic compounds such as acetosyringone. Induction leads to the expression of virulence (*vir*) genes located on a resident Ti plasmid (10). Of these *vir* genes, the 11 genes of the *virB* operon are involved in the synthesis and assembly of the T pilus (5). The T pilus is composed mainly of processed VirB2 protein (6), with VirB5 (12) and VirB7 (11) as associated components. Jones et al. (4) first characterized the processing steps of VirB2 that is cleaved into a 7.2-kDa product in both *Escherichia coli* and *A. tumefaciens*. Further studies by mass spectrometry (MS) demonstrated that the processed VirB2 product is composed of 74 amino acid residues linked by a peptide bond between the amino- and carboxyl-terminal residues to generate a cyclic peptide (the T pilin) (1). T pilin subunits are transported across the bacterial membranes and assembled into an exocellular, flexuous T pilus filament 10 nm in diameter protruding from the bacterial cell wall (5–7). Transport of T pilin subunits to the cell surface requires each of the 11 genes of the *virB* operon (5), while propilin cleavage can occur independently of any other gene of the Ti plasmid (1). The 47-residue signal peptide is cleaved off amino terminally from VirB2 propilin, most likely by a general signal peptidase I, LepB (1, 4, 13). Whether peptidyl cyclization of the resulting 74-residue peptide takes place simultaneously or is an individual reaction remains unclear.

In the present study, we examined the propilin cleavage and cyclization reactions that lead to T pilin formation in *E. coli* and *A. tumefaciens* and addressed the roles of these reactions in T pilus biogenesis and tumorigenesis.

T pilin is cyclized in *A. tumefaciens* but not in *E. coli*. Based on immunoblotting and pulse-chase analyses, it was demonstrated that VirB2 propilin is cleaved in *A. tumefaciens*, as well

as in *E. coli* (4). To determine whether the processed VirB2 protein is cyclized in *E. coli* like that in *A. tumefaciens*, we expressed *virB2* under *tac* promoter control in both *E. coli* and *A. tumefaciens* as previously described (1). VirB2 was induced by isopropyl- β -D-thiogalactopyranoside (IPTG) in *E. coli* and *A. tumefaciens* (in the absence of the Ti plasmid), and cells were analyzed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS and by immunoblotting with anti-VirB2 serum as previously described (1, 6). Briefly, 0.3 μ l of either cell suspension or pilus preparation was cocrystallized with *trans*-3-indolylacrylic acid and analyzed with a Bruker Reflex-II time-of-flight spectrometer (Bruker-Daltonik). Processed VirB2 was detected at m/z 7,202.2 when expressed in *E. coli* but found at m/z 7,184.3 when expressed in *A. tumefaciens* (Fig. 1A). The difference in mass of 18 Da is explained by the loss of one molecule of H₂O, caused by the formation of a new peptide bond (head-to-tail linkage) in the processed VirB2 protein, yielding the cyclized T pilus subunit (1). The results suggest that VirB2 is cleaved by a general signal peptidase in both *E. coli* and *A. tumefaciens* but that the product is only cyclized in *A. tumefaciens*. As cleavage and cyclization take place in the absence of the Ti plasmid, we concluded that Ti plasmid genes other than *virB2* are dispensable for VirB2 propilin processing. Interestingly, the linear and cyclic molecules also show different mobilities when fractionated by Tricine-sodium dodecyl sulfate (SDS)-16.5% polyacrylamide gel electrophoresis (PAGE). As shown in Fig. 1B, the linear form of cleaved VirB2 (m/z 7,202.2) has a mobility slower than that of T pilin (m/z 7,184.3). A mass difference of 18 Da cannot be resolved by SDS-PAGE; the difference in mobility is likely due to a difference in peptide conformation between the linear and circularized forms of the molecules.

For the conjugative R pilus of IncP plasmids, we have shown previously that propilin processing includes triple truncation and a cyclization step leading to the production of R pilus subunits in *E. coli* (1). Thereby, TraF, a plasmid-encoded protein, catalyzes the last step in this maturation cascade (3), acting as a leader peptidase-like protease in a catalytic dyad-like mechanism (2). In contrast, T pilin cyclization is independent from other Ti plasmid genes. The fact that VirB2 is processed in both *A. tumefaciens* and *E. coli* but is only cyclized

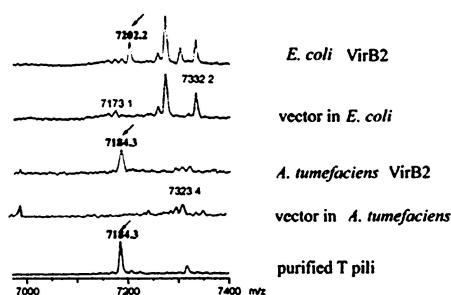
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A. MALDI-TOF



B. Immunoblot

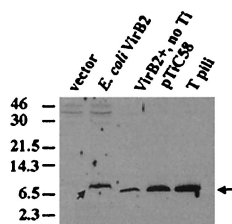
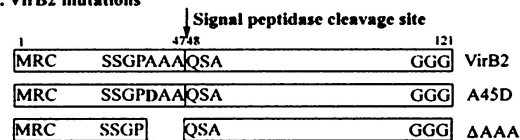


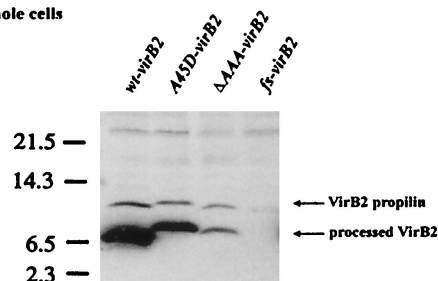
FIG. 1. Cyclization of T pilin occurs in *A. tumefaciens* but not in *E. coli*. (A) MALDI-TOF MS data derived from sample preparations with whole cells of *E. coli* DH5 α expressing VirB2 (pUCD4805) versus *A. tumefaciens* NT1REB expressing VirB2 (pUCD4813) in the absence of its Ti plasmid (1). Purified T pili and vectors served as controls. The signal at m/z 7,202.2 corresponds to linear processed VirB2, while the signal at m/z 7,184.3 corresponds to T pilin (indicated by arrows). (B) Immunoblot of linear VirB2 and T pilin. Whole-cell lysates from *E. coli* DH5 α containing either pTTQ18 (vector) or pUCD4805 (VirB2) or from *A. tumefaciens* NT1REB containing either pUCD4813 (VirB2, no Ti) or pTiC58 were fractionated by Tricine-SDS-16.5% PAGE and analyzed by immunoblotting using anti-VirB2 antibody. Molecular masses of protein size markers (in kilodaltons) are indicated on the left. Positions of linear processed VirB2 and T pilin are marked by arrows.

in *A. tumefaciens* suggests that peptide cyclization might be catalyzed by a second enzyme that is present in *A. tumefaciens* but not in *E. coli*. However, the lack of a linear processed T pilin signal (m/z 7,202.2) in *A. tumefaciens* implies either that the reactions of the signal peptidase cleavage and peptide bond formation occur by two efficient, almost simultaneous steps or, alternatively, that these two reactions are catalyzed by the very same enzyme. We have examined the recently completed *A. tumefaciens* genome sequences by performing a BLAST search using the *E. coli* LepB (signal peptidase I) protein sequence as a string and found one copy of LepB encoded by the *A. tumefaciens* circular chromosome (accession number AAK86842). No additional TraF homolog was found on either the chromosomes or the cryptic plasmid other than the known TraF protein (accession number AAK91092) encoded by the Ti plasmid. The potential LepB homolog of chromosomal origin is a good candidate for VirB2 processing and cyclization. Alternatively, a second enzyme that has no sequence homology to LepB or TraF might be required for cyclization of the processed VirB2 protein into T pilin in *A. tumefaciens*.

A. VirB2 mutations



B. Whole cells



C. Supernatant

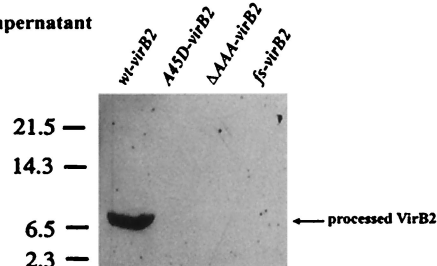


FIG. 2. Processing and exportation analyses of VirB2 mutant proteins in *A. tumefaciens*. (A) Mutations in the signal peptidase cleavage sequences of VirB2. Proteins are drawn as boxes, the first and last amino acid residues are numbered, and the signal peptidase cleavage site A47-Q48 is indicated. The A45D mutant carries a substitution mutation that replaces Ala-45 (A) with Asp (D) (in bold), and the Δ AAA mutation is a deletion of three Ala residues at positions 45 to 47. (B and C) Strain NT1RE containing either pJK270 (wild type; wt-virB2), pUCD4605 (A45D-virB2), pUCD4606 (Δ AAA-virB2), or pUCD4607 (frameshift mutant, fs-virB2) was induced with acetosyringone in I medium at 19°C for 72 h (6). Proteins from whole-cell lysates (B) and from their concentrated supernatants (C) were fractionated by Tricine-SDS-16.5% PAGE and immunoblotted with anti-VirB2 antibody. Molecular masses of protein markers are shown on the left side of each immunoblot. Unprocessed and processed VirB2 proteins are indicated.

Requirement of the signal peptidase recognition sequence for T pilin synthesis. A signal peptidase cleavage within VirB2 was predicted to occur between Ala-47 (A47) and Gln-48 (Q48) (13) (Fig. 2A). When two virB2 mutants, one coding for an amino acid substitution (A45D) and the other coding for a three-amino-acid deletion (A45 to A47, Δ AAA) in the signal peptidase cleavage sequence, were induced and tested for virulence in a previous study, both mutants were avirulent and neither VirB2 propilin nor processed VirB2 was detected (4). We have now examined the effects of these mutations in *A. tumefaciens* strains grown under optimal T pilus induction conditions at 19°C (6) instead of at 28°C (4). After induction with acetosyringone in I medium at 19°C for 72 h (6), we were able to detect VirB2 products from both the A45D mutant and Δ AAA deletion mutants (Fig. 2B). No VirB2 signals were detected from a frameshift mutant used as a negative control. This frameshift mutant was created by PCR to generate a 1-bp

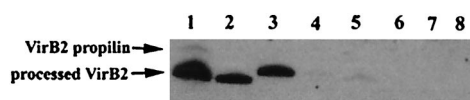


FIG. 3. Localization of mutant and wild-type VirB2 proteins in *A. tumefaciens* NT1RE. Strain NT1RE contained either pJK270 (wild-type *virB2*) (lanes 1 and 2), pUCD4605 (A45D-*virB2*) (lanes 3 and 4), pUCD4606 (Δ AAA-*virB2*) (lanes 5 and 6), or pUCD4607 (frameshift mutation *virB2*) (lanes 7 and 8). Isolated membrane vesicles from each strain were treated with 2% Sarkosyl to separate inner membrane (lanes 1, 3, 5, and 7)- and outer membrane (lanes 2, 4, 6, and 8)-associated proteins. Proteins were fractionated by Tricine-SDS-16.5% PAGE and analyzed by immunoblotting with anti-VirB2 antibody. Unprocessed and processed VirB2 proteins are indicated.

deletion at nucleotide 119 of the *virB2* open reading frame (TTT instead of TTTT) and introduced into Ti plasmid pJK270 by markerless gene replacement performed as previously described (4). Although mutant A45D and Δ AAA VirB2 proteins were produced and processed, they accumulated at lower levels than the wild-type protein, displaying electrophoretic mobilities slower than that of T pilin. In addition to processed VirB2, we were also able to detect the propilin although the propilins were not consistently detectable due to the quick turnover of the precursor. This result coincides with the previous observation that both A45D and Δ AAA mutants are also processed when they are expressed in *E. coli* (4). Although the VirB2 mutant proteins were processed, they appeared not to be exported out of *A. tumefaciens* cells, as judged by the absence of VirB2 in concentrated cell supernatants (containing the secreted and surface proteins) obtained by trichloroacetic acid precipitation performed as previously described (5) (Fig. 2C). Furthermore, T pili were not detected by electron microscopy of these mutants (data not shown). These data suggest that the VirB2 signal peptidase cleavage sequence plays a role important for correct processing to produce T pilin subunits for their export and assembly into T pili.

To determine the localization of mutant VirB2, total membrane fractions were isolated (13) and treated with 25 mM HEPES buffer (pH 7.6) containing 2% Sarkosyl (lauryl sarcosinate) on ice for 30 min and centrifuged at $40,000 \times g$ and 4°C for 30 min (8, 14). The inner membrane proteins were present in the supernatant, and the outer membrane proteins were sedimented in the pellet. By immunoblotting analysis with anti-VirB2 antibody, A45D mutant proteins accumulated mainly in the inner membrane fraction (Sarkosyl soluble), with trace amounts appearing in the outer membrane fraction (Sarkosyl insoluble), and AAA mutant proteins were detected only in the inner membrane fraction (Fig. 3). Wild-type T pilin molecules associated with both the inner and outer membrane fractions and appeared to be exocellularly assembled into the T pilus filament.

Intact *A. tumefaciens* NT1RE cells producing either wild-type or mutant VirB2 were induced with acetosyringone in I medium at 19°C for 72 h and analyzed by MALDI-TOF MS for detection of VirB2 products formed on the bacterial surface as described previously (1). Whereas a signal at m/z 7,184.3 (the m/z of T pilin) was detected from the wild-type strain, no signal matching the theoretical mass of the cleaved A45D or Δ AAA mutant protein was obtained (data not shown), suggesting that little mutant VirB2 is on the bacterial surface.

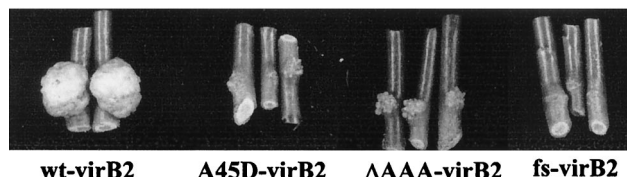


FIG. 4. Tumorigenicity assay on *D. stramonium*. Representative sections of the stem of each test plant inoculated with the indicated strain are shown at 4 to 5 weeks postinoculation. The inoculated strains of *A. tumefaciens* NT1RE contained either pJK270 (wild-type *virB2* [wt-*virB2*]), pUCD4605 (A45D-*virB2*), pUCD4606 (Δ AAA-*virB2*), or pUCD4607 (*virB2* frameshift mutant [fs-*virB2*]).

Virulence is severely affected by alterations in the signal peptide cleavage sequence. To determine the effects of mutations at the signal peptidase cleavage sequences of VirB2 on virulence, the tumor-inducing activity of wild-type and mutant *virB2* strains was assessed by inoculation of Jimson weed (*Datura stramonium*) seedlings as previously described (4). Three independent assays were carried out with each strain by using three plant replicates. Tumors were induced by wild-type *A. tumefaciens* NT1RE(pJK270) but not by the mutant strains at 2 weeks postinoculation. However, tiny tumors (1 to 2 mm in diameter) were induced by both A45D and Δ AAA mutants when tumorigenesis was assessed at 4 to 5 weeks postinoculation (Fig. 4). The frameshift mutation completely inhibited tumor formation. The tiny-tumor formation might be caused by a reversion mutation of A45D or Δ AAA within a large population of avirulent mutant cells. The frameshift mutant consistently failed to produce tumors. Similar results were obtained in independent tests using a root disk assay performed with root tissue derived from *Pastinaca sativa* (parsnip) in accordance with published protocols (data not shown) (9).

The signal peptidase cleavage sequence of VirB2 appears to play a critical role during virulence. Mutations near or at the signal peptide cleavage site (A47-Q48) result in severe attenuation of virulence. The avirulence of the frameshift mutant underlines the essential function of VirB2 in virulence. Within strains bearing either the A45D or the Δ AAA mutation, VirB2 processing still occurs but the processed product is degraded and not exported; thus, the biogenesis of the T pilus is hampered. Interestingly, the mutations did not completely abolish virulence since very tiny tumors arose at 4 to 5 weeks postinoculation, thus raising the question of the role of the T pilus during tumorigenesis. It is possible that a vestigial and partially functional pilus is formed only in planta and not on induction media. However, intriguingly, a nonpolar *virB1* mutant also reveals a phenotype similar to that of the A45D and Δ AAA mutants. We have previously shown that the nonpolar *virB1* mutant, which only causes the attenuation on tumorigenesis, is unable to export T pilin and assemble a T pilus (5). In conclusion, it is certain that VirB2 is the indispensable component of the VirB channel, which is essential for transport of the T DNA-protein complex from *A. tumefaciens* into the plant cell. However, the T pilus may not be required for T-DNA transfer but definitely plays an important role in sufficient T DNA-protein complex transport through the VirB channel or the T pilus is important but not essential for intimate contact (at-

tachment) between the *Agrobacterium* cell and the host plant cell for T DNA transfer.

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REFERENCES

1. Eisenbrandt, R., M. Kalkum, E. M. Lai, R. Lurz, C. I. Kado, and E. Lanka. 1999. Conjugative pili of IncP plasmids and the Ti plasmid T pilus are composed of cyclic subunits. *J. Biol. Chem.* **274**:22548–22555.
2. Eisenbrandt, R., M. Kalkum, R. Lurz, and E. Lanka. 2000. Maturation of IncP pilin precursors resembles the catalytic dyad-like mechanism of leader peptidases. *J. Bacteriol.* **182**:6751–6761.
3. Haase, J., and E. Lanka. 1997. A specific protease encoded by the conjugative DNA transfer systems of IncP and Ti plasmids is essential for pilus synthesis. *J. Bacteriol.* **179**:5728–5735.
4. Jones, A. L., E. M. Lai, K. Shirasu, and C. I. Kado. 1996. VirB2 is a processed pilin-like protein encoded by the *Agrobacterium tumefaciens* Ti plasmid. *J. Bacteriol.* **178**:5706–5711.
5. Lai, E. M., O. Chesnokova, L. M. Banta, and C. I. Kado. 2000. Genetic and environmental factors affecting T pilin export and T pilus biogenesis in relation to flagellation of *Agrobacterium tumefaciens*. *J. Bacteriol.* **182**:3705–3716.
6. Lai, E. M., and C. I. Kado. 1998. Processed VirB2 is the major subunit of the promiscuous pilus of *Agrobacterium tumefaciens*. *J. Bacteriol.* **180**:2711–2717.
7. Lai, E. M., and C. I. Kado. 2000. The T pilus of *Agrobacterium tumefaciens*. *Trends Microbiol.* **8**:361–369.
8. Lamber, P. A. 1988. Isolation and purification of outer membrane proteins from Gram-negative bacteria, p. 110–121. *In* I. Hancock and I. Poxton (ed.), *Bacterial cell surface techniques*. John Wiley & Sons, Inc., New York, N.Y.
9. Pueppke, S. G., and U. K. Benny. 1984. Adsorption of tumorigenic *Agrobacterium tumefaciens* cells to susceptible potato tuber tissues. *Can. J. Microbiol.* **30**:1030–1037.
10. Rogowsky, P. M., T. J. Close, J. A. Chimera, J. J. Shaw, and C. I. Kado. 1987. Regulation of the *vir* genes of *Agrobacterium tumefaciens* plasmid pTiC58. *J. Bacteriol.* **169**:5101–5112.
11. Sagulenko, V., E. Sagulenko, S. Jakubowski, E. Spudich, and P. J. Christie. 2001. VirB7 lipoprotein is exocellular and associates with the *Agrobacterium tumefaciens* T pilus. *J. Bacteriol.* **183**:3642–3651.
12. Schmidt-Eisenlohr, H., N. Domke, C. Angerer, G. Wanner, P. C. Zambryski, and C. Baron. 1999. Vir proteins stabilize VirB5 and mediate its association with the T pilus of *Agrobacterium tumefaciens*. *J. Bacteriol.* **181**:7485–7492.
13. Shirasu, K., and C. I. Kado. 1993. Membrane location of the Ti plasmid VirB proteins involved in the biosynthesis of a pilin-like conjugative structure on *Agrobacterium tumefaciens*. *FEMS Microbiol. Lett.* **111**:287–294.
14. Thorstenson, Y. R., G. A. Kuldau, and P. C. Zambryski. 1993. Subcellular localization of seven VirB proteins of *Agrobacterium tumefaciens*: implications for the formation of a T-DNA transport structure. *J. Bacteriol.* **175**:5233–5241.