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VirB2 Is a Processed Pilin-Like Protein Encoded by the *Agrobacterium tumefaciens* Ti Plasmid

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The mechanism of DNA transmission between distinct organisms has remained a subject of long-standing interest. *Agrobacterium tumefaciens* mediates the transfer of plant oncogenes in the form of a 25-kb T-DNA sector of a resident Ti plasmid. A growing body of evidence leading to the elucidation of the mechanism involved in T-DNA transfer comes from studies on the *vir* genes contained in six major operons that are required for the T-DNA transfer process. Recent comparative amino acid sequence studies of the products of these *vir* genes have revealed interesting similarities between Tra proteins of *Escherichia coli* F factor, which are involved in the biosynthesis and assembly of a conjugative pilus, and VirB proteins encoded by genes of the *virB* operon of *A. tumefaciens* pTiC58. We have previously identified VirB2 as a pilin-like protein with processing features similar to those of TraA of the F plasmid and have shown that VirB2 is required for the biosynthesis of pilin on a flagella-free *Agrobacterium* strain. In the present work, VirB2 is found to be processed and localized primarily to the cytoplasmic membrane in *E. coli*. Cleavage of VirB2 was predicted previously to occur between alanine and glutamine in the sequence -Pro-Ala-Ala-Ala-Glu-Ser-. This peptidase cleavage sequence was mutated by an amino acid substitution for one of the alanine residues (D for A at position 45 [A45D]), by deletion of the three adjacent alanines, and by a frameshift mutation 22 bp upstream of the predicted Ala-Glu cleavage site. With the exception of the frameshift mutation, the alanine mutations do not prevent VirB2 processing in *E. coli*, while in *A. tumefaciens* they result in VirB2 instability, since no holo- or processed protein is detectable. All of the above mutations abolish virulence. The frameshift mutation abolishes processing in both organisms. These results indicate that VirB2 is processed into a 7.2-kDa structural protein. The cleavage site in *E. coli* appears to differ from that predicted in *A. tumefaciens*. Yet, the cleavage sites are relatively close to each other since the final cleavage products are similar in size and are produced irrespective of the length of the amino-terminal portion of the holoprotein. As we observed previously, the similarity between the processing of VirB2 in *A. tumefaciens* and the processing of the propilin TraA of the F plasmid now extends to *E. coli*.

Plant transformation by *Agrobacterium tumefaciens* requires the presence of the Ti plasmid, whose genes mediate the transmission of an oncogene-bearing plasmid sector (T-DNA) from the infecting bacteria to plant cells. The *vir* genes required for T-DNA processing and transfer are contained in a 29-kb regulon on the Ti plasmid, which is arranged as six major operons in the linear order *virA*, *virB*, *virG*, *virC*, *virD*, and *virE* (20). The *virA* and *virG* gene products are part of a conserved two-component signal transducing system conferring positive transcriptional regulation of the remaining operons (29). The genes of the remaining operons confer activities essential for the processing and transfer of the 25-kb T-DNA.

These processing and transfer mechanisms have many features in common with conjugational DNA transfer occurring between bacteria. Sequence comparison between *vir* genes and genes essential for conjugative transfer of plasmids in other bacterial systems have provided valuable insights into the potential roles of the products of these *vir* genes. The most striking case involves the amino acid sequence identities between the proteins encoded by the *virB* operon and those known to be involved in the synthesis and assembly of a conjugative pilus, such as the pilus of plasmid F of the plasmid incompatibility group *Inc*FI (12, 22, 23). Further remarkable similarities have been observed between VirB and VirD proteins and those encoded by plasmid transfer genes of conjugative plasmids (11, 12, 15–18, 21) and genes involved in secretion of peptide toxin from *Bordetella pertussis* (3, 21, 27). The VirB2 protein shares 48% amino acid sequence similarity with TraA of plasmid F. TraA is the propilin protein that is the major constituent of the F conjugative pilus. The products of at least 10 other F *tra* genes are required for synthesis and assembly of the conjugative-pilus structure (4, 9). This observation suggested that VirB2 is also a pilin protein that may feasibly form a pilus with the other VirB proteins required for the elaboration and assembly of such a structure, which may facilitate T-DNA transfer (12, 13). VirB-dependent pili have been observed in a flagellum-free strain of *A. tumefaciens* (13, 13a). The results of these sequence studies, along with those of earlier genetic analyses (1, 2, 7, 25), have strongly implicated a conjugative mechanism for T-DNA transfer from *A. tumefaciens* to plants.

In F-mediated transfer, the 12.8-kDa propilin protein TraA is processed into a 7.2-kDa N-acetylated F-pilin subunit (4, 9). Likewise, the 12.3-kDa protein VirB2 appears to be processed into a 7.2-kDa polypeptide (22). We have investigated the VirB2 processing mechanism further and show in this report that the processing reaction that occurs in *A. tumefaciens* also occurs in *Escherichia coli* and that the 12.3-kDa holoprotein

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains		
DH5α	F$^-$ *endA1 hsdR17* (r$^-$ m$^+$) *supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1 deoR* Δ(*lacZYA-argF*)U169 ϕ80d*lacZ*Δ*M15*	8
BL21(DE3)	F$^-$ *ompT* (r$^-$ m$^-$)	26
S17-1	*thi pro hsdR hsdM recA* mod$^+$ Tp^r Sm^r res$^-$	24
A. tumefaciens strains		
NT1RE	Rm^r Em^r onc$^-$	14
LBA4301RM	Rec$^-$ Rm^r onc$^-$	14
Plasmids		
pJQ200SK	Derived from pJQ200 containing *sacB* from pUCD800 via pUM24; Gm^r *traJ oriT*	19
pET3a	*bla Bam*HI cloning site 29 bp downstream from T7 RNAP start of ϕ10 promoter	26
pJK270	pTiC58Tra^c with *Tn*5 insertion near left border of the T-DNA	14
pUCD2001	pTAR-ori pBR322 ori Tc^r Km^r λcos Ap^r	5
pUCD800	Km^r *sacB* λcos Sa ori 322-ori	6
pUCD3412	*virB1-B3* in pTZ18R	23
pUCD3432	*virB2* under gene ϕ10 promoter control	This study
pUCD4591	*virB2* under *virB* promoter control in pUCD2001	This study
pUCD4598	*virB2* containing a frameshift mutation 22 bp upstream of A47	This study
pUCD4605	*virB2* in pJK270 containing an A45D substitution	This study
pUCD4606	*virB2* in pJK270 containing an A-45 to A-47 deletion	This study
pUCD4726	*virB2* in pET3a containing an A45D substitution	This study
pUCD4743	*virB2* in pET3a containing an A-45 to A-47 deletion	This study

remains unprocessed in vitro. We demonstrate that VirB2 cleavage occurs in *E. coli* irrespective of amino acid substitution and deletion mutations in the putative cleavage site. These mutations cause the complete loss of oncogenicity and protein instability rather than leaving a residual uncleaved product in *A. tumefaciens.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. *E. coli* BL21(DE3) (26), S17-1 (24), and DH5α (8) were grown at 37°C in LB medium (10 g of tryptone [Difco], 5 g of yeast extract, and 3 g of NaCl per liter) with vigorous aeration or on LB containing 1.5% agar. When specified, M9 medium (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1 g of NH_4Cl, 0.1 g of $MgSO_4 \cdot 7H_2O$, and 0.1 g of $CaCl_2$ per liter with 0.2% glucose; pH 7.4) was also used. *A. tumefaciens* NT1RE and LBA4301RM, described previously (14), were grown at 28°C in medium 523 (10 g of sucrose, 8 g of tryptone [Difco], 4 g of yeast extract, 3 g of K_2HPO_4, and 0.3 g of $MgSO_4 \cdot 7H_2O$ per liter) with vigorous aeration or on 523 containing 1.5% agar.

pJK270 is plasmid pTiC58 containing a Tn5 insertion near the left border of the T-DNA (14). Plasmid pUCD3432 contains the *virB2* gene derived from pUCD2619, which contains the entire *vir* regulon of pTiC58 (20). The construction of plasmid pUCD3432 is described in the next section. pUCD3412 contains *virB1*, *virB2*, and *virB3* genes in pTZ18R (United States Biochemical, Cleveland, Ohio); pUCD4591 contains the promoter of *virB*, including *virB1* and *virB2* cloned in pUCD2001 (5). Plasmid pJQ200SK, containing the original *sacB* gene from Gay et al. for positive selection (6), was kindly provided by Michael F. Haynes (19). The expression plasmid pET3a, which was used in the preparation of VirB2 protein, was described previously (26).

Construction of VirB2 expression plasmids. pUCD3432 was constructed by amplifying the *virB2* gene of pUCD2619 by PCR with primers A (5′-CGCGGA TCCGCAATAATGCGATGCTT-3′) and B (5′-CGCGGATCCAGAC GATC ATTCATTAG-3′), both of which have *Bam*HI sites incorporated at their termini. The *virB2* PCR product was digested with *Bam*HI and inserted and ligated into the *Bam*HI site of pET3a. The correct orientation of the *virB2* insert with respect to the phage T7 promoter was verified by restriction endonuclease mapping and sequencing of the recombinant plasmid. The *virB2* gene is oriented such that the expression should result in the production of a fusion protein connecting the first 12 amino acids of the T7 gene ϕ10 to VirB2. Protein expression was regulated by using isopropyl-β-D-thiogalactopyranoside (IPTG) (Research Products, Mt. Prospect, Ill.).

In vitro expression of VirB2. DNAs of *virB2* and *virB2* derivatives were purified by CsCl density gradient centrifugation and used as the templates for coupled transcription-translation reactions to generate proteins in vitro. A commercial *E. coli* S30 preparation (Promega, Madison, Wis.) was used with 1 U each of T7 RNA polymerase and RNasin (Promega) per 1 to 4 μg of the DNA template. Proteins labeled with [^{35}S]methionine were precipitated with acetone and fractionated by Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 16.5% acrylamide–3% bisacrylamide gels. Tricine buffer contains 0.1 M Tricine and 0.1% SDS at pH 8.25. The proteins were analyzed by autoradiography and immunoblotting (see below).

Immunoblot analysis. The production of rabbit-borne polyclonal antibodies to VirB2 and VirB9 was described previously (10). To detect specific VirB2 expression in *E. coli*, antibody raised against the VirB2 peptide was cross-absorbed by incubating the antibody with a sonicated *E. coli* DH10B lysate for 4 h. The cross-absorbed antibody was preserved with 0.05% sodium azide at 4°C. The presence of VirB2 and VirB9 proteins in whole-cell extracts, subcellular fractions, and VirB2 synthesized in vitro was determined by immunoblotting (Western blotting) (10). Specific antiserum reactions were visualized with the ECL chemiluminescence system employing horseradish peroxidase-conjugated donkey antibodies to the VirB2 rabbit antibodies (Amersham, Arlington Heights, Ill.).

Pulse-chase analysis. *E. coli* BL21(DE3)(pLysS) cells containing either pUCD3432, pUCD4726, pUCD4598, pUCD4743, or pET3a were grown in M9 minimal medium with appropriate antibiotics at 37°C to early log phase. Cells in early log phase were induced with IPTG at a final concentration of 1 mM for 1 h and then treated with rifampin (100 μg/ml) for an additional 1 h. The cells were then labelled with 10 μCi of [^{35}S]methionine for 1 min, and 250 μl of the cell suspension was centrifuged at 10,000 × *g* for 2 min. Two volumes of fresh M9 medium containing 1.2 mM unlabelled L-methionine were added to the remaining labelled culture, and 750-μl aliquots were removed at timely intervals until 2 h after addition of the label. The labelled cells were collected by centrifugation at 10,000 × *g* for 2 min and quickly frozen in a solid CO_2-ethanol bath. The cells were thawed, resuspended in 50 μl of Tricine solubilization buffer (Bio-Rad, Hercules, Calif.), and placed in a boiling water bath for 5 min. The solubilized proteins were fractionated by SDS-PAGE and autoradiographed.

Site-directed mutagenesis and gene replacement. Site-directed mutagenesis employing oligonucleotides was carried out according to the supplier of the Mutagene kit (Bio-Rad, Richmond, Calif.). The oligonucleotide 5′-TTCTCCT CTGGGCCGCAA TCTGCAGGTGGC-3′ was used in the construction of B2D45AAA47, and 5′-GGGCCGGACGCGGCCCAATCT-3′ was employed in the construction of B2A45D. The template plasmid for mutagenesis was pUCD3412, generating pUCD4596 (with a substitution of D for A at position 45 [A45D]) and pUCD4597 (AAA deleted). The mutations were confirmed by nucleotide sequencing. Markerless gene replacement, which was used to introduce these mutations into the whole Ti plasmid, required pJQ200SK and is described in Results.

Membrane fractionation. As described previously (10, 22, 23), cells from 500 ml of culture were suspended in 10 ml of solution A {25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.6]), 2 g of sucrose, 0.2 M KCl, 0.2 mM dithiothreitol, 0.2 mg of DNase per ml, 0.2 mg of RNAse A per ml} and passed through a French pressure cell at 16,000 lb/in^2 three times. Lysozyme (5 mg) was then added to the cracked cells, which were then mixed and incubated on crushed ice for 30 min. Cell debris was removed by centrifugation (2,000 × *g*, 10 min, 4°C), and the supernatant was recentrifuged (110,000 × *g*, 1 h, 4°C) to pellet the membrane fraction. The supernatant was discarded, and the pellet was

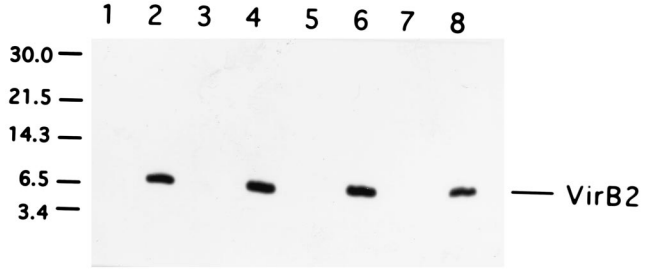

FIG. 1. VirB2 processing in *E. coli* BL21(DE3): immunoblot of proteins fractionated electrophoretically and blotted onto nitrocellulose. Either proteins were expressed in cells containing pET3a without IPTG induction (lane 1), pET3a with IPTG induction for 1 h (lane 3), pET3a with IPTG induction for 2 h (lane 5), pUCD3432 without IPTG induction (lane 2), pUCD3432 with IPTG induction for 1 h (lane 4), or pUCD3432 with IPTG induction for 2 h (lane 6) or they were from acetosyringone-induced *A. tumefaciens* NT1RE cells in the absence (lane 7) or presence of pTiC58 as pJK270 (lane 8). Molecular mass markers (in kilodaltons) are shown on the left.

thoroughly resuspended in 1.0 ml of solution B (5 mM EDTA [pH 7.5], 0.2 M dithiothreitol, 0.2 g of sucrose). Over the suspension was layered 1.0 ml of 70% sucrose in 5 mM EDTA, pH 7.5, and then 3.0 ml of 53% sucrose in 5 mM EDTA, pH 7.5. The discontinuous gradient was centrifuged (100,000 × *g*, 15 h, 4°C) and fractionated into subcellular components (22, 23).

RESULTS

VirB2 is processed in *E. coli*. Previously, we examined the production of the VirB2 protein as part of a study in which various *virB* nonpolar mutants were analyzed with respect to interdependencies between VirB proteins for specific membrane associations (10). The *virB* mutations examined had no effect on the accumulation of VirB2 protein, but in all cases, Western blot analyses revealed the presence of a 7.2-kDa protein rather than the 12.3-kDa protein predicted from its open reading frame (22). This suggested that VirB2 might be processed by a specific peptidase in *A. tumefaciens*. The specificity of this reaction was therefore studied in the absence of *A. tumefaciens* gene products by producing the VirB2 protein in *E. coli*. Plasmid pUCD3432, containing the *virB2* gene under the control of a T7 gene ϕ10 promoter, was transformed in *E. coli* BL21(DE3), and the VirB2 protein was analyzed in four randomly selected transformants. Samples were taken at time points before and after induction with IPTG, and the proteins were fractionated by SDS-PAGE and analyzed by Western blotting with VirB2-specific antisera. When extracts of *E. coli* containing pUCD3432 were analyzed, a 7.2-kDa protein was observed even in the absence of IPTG induction (Fig. 1). Expression of the T7 polymerase gene in BL21(DE3) is controlled by the *lacUV* promoter, which is not tightly regulated; hence, the presence of VirB2 in the uninduced sample is not surprising. The predicted size of VirB2 is 12.3 kDa, while that of the VirB2 fusion protein (joined with the first 12 amino acids of the T7 gene ϕ10; see Materials and Methods) is 13.8 kDa; yet, VirB2 is detected as a 7.2-kDa product. This result suggests that VirB2 is specifically cleaved in *E. coli*, with an end product similar in size to that produced in *A. tumefaciens*. That is, since in *E. coli* the VirB2 protein is expressed and processed in the absence of both Ti plasmid genes and *A. tumefaciens* chromosomal genes, there is apparently a signal peptidase in *E. coli* which recognizes a signal sequence in VirB2 to generate the 7.2-kDa product.

Location of VirB2 protein in *E. coli*. Our previous studies on the subcellular localization of VirB proteins in *A. tumefaciens* showed that the 7.2-kDa VirB2 protein is associated primarily with the inner membrane but is also associated, albeit at much lower concentrations, with the outer membrane (22). In the present work with *E. coli*, the processed 7.2-kDa VirB2 protein is also associated with both the inner and outer membranes, with greater association occurring with the inner membrane than with the outer membrane fraction (Fig. 2). Thus, the similarities in VirB2 processing extend to similar subcellular localization of the processed protein.

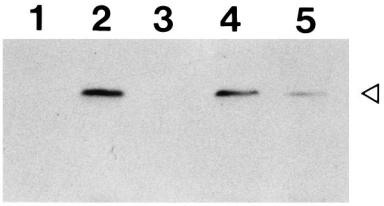

FIG. 2. Localization of the VirB2 protein to the inner and outer membrane fractions of *E. coli* BL21(DE3) induced with IPTG for 2 h. A Western blot with VirB2-specific antibody is shown. VirB2 is indicated by the open arrowhead. Lane 1, proteins from cells containing pET3a only. Analysis of components from cells containing pUCD3432: lane 2, whole-cell extract; lane 3, soluble fraction; lane 4, inner membrane fraction; lane 5, outer membrane fraction. All lanes were loaded with identical concentrations of membrane proteins. The homogeneity of the membrane fractions was verified by the association of NADH oxidase activity as done previously (22).

Alteration of the signal peptidase cleavage site affects VirB2 accumulation and virulence. Previously, cleavage of VirB2 in *A. tumefaciens* was predicted to occur between alanine and glutamine in the sequence -Pro-Ala-Ala-Ala-Glu-Ser- (residues 44 to 49) of the VirB2 holoprotein (22). Mutant clones pUCD4726, encoding VirB2 with a substitution of Asp at Ala-45 (A45D); pUCD4743, encoding VirB2 with the three adjacent alanine residues removed; and pUCD4598, containing a frameshift mutation, were placed under the control of the T7 gene ϕ10 promoter by subcloning the mutant genes into pET3a. When pUCD4726 was expressed in *E. coli* BL21(DE3) (pLysS) and the resulting proteins were fractionated by SDS-PAGE, the mutant VirB2 was detected by immunoblotting (Fig. 3). Likewise, analysis of pUCD4743 produced proteins of the same size. On the other hand, pUCD4598, bearing a frameshift mutation, did not produce VirB2 (Fig. 3). We interpret these results as showing that the peptidase cleavage occurs close to (owing to the size of the processed product) and includes the three alanine residues.

To determine the effects of these mutations in *A. tumefaciens*, each mutant *virB2* gene was introduced into the TiC58 plasmid pJK270, harbored in strain NT1RE, by reciprocal double recombination as follows. A 2-kb *Sac*I-*Pvu*II DNA fragment including the mutated portion of the *virB2* gene was cloned into the *Sac*I and SmaI sites in pJQ200SK DNA to form

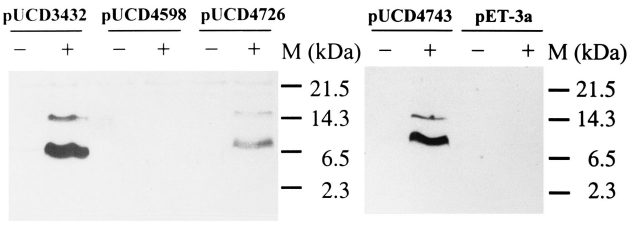

FIG. 3. Immunoblot analysis of VirB2 protein processing in *E. coli* cells. Wild-type and mutant VirB2 was expressed in *E. coli* cells induced (+) with IPTG. Uninduced (−) cells show no VirB2 production. The frameshift mutant pUCD4598 also does not produce VirB2, while VirB2 bearing the A45D and ΔAAA mutations (pUCD4726 and pUCD4743, respectively) was still processed. Molecular mass markers (M) are shown to the right of each panel.

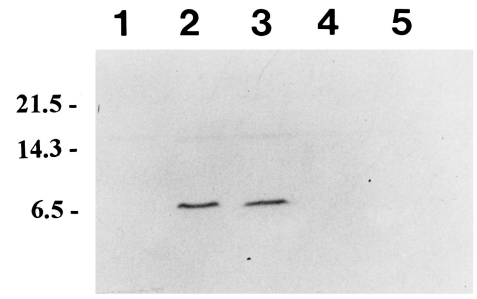

FIG. 4. Instability of VirB2 containing either the A45D or the ΔAAA mutation. Shown is a Western blot of *A. tumefaciens* NT1 induced with acetosyringone overnight and containing the following plasmids: lane 1, none; lane 2, pJK270; lane 3, pJK270 with a double-crossover recombination that did not affect *virB2*; lane 4, pUCD4605; lane 5, pUCD4606. Molecular mass markers (in kilodaltons) are shown on the left.

pUCD4605 and pUCD4606, respectively. pJQ200SK allows for positive selection for double-crossover recombination by virtue of the *sacB* gene conferring sucrose sensitivity (6, 19). *E. coli* S17-1 was transformed with the plasmids individually, and the resulting transformants were mated with *A. tumefaciens* NT1RE(pJK270) overnight on LB agar. Gentimicin-resistant, sucrose-sensitive transconjugants were recovered by first plating them on LB agar containing gentimicin, then growing them without selection for 20 generations in LB medium, and then plating them on LB agar containing 5% sucrose. Colonies that were sucrose resistant and gentimicin sensitive had undergone double recombination, and the insertion of either mutant *virB2* gene into pJK270 was confirmed by restriction mapping (data not shown).

Analysis of protein extracts from *A. tumefaciens* NT1RE containing either pUCD4605 or pUCD4606 revealed that there was no detectable VirB2, either as a holoprotein or the processed protein (Fig. 4). As a control, we included a pJK270 derivative that had undergone the double-recombination process but had not incorporated a mutation. Here, the VirB2 protein is detected as expected (Fig. 4, lane 3). Virulence conferred by pUCD4605 and pUCD4606 was completely abolished, and each of these *virB2* mutant plasmids could be complemented with pUCD4591 containing *virB2* (Fig. 5). The predicted peptidase cleavage sequence is therefore critically needed in the VirB2 processing step that leads to T-DNA transfer (as judged by the virulence assays).

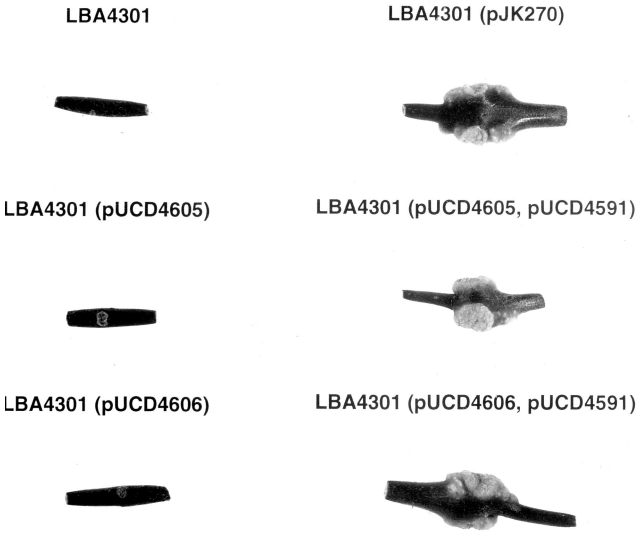

FIG. 5. Virulence assay on *Datura stramonium*. A representative inoculated portion of the stem of each test plant is shown. Inoculations were performed as they had been before (20). Tumor development was noted at 6 weeks postinoculation. Inoculations were made with *A. tumefaciens* LBA4301 with and without the respective test plasmid(s) as shown.

Kinetics of VirB2 processing in *E. coli*. The processing of VirB2 in *E. coli* was monitored as a function of time. The three *virB2* mutant products were labelled for 1 min with [^{35}S]methionine and then chased with excess unlabelled L-methionine after 1 h of IPTG induction followed by 1 h of rifampin treatment in M9 medium. As shown in Fig. 6, VirB2 processing took place with pUCD4726 and pUCD4743 within 1 min. No processing was observed with pUCD4598, which contains the frameshift mutation 22 bp upstream of the putative cleavage site AAA ↓ Q. These results verify the above-cited observation that VirB2 lacking the three alanine residues or containing an amino acid substitution is still recognized by the signal peptidase in *E. coli.*

Holo-VirB2 protein is produced in vitro. To verify our hypothesis that the *virB2* gene product is processed into a 7.2-kDa protein, both the wild-type and mutant *virB2* genes were analyzed in vitro for their respective products in a coupled transcription-translation system. As shown in Fig. 7, the *virB2* gene cloned in pUCD3432 is fully expressed as a 13.8-kDa predicted fusion product (lane 1). A protein of the same size appears with the *virB2* mutants bearing the site-specific amino acid substitution mutation (A45D) (lane 2). When pUCD4743 encoding VirB2 lacking the three alanine residues was examined, a protein slightly smaller than the 13.8-kDa product was observed, presumably because of shortening of the protein by the removal of these residues (lane 3). Analysis of the frameshift mutant pUCD4598 showed the presence of the 13.8-kDa protein, but the VirB2 protein could not be detected with VirB2 antiserum that recognizes the amino-terminal sequence of the processed 7.2-kDa protein, most likely because a heterologous protein was produced in its place (Fig. 7B, lane 4). The vector pET3a alone did not produce a 13.8-kDa protein (lane 5). Hence, in vitro, the *virB2* gene encodes a full-length protein product that remains unprocessed.

DISCUSSION

The VirB2 protein displays strong amino acid sequence homology (48% identity) to the TraA propilin encoded by the *traA* gene of plasmid F (22). The similarity also extends to other Tra proteins involved in the conjugative transfer of F-plasmid DNA. For example, VirB4 and TraC (23), VirB3 and TraL (22), VirB5 and TraE (12), and VirB10 and TraB (12) are all involved in the assembly of the F conjugative pilus provided by the TraA pilin structural gene (4, 9, 28). These similarities also extend to protein processing, with the VirB2 protein being cleaved like TraA propilin, resulting in a 7.2-kDa product like that observed with F propilin. Like TraA, VirB2 is also localized primarily to the inner (cytoplasmic) membrane (22). The overall similarities, both in structure and function, provide a strong argument that VirB2 encodes as a T-DNA transfer apparatus a conjugative pilus-like structure, as predicted previously (12, 13, 22). Indeed, our recent studies on a flagella-free strain have revealed the presence of pili whose appearance is dependent on *virB2* (13, 13a). At least two pilus types are produced; one is a long, thin pilus, while the other is a rather thick, stiff pilus, much like that produced by IncW plasmid pSa (13, 13a).

In the present work, we find that VirB2 is also processed in

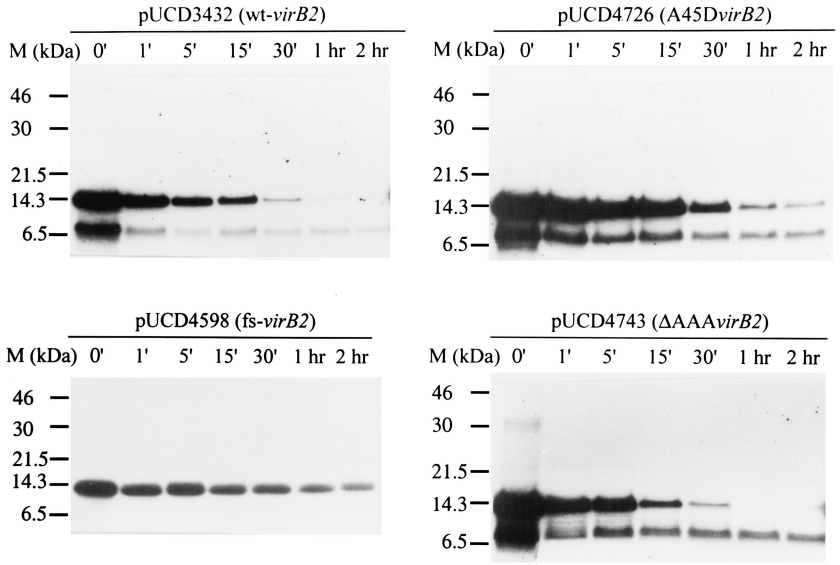

FIG. 6. Pulse-chase analyses of VirB2 processing. The protein was labelled with [^{35}S]methionine for 1 min and then chased with excess unlabelled L-methionine. Aliquots were taken at the times indicated for wild-type pUCD3432 (wt-*virB2*) and mutants pUCD4726, pUCD4598, and pUCD4743. No 7.2-kDa protein was generated when the frameshift mutation (fs-*virB2*) was present. Molecular mass markers (M) are shown to the left of each panel.

E. coli and is localized primarily to the inner membrane. The cleavage reaction is specific and remains unaltered even in the presence of 12 additional amino acid residues (originating from T7 gene ϕ10) attached to the amino terminus of VirB2, generating a fusion protein of slightly larger size than that of VirB2 itself. Yet, as in *A. tumefaciens* (22), the processed end product generated in *E. coli* is a 7.2-kDa polypeptide.

Alteration of the predicted signal peptidase sequence -Pro-Ala-Ala-Ala-Glu-Ser- (residues 44 to 49) by a single amino acid substitution (A45D) or by deletion of the three alanine residues results in the complete loss of virulence of *A. tumefaciens*. We also find from these studies that rather than causing the appearance of an unprocessed holoprotein, either mutation causes the loss of VirB2 protein in vivo in *A. tumefaciens*. On the other hand, in *E. coli*, the A45D substitution and AAA deletion mutations did not affect either the production of the holoprotein or its processing. The absence of a processed protein or holoprotein in *A. tumefaciens* suggests that there may be protein degradation occurring when VirB2 contains these mutations. In *E. coli*, on the other hand, the processing reaction remains unaffected by these mutations, which suggests that the signal peptidase cleavage site differs from that recognized by a signal peptidase in *Agrobacterium* cells.

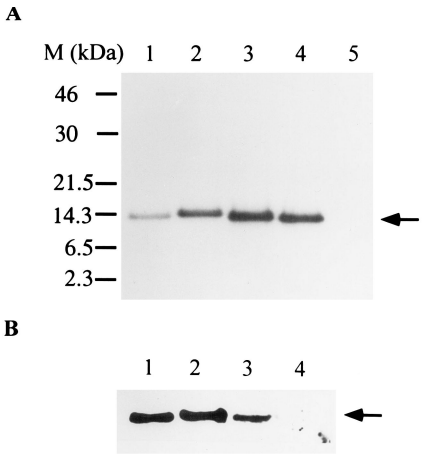

FIG. 7. Lack of processing of VirB2 in vitro. (A) Autoradiograph of VirB2 synthesized in S30 extract containing T7 RNA polymerase and labelled with [^{35}S]methionine. Templates for the in vitro polypeptide synthesis reactions were as follows: lane 1, pUCD3432; lane 2, pUCD4726; lane 3, pUCD4743; lane 4, pUCD4598; and lane 5, pET3a. (B) Western blot with VirB2-specific antiserum. The lanes are as for panel A. Lane 4 shows that no VirB2-specific protein was encoded by the VirB2 frameshift mutation in pUCD4598. The nonprocessed VirB2 proteins are indicated by the arrows.

With the F-plasmid pilin synthesis and assembly system, the TraA propilin is processed into a 7.2-kDa pilin subunit before its congregative assembly into a conjugative pilus. Additional genes encoding a transacetylase (TraX) and a pilus maturation factor (TraQ) are required for final pilus assembly (4, 9). Such enzymes and factors have not been identified for the VirB2 system, and they may or may not be required. Further analysis of the N-terminal sequence of the processed VirB2 protein should shed some light on whether or not the N-terminal amino acid is capped with an acetyl group. The recalcitrant nature of the N terminus with regard to sequencing seems to suggest this (14a).

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