The *Agrobacterium tumefaciens* *virB7* Gene Product, a Proposed Component of the T-Complex Transport Apparatus, Is a Membrane-Associated Lipoprotein Exposed at the Periplasmic Surface

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The *Agrobacterium tumefaciens* *virB7* gene product contains a typical signal sequence ending with a consensus signal peptidase II cleavage site characteristic of bacterial lipoproteins. VirB7 was shown to be processed as a lipoprotein by (i) *in vivo* labeling of native VirB7 and a VirB7::PhoA fusion with [³H]palmitic acid and (ii) inhibition of VirB7 processing by globomycin, a known inhibitor of signal peptidase II. A VirB7 derivative sustaining a Ser substitution for the invariant Cys-15 residue within the signal peptidase II cleavage site could not be visualized immunologically and failed to complement a $\Delta virB7$ mutation, establishing the importance of this putative lipid attachment site for VirB7 maturation and function. VirB7 partitioned predominantly with outer membrane fractions from wild-type A348 cells as well as a $\Delta virB$ operon derivative transformed with a *virB7* expression plasmid. Expression of *virB7* fused to *phoA*, the alkaline phosphatase gene of *Escherichia coli*, gave rise to high alkaline phosphatase activities in *E. coli* and *A. tumefaciens* cells, providing genetic evidence for the export of VirB7 in these hosts. VirB7 was shown to be intrinsically resistant to proteinase K; by contrast, a VirB7::PhoA derivative was degraded by proteinase K treatment of *A. tumefaciens* spheroplasts and remained intact upon treatment of whole cells. Together, the results of these studies favor a model in which VirB7 is topologically configured as a monotopic protein with its amino terminus anchored predominantly to the outer membrane and with its hydrophilic carboxyl domain located in the periplasmic space. Parallel studies of VirB5, VirB8, VirB9, and VirB10 established that each of these membrane-associated proteins also contains a large periplasmic domain whereas VirB11 resides predominantly or exclusively within the interior of the cell.

Agrobacterium tumefaciens incites disease by processing a segment of its genome (T-DNA) into a translocation-competent nucleoprotein particle (T-complex) which is then delivered across the bacterial envelope to a susceptible plant cell (for recent reviews, see references 28 and 81). In recent years, studies of two early events in the infection process, T-DNA processing and T-complex transport across the *A. tumefaciens* envelope, and parallel studies of analogous events associated with the conjugative transfer of the IncP, IncN, and IncW broad-host-range plasmids have provided strong evidence that these interkingdom and interbacterial DNA transfer systems are evolutionarily related (see reference 40). Both the T-DNA and RP4 (an IncP α plasmid) processing reactions have been examined in detail. These studies have shown that transfer is initiated by a strand- and site-specific cleavage at nick sites found within T-DNA border sequences and transfer origins (*oriT*), respectively. The nick sites of these elements are highly conserved, as are the active-site motifs of the relaxases, VirD2 and TraI, that catalyze the nicking events at T-DNA borders and *oriT* of RP4, respectively (48, 49). Purified VirD2 and TraI cleave at the nick sites within T-DNA and *oriT*, respectively, although VirD2 also cleaves at *oriT* while TraI fails to cleave at T-DNA borders. Both proteins remain covalently bound to the

5' phosphoryl end of the nicked DNA, and both proteins catalyze a joining activity reminiscent of that of type I topoisomerases (48, 49). In both transfer systems, the translocation-competent form of DNA is proposed to consist of a single-stranded DNA molecule covalently bound at the 5' end by the relaxase and coated along its length with a single-stranded DNA-binding protein. In the case of T-complexes, both VirD2, the 5' capping protein, and VirE2, the single-stranded DNA-binding protein, also possess functional nuclear localization sequences for the delivery of T-complexes to eukaryotic nuclei (see reference 80).

The apparatus responsible for exporting translocation-competent T-complexes across the *A. tumefaciens* envelope has been proposed to consist of most or all of the 11 products of the ~9.5-kb *virB* operon and the *virD4* gene (2, 35, 58, 66, 71, 72). Recent sequence analyses led to the discovery that products of the *virB* operon are highly similar to products of the conjugal transfer operons of the IncN, IncP, and IncW systems (32, 38, 50). Each of the 11 VirB proteins has a Tra homolog coded for by the *tra* operon of pKM101 (IncN) (50), and each of 6 VirB proteins has a Trb homolog coded for by the *trb* operon of RP4 (IncP α) (38). With few exceptions, genes for the homologous proteins are colinear in each of these transfer operons (38, 50). Interestingly, the protein components of each of these DNA transport systems also bear strong sequence similarities to proteins that are coded for by the *ptl* operon and that are proposed to assemble into a transport apparatus for exporting pertussis toxin across the *Bordetella pertussis* envelope. Each of eight VirB proteins exhibits sequence similarity

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to a Ptl protein (13, 34, 76), and recently, a ninth *ptl* gene that likely encodes a functional analog of VirB7 was identified (77).

The T-complex and broad-host-range plasmid transfer systems also are functionally related, as was demonstrated by the ability of each system to deliver mobilizable plasmids of the IncQ incompatibility group to recipient cells (2, 8, 26, 39, 79). In fact, the T-complex transporter will deliver IncQ plasmids to both plant and bacterial recipient cells, although interbacterial transfer of IncQ plasmids by this system occurs at appreciably lower frequencies (10^{-3} to 10^{-4} per donor cell) than those by the broad-host-range plasmid transfer systems (10^{-1} per donor) (2, 8, 26, 39, 79). The T-complex transport system apparently has evolved to be optimally adapted for DNA delivery to plant cells but also has retained a functional vestige of its ancestral operon in its ability to direct DNA transfer to bacterial recipients (77). The VirB transport system may also be functionally related to the Ptl transport system, given evidence that the VirB transport apparatus is capable of exporting VirE2 to either plant or bacterial recipients independently of DNA transfer (6, 12, 46).

At present, there is no detailed information about the structures of any of these macromolecular transport systems or their modes of action with respect to substrate recognition or the energetics of the transport process. With a goal of developing useful constructs for a structure-function analysis of the T-complex transporter, we generated a set of 11 *virB* mutants of wild-type strain A348, with each sustaining a precise *virB* gene deletion. With these mutants and a corresponding set of *virB* expression plasmids, we demonstrated that *virB2* through *virB11* are essential virulence genes, whereas *virB1* is important but non-essential for *A. tumefaciens* infection (4, 5). During the course of these investigations, we discovered that a precise deletion of *virB7* has a profound effect on the ability of cells to accumulate other VirB proteins (5). This discovery prompted us to test a hypothesis that VirB7 plays an important role in stabilizing VirB proteins during assembly of the T-complex transport apparatus. Interestingly, the amino terminus of VirB7 consists of a typical bacterial signal sequence that ends with Ala-Leu-Ser-Gly-Cys, a motif that resembles the consensus signal peptidase II (SPII) cleavage and lipid modification sequence of bacterial lipoproteins (27). In this report, we tested for SPII processing and modification of VirB7 in vivo, and we characterized the topology of VirB7 as well as the topologies of other VirB proteins whose steady-state levels are influenced by the synthesis of VirB7 (see references 5, 19, and 59). Our findings indicate VirB7 is topologically configured as a monotopic protein, with its amino terminus anchored predominantly to the outer membrane and its hydrophilic carboxyl domain located in the periplasmic space. Furthermore, we provide evidence that all of the VirB proteins, with the possible exceptions of the cytoplasmic membrane-associated ATPases VirB4 and VirB11, contain periplasmic domains or reside extracellularly. In the accompanying article (19) and elsewhere (59), we show that VirB7 interacts directly with VirB9 and that this interaction promotes the stabilization of a complex of VirB proteins that includes VirB4, VirB9, VirB10, and VirB11.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists bacterial strains and plasmids used in this study. Bacterial growth media and bacterial growth conditions have been previously described (11, 12). For induction of the *vir* genes, *A. tumefaciens* cells were grown in MG/L (62) medium to an optical density at 600 nm (OD_{600}) of 0.5, harvested by centrifugation, and inoculated at an initial OD_{600} of 0.2 into induction medium (IM; AB minimal medium, pH 5.5, containing 1 mM phosphate [see reference 12]) supplemented with 200 μ M acetosyringone (AS). Cultures were incubated with shaking at 28°C for 18 h, and cells were harvested for protein analysis (12). In *A. tumefaciens* and *Escherichia coli*, plasmids were maintained by the addition of carbenicillin (50 μ g/ml), kanamycin (50 μ g/ml), or tetracycline (5 μ g/ml) to the growth medium.

DNA techniques. *A. tumefaciens* cells were transformed by electroporation as previously described (4). Plasmids were recovered from *A. tumefaciens* by the boiling miniprep method of Birnboim and Doly as described by Sambrook et al. (55). Double-stranded DNA sequencing was performed with Sequenase according to the manufacturer's instructions (U.S. Biochemicals, Cleveland, Ohio). PCR amplification was performed with a Perkin-Elmer Cetus DNA thermocycler and Taq DNA polymerase from Perkin-Elmer (Madison, Wis.). Oligonucleotides were synthesized with a BioSearch 8600 DNA synthesizer. The Cys-15 residue of VirB7 was replaced with a serine (C15S) by a two-step PCR mutagenesis protocol (see reference 30). First, the 5' end of VirB7 was amplified with mutagenic oligonucleotide TGTCTGGGATCCGCTCAAAGC and an oligonucleotide that was complementary to vector DNA residing upstream of *virB7* on the template plasmid, pPCB975 (Table 1). The amplified product was gel isolated and then used as an upstream primer along with a T7 primer for the amplification of full-length *virB7C15S* and *virB8* from a pPCB975 template. The amplified product was digested with *NdeI* (at the 5' end of *virB7*) and *XhoI* (downstream of *virB8*), and the resulting fragment was cloned into pBSISK⁺ *NdeI* (Table 1), resulting in pXZ16. Plasmid pXZ16 was linearized by digestion with *NdeI*, converted to blunt ends with the Klenow subunit of *E. coli* DNA polymerase, and then digested with *BglII* to liberate a 123-bp fragment encoding residues 1 through 41 of VirB7C15S. This fragment was cloned in frame into the *EcoRV* and *BamHI* sites of pUI1156 (Table 1), resulting in plasmid pXZ17, which encodes the VirB7 derivative, VirB7C15S::PhoA41. The C15S mutation was confirmed by sequencing the length of *virB7C15S*. In-frame fusions of *virB* genes to '*phoA*', the alkaline phosphatase gene devoid of its signal sequence (42), were constructed by cloning (Table 1). To construct *virB2::phoA121*, the *virB2* coding sequence was amplified by PCR with an oligonucleotide, TGGCAGCTAAGGA GATAACAT, that is complementary to the sequence residing just upstream of *virB1* and a second oligonucleotide, ATTCTAGAACTACCGCAGTGAAGCG, that is complementary to the sequence at the 3' end of *virB2*, with plasmid pPC917 being used as a template. The second oligonucleotide contains a change in the nucleotide sequence so that an *XbaI* site replaced the translational stop codon. The amplified product was digested with *PvuII* (within *virB1*) and *XbaI* and the gel-isolated fragment was cloned into pUI1158 at the *EcoRV* and *XbaI* sites, resulting in a full-length *virB2* fused in frame to '*phoA*'.

Protein analysis and immunoblotting. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (12). Vir proteins and PhoA fusion proteins were visualized by SDS-PAGE, protein transfer to nitrocellulose membranes, and immunoblot development with goat anti-rabbit antibodies conjugated to alkaline phosphatase (AP) (11, 12). The reactivities of antisera to the VirE2 (12), VirB4 (73), VirB5 (4), VirB9 (73), VirB10 (73), and VirB11 (11) proteins were previously demonstrated. Anti-DnaA antiserum (43) was kindly provided by William Margolin. Anti-VirB7 and anti-VirB8 antisera were raised by overproducing both proteins in *E. coli* BL21(DE3, pPC979) (Table 1). A 100-ml culture was grown to an OD_{600} of 0.3, isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a final concentration of 1 mM to induce the expression of *virB7* and *virB8* from the bacteriophage T7 gene 10 promoter, and the cells were incubated with shaking for 16 h at 37°C. Extracts prepared from IPTG-induced cells disrupted in a French pressure cell were centrifuged at $30,000 \times g$ for 30 min, which resulted in the pelleting of large amounts of VirB7 and VirB8 proteins. Insoluble material was boiled in electrophoresis buffer and subjected to preparative-scale SDS-PAGE by using a Tricine buffer system (56). The VirB7 and VirB8 proteins were eluted from the gels as described previously (11) and sent to Cocalco Biologicals, Inc. (Reamstown, Pa.) for injection into New Zealand White rabbits. For comparisons of steady-state levels of VirB proteins, immunoblots were scanned with a scanning densitometry system from PDI, Inc. (Huntington Station, N.Y.). Protein loading on SDS-polyacrylamide gels routinely was normalized with respect to total protein content as well as VirE2 protein abundance to ensure that the *vir* genes were induced equivalently in all strains (5).

For subcellular localization studies, cells were lysed with a French pressure cell ($14,000 \text{ lb/in}^2$), and cell debris and unbroken cells were removed by low-speed centrifugation at $7,000 \times g$. The cleared lysate was resolved into soluble (cytoplasmic and periplasmic) and insoluble (membrane) material by centrifugation at $150,000 \times g$ for 1 h at 4°C with an SW55 Ti rotor. Total membrane material was further fractionated into cytoplasmic and outer membrane fractions by isopycnic sucrose gradient centrifugation at $120,000 \times g$ for 22 h at 4°C according to the method of de Maagd and Lutgenberg (17) with modifications as described previously (11, 12). The efficiency of fractionation was assessed by measuring the cytoplasmic marker, malate dehydrogenase, the cytoplasmic membrane marker, NADH oxidase, and the outer membrane marker, 2-keto-3-deoxyoctonate (see reference 17). Total membrane material also was fractionated into cytoplasmic and outer membrane material by selective solubilization of the cytoplasmic membrane with *N*-lauryl sarcosine (Sarkosyl) and then by sedimentation of the outer membrane material by centrifugation at $40,000 \times g$ for 45 min at 4°C (36).

Protease susceptibility studies. *A. tumefaciens* whole cells and spheroplasts were prepared for treatment with proteinase K according to the method of Winans et al. (78) with the following modifications. Cells from a 12.5-ml culture induced with AS for 18 h were suspended in 500 μ l of spheroplast buffer (20% sucrose, 20 mM Tris [pH 8]) and aliquoted into 50- μ l volumes. To make spheroplasts, lysozyme at a final concentration of 200 μ M was added and the cells were incubated on ice for 5 min. Then, EDTA at a final concentration of 2 mM was added, and the cells were incubated on ice for 45 min. To monitor the protease

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	λ^- ϕ 80d/ <i>lacZ</i> Δ M15 Δ (<i>lacZYA^- argF</i>)U169 <i>recA1 endA1 hsdR17</i> ($r_K^- m_K^+$) <i>supE44 thi-1 gyrA relA1</i>	55
CC118	<i>araD139</i> Δ (<i>ara leu</i>)7697 Δ <i>lacX74 phoA</i> Δ 20 <i>galE galK thi rpsE rpoB argE</i> (Am) <i>recA1</i>	42
BL21(λ DE3)	F $^-$ <i>ompT</i> $r_B^- m_B^-$	Novagen
<i>A. tumefaciens</i>		
A136	Strain C58 cured of the pTiC58 plasmid	75
A348	A136 containing octopine-type Ti plasmid pTiA6	23
PC1000	A348 derivative sustaining a <i>virB</i> operon deletion from pTiA6	This study
PC1007	Formerly A348 Δ <i>virB7</i> ; A348 derivative sustaining a <i>virB7</i> gene deletion from pTiA6	5
226Mx	A136 containing pTiA6 <i>virA</i> ::Tn3-HoHo1	61
243Mx	A136 containing pTiA6 <i>virB1</i> ::Tn3-HoHo1	61
379Mx	A136 containing pTiA6 <i>virC1</i> ::Tn3-HoHo1	61
306Mx	A136 containing pTiA6 <i>virD1</i> ::Tn3-HoHo1	61
361Mx	A136 containing pTiA6 <i>virE1</i> ::Tn3-HoHo1	61
363Mx	A136 containing pTiA6 <i>virG</i> ::Tn3-HoHo1	61
Plasmid vectors		
pBSIISK $^+$	Crb $^+$; cloning vector	Stratagene
pBCSK $^+$	Cam $^+$; cloning vector	Stratagene
pBSIISK $^+$ <i>NdeI</i>	Crb $^+$; pBSIISK $^+$ containing an <i>NdeI</i> restriction site at the translational start site of <i>lacZ</i>	5
pBCSK $^+$ <i>NdeI</i>	Cam $^+$; pBCSK $^+$ containing an <i>NdeI</i> restriction site at the translational start site of <i>lacZ</i>	5
pBKSK $^+$ <i>NdeI</i>	Kan $^+$; pBCSK $^+$ containing an <i>NdeI</i> site at the translational start site of <i>lacZ</i> and the <i>nptII</i> gene from pUC4K (Pharmacia) introduced at the unique <i>XmnI</i> site	5
pSW172	Tet r ; broad-host-range IncP plasmid containing <i>P_{lac}</i> and polylinker sequence from pIC19	9
pT7-SC11	Crb $^+$; translation vector carrying the T7 <i>lac</i> promoter	Novagen
pUI310	Crb $^+$; pUC19 carrying <i>phoA</i> from which the signal sequence has been deleted (' <i>phoA</i>)	70
pUI1156	Crb $^+$; pBSIISK $^+$ carrying ' <i>phoA</i> ' from pUI310 introduced as an <i>XbaI</i> and <i>SacI</i> fragment; ' <i>phoA</i> ' is in reading frame one with respect to the upstream polylinker sequence	M. Wood and S. Kaplan
pUI1158	Crb $^+$; pBSIISK $^+$ carrying ' <i>phoA</i> ' from pUI320 introduced as an <i>XbaI</i> and <i>SacI</i> fragment; ' <i>phoA</i> ' is in reading frame two with respect to the upstream polylinker sequence	M. Wood and S. Kaplan
pUI1160	Crb $^+$; pUI1158 with a <i>BamHI</i> site made blunt ended with Klenow subunit of <i>E. coli</i> DNA polymerase and religated; ' <i>phoA</i> ' is in reading frame three with respect to the upstream polylinker sequence	J. Eraso and S. Kaplan
<i>virB</i> expression plasmids		
pPC914KS $^+$	Crb $^+$; pBSIISK $^+$ with a 1.37-kb <i>HindIII</i> fragment from pPC917 containing <i>P_{virB}</i> and <i>virB1</i> with an <i>NdeI</i> site at its translational start site	This study
pPC917	Crb $^+$; pBSIISK $^+$ with a 2.8-kb <i>SalI-BamHI</i> fragment containing <i>P_{virB}</i> through the 5' end of <i>virB4</i> with an <i>NdeI</i> site engineered at the <i>virB1</i> translational start site	5
pPC937	Crb $^+$; pBSIISK $^+$ with a 2.8-kb <i>SalI-BamHI</i> fragment containing <i>P_{virB}</i> through the 5' end of <i>virB4</i> with an <i>NdeI</i> site engineered at the <i>virB3</i> translational start site	5
pPC958	Cam $^+$; pBCSK $^+$ <i>NdeI</i> with a 0.7-kb <i>NdeI-SacI</i> fragment containing <i>virB5</i> introduced into corresponding sites downstream of <i>P_{lac}</i>	5
pPC961	Crb $^+$; pBSIISK $^+$ with a 1.1-kb <i>SacI-BglII</i> fragment containing <i>virB6</i> introduced into <i>SacI</i> and <i>BamHI</i> sites downstream of <i>P_{lac}</i>	5
pPC974	Crb $^+$; pPC914 with a 0.29-kb <i>NdeI-NruI</i> fragment containing <i>virB7</i> introduced into <i>NdeI</i> and <i>EcoRV</i> sites downstream of <i>P_{virB}</i>	5
pPC978	Cam $^+$; pBCSK $^+$ <i>NdeI</i> with a <i>NdeI-NruI</i> fragment containing <i>virB7</i> introduced into <i>NdeI</i> and <i>EcoRV</i> sites downstream of <i>P_{lac}</i>	5
pPC979	Crb $^+$; pT7-SC11 with a 0.96-kb <i>NdeI-BamHI</i> fragment containing <i>virB7</i> and <i>virB8</i> from pPC977 (5) in corresponding sites downstream of the T7 <i>lac</i> promoter	This study
pPC983	Kan $^+$; pBKSK $^+$ <i>NdeI</i> with a 0.79-kb <i>NdeI-XhoI</i> fragment containing <i>virB8</i> in corresponding sites downstream of <i>P_{lac}</i>	5
pBB20	Crb $^+$; pUC118 with a 4.27-kb <i>SphI</i> fragment from pJW239 (73) containing the 3' ends of <i>virB4</i> through <i>virB8</i> downstream of <i>P_{lac}</i>	5
pXZ16	Crb $^+$; pBSIISK $^+$ with a 0.96-kb PCR fragment as an <i>NdeI-XhoI</i> fragment containing <i>virB7C15S</i> and <i>virB8</i> downstream of <i>P_{lac}</i>	This study
pJW327	Crb $^+$; pUC119 with a 2.2-kb <i>EcoRI</i> fragment containing <i>virB9</i> and <i>virB10</i> downstream of <i>P_{lac}</i>	73
pJW369	Cam $^+$; pUC19-cam with a 1.26-kb <i>EcoRI</i> fragment containing <i>virB10</i> downstream of <i>P_{lac}</i>	73
<i>virB::phoA</i> plasmids		
pCW814	Crb $^+$; a 1.23-kb <i>SalI-BsaAI</i> fragment containing <i>virB1</i> from pPC917 introduced at <i>SalI-EcoRV</i> sites of pUI1158	This study

Continued on following page

TABLE 1—Continued.

Bacterial strain or plasmid	Relevant characteristics	Source or reference
pPC813	Kan ^r ; 1.89-kb <i>NdeI</i> - <i>KpnI</i> fragment containing <i>virB1::phoA</i> from pPC813 introduced at corresponding sites of pBKS ⁺ <i>NdeI</i> , placing <i>virB1::phoA164</i> downstream of <i>P_{lac}</i>	This study
pXZ824	Crb ^r ; a 0.59-kb <i>PvuII</i> - <i>XbaI</i> PCR fragment containing entire <i>virB2</i> gene introduced at <i>EcoRV</i> - <i>XbaI</i> sites of pUI1158, placing <i>virB2::phoA121</i> downstream of <i>P_{lac}</i>	This study
pCW834	Crb ^r ; a 0.44-kb <i>EcoRV</i> - <i>XmnI</i> fragment containing <i>virB3</i> from pPC937 introduced at the <i>EcoRV</i> site of pUI1156, placing <i>virB3::phoA447</i> downstream of <i>P_{lac}</i>	This study
pPC833	Kan ^r ; a 1.54-kb <i>NdeI</i> - <i>KpnI</i> fragment containing <i>virB3::phoA</i> from pCW834 introduced at corresponding sites of pBKS ⁺ <i>NdeI</i> , placing <i>virB3::phoA447</i> downstream of <i>P_{lac}</i>	This study
pAH858	Chl ^r ; a 1.4-kb <i>Sall</i> - <i>KpnI</i> fragment containing <i>phoA</i> from pUI1156 introduced at corresponding sites of pPC858, placing <i>virB5::phoA93</i> downstream of <i>P_{lac}</i>	This study
pAH853	Kan ^r ; ~1.68-kb <i>NdeI</i> - <i>KpnI</i> fragment containing <i>virB5::phoA</i> from pAH858 introduced at corresponding sites of pBKS ⁺ <i>NdeI</i> , placing <i>virB5::phoA</i> downstream of <i>P_{lac}</i>	This study
pPC878	Chl ^r ; a 1.4-kb <i>BamHI</i> - <i>KpnI</i> fragment containing <i>phoA</i> from pUI1310 introduced at <i>BglII</i> - <i>KpnI</i> sites of pPC978, placing <i>virB7::phoA41</i> downstream of <i>P_{lac}</i>	This study
pPC875	Kan ^r ; an ~1.52-kb <i>NdeI</i> - <i>KpnI</i> fragment containing <i>virB7::phoA</i> from pPC878 introduced at corresponding sites of pBKS ⁺ <i>NdeI</i> , placing <i>virB7::phoA41</i> downstream of <i>P_{lac}</i>	This study
pPC873	Crb ^r ; an ~1.52-kb <i>NdeI</i> - <i>KpnI</i> fragment containing <i>virB7::phoA41</i> from pPC878 introduced at corresponding sites of pBSISK ⁺ <i>NdeI</i> , placing <i>virB7::phoA</i> downstream of <i>P_{lac}</i>	This study
pXZ17	Crb ^r ; a 0.12-kb <i>NdeI</i> - <i>BglII</i> fragment from pXZ16 into pUI1156, placing <i>virB7C15S::phoA41</i> downstream of <i>P_{lac}</i>	This study
pAH888	Chl ^r ; a 1.4-kb <i>EcoRV</i> - <i>KpnI</i> fragment containing <i>phoA</i> from pUI1158 introduced at <i>HincII</i> - <i>KpnI</i> sites of pPC983, placing <i>virB8::phoA72</i> downstream of <i>P_{lac}</i>	This study
pPC883	Kan ^r ; a 1.62-kb <i>NdeI</i> - <i>KpnI</i> fragment containing <i>virB8::phoA</i> from pAH888 introduced at corresponding sites of pBKS ⁺ <i>NdeI</i> , placing <i>virB8::phoA72</i> downstream of <i>P_{lac}</i>	This study
pAH894	Crb ^r ; a 450-bp <i>Sall</i> - <i>BglII</i> fragment from pJW327 introduced at <i>Sall</i> - <i>BamHI</i> sites of pUI1156, placing <i>virB9::phoA74</i> downstream of <i>P_{lac}</i>	This study
pCM2	Crb ^r ; a 980-bp <i>Sall</i> fragment from pJW369 introduced at the <i>Sall</i> site of pUI1158, placing <i>virB10::phoA276</i> downstream of <i>P_{lac}</i>	This study

susceptibility of proteins in lysed spheroplasts, the spheroplasts were treated with 0.05% Triton X-100 or with 2% Triton X-114, sonicated briefly, and maintained on ice for 30 min. Identical results were obtained by lysis with each detergent; therefore, data are reported only for lysis with 2% Triton X-114. Proteinase K from a freshly prepared stock (10 mg/ml) was added to whole cells, spheroplasts, and detergent-lysed spheroplasts at a final concentration of 100, 200, or 400 µg/ml, and then the reaction mixtures were incubated on ice for 45 min. The reactions were quenched by addition of the protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), at a final concentration of 2 mM, and the quenching was followed by incubation on ice for 5 min. Whole cells and spheroplasts also were pretreated with 2 mM PMSF before the addition of proteinase K to ensure that PMSF effectively inhibited proteinase K under the experimental conditions used in this study. The extent of proteolysis of VirB proteins was analyzed by SDS-PAGE and immunostaining.

AP and β-galactosidase activity assays. AP activity was detected on agar plates supplemented with 40 µg of the chromogenic indicator XP (5-bromo-4-chloro-3-indolyl phosphate) per ml. For measurements of the AP activities of *E. coli* CC118 cells expressing *virB::phoA* fusions, the cells were grown in L broth to an OD₆₀₀ of 0.2, IPTG was added to a final concentration of 1 mM, and the cells were incubated for an additional 1 h at 37°C. β-Galactosidase and AP activities were determined for *A. tumefaciens* cells incubated in IM medium both in the absence and presence of AS. AP activity was quantitated by measuring the production of *p*-nitrophenol from *p*-nitrophenyl phosphate as described by Manoil (41). β-Galactosidase activity was quantitated by measuring the production of *o*-nitrophenol from *o*-nitrophenyl-β-D-galactoside as described by Miller (45).

Lipoprotein analysis. Cellular lipoproteins of *E. coli* BL21(pPC979, DE3) and DH5α(pPC873) (Table 1) were labeled with [³H]palmitic acid essentially as described by Jung et al. (31). One hundred microliters of overnight cultures was inoculated into 2 ml of L broth containing carbenicillin (50 µg/ml), and the cultures were grown to an OD₆₀₀ of 0.3 at 37°C. The cells were harvested, washed once with 1 ml of M9 medium, and resuspended in 2.5 ml of M9 medium containing carbenicillin (50 µg/ml) and Casamino Acids (1%, wt/vol). The cells were incubated for 30 min at 37°C, IPTG was added to a final concentration of 1 mM, and the cells were incubated for an additional 25 min at 37°C. To inhibit host RNA polymerase in BL21(pPC979, DE3) cells and allow for the selective overexpression of *virB7* from the bacteriophage T7 gene 10 promoter, rifampin was added at a final concentration of 200 µg/ml and the cells were incubated for an additional 20 min at 37°C. This step was omitted for the DH5α(pPC873) cells, since *P_{lac}* was used to transcribe *virB7::phoA41*. Next, [9,10(*n*)-³H]palmitate (TRK909; 40 to 60 Ci/mmol; Amersham, Arlington Heights, Ill.) was added at 10 µCi/ml, and the cells were incubated for 2 h at 37°C. Phospholipids labeled with

[³H]palmitate were extracted according to the method of Takase et al. (65) as follows. The cells were harvested by centrifugation at 10,000 × *g* for 5 min at 4°C, washed once in 1 ml of ice-cold 10 mM sodium phosphate buffer (pH 7.0), and resuspended in 0.4 ml of 10 mM sodium phosphate buffer (pH 7.0). The cells were lysed by three cycles of freezing and thawing, briefly sonicated on ice, and centrifuged at 1,000 × *g* to remove cell debris. The supernatant was transferred to a new Eppendorf tube, 0.04 ml of 4 M NaCl was added, and the resulting mixture was incubated on ice for 10 min. Membranes were recovered by centrifugation at 14,000 × *g* for 1 h. To solubilize lipids, the membrane pellet was resuspended in 0.5 ml of chloroform-methanol (2:1, vol/vol) and shaken at room temperature for 20 min. Membrane proteins were pelleted by centrifugation at 14,000 × *g* for 30 min and subjected to two more cycles of solvent extraction. The air-dried pellet was resuspended in 50 µl of 10 mM Tris buffer (pH 7.0) and 50 µl of Laemmli's protein sample buffer (55). Proteins were resolved by SDS-PAGE, and labeled proteins were detected by autoradiography.

To examine the effect of globomycin (Sankyo Co. Ltd., Tokyo, Japan) on VirB7 processing, *A. tumefaciens* PC1007(pPCB976) cells (Table 1) were induced for *vir* gene expression by incubation in the presence of IM with 200 µM AS for 5.5 h. Globomycin was added at a final concentration of 200 µg/ml, and the cells were incubated for 30 min at 28°C. At this point (0 min), IPTG was added at a final concentration of 1 mM, and the cells were harvested at 15, 30, 45, and 90 min and 14 h for analysis by SDS-PAGE and immunoblotting with VirB7 antiserum.

Construction of a *virB* operon deletion derivative of A348. The *virB* operon was precisely deleted from the pTiA6NC plasmid by a mutagenesis protocol described previously for the generation of nonpolar deletions of each of the *virB* genes (5). A Δ *virB* operon mutation was constructed in vitro by subcloning as follows. Plasmid pPC9118 (Table 1) was digested with *Bam*HI (at bp 9154 with respect to the *Sall* site residing upstream of the *virB* operon of pTiA6NC, as defined by Ward et al. [71, 72]) and *Bgl*II (at bp 10113) and religated. From the resulting plasmid, a 453-bp *NdeI*-*KpnI* fragment containing 50 bp of the 3' end of the *virB11* gene and 403 bp of the pTiA6NC sequence residing downstream of *virB11* was isolated. This fragment was inserted into pPC914KS⁺ (Table 1) to substitute for the *virB1* coding sequence carried on this plasmid. This insertion placed 742 bp of the sequence residing immediately upstream of the *virB1* translational start site (5' flanking sequence) in juxtaposition with 50 bp of the 3' end of *virB11* and 403 bp of the sequence residing immediately downstream of *virB11* (3' flanking sequence).

An ~1-kb *Hind*III fragment carrying 550 bp of the 5' flanking sequence and 453 bp of the 3' flanking sequence was introduced into plasmid pBB50 (5). This plasmid is a ColE1 replicon that carries an *nptII* gene and a *sacB* gene for

FIG. 1. [^3H]palmitate labeling of VirB7 and the VirB7::PhoA41 fusion protein. Extracts of *E. coli* cells grown in M9 medium and labeled with [^3H]palmitate for 2 h were analyzed by Tricine-SDS-PAGE and autoradiographed. Lane 1, BL21(DE3, pT7-SC11); lane 2, BL21(DE3, pPC979); lane 3, DH5 α (pUI1156); lane 4, DH5 α (pPC873). The positions of VirB7 and VirB7::PhoA41 are indicated at the left; the positions and sizes (in kilodaltons) of protein standards are indicated at the right.

plasmid counterselection in *A. tumefaciens*. The resulting plasmid was introduced by electroporation into *A. tumefaciens* A348. Kan r transformants presumably arose by plasmid integration into the pTi plasmid, since ColE1 plasmids do not replicate in *A. tumefaciens*. Cells were grown overnight in the absence of antibiotic selection and then for a second night in the presence of sucrose to counterselect for cells in which the ColE1 plasmid was lost by a second crossover event. Kan s Suc r cells were analyzed by Southern blot hybridization and immunostaining to identify the *virB* operon deletion strain designated PC1000 (data not shown).

RESULTS

The *virB7* gene product is a lipoprotein. Studies of Lpp, the major outer membrane lipoprotein of *E. coli*, have led to the proposal that bacterial lipoproteins are modified by the addition of a thioether-linked diglyceride to the invariant Cys residue within the SPII consensus recognition sequence, Leu-Leu-Ala-Gly-Cys. This addition is followed by the addition of two fatty acids to the diglyceride via ester linkages, SPII cleavage just before the modified Cys residue, and further acylation at the Cys residue, most often by amide linkage to palmitic acid (27). Processing of VirB7 by this pathway would yield a 41-amino-acid polypeptide with a lipid-modified amino-terminal Cys residue. We attempted to gain biochemical evidence for the modification of VirB7 *in vivo* by incubation of AS-induced *A. tumefaciens* A348 in the presence of [^3H]palmitate. These efforts were unsuccessful because of a high level of background in the fluorographs encompassing the region of the gel where the ~4.5-kDa VirB7 protein migrated. We attribute this background to the incorporation of labeled palmitate into the abundant lipopolysaccharides synthesized by *A. tumefaciens* cells. To circumvent this problem, we selectively overexpressed *virB7* from the strong bacteriophage T7 gene 10 promoter in *E. coli* BL21(DE3, pPC979) (Table 1). The fluorographs revealed the presence of a prominently labeled species with a size of ~4.5 kDa in BL21(DE3, pPC979) (Fig. 1, lane 2). This species corresponds in size to the processed form of VirB7, and it was missing from the vector-only strain, BL21(DE3, pT7-SC11) (Fig. 1, lane 1). A faintly labeled species migrating at ~14 kDa also was detected in BL21(DE3, pPC979) cells and not in the isogenic control strain. This species may correspond to a small amount of VirB7 migrating aberrantly because of lipid modification or assembly into a detergent-resistant oligomer.

In these experiments, presumptively labeled VirB7 migrated only slightly more slowly than heavily labeled polysaccharides in the SDS-polyacrylamide gels (Fig. 1). To gain further evi-

FIG. 2. Globomycin sensitivity of VirB7 synthesized by *A. tumefaciens* PC1007(pPCB976). Cells incubated in the absence (–) or presence (+) of globomycin (200 $\mu\text{g}/\text{ml}$) were harvested for analysis after 15 and 30 min of incubation. Total cellular proteins were resolved by Tricine-SDS-PAGE and examined for VirB7 protein content by immunostaining. The positions of VirB7 and the putative proform of VirB7 are indicated at the left; the positions and sizes (in kilodaltons) of protein standards are indicated at the right.

dence for lipid modification of VirB7, we incubated *E. coli* DH5 α (pPC873), which expresses the *virB7::phoA41* chimeric gene, in the presence of labeled palmitate. An ~51-kDa species, the expected size of the VirB7::PhoA41 fusion protein, was labeled (Fig. 1, lane 4). This labeled species was not detected in the vector-only control strain, DH5 α (pUI1156) (Fig. 1, lane 3). Other labeled species were found to be present in both DH5 α strains. The difference in background labeling observed with BL21 and DH5 α cells was attributed to the use of rifampin to inhibit host RNA polymerase prior to the palmitate labeling of BL21 cultures (see Materials and Methods). The 'PhoA moiety does not incorporate palmitate (1). Therefore, the VirB7 portion of the VirB7::PhoA fusion protein was specifically labeled in these experiments.

The cyclic peptide antibiotic globomycin prevents lipoprotein processing in bacteria by inhibiting SPII activity (29). Initial experiments showed that globomycin-treated *A. tumefaciens* A348 induced for *vir* gene expression with AS for various times failed to accumulate detectable levels of either the unprocessed or processed forms of VirB7. These findings suggested that globomycin inhibited the maturation of VirB7 and, furthermore, that the prolipoprotein was unstable. To determine whether unprocessed VirB7 could be visualized soon after synthesis, we introduced *lacI^q* onto plasmid pPC975 (Table 1) for the IPTG-dependent expression of *virB7*. We show below that *A. tumefaciens* PC1007 cells carrying the resulting plasmid, pPCB976, possessed wild-type levels of VirB7 after being induced with 1 mM IPTG. PC1007(pPCB976) cells were incubated in AS-containing IM medium for 5.5 h to account for a possible *vir*-encoded function required for VirB7 processing. The cells were then treated with globomycin for 30 min, IPTG was added to induce *virB7* expression, and aliquots of cells were harvested at various times for analysis of VirB7 protein content.

As was observed with A348 cells, the mature form of VirB7 could not be detected in globomycin-treated PC1007(pPCB976) cells (Fig. 2, lanes 2 and 4). However, a small amount of an immunoreactive protein that migrated higher in SDS-polyacrylamide gels than the mature form of VirB7 was detected in globomycin-treated cells harvested 15 min after IPTG induction. The difference in molecular mass between the immunoreactive proteins present in untreated and globomycin-treated cells was ~2 kDa, the expected size of the signal peptide. This larger, putative precursor form of VirB7 was not detected in globomycin-treated cells harvested at 30 (Fig. 2, lane 4), 45, or 90 min or 14 h (data not shown) after IPTG induction. In a corresponding analysis of *E. coli* BL21(DE3, pPC979) (Table 1), we determined that globomycin-treated cells also failed to accumulate the mature form of

VirB7 and that the putative prolipoprotein was not abundant. However, the presence of material that was cross-reactive with the VirB7 antiserum prevented our detection of low levels of the prolipoprotein in *E. coli* extracts (data not shown).

On the basis of analogy with other bacterial lipoproteins (27), we predicted that the Cys-15 residue within the consensus recognition site for SPII cleavage is critical for the maturation and function of VirB7. We replaced Cys-15 with Ser and examined the effect on VirB7 processing and on *A. tumefaciens* virulence. VirB7C15S did not accumulate to detectable levels in AS-induced PC1007(pXZB16) cells, as determined by immunoblot analyses (data not shown). We next examined the effect of a C15S substitution on the stability of VirB7::PhoA41. *E. coli* CC118(pXZB17) and *A. tumefaciens* PC1007(pXZB17) and A348(pXZB17) cells synthesizing the mutant derivative, VirB7C15S::PhoA41, reproducibly exhibited AP activities of 75 to 100 U per OD₆₀₀ unit, which, as shown below, is considerably lower than the AP activities of cells expressing the unmutated *virB7::phoA41* chimeric gene. However, these AP levels still are significantly higher than the background AP activities of parental *E. coli* CC118 and *A. tumefaciens* A348 cells (0 to 2 U per OD₆₀₀ unit), suggesting that the C15S and C15S::PhoA41 derivatives are exported across the cytoplasmic membrane but then are rapidly degraded in the periplasm. As was expected, PC1007(pXZB16) and PC1007(pXZB17) cells were avirulent after being inoculated onto *Kalanchoe daigremontiana* leaves (see reference 19). Taken together, our data confirm predictions from DNA sequence analyses that VirB7 is processed as a lipoprotein. Furthermore, only the mature form of VirB7 is stable and competent to function as a virulence factor.

Subcellular localization of VirB7 and other VirB proteins in A348. Fatty acylation at the amino terminus serves to tether most bacterial lipoproteins to the membrane. Typically, such lipoproteins as VirB7 that lack a sorting signal consisting of an Asp at the +2 position of the mature protein localize to the inner face of the outer membrane (24, 25, 51). We fractionated AS-induced A348 cells into soluble (cytoplasm and periplasm) and insoluble (total membrane) material and examined the materials for the presence of VirB7 by SDS-PAGE and immunostaining of blots with VirB7 antiserum (see Materials and Methods). We detected VirB7 only in the total membrane fraction (Fig. 3B). We further resolved membrane material into cytoplasmic and outer membranes by two different techniques, isopycnic sucrose gradient centrifugation (Fig. 3) and Sarkosyl solubilization (data not shown). By both techniques, we detected VirB7 primarily in the outer membrane fractions, although a significant amount of protein also partitioned with the cytoplasmic membrane fractions. In several repetitions of these experiments, we consistently found ~60% of total VirB7 protein in outer membrane fractions and ~40% in the cytoplasmic membrane fractions, as determined by densitometry scanning of the immunoblots. NADH oxidase activity, a cytoplasmic membrane marker, was found only in cytoplasmic membrane fractions (Fig. 3A). However, although most of the 2-keto-3-deoxyoctonate, an outer membrane marker, was present in the outer membrane fraction, we reproducibly detected a small amount in cytoplasmic membrane fractions (Fig. 3A). After being corrected for any possible contamination of the cytoplasmic membrane fractions with outer membrane, the amount of VirB7 in the cytoplasmic fraction was estimated to be ~32%. Therefore, we suggest that VirB7 displays a property uncharacteristic of most lipoproteins (see reference 27) by sorting to both the cytoplasmic and outer membrane fractions.

To test whether VirB7 partitioning is influenced by the presence of other VirB proteins, we examined subcellular fractions

FIG. 3. Localization of VirB proteins in *A. tumefaciens* A348. Cells were induced for *vir* gene expression with AS (200 μM) for 18 h and then fractionated as previously described (12). (A) Membrane separation profile, indicating the A_{280} , NADH oxidase activity, sucrose refractive index (p), and 2-keto-3-deoxyoctonate (KDO) values of fractions obtained after isopycnic sucrose density gradient centrifugation. OM, outer membrane; IM, inner membrane. (B) Proteins of the various subcellular fractions were resolved by SDS-PAGE and examined for the presence of VirB proteins by immunostaining. Immunoblots were developed with antibodies reactive to the VirB proteins listed. TP, total cellular protein; TC/P, total soluble (cytoplasmic and periplasmic) protein; TM, total membrane protein; IM, inner membrane protein; OM, outer membrane protein. (C) Localization of VirB7 in *A. tumefaciens* PC1000(pPCB974). Subcellular fractionation, the estimation of the quality of fractionation, and the detection of VirB7 by SDS-PAGE and immunostaining were performed as for A348.

of the *virB* operon deletion derivative, PC1000, transformed with the *virB7* expression plasmid, pPCB974 (Table 1). The quality of the fractionation was assessed with the same criteria used for the A348 fractionation (data not shown). Interestingly, we consistently found a slightly greater amount of VirB7, >80% of total protein, in the outer membrane fractions than was detected in the outer membrane fractions of wild-type cells (Fig. 3C). These findings clearly show that VirB7 sorts to the outer membrane independently of other VirB proteins. Furthermore, our data indicate that the VirB proteins enhance, to a minor extent, the accumulation of VirB7 at the cytoplasmic membrane.

We used our collection of VirB antisera to examine partitioning of other VirB proteins. Several VirB proteins, including VirB5, VirB9, and VirB10, partitioned almost equally between the cytoplasmic and outer membrane fractions (Fig. 3B). Others, including VirB4, VirB8, and VirB11, partitioned predominantly with the cytoplasmic membrane (Fig. 3B). We and others have demonstrated that the VirB4 and VirB11 ATPases associate with the cytoplasmic membrane in the absence of other *vir*- and pTi plasmid-encoded proteins and that the mem-

brane association of both proteins is resistant to various chemical treatments, suggesting that these proteins interact directly with the cytoplasmic membrane (4, 14, 22, 54, 63). Electron microscopy studies by Thorstenson et al. (67) also have shown that VirB8 from pTiC58 associates with the cytoplasmic membrane. Together, these findings indicate that VirB4, VirB8, and VirB11 localize exclusively at the cytoplasmic membrane. By contrast, other VirB proteins, including VirB5, VirB9, and VirB10, may interact directly with both membranes, as was postulated above for VirB7. Alternatively, it is possible that the assembly of these proteins into a tightly interacting protein complex that spans both membranes influences the partitioning of the proteins during subcellular localization.

Protease susceptibility of VirB7 and other VirB proteins.

Most of the VirB proteins contain characteristic signal sequences or hydrophobic domains, leading to predictions that they are partly or entirely exported across the cytoplasmic envelope (35, 58, 66, 71, 72). We used two additional approaches, susceptibility to protease and analysis of VirB::PhoA fusion proteins, to test for the export of VirB7 and other VirB proteins. For the protease susceptibility studies, A348 whole cells, spheroplasts, and detergent-lysed spheroplasts, prepared as described above (see Materials and Methods), were incubated in the presence of proteinase K. In preliminary experiments, we determined that the treatment of whole cells with 400 μ g of proteinase K per ml and of spheroplasts with 100 and 200 μ g of proteinase K per ml caused no detectable loss of DnaA, a cytoplasmic control protein (Fig. 4A, lanes 2, 5, and 6). Treatment of spheroplasts with 400 μ g of proteinase K per ml caused a slight reduction in DnaA protein abundance (Fig. 4A, lane 7), indicating that protease at this concentration somehow gains access to cytoplasmic proteins.

Protease treatment of A348 whole cells as well as spheroplasts caused no detectable loss of VirB7 (Fig. 4A). Interestingly, we also determined that treatment of spheroplasts lysed by detergent and sonication caused only a slight reduction in the levels of VirB7 (Fig. 4A, lane 10). By contrast, treatment of lysed spheroplasts caused the complete degradation of each of the other VirB proteins as well as DnaA (Fig. 4A, lane 10). This result shows that spheroplasts were effectively lysed by detergent treatment and, furthermore, that proteinase K is active in the presence of low concentrations of the nonionic detergents used for lysis. We also determined that VirB7 is resistant to other proteases, including trypsin, *Staphylococcus aureus* V8, and chymotrypsin (data not shown). Therefore, on the basis of these findings, we conclude that VirB7 adopts a protease-resistant conformation upon insertion into the *A. tumefaciens* envelope. Interestingly, other exported proteins in bacteria also are intrinsically resistant to proteases, most notably *E. coli* Lpp lipoprotein assembled as a trimer in the outer membrane (10, 44).

Native PhoA also is protease resistant, but fusion junctions between PhoA and the proteins of interest often are susceptible to proteinase K treatment (69). This fact led us to investigate whether the PhoA derivative VirB7::PhoA41 was degraded by proteinase K. Figure 4B shows that proteinase K treatment of whole cells of A348(pPCB873) failed to cause a detectable reduction in the amount of the 51-kDa VirB7::PhoA41 fusion protein, as assessed by immunodevelopment with VirB7 and with PhoA antiserum. By contrast, proteinase K treatment of spheroplasts caused a reduction in the amount of intact VirB7::PhoA41, as clearly shown by the development of blots with VirB7 antiserum. Interestingly, the development of blots with PhoA antiserum showed only a slight reduction in the levels of an ~50-kDa immunoreactive species. We suggest that this species probably corresponds to the protease-resistant PhoA moi-

FIG. 4. Susceptibility of VirB proteins to protease digestion in spheroplasts of *A. tumefaciens*. AS-induced *A. tumefaciens* A348 (A) and A348(pPCB873) (B) were converted to spheroplasts as described in Materials and Methods. Spheroplasts were lysed by the addition of 2% Triton X-114, which was followed by a brief sonication. Treated samples were subjected to SDS-PAGE and the immunodevelopment of blots with the VirB, PhoA, and DnaA antisera to identify the proteins listed at the right. The corresponding molecular masses (in kilodaltons) are in parentheses below each of the listed proteins. (A) Lanes M, molecular size standards, with sizes (in kilodaltons) listed at the left; lanes 1, whole cells without protease; lanes 2, whole cells with proteinase K added to 400 μ g/ml; lanes 3, whole cells with PMSF added to 2 mM and then proteinase K added to 400 μ g/ml; lanes 4, spheroplasts without protease; lanes 5, spheroplasts with proteinase K added to 100 μ g/ml; lanes 6, spheroplasts with proteinase K added to 200 μ g/ml; lanes 7, spheroplasts with proteinase K added to 400 μ g/ml; lanes 8, spheroplasts with PMSF added to 2 mM and then proteinase K added to 400 μ g/ml; lanes 9, lysed spheroplasts with proteinase K added to 100 μ g/ml. (B) The lanes are the same as those in panel A, except that the order of lanes 5 and 7 is reversed.

ety (~48 kDa) of the VirB7::PhoA41 fusion protein that was released by proteolysis at the fusion junction. Interestingly, this form of PhoA was completely degraded upon protease treatment of cells lysed with 2% Triton X-114 and sonication (Fig. 4B, lane 10). Results of these studies indicate that VirB7::PhoA41 resides predominantly or exclusively in the periplasmic space. Evidence presented elsewhere (59) shows that VirB7::PhoA41 localizes to the same cellular compartment as native VirB7 (see Discussion).

FIG. 5. AP activities of VirB::PhoA fusion proteins in *E. coli* CC118 and *A. tumefaciens* A348. VirB::PhoA proteins were constructed by cloning as described in the text. The positions of the junction sites are indicated with arrows above the *virB* operon. The fusion proteins are identified according to the most carboxyl-terminal amino acid residues of the VirB proteins (as defined by Ward et al. [71, 72]) prior to the PhoA sequence. The AP activities of strains carrying different plasmids were measured in permeabilized cells (41). AP activities in units per OD₆₀₀ unit are presented for each VirB::PhoA fusion protein expressed in *E. coli* CC118 and *A. tumefaciens* A348. The reported activity levels represent an average of three independent assays from a single experiment. The experiments were repeated three times with similar results. *E. coli* cells were incubated in Luria broth with IPTG for the induction of *virB::phoA* genes, and *A. tumefaciens* cells were incubated in IM with and without AS (200 μ M), as described in the text. The numbers below the *virB* operon are a compilation of VirB::PhoA fusions exhibiting AP activity according to Beijersbergen et al. (3) (thin lines) and Ward et al. (74) (thick lines).

Figure 4A also shows that treatment of A348 whole cells failed to cause noticeable reductions in the levels of other VirB proteins, including VirB5 and VirB8 through VirB11. By contrast, treatment of spheroplasts resulted in the complete or nearly complete degradation of VirB5, VirB8, VirB9, and VirB10. In each case, the native protein was significantly reduced in abundance in spheroplasts treated with 100 μ g of proteinase K per ml and was undetectable or present at very low levels in spheroplasts treated with 200 or 400 μ g of proteinase K per ml. The disappearance of native VirB5 or VirB8, VirB9, or VirB10 did not result in a corresponding appearance of low-molecular-weight species reactive with antisera to these VirB proteins. This result indicates that VirB5, VirB8, VirB9, and VirB10 do not contain large domains sequestered from protease because of, for example, a cytoplasmic localization. Such cytoplasmic domains should have been immunologically detectable, given that treatment of spheroplasts with 100 or 200 μ g of proteinase K per ml failed to cause the degradation of DnaA. Therefore, we suggest that VirB5, VirB8, VirB9, and VirB10 possess large periplasmic domains. Additional evidence for the extracytoplasmic localization of these proteins is presented below.

Protease treatment of whole cells caused no detectable reduction in the levels of native VirB11 (Fig. 4A, lane 3). Treatment of spheroplasts with 100 or 200 μ g of proteinase K per ml also did not affect levels of VirB11, but treatment with 400 μ g of proteinase K per ml caused a slight reduction in the levels of VirB11, as was observed for DnaA (Fig. 4A, lane 7). VirB11 is not intrinsically resistant to proteinase K, as was demonstrated by its complete degradation upon the treatment of lysed spheroplasts (Fig. 4A, lane 9). Therefore, we suggest that VirB11, in contrast to the other VirB proteins examined in this study, resides predominantly or exclusively within the interior of the cell.

Evidence for export of VirB proteins by construction of VirB::PhoA fusions. As a complementary genetic approach, we quantitated the AP activities of a collection of VirB::PhoA fusions. We introduced *'phoA* into restriction sites at positions in *virB1* through *virB3*, *virB5*, and *virB7* through *virB10* that possessed a high probability of encoding an exported domain, as determined by sequence analysis (71, 72). A corresponding analysis of a *virB11::phoA* fusion previously failed to provide evidence for the export at least of the carboxyl end of VirB11

(11); results of our studies of *virB4* and *virB6* fused to *'phoA* will be described elsewhere (14). Figure 5 shows the positions of the fusion junctions. During cloning in *E. coli*, each *virB::phoA* fusion was identified as a blue colony on XP plates. Quantitative assays showed that each of the fusion proteins generated in this study exhibited intermediate (50 to 200 U per OD₆₀₀ unit) to high (>200 U per OD₆₀₀ unit) levels of AP activity (Fig. 5). Both *E. coli* CC118 and *A. tumefaciens* A348 host cells reproducibly possessed background (0 to 2 U per OD₆₀₀ unit) levels of AP activities. These findings provide genetic evidence that each of the VirB proteins, VirB1, VirB2, VirB3, VirB5, and VirB7 through VirB10, contains exported domains.

During the course of these studies, we observed that VirB7::PhoA41 displayed an unusual property that distinguished it from the other VirB::PhoA fusion proteins. A348(pPCB873) cells expressing *virB7::phoA41* in *trans* exhibited a relatively low level of AP activity after being incubated in IM without AS. By contrast, the cells exhibited a dramatic ~35-fold enhancement in AP activity after being incubated in IM containing AS (Fig. 5). VirB7::PhoA41 protein content also increased substantially in AS-induced cells, as was shown by the development of immunoblots with anti-VirB7 (Fig. 6) or anti-PhoA antiserum (data not shown). Since pPCB873 constitutively expresses *virB7::phoA41* from *P_{lac}*, it seemed unlikely that the AS-dependent accumulation of VirB7::PhoA41 was due to an increase in the transcription of *virB7::phoA41*. To rule out a possibility that an AS-responsive promoter was somehow introduced upstream of *virB7::phoA41*, we introduced other *virB::phoA* chimeric genes into the pBlue-script II vector used to construct pPCB873 (Table 1). This exchange was conveniently carried out, because several of the *virB::phoA* chimeric genes, in addition to *virB7::phoA41*, are flanked at the 5' and 3' ends by *NdeI* and *KpnI* sites, respectively (Table 1). None of the resulting constructs gave rise to enhanced levels of AP activities or of the corresponding fusion protein upon AS induction of cells transformed with these plasmids (data not shown). This result led us to test the possibility that a Vir protein was involved in stabilizing VirB7::PhoA41. Toward the identification of such a stabilizing element, we introduced the *virB7::phoA41* expression plasmid, pPCB875 (Table 1), into mutants sustaining polar Tn3-HoHo1 insertions near the 5' ends of the essential *vir* operons, *virA* (226Mx), *virB* (243Mx), *virC* (379Mx), *virD* (306Mx), *virE* (361Mx), and *virG* (363Mx) (Table 1). After being assayed for AP activity, mutants with Tn3-HoHo1 inser-

FIG. 6. AP activity (histogram) and VirB7::PhoA41 protein content (immunoblot) of various A348 derivatives transformed with pPCB875. The Tn3-HoHo1 insertion mutations mapped to the 5' ends of the *vir* operons (see reference 61) were *virA* (226Mx), *virG* (363Mx), *virB1* (243Mx), *virC1* (379Mx), *virD1* (306Mx), and *virE1* (361Mx). *ΔvirB* (PC1000) is a *virB* operon deletion mutant constructed as described in the text. The reported AP activity levels represent an average of three independent assays from a single experiment. The experiments were repeated three times with similar results. The immunoblots were developed with VirB7 antiserum so that the VirB7::PhoA41 protein levels in each of the A348 derivatives could be compared with one another.

tions in the *virB*, *virC*, *virD*, and *virE* operons still accumulated high levels of VirB7::PhoA41 protein and AP activity (Fig. 6). Interestingly, two mutants inactivated for the expression of all *virB* genes, 243Mx(pPCB875) and PC1000(pPCB873) (Table 1), exhibited slightly lower AP activities than strains expressing the *virB* genes, indicating that the presence of VirB proteins has a modest effect in promoting the accumulation of VirB7::PhoA41. By contrast, *virA* and *virG* mutants exhibited no AS-dependent enhancement of AP activity or VirB7::PhoA41 protein content (Fig. 6). Therefore, the synthesis of VirA and/or VirG is absolutely required for the stabilization of the fusion protein. For reasons cited below (see Discussion), we favor a model in which VirA and VirG stabilize the fusion protein not through direct protein interactions but instead by activating the expression of an unidentified Vir protein that performs this function.

DISCUSSION

Early molecular genetic and sequence analyses led to the view that the VirB proteins function at a late stage in the infection process, probably as components of a transport apparatus required for exporting T-complexes across the bacterial envelope (35, 58, 60–62, 66, 71, 72). VirB7 is unique among the VirB proteins in that it possesses a consensus sequence for recognition by SPII and lipid modification. In the present study, we provided evidence that (i) VirB7 is processed as a lipoprotein *in vivo*, (ii) VirB7 is anchored predominantly to the outer membrane and resides within the periplasmic space, and (iii) several VirB proteins dependent on VirB7 for accumulation to wild-type levels (see references 19 and 59) contain large periplasmic domains while others, e.g., VirB11, localize predominantly or exclusively in the cytoplasm.

VirB7 is a membrane-associated lipoprotein. VirB7 and VirB7::PhoA41 were modified by fatty acylation *in vivo*, as was demonstrated by the incorporation of [³H]palmitate (Fig. 1). This result fulfills one of two biochemical criteria used to confirm lipoprotein processing. The second, inhibition of

VirB7 processing by globomycin, a known inhibitor of SPII, was demonstrated with a derivative of A348 expressing *virB7* upon induction with IPTG. Results of these globomycin experiments also indicated that processing failure or perhaps aberrant splicing by signal peptidase I (SPI) rendered the pro-lipoprotein extremely unstable. The C15S mutation mimicked the destabilizing effect of globomycin treatment, and as was expected, VirB7C15S failed to complement the *ΔvirB7* mutation in virulence assays. It is notable that this property, the instability of the unprocessed form of the lipoprotein, distinguishes VirB7 from a number of other lipoproteins in which precursors resulting from the treatment of cells with globomycin are readily visualized (27, 29, 31, 65). Elsewhere, we describe a second property that distinguishes VirB7 from most other VirB proteins, namely, that the mature form of VirB7 is stabilized by the formation of intermolecular cross-links (59). Taken together, our data show that two posttranslational processing pathways, a lipoprotein biosynthetic pathway and a disulfide bond formation pathway, are essential for the stabilization of VirB7.

Lipoproteins devoid of the Asp sorting signal at position +2 typically localize to the outer membrane, as was exemplified by PulS, a lipoprotein containing a Gln at the +2 position associated with the pullulanase secretion system of *Klebsiella oxytoca* (15, 52). VirB7 also contains a Gln at the +2 position, but in contrast to PulS, it reproducibly partitioned with the cytoplasmic membrane as well as the outer membrane (Fig. 3). This fractionation profile was observed in wild-type A348 as well as an A348 derivative synthesizing VirB7 in the absence of VirB proteins, indicating that VirB7 possesses information intrinsic to its own sequence for sorting to both membranes. While this property is unusual among lipoproteins, a family of small lipoproteins, the bacteriocin release lipoproteins, also has been reported to copartition with both the cytoplasmic and outer membranes (see references 16 and 47). Interestingly, VirB7 and these bacteriocin release lipoproteins exhibit sequence similarities (58), which raises the possibility that these proteins adopt similar conformations at the bacterial envelope. If so, however, in this instance it appears that the conservation of structure does not extend to that of function. The bacteriocin release lipoproteins are thought to promote the export of bacteriocins across the outer membranes by partially lysing the *E. coli* envelope (16). Our studies have shown that VirB7 instead contributes to the stabilization of VirB proteins during the assembly of a multicomponent transport apparatus (19, 59). This outcome strongly argues that VirB7 does not function in the lysis of either the *A. tumefaciens* envelope or the plant membrane.

The hydropathy profile of VirB7 consists of a stretch of 10 hydrophobic residues near the amino terminus and a large hydrophilic carboxyl domain. Others have noted that bacterial lipoproteins bound to the membrane exclusively by fatty acylation are easily removed from the membranes during cell fractionation (7, 53). We were unable to detect significant levels of VirB7 in the culture supernatant (data not shown) or soluble fractions (Fig. 3), indicating that the amino-terminal hydrophobic residues may serve to strengthen the affinity of VirB7 for membranes. The intrinsic protease resistance of native VirB7 prevented us from demonstrating directly that VirB7 resides within the periplasm. However, VirB7::PhoA41 localized to the periplasm, as was evidenced by its susceptibility to proteases in treated spheroplasts but not whole cells. Furthermore, we have shown elsewhere that (i) native VirB7 is stabilized by forming a disulfide-cross-linked homodimer and (ii) VirB7::PhoA41 cross-links both with itself and with native VirB7 (59). These findings provide compelling evidence that

native VirB7 and VirB7::PhoA41 colocalize in the reducing environment of the periplasmic space. Taken together, the results from these subcellular localization, protease accessibility, and PhoA fusion studies favor a model in which VirB7 is topologically configured as a monotopic protein with an amino-terminal anchor in the cytoplasmic or outer membranes and a periplasmically exposed carboxyl domain. Whether VirB7 also shares a characteristic of many lipoproteins by associating with the peptidoglycan (37) remains to be examined.

Other membrane-associated VirB proteins contain periplasmic domains. To gain information about the structure of the proposed VirB complex and possible contacts between VirB7 and other VirB proteins, we characterized the topologies of other VirB proteins at the membrane. Several VirB proteins, including VirB5, VirB7, VirB9, and VirB10, cofractionated with the cytoplasmic and outer membranes, while VirB4, VirB8, and VirB11 fractionated predominantly with the cytoplasmic membrane (Fig. 3). These findings are in general agreement with VirB protein localization data reported by others, although in specific instances, the data differ with respect to the relative abundances of VirB protein in the cytoplasmic and outer membrane fractions. For example, our findings contrast with those of Thorstenson et al. (67), who reported that VirB4, VirB8, and VirB11 partitioned equally between the cytoplasmic and outer membranes, Ward et al. (74), who reported that VirB10 fractionated almost exclusively with the cytoplasmic membrane, and Shirasu and Kado (57), who reported that VirB9 fractionated predominantly with the outer membrane. We have provided genetic evidence (19) in support of the proposal that most of the VirB proteins assemble as a multimeric complex. Furthermore, this complex is assembled as a stabilized structure only when VirB7 first forms a covalent dimer with VirB9 (19, 59). Thus, it is conceivable that subtle differences in experimental protocols that influence the integrity of the VirB7-VirB9 cross-link may well have an effect on the distribution of individual VirB proteins in a sucrose gradient. Whether these differences account for the apparent discrepancies in the VirB protein localization data remains to be explored.

It is clear from our analyses and investigations by other groups that subcellular localization studies have provided a limited view of the organization of the VirB proteins at the *A. tumefaciens* envelope. We gained additional information about the topological configurations of VirB proteins by two complementary approaches. Our protease susceptibility studies showed that each of the VirB proteins examined in this study, VirB5 and VirB8 through VirB11, remained intact upon proteinase K treatment of whole cells, suggesting that these proteins do not reside extracellularly. By contrast, each of these proteins, with the exception of VirB11, was completely or nearly completely degraded upon protease treatment of spheroplasts. Furthermore, the disappearance of these proteins was not accompanied by the appearance of lower-molecular-weight species potentially representative of protease-inaccessible cytoplasmic domains. Together, these findings indicate that VirB5, VirB8, VirB9, and VirB10 but not VirB11 contain large periplasmic domains. This interpretation is consistent with the available information for each of these proteins. (i) VirB5 and VirB9 have been predicted by sequence analyses to localize extracytoplasmically by virtue of the presence of an amino-terminal signal sequence and the lack of additional hydrophobic regions (35, 58). (ii) At least one immunoreactive domain of VirB8 is localized on the periplasmic surface of the cytoplasmic membrane, as was shown by immunoelectron microscopy studies (68). (iii) Both VirB5 and VirB8 are released from the membrane by exposure to urea, indicating that both proteins are peripherally associated with the membrane (20). (iv) VirB10

has been predicted by sequence analyses (35, 58, 71, 72) to contain one transmembrane domain near its amino terminus and by PhoA fusion studies to contain a large periplasmic carboxyl domain (74). (v) VirB11 has been predicted to remain in the cell interior on the bases of its overall hydrophilicity, the absence of a characteristic signal sequence or obvious transmembrane domains, and lack of AP activity for a VirB11::PhoA fusion (11, 35, 58, 66, 71, 72).

Our VirB::PhoA fusion studies provided evidence for the export of each of the VirB proteins, VirB1, VirB2, VirB3, VirB5, VirB7, VirB8, VirB9, and VirB10, both in *E. coli* and in *A. tumefaciens*. Interestingly, however, as a group, the VirB::PhoA fusions displayed a wide range of AP activity levels ranging from 82 U per OD₆₀₀ unit for VirB7::PhoA41 in uninduced *A. tumefaciens* to 3,070 U per OD₆₀₀ unit for VirB7::PhoA41 in AS-induced *A. tumefaciens*. This variation most probably can be attributed to intrinsic differences in the steady-state levels of the fusion proteins. While levels of these fusion proteins may or may not be correlated with levels of the corresponding native VirB proteins, it is interesting to note from immunoblot studies that the VirB proteins vary widely in abundance despite their apparent cosynthesis from the same *virB* transcript (5, 19). Alternatively, it is possible that the variation in AP activities is explained by the folding of certain VirB::PhoA fusions into conformations that impede the dimerization of PhoA moieties, a prerequisite for AP activity. A third possibility, which could account for the low AP activities of certain VirB::PhoA fusion proteins, e.g., VirB3::PhoA47, is that the fusion junction was located within a region of contiguous hydrophobic residues which in the native protein fold as a cytoplasmic domain but in the fusion protein partially or completely traverse the membrane, thus allowing for the artifactual export of the PhoA moiety. As a precedent, certain 'PhoA fusions within a hydrophobic domain (residues 79 to 94) of mannitol permease resulted in low AP activities, although other data favored a cytoplasmic localization of this region (64).

Results of our VirB::PhoA fusion studies agree with and complement the VirB::PhoA fusion data from Beijersbergen et al. (3) and Ward et al. (74). As can be seen from Fig. 5, these earlier reports provided evidence for the export of VirB1, VirB2, VirB4, VirB5, VirB6, VirB7, and VirB10, although quantitative data were presented only for VirB10 (74). This list now can be extended to include VirB3, VirB8, and VirB9. Our findings also agree with the PhoA fusion data reported by Pohlman et al. (50) for the homologous Tra proteins of the IncN plasmid, pKM101, which further establishes the relatedness of these two systems. On the basis of the results of the PhoA and protease susceptibility studies, a picture emerges in which many of the VirB proteins, including VirB5, VirB7, VirB8, VirB9, and VirB10, reside predominantly or exclusively within the periplasmic space. VirB1, VirB2, and VirB3 may also localize in the periplasmic space, but these also appear to be the only candidates among the VirB proteins for localization at the cell exterior. Of the three remaining VirB proteins, VirB6 is predicted by sequence analysis to span the cytoplasmic membrane at least six times (see reference 81). VirB6 appears to have a stabilizing activity on other VirB proteins (5). These features suggest that VirB6 is a key structural component of the presumed VirB protein complex at the cytoplasmic membrane. The VirB4 and VirB11 ATPases like other ATPase subunits of many eubacterial transporters (18), are proposed to reside predominantly or exclusively within the cell interior.

Our studies do not exclude the possibility that a VirB protein can be redistributed to the cell exterior during the infection process, for example, in response to a plant or bacterial mating

signal. This point is an important point in view of the proposal that the T-complex transport system may consist not only of a transmembrane channel for the export of T-complexes but also of a pilus for the attachment of agrobacterial donors to competent recipients (33, 57). By analogy, TraA pilin subunits of the *E. coli* F pili have been shown to accumulate in the cytoplasmic membrane, but upon receipt of an unknown signal, they are recruited to the cell exterior for the polymerization of F pili (21).

A stabilizing activity for the VirB7::PhoA41 fusion protein. Construction of the VirB::PhoA fusions served to demonstrate that these proteins contain export signals. However, we also sought to test the notion that PhoA could serve as a useful reporter for the identification of stabilizing interactions between a VirB component of a VirB::PhoA fusion protein and another Vir protein. Among the VirB::PhoA fusion proteins analyzed, only VirB7::PhoA41 displayed the phenotype consistent with this possibility, that is, enhanced levels of AP activity upon AS induction. Given the position of *virB7* in the *virB* operon, we expected that VirB proteins would contribute to the stabilization of VirB7::PhoA41. Surprisingly, however, the fusion protein also accumulated to high levels in AS-induced PC1000(pPCB873) as well as in each of the 11 $\Delta virB$ gene mutants carrying pPCB873 (data not shown). Among the essential Vir proteins that we tested, only VirA and VirG were absolutely required for the accumulation of the fusion protein. VirA or VirG potentially stabilizes the fusion protein through direct interactions, but such interactions likely would reciprocally influence VirA-VirG-mediated transcription of *vir* promoters. However, this two-component system functions at wild-type levels in strains with mutations for the expression of native *virB7* as well as the other *virB* genes (61). Furthermore, we have determined that the Tn3-HoHo1 strains, 243Mx, 379Mx, 306Mx, and 361Mx, which contain $P_{vir}::lacZ$ transcriptional or translational fusions exhibit levels of β -galactosidase activity comparable to those of the isogenic strains transformed with the *virB7::phoA41* expression plasmid pPCB875 (data not shown). This result shows that VirA and VirG activities also are unaffected by the synthesis of large amounts of VirB7::PhoA41. On the basis of these observations, we suggest that VirA and VirG activate the transcription of an unidentified *vir* gene whose product interacts with and stabilizes VirB7::PhoA41. The proposed protein may stabilize VirB7::PhoA41 through a direct interaction. By analogy, Lpp has been proposed to be stabilized through interactions with OmpA or another receptor required for assembly as a trimer in the outer membrane (10, 44). Alternatively, it is possible that the proposed Vir protein functions as a component of the VirB7 maturation pathway. In this regard, it is interesting to note that VirB7C15S::PhoA41 exhibits low levels of AP activity and, unlike VirB7::PhoA41, is unresponsive to AS induction. This result could imply that the proposed stabilizing factor fails to recognize or interact productively with the C15S derivative. Toward the identification of the proposed stabilizing factor, we currently are screening for mutations that abolish the AS-dependent accumulation of VirB7::PhoA41.

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