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Genetic Complementation Analysis of the *Agrobacterium tumefaciens virB* Operon: *virB2* through *virB11* Are Essential Virulence Genes

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The *Agrobacterium tumefaciens virB* gene products are proposed to assemble into a transport system capable of exporting complexes of DNA and protein across the bacterial envelope en route to plant cells. Nonpolar null mutations were constructed in each of the 11 *virB* genes of the *A. tumefaciens* pTiA6NC plasmid. In tumorigenicity assays, Δ*virB1* mutants exhibited severely attenuated virulence and Δ*virB2* through Δ*virB11* mutants exhibited avirulence. *Nde*I restriction sites introduced at the predicted translational start sites of the *virB* genes were used to subclone each of the *virB* genes downstream of the *lacZ* or *virB* promoter on broad-host-range plasmids. *virB* gene expression plasmids were used to define promoter and general sequence requirements for genetic complementation of the deletion mutations. Whereas *virB1* and *virB2* complemented Δ*virB1* and Δ*virB2*, respectively, only when expressed in *trans* from the *virB* promoter, *virB3* through *virB11* complemented the corresponding deletion mutations when expressed in *trans* from either the *lacZ* or *virB* promoter. Several *virB* genes required additional upstream or downstream sequences for complementation: (i) *virB2* complemented the Δ*virB2* mutation only when the complementing plasmid coexpressed *virB1* and *virB2*, (ii) *virB6* and *virB9* complemented the Δ*virB6* and Δ*virB9* mutations only when the complementing plasmids carried at most 55 and 230 bp of sequences residing 5′ of these genes, respectively, and (iii) *virB7* and *virB8* complemented the Δ*virB7* and Δ*virB8* mutations only when the complementing plasmid coexpressed *virB7* and *virB8*. These studies established that *virB1* is an accessory virulence determinant and *virB2* through *virB11* are absolutely essential for the *A. tumefaciens* infection process.

Agrobacterium tumefaciens incites susceptible plants to develop crown gall tumors by a DNA transfer event which is unique among known bacterial pathogens (for reviews, see references 8, 18, 47, and 49). In response to phenolic (32) and sugar signals (4, 26) released from wounded plant tissues, *A. tumefaciens* processes a specific segment of its DNA (T-DNA) residing on a large plasmid (pTi) into a translocation-competent complex of T-DNA and protein. This T-complex is delivered from the bacterium to the plant nucleus, where the T-DNA integrates, apparently by illegitimate recombination (14), into the plant nuclear genome. Expression of oncogenes encoded by the T-DNA disrupts endogenous plant hormone levels, resulting in cellular proliferation and the formation of characteristic galls.

Translocation-competent T-complexes have been proposed to consist of single-stranded T-DNA molecules (T-strands) and several proteins. These proteins include VirE2 (60 kDa), a single-stranded DNA-binding protein which noncovalently associates along the length of the T-strand, and VirD2 (56 kDa), one of two subunits composing the VirD endonuclease which likely binds covalently to the 5′ end of the T-strand (for reviews, see references 8, 18, 47, and 49). The estimated molecular mass of a T-complex exceeds 50×10^6 Da (8). Each complex constitutes an extremely large macromolecule that must transit several prokaryotic and eukaryotic membranes to reach its final destination in the plant cell nucleus. Several lines of genetic and biochemical evidence suggest that the ~9.5-kb *virB* operon encodes the structural components of the transport apparatus dedicated to exporting T-complexes across the *A. tumefaciens* envelope (1, 2, 11, 20, 28–30, 37–39, 41–45, 49). Early genetic studies demonstrated that the VirB proteins function at a late stage, following T-strand synthesis, in the infection process (34). More recently, the importance of at least some VirB proteins has been demonstrated for the interkingdom transfer of T-complexes as well as broad-host-range IncQ plasmids (43). In fact, VirB proteins have also been shown to direct interbacterial transfer of IncQ plasmids (1) and to enhance interbacterial transfer of pTi plasmids (13, 36). Consistent with their proposed transport functions, most, if not all, of the VirB proteins associate with the bacterial envelope (2, 6, 11, 28, 29, 38, 39, 45).

The VirB proteins resemble protein subunits of other systems dedicated to macromolecular transport. For example, by the criteria of amino acid sequence similarity, hydropathy, and cytoplasmic membrane localization, VirB11, an ATPase with autophosphorylating activity (6), resembles nucleoside triphosphate (NTP)-binding subunits associated with a large number of bacterial transport systems. The conserved NTP-binding proteins likely supply the energetics, through NTP hydrolysis and/or phosphorylation, to drive the export or import of macromolecules as diverse as mono- and multimeric proteins, naked DNA, and DNA-protein complexes (see references 6, 15, and 25). In addition, extensive sequence similarities between most of the 11 VirB proteins and components of two distinct classes of bacterial transporters recently have been shown to exist. The first class consists of the conjugal transfer systems of IncP, IncN, and IncW broad-host-range plasmids (18, 21, 47a). The *trb* operon of the IncP plasmid, pRP4, encodes proteins which exhibit sequence similarities to six VirB proteins (21, 22). Transfer operons of the IncN (47a) and IncW (see reference 18) plasmids encode proteins which exhibit sequence similarities to most, if not all, of the 11 VirB proteins. Furthermore, with few exceptions, genes encoding

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similar proteins are colinear in the *virB* and the IncP, IncN, and IncW transfer operons (18, 21, 47a). These similarities have served to strengthen a hypothesis that DNA transport to plants is an evolved form of interbacterial conjugation (3, 35). The discovery that VirB proteins are required for both the interbacterial (1) and interkingdom (43) transport of IncQ plasmids provides experimental evidence in support of this conjugation model.

The second related transport system, encoded by the *Bordetella pertussis ptl* operon (9, 46), directs the export not of DNA-protein complexes but of a complex of six proteins comprising pertussis toxin. The *B. pertussis ptl* operon consists of at least eight genes whose products have sequence similarities with VirB and broad-host-range plasmid transfer proteins. Again, with few exceptions, these genes are colinear with related genes encoded by the *virB* and the broad-host-range transfer operons (18, 21, 27, 46, 47a). The apparently common ancestry of these macromolecular transporters suggests that conserved structural and mechanistic features likely exist among these and perhaps other transporters dedicated to the export of substrates ranging from large protein complexes to complexes of DNA and protein. At present, little information is available regarding the assembly or the final structures of these transporters at the gram-negative envelope. Virtually nothing is known about the signals that confer substrate recognition or the energetics of the transport process.

To initiate studies aimed at elucidating the structural, functional, and energetic features of the T-complex transport system of *A. tumefaciens*, we have constructed 11 nonpolar *virB* null mutants. Constructs consisting of *virB* genes expressed from a cloned *virB* promoter or from the *lacZ* promoter were tested for the ability to genetically complement the null mutations. Our results show that *virB2* through *virB11* are essential virulence genes, whereas *virB1* is important but nonessential for *A. tumefaciens* infection. These findings are discussed in the context of recent studies of the VirB, Tra, and Ptl transporters.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 lists bacterial strains and plasmids used in this study. *A. tumefaciens* cells were transformed by electroporation as previously described (2). Recombinant broad-host-range plasmids expressing *virB* genes were recovered from *A. tumefaciens* by the boiling minipreparation method and then introduced into *Escherichia coli* DH5α for preparative-scale DNA isolation (2). Bacterial growth media, bacterial and phage growth conditions, and procedures for *vir* gene induction with acetosyringone have been previously described (2, 6, 7, 17). In *E. coli*, plasmids were maintained by addition of carbenicillin (100 μg/ml), kanamycin (100 μg/ml), chloramphenicol (50 μg/ml), or tetracycline (10 μg/ml) to the growth medium. In *A. tumefaciens*, plasmids were maintained by addition of carbenicillin (100 μg/ml), kanamycin (100 μg/ml), or tetracycline (10 μg/ml).

Protein analysis and immunoblotting. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (2, 7). Vir proteins were visualized by SDS-PAGE, protein transfer to nitrocellulose membranes, and immunoblot development with goat anti-rabbit or anti-mouse antibodies conjugated to alkaline phosphatase (7). Polyclonal antiserum specificities were previously documented for the VirE2 (7), VirB4 (2), VirB9 (44), VirB10 (44), and VirB11 (6) proteins.

In vitro construction of Δ*virB* mutations. Each of the *virB* genes, *virB1* through *virB11*, was precisely deleted from the pTiA6NC plasmid by using a combination of site-directed mutagenesis or subcloning and marker exchange-eviction mutagenesis, essentially as described previously for construction of a Δ*virB4* mutation (2). Plasmids sustaining *virB* gene deletions with 300 to 800 bp of flanking sequence were generated as follows. Deletions of *virB1* through *virB8* were generated by site-directed mutagenesis with oligonucleotides and uracil-incorporated template DNA derived from plasmids listed in Figure 1. The oligonucleotides (30-mers) were designed so that the 5′ half was complementary to the first ~15 bases of the gene downstream of the target gene to be deleted and the 3′ half was complementary to sequences immediately upstream of the target gene. Gene deletions were constructed in vitro by annealing oligonucleotides to template DNAs and then synthesizing the complementary strands with T4 or T7 DNA polymerase. The desired products of in vitro synthesis consisted of annealed uracil- and non-uracil-containing strands and a single-stranded, uracil-containing loop conforming to the *virB* gene targeted for deletion. Reaction products were introduced into *E. coli* DH5α for selective degradation of uracil-containing DNA and replication of the non-uracil-containing strand. At least two putative *virB* gene deletion mutations were identified in a screen of 10 transformants by restriction endonuclease mapping and by DNA sequencing across the deletion junction. DNA sequence analyses also confirmed that the flanking sequences used for recombination of each Δ*virB* mutation onto the pTi plasmid (Fig. 1) sustained no sequence alterations other than the desired *virB* gene deletion.

The Δ*virB9* mutation was constructed by precisely removing the *virB9* coding sequence carried on a 900-bp *Nde*I-*Hin*dIII fragment from plasmid pPC997 (Table 1; see below) and substituting in its place *virB10* carried on a 1.2-kb *Nde*I-*Hin*dIII fragment isolated from pPC9108 (Table 1). The resulting plasmid, pBB991, sustains a precise replacement of *virB9* by the *virB10* coding sequence.

The Δ*virB10* mutation was constructed by introducing two fragments, a 0.69-kb *Sac*II-*Bam*HI fragment containing the 3′ end of *virB9* from pJW283 (Table 1) and a 1.47-kb *Eco*RI-*Hin*dIII fragment containing *virB11* from pJW322 (Table 1), into corresponding sites within pBCSK$^+$. The resulting plasmid, pBB9102, contains the 3′ end of *virB9*, only 42 bp of the 5′ end of *virB10*, and *virB11*.

The Δ*virB11* mutation was constructed by digesting pJW322 (Table 1) with *Bam*HI and *Bgl*II and religating the plasmid. The resulting plasmid, pBB9112, contains only 40 bp of *virB11* coding sequence.

Marker-exchange eviction mutagenesis. The Δ*virB* mutations and approximately equal lengths of flanking DNA were subcloned into the suicide vector pBB50 (Table 1) for delivery into *A. tumefaciens*. Two features of pBB50, its failure to replicate in *A. tumefaciens* and the presence of the counterselectable *sacB* gene, allow for the rapid generation of chromosomal or pTi plasmid allelic replacements (see reference 2). The suicide plasmid carrying Δ*virB1* was constructed as follows. First, a *Sal*I-*Bgl*II fragment sustaining the Δ*virB1* mutation was introduced into the *Sal*I and *Bam*HI sites in the pBCKS$^+$ polylinker. Next, a *Bam*HI restriction fragment carrying the *sacB* and *nptII* genes was isolated from pAS505a (Table 1), made blunt ended with Klenow fragment, and introduced into the plasmid at the unique *Sca*I site in the *cat* gene. The suicide plasmids carrying the Δ*virB2* through Δ*virB11* mutations were constructed by introducing the fragments shown in Fig. 1, each containing blunt ends at their 5′ and 3′ termini, into the unique *Sca*I site in the *cat* gene of pBB50. This series of plasmids was designated pBB1X0, where

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	λ⁻ ϕ80d/*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA relA1*	GIBCO BRL
CJ236	*dut ung thi relA*; pCJ105 (Cam^r)	Bio-Rad
A. tumefaciens		
A136	Strain C58 cured of pTi plasmid	Laboratory stock
A348	A136 containing pTiA6NC	Laboratory stock
A348Δ*virBX*	A348 derivatives, each sustaining a *virB* gene deletion from pTiA6NC, where *X*, numbered 1 through 11, represents the deleted *virB* gene	This study
Plasmid vectors		
pBSIISK$^+$	Carb^r, cloning vector	Stratagene
pBCSK$^+$	Cam^r, cloning vector	Stratagene
pBSIISK$^+$.*Nde*I	Carb^r, pBIISK$^+$ containing an *Nde*I restriction site at the translational start site of *lacZ'*	This study
pBCSK$^+$.*Nde*I	Cam^r, pBCSK$^+$ containing an *Nde*I restriction site at the translational start site of *lacZ'*	This study
pBKSK$^+$.*Nde*I	Kan^r Cam^s, pBCSK$^+$.*Nde*I containing the *nptII* gene from pUC4K introduced at the unique *Sca*I site within the *cat* gene	This study
pUC4K	pUC7 carrying the *nptII* gene from Tn*5*	Pharmacia
pAS505a	Carb^r Kan^r, ~3-kb *Bam*HI fragment containing *nptII* and the *Bacillus subtilis sacB* gene in pUC7	A. Sawato
pBB50	Kan^r, ~3-kb *Bam*HI fragment containing the *nptII* and *sacB* genes from pAS505A introduced at the *Bam*HI site within the polylinker of pBCSK$^+$	This study
pSW172	Tet^r, broad-host-range IncP plasmid containing *lacZ* promoter and polycloning region from pIC19	5
pED33	Kan^r, 0.6-kb *Xho*I fragment *virB* promoter cassette from pED31 in *Sal*I site of pTJS75-kan	44
Plasmid sources of *virB* genes		
pZD1	Carb^r, 3.71-kb *Eco*RV-*Sac*I fragment containing *virB2* through *virB5* cloned downstream of *lacZ* promoter in pBSIIKS$^+$	2
pJW239	Carb^r, 5.15-kb *Bam*HI-14 fragment of pTiA6NC containing 3′ end of *virB4-virB9* cloned downstream of the *lacZ* promoter in pTZ19R	2
pJW283	Carb^r, 2.22-kb *Eco*RI-*Bam*HI fragment containing *virB7* through *virB9* in pUC118 downstream of the *lacZ* promoter	J. Ward
pJW322	Carb^r, *virB11* cloned downstream of the *lacZ* promoter	6
pJW327	Carb^r, *virB9* and *virB10* cloned downstream of the *lacZ* promoter	44
pUC8Sal13a	Carb^r, 3.5-kb *Sal*I fragment 13a of pTiA6NC in pUC8	2
pBB2	Cam^r, 2.87-kb *Sal*I-*Bam*HI fragment from pUC8Sal13a containing *virB1* through 5′ end of *virB4* in pUC13CM, placing these genes under *lacZ* promoter control	This study
pBB5	Carb^r, *Eco*RV-*Sac*I fragment containing *virB2* through *virB5* deleted of a 668-bp *Bgl*II-*Bam*HI fragment within *virB4* in pBSIIKS$^+$	2
pBB8	Carb^r, 2.87-kb *Sal*I-*Bam*HI fragment from pBB2 containing *virB* promoter through 5′ end of *virB4* in pBSIIKS$^+$, placing these genes under *lacZ* promoter control	This study
pBB20	Carb^r, 4.27-kb *Sph*I fragment from pJW239 containing the 3′ end of *virB4* through *virB8* in pUC118 downstream of the *lacZ* promoter	This study
Plasmids used for constructing *virB* gene deletions		
pBB991	Carb^r, 1.14-kb *Nde*I-*Hin*dIII fragment containing *virB10* from pPC9108 inserted into similarly digested pPC997, resulting in a deletion of *virB9*	This study
pBB9102	Cam^r, 0.69-kb *Sac*II-*Bam*HI fragment containing *virB9* from pJW283 and a 1.47-kb *Eco*RI-*Hin*dIII fragment containing *virB11* from pJW322 introduced into pBCSK$^+$, resulting in a deletion of *virB10*	This study
pBB9112	Carb^r, pJW322 deleted of a 996-bp *Bgl*II-*Bam*HI fragment, resulting in a deletion of *virB11*	This study
pBB1X0 series	Plasmid pBB50 containing each of the *virB* gene deletions and flanking *virB* sequences; the *virB* gene designated by X, numbered 1 through 11, was deleted by in vitro mutagenesis or by subcloning	This study

Continued on following page

TABLE 1—*Continued*

Bacterial strain or plasmid	Relevant characteristics	Source or reference
Plasmids used for complementation studies		
pPC9X7 series	Carb^r, pBluescript or pUC plasmids carrying *virB* genes mutated to carry *Nde*I restriction sites at the translational start sites of each *virB* gene; the *virB* gene designated by X, numbered 1 through 11, carries an *Nde*I restriction site introduced at the translational start site predicted by Ward et al. (41, 42)	This study
pPC9X8 series	Cam^r, each *virB* gene designated by X, numbered 1 through 11, isolated from pPC9X7 with *Nde*I and a second enzyme and introduced into pBCSK$^+$.*Nde*I	This study
pPC9X3 series	Kan^r, pPC9X8 plasmids containing the *nptII* gene from pUC4K at the *Sca*I site within the *cat* gene	This study
pPC9X4 series	Carb^r, *virB* genes designated by X, numbered 1 through 11, isolated from pPC9X7, deleting *virB1.Nde*I and placing the *virB.Nde*I gene of interest under *virB* promoter control	This study
pPC914	Carb^r, 1.37-kb *Hin*dIII fragment from pPC917 containing the *virB* promoter and *virB1* with an *Nde*I site at its translational start site in pBSIISK$^+$; *virB1* is under *virB* promoter control	This study
pPC924	Carb^r, 1.4-kb *Nde*I-*Bam*HI fragment containing *virB2* and *virB3* from pPC927 into pPC914, substituting for the 0.82-kb *Nde*I-*Bam*HI fragment containing *virB1* and placing *virB2* and *virB3* under *virB* promoter control	This study
pPC964	Carb^r, 1.04-kb *Nde*I-*Bgl*II fragment containing *virB6* from pPC967 into the *Nde*I and *Bam*HI sites of pPC914, substituting for the 0.82-kb *Nde*I-*Bam*HI fragment containing *virB1* and placing *virB6* under *virB* promoter control	This study
pPC974	Carb^r, 0.29-kb *Nde*I-*Nru*I fragment containing *virB7* from pPC977 into the *Nde*I and *Eco*RV sites of pPC914, substituting for the 0.82-kb *Nde*I-*Bam*HI fragment containing *virB1* and placing *virB7* under *virB* promoter control	This study
pPC994	Carb^r, 0.92-kb *Nde*I-*Bam*HI fragment containing *virB9* from pPC997 into pPC914, substituting for the 0.82-kb *Nde*I-*Bam*HI fragment containing *virB1* and placing *virB9* under *virB* promoter control	This study
pPC912	Carb^r, 2.13-kb *Nde*I-*Bam*HI fragment containing *virB1*, *virB2*, and *virB3* from pPC917 in pBSIISK$^+$.*Nde*I; these genes are under *lacZ* promoter control	This study
pPC915	Carb^r, 0.89-kb *Hin*cII-*Hin*dIII fragment containing *virB1* from pBB8 in the *Eco*RV-*Hin*dIII sites of pBSIISK$^+$, placing *virB1* under *lacZ* promoter control	This study
pPC916	Carb^r, 2.13-kb *Nde*I-*Bam*HI fragment containing *virB1*, *virB2*, and *virB3* from pPC917 in pPC914, substituting for the 0.82-kb *Nde*I-*Bam*HI fragment containing *virB1* and placing these genes under *virB* promoter control	This study
pPC922	Kan^r, 1.7-kb *Sac*II-*Bam*HI fragment containing *virB2* and *virB3* from pBB2 into pBKSK$^+$.*Nde*I, placing these genes under *lacZ* promoter control	This study
pPCB925	Carb^r Kan^r, pBB8 digested with *Sac*II and ligated with similarly digested pED33, placing *virB2* and *virB3* under *virB* promoter control	This study
pPC961	Carb^r, 1.1-kb *Sac*I-*Bgl*II fragment containing *virB6* from pBB20 into *Sac*I and *Bam*HI sites of pBSIISK$^+$, placing *virB6* under *lacZ* promoter control	This study
pPC962	Carb^r, 1.047-kb *Cla*I-*Bgl*II fragment containing *virB6* from pBB20 into pBSIIKS$^+$, placing *virB6* under *lacZ* promoter control	This study
pPC975	Kan^r, 0.96-kb *Nde*I-*Hin*dIII fragment containing *virB7* and *virB8* from pPC977 into pBSIIKSK$^+$. *Nde*I, placing *virB7* and *virB8* under *lacZ* promoter control	This study
pPC981	Kan^r, 0.88-kb *Nde*I-*Hin*dIII fragment containing *virB8* from pPC986 in pBSIIKSK$^+$.*Nde*I, placing *virB8* under *lacZ* promoter control	This study
pPC982	Carb^r, 0.84-kb *Bgl*II-*Hin*dIII fragment containing *virB8* from pBB20 in pBSIISK$^+$, placing *virB8* under *lacZ* promoter control	This study
pPCB985	Carb^r Kan^r, pBB20 digested with *Bgl*II and ligated with pED33 digested with *Bam*HI, placing *virB8* under *virB* promoter control	This study
pPC986	Carb^r, pBB20 carrying an *Nde*I site introduced by site-directed mutagenesis at bp 6325	This study
pED9	Tet^r, *virB9*, *virB10*, and *virB11* cloned downstream of the *virB* promoter	44
pPCB992	Carb^r Tet^r, pJW327 digested with *Sna*BI and ligated to pSW172 digested with *Eco*RV, placing *virB9* under *lacZ* promoter control	This study
pPCB995	Tet^r, pED9 digested with *Pst*I and religated, resulting in a deletion of *virB10* and *virB11*; *virB9* is under *virB* promoter control	This study

X, numbered 1 through 11, represents the cloned Δ*virB* mutation (Table 1).

Each of the pBB1X0 suicide plasmids was introduced by electroporation into the wild-type strain A348 with selection for Kan^r transformants. Kan^r transformants that were also sucrose sensitive were presumed to have arisen by plasmid integration as a result of a single-crossover event between homologous *virB* sequences on the pBB1X0 plasmids and the pTi plasmid. Five Kan^r Suc^s strains derived from each of the 11 transformations were grown overnight in 2 ml of antibiotic-free

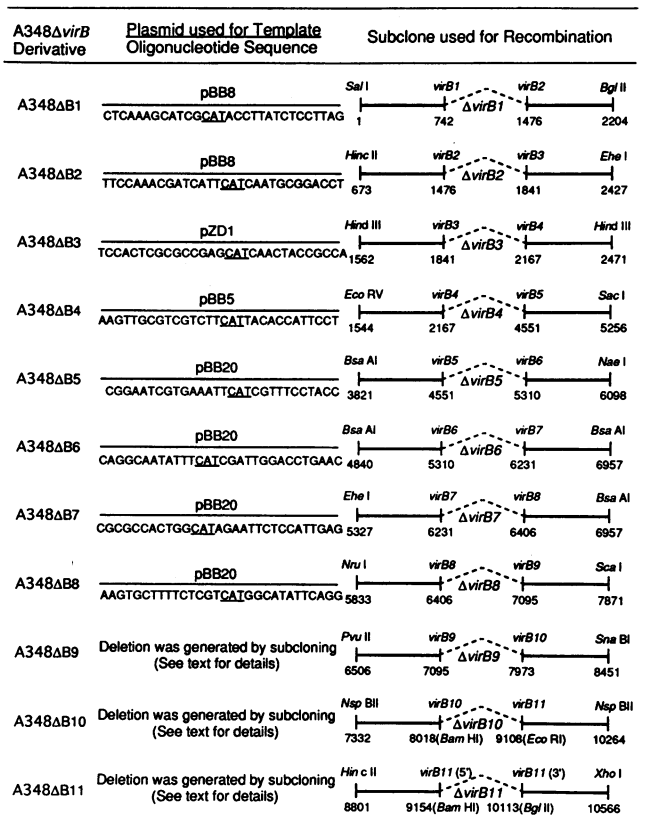

FIG. 1. Construction of A348Δ*virB* strains. The *virB* gene deletions listed at the left were constructed by site-directed mutagenesis or subcloning, as described in the text. The plasmid sources for template DNA (Table 1) and the mutagenic oligonucleotides used for site-directed mutagenesis are shown for each *virB* gene deletion. In each case, the deletion junctions reside immediately 3′ of the underlined CAT sequences. Restriction sites residing approximately equidistant from the Δ*virB* mutations were used to subclone each gene deletion onto the suicide vector, pBB50. Positions of restriction sites and translational start sites as predicted by Ward et al. (41, 42) are shown by use of the left junction of *Sal*I fragment 13a of pTiA6NC as a reference point. The resulting plasmids, designated pBB1X0, where X represents *virB1* through *virB11* (Table 1), were used to evict each *virB* gene from pTiA6NC with a two-step recombination procedure described in the text.

MG/L (33) medium to allow for excision of vector sequences by a second crossover event. The desired double recombinants were enriched by diluting cultures 100-fold into MG/L medium supplemented with 5% sucrose as a counterselection for cells in which the *sacB* lethality gene had been excised. Overnight cultures were spread or streaked on MG/L plates containing 5% sucrose, and 20 to 50 Suc^r colonies from each of the five parental strains were screened for Kan^s. Sucrose counterselection resulted in recovery of the desired Suc^r Kan^s double recombinants (see below), as well as sucrose-resistant colonies that may have arisen through inactivation of *sacB* or another gene associated with sucrose metabolism.

Identification of nonpolar Δ*virB* mutations in pTiA6NC. *virB* gene deletion mutants were identified as follows. For each *virB* gene, three Suc^r Kan^s strains derived from each of the five Suc^s Kan^r parental strains, for a total of 15 strains, were examined by Southern blot hybridization. Total DNAs extracted from the putative mutants and from wild-type A348 were digested with restriction enzymes whose cleavage patterns on gels were predicted to be altered by the *virB* deletion of interest. To identify mutants sustaining deletions of the expected size, blots were probed with radioactively labeled restriction fragments that were complementary to sequences flanking the deletion junction. To confirm the gene deletion, blots were probed with sequences complementary to the target gene. Finally, to verify that the deletion mutants sustained no other alterations in the *virB* operon, blots were probed with the entire *virB* operon that we have cloned onto a Bluescript plasmid. These analyses resulted in the identification of at least three mutants for each *virB* gene that sustained a deletion of the expected size and possessed no other detectable alterations in the *virB* operon (data not shown).

Introduction of *Nde*I restriction sites at the predicted start codons of the *virB* genes. Figure 2 lists the template sources and the oligonucleotides used to introduce *Nde*I restriction sites at the translational start sites of each of the 11 *virB* genes as predicted by Ward et al. (41, 42). Throughout this paper, nucleotide positions are numbered with reference to the left junction of *Sal*I fragment 13a of pTiA6NC, according to the system of Ward et al. (41, 42). Mutagenic oligonucleotides were designed to contain an *Nde*I site (CATATG) flanked by sequences complementary to *virB* sequences residing 5′ and 3′ of each *virB* start codon. The mutagenesis scheme essentially was the same as that used for construction of the Δ*virB* mutations. Transformants were screened by endonuclease digestions and DNA sequence analyses for the presence of a correctly positioned *Nde*I site. Plasmids were designated pPC9X7, where X, numbered 1 through 11, represents the *virB* gene possessing an *Nde*I site at the predicted start codon (Table 1).

The *virB* genes were subcloned from the pPC9X7 plasmids downstream of the *lacZ* promoter and, in some instances, the *virB* promoter, by using *Nde*I and the restriction enzymes shown in Fig. 2. *virB* genes cloned in expression plasmids by use of *Nde*I and a 3′-cutting enzyme were designated *Nde*I.*virBX*, where *X*, numbered 1 through 11, represents the *virB* gene of interest. Restriction fragments carrying each *virB* gene were placed under transcriptional control of the *lacZ* promoter by cloning into similarly digested pBCSK$^+$.*Nde*I (Table 1). This is a derivative of pBCSK$^+$ engineered by site-directed mutagenesis to contain an *Nde*I restriction site at the start codon of *lacZ′* residing 55 bp upstream of the polylinker sequences. All pBluescript plasmid derivatives engineered in these studies to carry *Nde*I sites at this position (Table 1) utilized the oligonucleotide 5′-GTAATCATGGTCATATGTGTTTCC-3′ for the mutagenesis.

The pBCSK$^+$.*Nde*I plasmids carrying the *virB* genes under *lacZ* promoter control were designated pPC9X8, where X, numbered 1 through 11, represents the subcloned *virB* gene. Plasmids pPC918 and pPC938 through pPC9118 express a single *virB* coding sequence from the *lacZ* promoter. Plasmid pPC928 expresses both the *virB2* and *virB3* coding sequences from the *lacZ* promoter.

Introduction of *virB* genes downstream of a *virB* promoter. *virB* genes were placed under transcriptional control of the *virB* promoter as follows. First, a 1.37-kb (bp 189 to 1562) *Hin*dIII fragment isolated from pPC917 (Table 1) was introduced in the orientation opposite that of the *lacZ* promoter in pBSIISK$^+$. The resulting plasmid, pPC914, carries the *virB* promoter and *virB1* with an *Nde*I site introduced at its translational start codon (bp 742). Additional *virB* genes were placed under *virB* promoter control by replacing the *virB1* coding sequence with a *virB* gene of interest. This was achieved by deleting *virB1* from pPC914

FIG. 2. Construction of *Nde*I.*virB* gene cassettes. *Nde*I restriction sites were introduced by site-directed mutagenesis at the translational start sites of the *virB* genes as predicted by Ward et al. (41, 42). The mutagenized sites are indicated by the long arrows, the positions of these sites are indicated in parentheses, and the plasmid sources for template DNA (Table 1) and the mutagenic oligonucleotides with the underlined *Nde*I restriction site are shown. The *Nde*I sites and other restriction sites, indicated by short arrows, were used to subclone the *virB* genes downstream of the *lacZ* or *virB* promoters, as described in the text. The set of plasmids shown, designated pPC9X8, where X is numbered 1 through 11, consists of the corresponding *virB* genes cloned in pBCSK$^+$.*Nde*I (Table 1) downstream of the *lacZ* promoter.

by digestion with *Nde*I and an enzyme that cleaves in the downstream polylinker sequence and then inserting a *virB* gene of choice isolated from the pPC9X7 or pPC9X8 series of plasmids as a fragment with compatible restriction enzyme sites. The resulting plasmids were designated pPC9X4, where X represents the cloned *virB* gene expressed from the *virB* promoter (Table 1). Of importance, the direction of transcription from the *virB* promoter is opposite that of the *lacZ* promoter in these plasmids. Furthermore, a translational stop codon (UAA) resides two codons upstream (bp 736) of *virB1* in pPC914. Therefore, expression of *virB* genes in these plasmids was dependent on translation initiation at or downstream of start sites predicted by Ward et al. (41, 42).

Introduction of cloned *virB* genes into *A. tumefaciens*. Each *virB* gene under control of the *lacZ* promoter was introduced into *A. tumefaciens* strains by use of the pPC9X8 plasmids. Because *A. tumefaciens* is intrinsically resistant to chloramphenicol, the *nptII* gene from pUC4K (Table 1) was introduced into the unique *Sca*I site of the *cat* gene carried by these plasmids. The resulting Kan^r plasmids were designated pPC9X3, where X, numbered 1 through 11, represents the cloned *virB* gene (Table 1). Plasmid pPC998 possesses two *Sca*I sites, one in the *cat* gene and a second in *virB9* (bp 7871), making it difficult to use this strategy to construct pPC993. Instead, the *virB9* coding sequence was introduced as a 922-bp *Nde*I (bp 7095)-*Bam*HI (bp 8017) fragment from pPC997 (Table 1) into a Kan^r derivative of pBCSK$^+$.*Nde*I. To construct pBKSK$^+$.*Nde*I, the *nptII* gene from pUC4K was introduced as a *Hin*cII fragment into the *Sca*I site of pBCSK$^+$.*Nde*I (Table 1).

Each ColE1-based, *virB* gene expression plasmid was introduced into *A. tumefaciens* by digestion with an enzyme which cleaved the plasmid at a site residing downstream of the promoter::*virB* gene construction. The linearized plasmid was ligated to a similarly digested IncP plasmid, pSW172 (Table 1), and the ligation mix was introduced by electroporation into *A. tumefaciens*. Transformants carrying resistance genes from both plasmids were presumed to carry a cointegrate of the narrow-host-range ColE1 and the broad-host-range pSW172 plasmids. This was confirmed by isolation of plasmids from *A. tumefaciens* and physical characterization by restriction endonuclease cleavage analysis. All cointegrate plasmids consisting of a ColE1 plasmid ligated to pSW172 were given the ColE1 plasmid designation plus B, for broad host range.

Virulence assays. Virulence assays were performed by inoculating wound sites of *Kalanchoe daigremontiana* leaves with $\sim 10^8$ CFU of the various bacterial strains according to a protocol described by Mantis and Winans (24). *Nicotiana xanthi* leaf discs were infected as previously described (16). Relative virulence was estimated for different strains by inoculating *K. daigremontiana* wound sites with 10-fold serial dilutions ranging from 10^2 to 10^8 CFU (24). Virulence was scored in terms of tumor size and time course of appearance, and photographs were taken 5 to 6 weeks after inoculation. Virulence of three independently derived deletion mutants for each *virB* gene was assayed at least 10 times, and tumor formation was monitored for up to 6 months with *K. daigremontiana* leaves and *N. xanthi* leaf discs. The ability of each plasmid construct described in the text to complement a Δ*virB* mutation was tested at least four times.

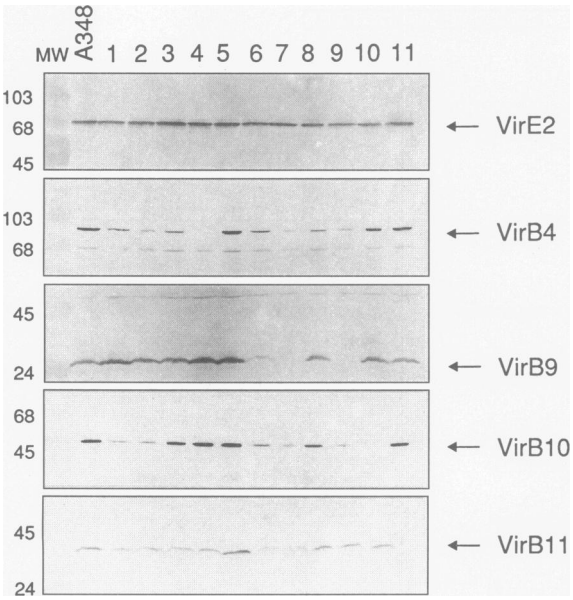

FIG. 3. Immunoblot analysis of the wild-type strain, A348, and a representative set of 11 Δ*virB* mutants. Each mutant sustained a deletion of the *virB* gene corresponding to the numbers 1 through 11 that identify each lane. Cell extracts prepared from acetosyringone-induced cultures were electrophoresed through SDS-polyacrylamide gels and examined by immunoblot analysis for Vir protein content by use of Vir-specific antibodies. Equivalent amounts (10 μg) of proteins from each of the cell extracts were loaded on the gels. The *vir* genes were expressed to similar extents, as deduced by examination of immunoblots for VirE2 protein content by use of VirE2 antibodies. MW, molecular mass markers; molecular masses (in kilodaltons) are indicated at the left.

RESULTS

Immunoblot studies of Δ*virB* mutants. Antisera specific for the VirB4, VirB9, VirB10, and VirB11 proteins were used in immunoblot (Western blot) analyses to examine the VirB protein content of the Δ*virB* mutants. Equivalent amounts (10 μg) of total proteins from cultures grown under *vir* gene induction conditions (see reference 7) were subjected to SDS-PAGE, electrophoretic transfer to nitrocellulose membranes, and immunodevelopment. First, cell extracts were examined for VirE2 protein content to ensure that the *vir* genes were induced to similar extents in these strains (Fig. 3). Next, extracts were examined for the presence of VirB4, VirB9, VirB10, and VirB11 proteins by use of available antisera (see Materials and Methods). The immunoblot studies showed that extracts of the Δ*virB4*, Δ*virB9*, Δ*virB10*, and Δ*virB11* mutants contained the expected VirB proteins and possessed no material reactive with antibodies specific for the product of the deleted gene (Fig. 3). These studies also showed that, with the exception of Δ*virB7* mutants (see below), extracts of the remaining Δ*virB* mutants contained each of the four VirB proteins, VirB4, VirB9, VirB10, and VirB11 (Fig. 3). These data, together with results of our Southern hybridization (see Materials and Methods) and genetic complementation (see below) studies, established that each of the Δ*virB* mutants sustains the expected *virB* gene deletion.

The immunoblot studies also showed that extracts of the Δ*virB* mutants contained VirB proteins encoded by genes distal to the deletion (Fig. 3). Therefore, each of the Δ*virB* mutations exhibits a nonpolar effect on expression of downstream *virB* genes. Results of the genetic complementation tests provided further evidence for the nonpolarity of these deletion mutations. As shown below, each of the Δ*virB* mutations could be complemented by expression in *trans* of the corresponding gene. This would not have been possible if any Δ*virB* mutation exhibited polarity, because the distal genes, *virB8*, *virB9*, *virB10*, and *virB11*, encode essential virulence factors (6, 10, 44).

We did, however, detect variation in steady-state levels of VirB4, VirB9, VirB10, and VirB11 in cell extracts of several Δ*virB* mutants (Fig. 3). Most notably, Δ*virB7* mutants possessed no detectable VirB9 protein and diminished levels of VirB4, VirB10, and VirB11 proteins (Fig. 3). We consistently were unable to detect VirB9 protein in extracts of any Δ*virB7* mutant, even when gels were overloaded with protein and blots were developed over a prolonged period with a high titer of antibody. To determine whether the presence of VirB7 affects VirB9 protein content, we examined extracts of Δ*virB7* mutants expressing *virB7* in *trans* from the *lacZ* promoter (pPCB973 [Table 1]), for the presence of VirB9 protein. Immunoblot studies showed that *virB7* expression in *trans* restored VirB9 protein content in the Δ*virB7* mutants to a detectable level, albeit still diminished relative to the wild-type strain A348. Interestingly, extracts of Δ*virB7* mutants coexpressing *virB7* and *virB8* from the *lacZ* promoter (pPCB975 [Table 1]) possessed wild-type levels of VirB9 protein (data not shown). These findings raise the intriguing possibility, which is also supported by results of our genetic complementation experiments (see below), that VirB7, VirB8, and VirB9 proteins interact and that this interaction somehow stabilizes VirB9 protein. Other mutants, including Δ*virB1*, Δ*virB2*, Δ*virB3*, Δ*virB6*, Δ*virB8*, and Δ*virB9*, also possessed diminished levels of one or more of the VirB4, VirB9, VirB10, and VirB11 proteins (Fig. 3 and Table 2). Whether the expression of additional *virB* genes in *trans* influences the steady-state contents of other VirB proteins is currently under investigation.

Virulence of Δ*virB* mutants. Each of the Δ*virB* mutants was examined for the ability to incite tumor formation on wounded *K. daigremontiana* leaves. Figures 4 through 9 show that mutants sustaining deletions of *virB2* through *virB11* failed to incite tumor formation. In contrast, by comparison with A348, Δ*virB1* mutants incited the formation of small tumors which appeared only after prolonged incubation (Fig. 4 and 5). Inoculation of wound sites of *K. daigremontiana* leaves or potato tuber discs (31) with 10-fold serially diluted bacterial cultures showed that virulence of the Δ*virB1* mutants was attenuated approximately 100-fold (data not shown). These studies established that *virB1* is not an essential virulence gene for infection of these dicot plants.

Genetic complementation of Δ*virB3*, Δ*virB4*, Δ*virB5*, Δ*virB10*, and Δ*virB11* mutations with *Nde*I.*virB* genes. We tested the ability of the pPCB9X3 series of plasmids to restore virulence to the corresponding Δ*virB* mutants. Each pPCB9X3 plasmid carries a *virB* gene cloned downstream of the *lacZ* promoter by use of an *Nde*I restriction site positioned at the translational start site of the gene (Fig. 2; see Materials and Methods). Figure 4 shows that 5 of the 11 Δ*virB* mutants, Δ*virB3*, Δ*virB4*, Δ*virB5*, Δ*virB10*, and Δ*virB11*, exhibited wild-type virulence when complemented with the corresponding *Nde*I.*virB* gene. Tumors incited by these strains were indistinguishable from those incited by A348, with respect both to morphology and to kinetics of appearance. Furthermore, no differences were detected in the numbers of tumors incited by these strains and by A348, as determined by virulence assays in which serial dilutions of these strains were inoculated onto wounded *K. daigremontiana* leaves (data not shown).

These complementation studies established that *virB3* and *virB5* are essential virulence genes and confirmed previous reports of the importance of *virB4* (2, 29), *virB10* (44), and *virB11* (6). Furthermore, the ability of *Nde*I.*virB* genes to restore wild-type levels of virulence suggests that the translational start site assignments by Ward et al. (41, 42) are correct for these genes. Finally, these data established that transcription from the *lacZ* promoter, which is constitutive in this system (5), of *virB3*, *virB4*, *virB5*, *virB10*, and *virB11* confers the same phenotypes as transcription of these genes from the inducible *virB* promoter in its native configuration on the pTi plasmid. This suggests that, in contrast to VirB1 and VirB2 (see below), the coordinated synthesis of these VirB proteins with the other VirB proteins is not essential for these proteins to function as virulence factors.

The pPCB9X3 plasmids expressing *virB1*, *virB2*, *virB6*, *virB7*, *virB8*, and *virB9* failed to restore the wild-type virulence phenotype to the corresponding Δ*virB* mutants (Fig. 5 to 9). We postulated that (i) inefficient transcription or translation of cloned *virB* genes, (ii) differences in the relative strengths of the *lacZ* and *virB* promoters, or (iii) disparate VirB protein synthesis kinetics could disrupt protein interactions critical to the assembly of a multisubunit T-complex transporter. With these possibilities in mind, we prepared and tested constructs shown in Fig. 5 to 9 for the ability to complement the Δ*virB1*, Δ*virB2*, Δ*virB6*, Δ*virB7*, Δ*virB8*, and Δ*virB9* mutations. First, we evaluated the importance of promoter choice, *lacZ* or *virB*, and second, we identified by further subcloning, the minimum sequences capable of complementation.

Genetic complementation of Δ*virB1*. Figure 5 shows constructs tested for the ability to complement the Δ*virB1* mutation. Constructs consisting of the *lacZ* and *virB* promoters tandemly arranged upstream of *virB1*, *virB2*, and *virB3* (pBBB8 and pPCB917 [Table 1]) restored virulence of Δ*virB1* mutants to wild-type levels (Fig. 5). The importance of (i) promoter choice and (ii) coupled expression of *virB1* with *virB2* and *virB3* was addressed by use of plasmids pPCB912 through pPCB915. When the *virB* promoter was used to transcribe *virB1* (pPCB914), Δ*virB1* mutants exhibited a wild-type virulence phenotype (Fig. 5). In contrast, transcription of *virB1* from the *lacZ* promoter failed to complement the Δ*virB1* mutation, regardless of the presence of (i) the *virB1* coding sequence only (pPCB913), (ii) ~50 bp of upstream sequence and *virB1* (pPCB915), or (iii) *virB1*, *virB2*, and *virB3* (pPCB912). Therefore, transcription of the *virB1* coding sequence from the *virB* promoter was necessary and sufficient for complementation of the Δ*virB1* mutation. The *virB* promoter requirement raises the possibility that the timing of VirB1 synthesis relative to the synthesis of other Vir proteins is important for VirB1 protein to function as a virulence factor. If so, VirB1 may stabilize or somehow activate another Vir protein through protein interactions established only when these proteins are coordinately synthesized. This, however, must be considered an accessory or a redundant function, since Δ*virB1* mutants retain the ability to incite tumor formation (Fig. 4 and 5).

An alternative explanation is that the *lacZ* promoter is considerably weaker than the *virB* promoter, resulting in insufficient VirB1 protein levels for complementation. In an

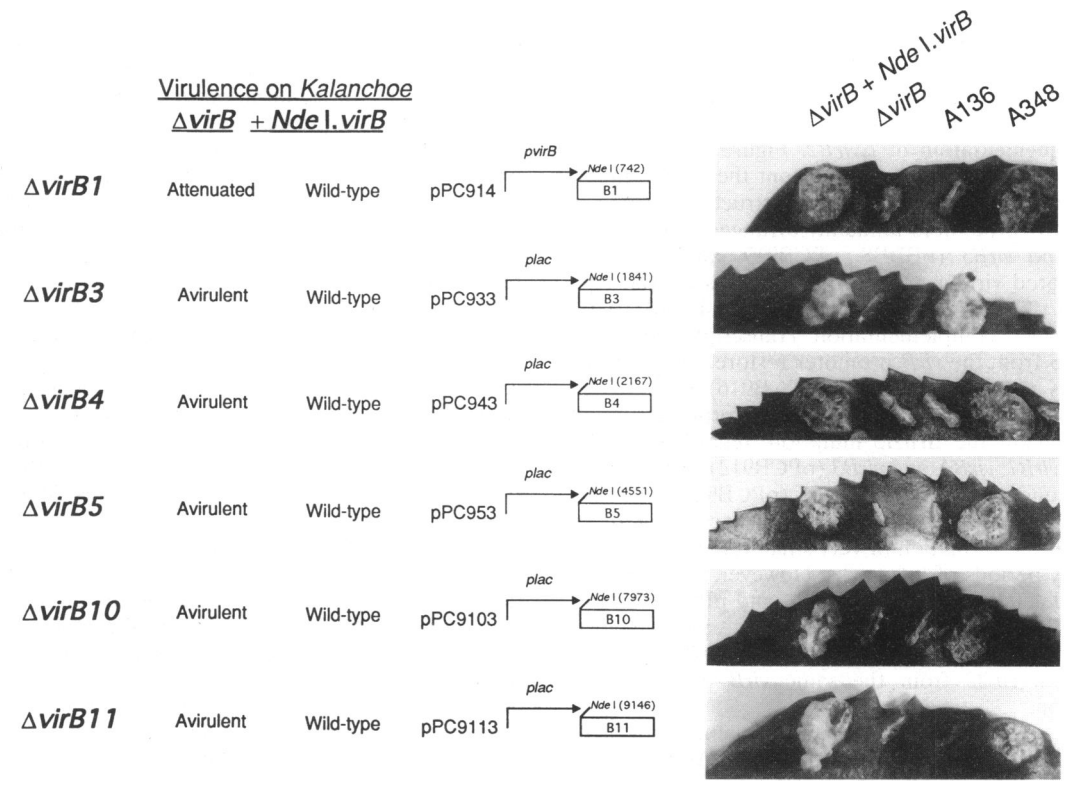

FIG. 4. Complementation of Δ*virB1*, Δ*virB3*, Δ*virB4*, Δ*virB5*, Δ*virB10*, and Δ*virB11* mutations assessed by virulence on *K. daigremontiana*. Complementing plasmids carried *virB* genes subcloned downstream of the *lacZ* or *virB* promoter by use of *Nde*I restriction sites introduced at their predicted translational start sites. Wound sites of each leaf were inoculated (see Materials and Methods) with ~10^8 CFU of wild-type strain A348; a Ti-plasmid-free strain, A136; and Δ*virB* mutants with or without plasmids carrying the constructs shown. Numbers in parentheses represent restriction site positions (Fig. 1). Plasmids used in the complementation studies were broad-host-range derivatives of plasmids listed in this and subsequent figures.

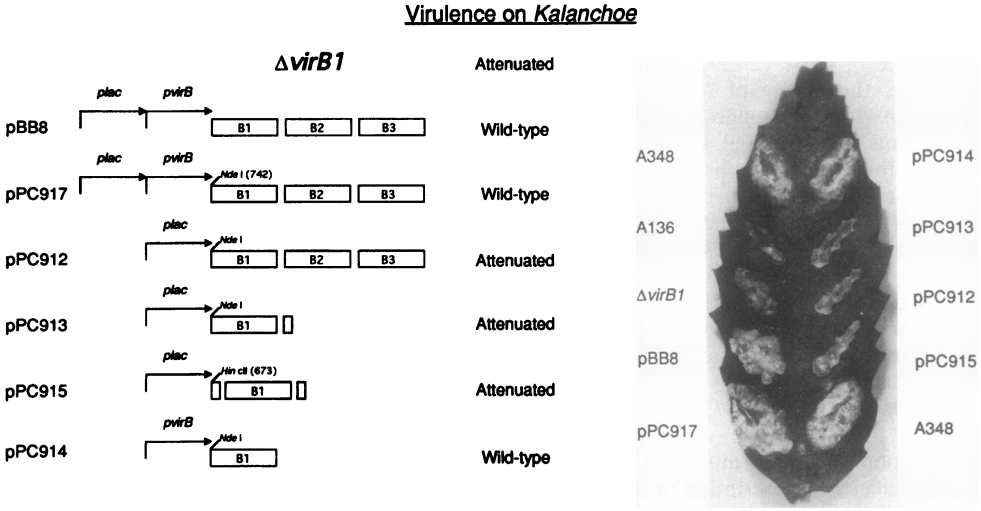

FIG. 5. Complementation of Δ*virB1* mutations assessed by virulence on *K. daigremontiana*, as described in the Fig. 4 legend. *virB1* expression plasmids were prepared as described (see Table 1 and Materials and Methods).

earlier study, the *lacZ* promoter in the absence of *lac* repressor was estimated to be two- to threefold stronger than the *virG* promoter (5). We have determined by immunoblot analysis that extracts from cells expressing *virB9*, *virB10*, and *virB11* from the constitutive *lacZ* (no *lac* repressor) and the inducible *virB* promoters possess similar levels of the VirB9, VirB10, and VirB11 proteins, respectively (data not shown). Therefore, although direct measurements of VirB1 content have not been possible because of the lack of VirB1-specific antisera, the available data suggest that variation in promoter strength alone cannot account for the observed *virB* promoter requirement for Δ*virB1* complementation.

Genetic complementation of Δ*virB2*. Figure 6 shows constructs tested for the ability to complement the Δ*virB2* mutation. As with Δ*virB1* complementation, constructs consisting of the *lacZ* and *virB* promoters tandemly arranged upstream of *virB1*, *virB2*, and *virB3* (pBBB8, pPCB917, and pPCB927 [Table 1]) restored virulence of Δ*virB2* mutants to wild-type levels (Fig. 6). The *virB* promoter also was found to be important for Δ*virB2* complementation. Transcription of *virB1*, *virB2*, and *virB3* from the *virB* promoter restored virulence of Δ*virB2* mutants to wild-type levels (pPCB916) (Fig. 6). In contrast, transcription of *virB2* from the *lacZ* promoter failed to fully complement the Δ*virB2* mutation, regardless of the presence of (i) *virB1*, *virB2*, and *virB3* (pPCB912), (ii) ~275 bp of upstream sequence, *virB2*, and *virB3* (pPCB922), and (iii) *virB2* and *virB3* (pPCB923). Δ*virB2* mutants carrying these plasmids incited the formation of very small tumors only after prolonged assay periods of >6 weeks. However, *virB2* expression plasmids deleted of *virB1* (pPCB924 and pPCB925) also conferred only a severely attenuated virulence phenotype to Δ*virB2* mutants (Fig. 6). This suggests that *virB1* must be coexpressed with *virB2* from the same *virB* promoter for complementation.

Coexpression of the entire *virB1* coding sequence with *virB2* appears necessary for complementation of the Δ*virB2* mutation. Transcription of as much as 275 bp of upstream sequence and *virB2* either from the *lacZ* (pPCB922) or the *virB* (pPCB925) promoter still failed to restore virulence of Δ*virB2* mutants to wild-type levels (Fig. 6). Furthermore, the importance of gene coexpression appears to be manifested at the level of protein cosynthesis, because a frameshift mutation introduced into the *virB1* coding sequence abolished complementation. The mutation was introduced into plasmid pBB2 (Table 1) by *Sac*II (bp 1215) digestion, removal of the 2-bp overhang with T4 DNA polymerase, and plasmid religation. The resulting plasmid, ligated to pSW172, failed to complement the Δ*virB1* or Δ*virB2* mutations (data not shown). These findings suggest that the *virB1* gene product, not *cis*-acting sequences within the *virB1* coding sequence, is important for complementation. Conceivably, VirB1 and VirB2 protein cosynthesis ensures that both proteins interact immediately upon their synthesis for stabilization or activation of function.

The minimal construct shown in this study to restore virulence of Δ*virB2* mutants to wild-type levels contained the *virB* promoter through *virB3* (pPCB916) (Fig. 6). We consider it unlikely that the two-step recombination procedure used to construct Δ*virB2* introduced a mutation in flanking coding sequences, which could also explain the gene coexpression requirements. DNA flanking the *virB2* gene deletion carried by pBB120 (Table 1), which was used to generate the Δ*virB2* mutants, was devoid of mutations (see Materials and Methods). Furthermore, we consistently obtained identical results in genetic complementation tests of three independently isolated Δ*virB2* mutants. Finally, in the absence of *virB1* coexpression, *virB2* still possessed the ability to partially restore virulence to Δ*virB2* mutants when expressed from either the *lacZ* (pPCB922 and pPCB23) or the *virB* (pPCB924 and pPCB925) promoter (Fig. 6).

Genetic complementation of Δ*virB6*. Figure 7 shows constructs tested for the ability to complement the Δ*virB6* mutation. Transcription from the *lacZ* promoter proved sufficient for complementation, as shown by the ability of constructs consisting of the *lacZ* promoter positioned upstream of *virB5* through *virB8* (pBBB20 and pPCB967) to fully restore virulence of Δ*virB6* mutants to wild-type levels (Fig. 7). However, transcription from the *lacZ* promoter of the *virB6* coding sequence alone (pPCB963) failed to complement the Δ*virB6* mutation (Fig. 7). To evaluate the importance of sequences residing upstream of the predicted *virB6* start site at bp 5310 (41, 42), *virB6* was cloned behind the *lacZ* promoter with the enzymes *Cla*I, which cleaves at bp 5306 (pPCB962), and *Sac*I, which cleaves at bp 5256 (pPCB961) (Fig. 7). The Δ*virB6* mutants carrying pPCB962 incited the formation of very small

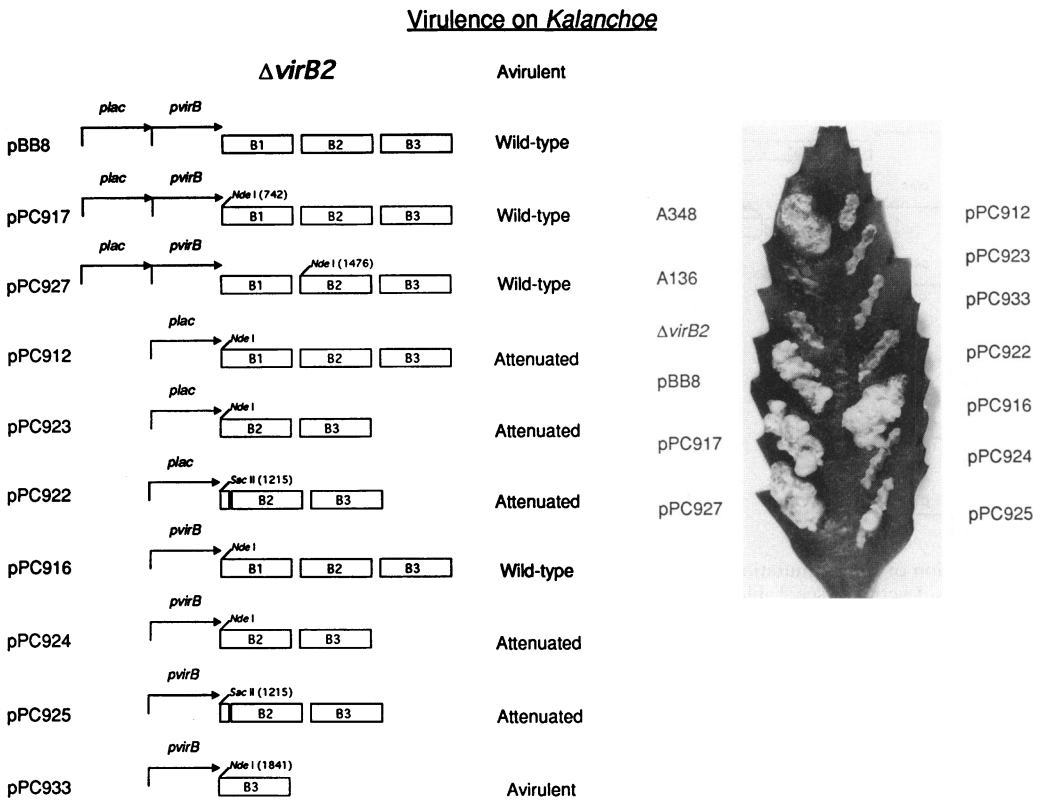

FIG. 6. Complementation of Δ*virB2* mutations assessed by virulence on *K. daigremontiana*, as described in the Fig. 4 legend. *virB2* expression plasmids were prepared as described (see Table 1 and Materials and Methods).

tumors only after a prolonged incubation period (Fig. 7). In contrast, the Δ*virB6* mutants carrying pPCB961 exhibited wild-type virulence (Fig. 7). These findings suggest that the ~55 bp of upstream sequence provides information which is essential for *virB6* expression.

This upstream region possesses no in-frame stop codons, establishing the potential for translation of *virB6* to initiate upstream of the site predicted by Ward et al. (41, 42). In fact, in their analysis of the pTiC58 plasmid, Kuldau et al. (20) noted that a potential alternative start site for *virB6* exists within the *virB5* coding sequence at a site which corresponds to bp 5140 on pTiA6NC. However, we favor the start site assignment by Ward et al. (41, 42), first, because an in-frame translational stop codon resides at bp 5211 on pTiA6NC, making the alternative start site at bp 5140 unlikely, and second, because no obvious alternative start sites exist in frame between the stop codon at bp 5211 and the predicted start site at bp 5310. We suggest instead that *cis*-acting sequences upstream of *virB6* contribute to efficient *virB6* expression, perhaps by stabilizing this region of mRNA or by providing binding sites for transcriptional or translational modulator proteins.

Genetic complementation of Δ*virB7* and Δ*virB8*. Figure 8 shows constructs tested for the ability to complement the Δ*virB7* and Δ*virB8* mutations. Transcription from the *lacZ* promoter proved sufficient for complementation, as shown by the ability of constructs consisting of the *lacZ* promoter positioned upstream of *virB5* through *virB8* (pBBB20, pPCB977, and pCB987) to fully restore virulence of Δ*virB7* and Δ*virB8* mutants to wild-type levels (Fig. 8). However, transcription from the *lacZ* promoter of either gene alone (pPCB973 or pPCB983) failed to fully complement the Δ*virB7* or Δ*virB8* mutation. Instead, as described in detail below, complementation of each mutation required transcription from the *lacZ* promoter of both the *virB7* and *virB8* coding sequences (pPCB975) (Fig. 8).

A *virB7* gene product previously has not been visualized. This, together with small size of the *virB7* coding sequence (165 bp), prompted Kuldau et al. (20) to speculate that *virB7* may represent a nontranslated, intercistronic region containing *cis*-acting sequences required for efficient *virB8* expression. To examine the potential for *virB7* to encode a virulence factor, we first attempted to visualize the *virB7* gene product. This was achieved in two ways. First, we overexpressed *virB7* in *E. coli* using a bacteriophage T7 expression system. Strains carrying the *virB7* overexpression construct overproduced a ~6-kDa protein (data not shown), the size predicted by DNA sequence analysis (20, 30, 41, 42). Second, we constructed an in-frame gene fusion between *virB7* and *phoA*, the gene encoding periplasmically active alkaline phosphatase. The *virB7*::*phoA* fusion was constructed by cloning the *phoA* gene devoid of a signal sequence as a *Bam*HI-*Kpn*I restriction fragment from pUI1310 (40) into pPC978 (Table 1) at the *Bgl*II site within the *virB7* coding sequence (bp 6353) and the *Kpn*I site in the downstream polylinker sequence. *E. coli* and *A. tumefaciens* cells expressing this gene fusion possessed high levels of alkaline phosphatase activity, as deduced by the rapid development of dark blue colonies when cells were incubated on plates containing 5-bromo-4-chloro-3-indolylphosphate (BCIP) (data not shown). These findings established that *virB7* encodes a secreted protein. Results of genetic complementa-

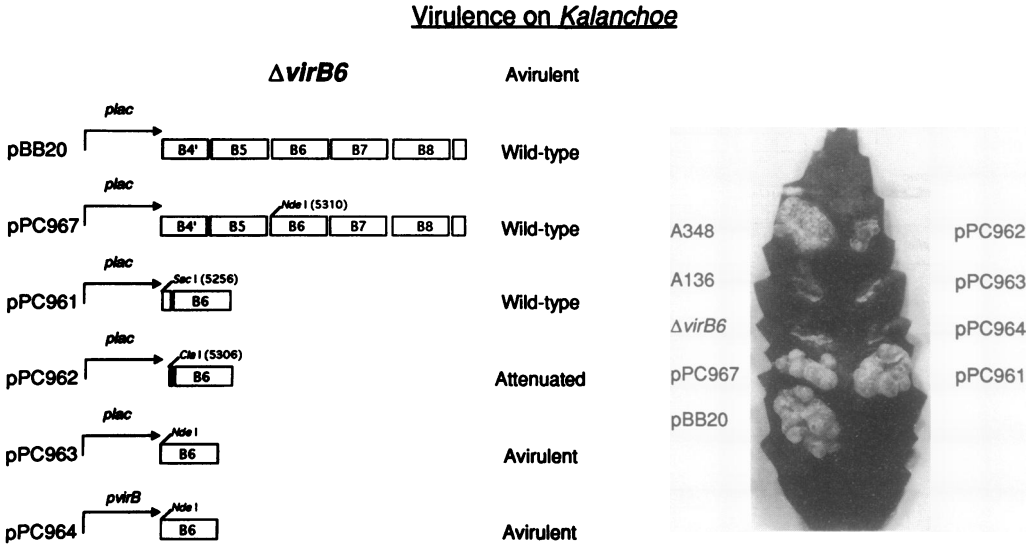

FIG. 7. Complementation of Δ*virB6* mutations assessed by virulence on *K. daigremontiana*, as described in the Fig. 4 legend. *virB6* expression plasmids were prepared as described (see Table 1 and Materials and Methods).

tion experiments (Fig. 8; see below) further established that *virB7* encodes an essential virulence factor.

Several groups (20, 30, 37) have postulated that the *virB8* coding sequence initiates upstream of the start site predicted by Ward et al. (41, 42). If so, our construction of the Δ*virB7* mutation would have deleted the 5′ end of *virB8*, necessitating that *virB7* and *virB8* be coexpressed for Δ*virB7* complementation (Fig. 8). The potential for *virB8* to initiate upstream of the start site predicted by Ward et al. (41, 42) was addressed by genetic complementation as follows. Initial studies showed that transcription of *virB8* initiating at bp 6406 from the *lacZ* promoter (pPCB983 [Table 1]) only partially complemented the Δ*virB8* mutation (Fig. 8). Therefore, this plasmid synthesizes at least a partially functional VirB8 protein. Next, we tested *virB8* expression constructs containing various lengths of upstream sequence for the ability to restore virulence of Δ*virB8* mutants. Constructs in which 53 bp of upstream sequence and *virB8* were placed under control of the *lacZ* (pPCB982 [Table 1]) and *virB* (pPCB985 [Table 1]) promoters, respectively, still conferred only an attenuated virulence phenotype to Δ*virB8* mutants (Fig. 8). Similarly, a construct in which 81 bp of upstream sequence and *virB8* were placed under control of the *lacZ* promoter (pPCB981 [Table 1]) also failed to restore wild-type virulence (Fig. 8). This upstream site represents a

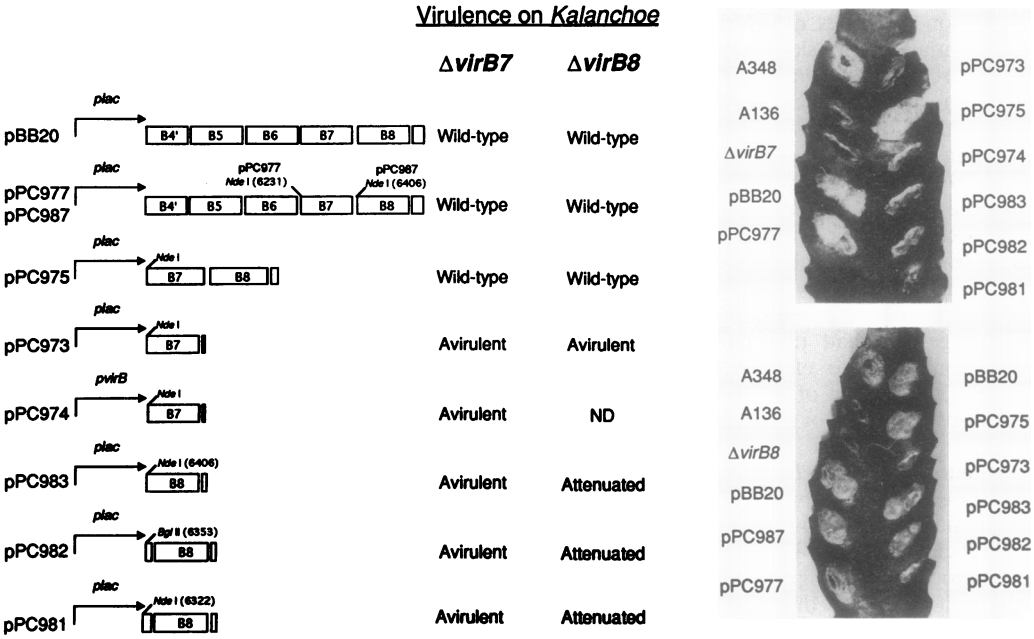

FIG. 8. Complementation of Δ*virB7* and Δ*virB8* mutations assessed by virulence on *K. daigremontiana*, as described in the Fig. 4 legend. *virB7* and *virB8* expression plasmids were prepared as described (see Table 1 and Materials and Methods).

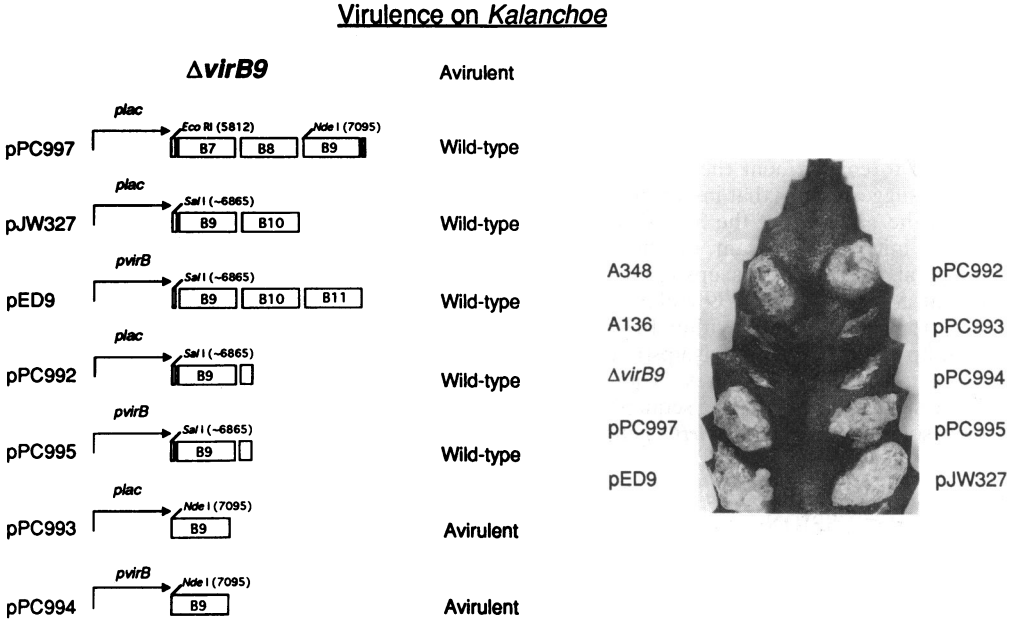

FIG. 9. Complementation of Δ*virB9* mutations assessed by virulence on *K. daigremontiana*, as described in the Fig. 4 legend. *virB9* expression plasmids were prepared as described (see Table 1 and Materials and Methods).

potential *virB8* start site as identified by Thompson et al. (37) in their analysis of the *virB* operon of the pTi plasmid, pTil5955.

These studies established that *virB8* expression containing as much as 81 bp of upstream sequence, representing almost half of the *virB7* coding sequence, only partially complemented the Δ*virB8* mutation. In agreement with these findings, a previous study showed that pED52, a plasmid equivalent to pPCB985, also only partially complemented a nonpolar *virB8* mutation (10). Thus, although we cannot exclude the possibility that an alternative upstream start site exists for *virB8*, our studies established that coexpression of the entire *virB7* and *virB8* coding sequences is essential for full complementation of both the Δ*virB7* and the Δ*virB8* mutations.

Finally, we examined whether VirB7 and VirB8 must be cosynthesized for complementation. For this, we introduced frameshift mutations into both the *virB7* and *virB8* coding sequences carried by plasmid pPC975. The frameshift mutation in the *virB7* coding sequence was constructed by digesting pPC975 with *Bgl*II (bp 6353), filling in the 4-bp overhang with Klenow fragment, and religating the plasmid. The frameshift mutation in the *virB8* coding sequence was constructed by digesting pPC975 with *Nco*I (bp 6563), filling in the 5-bp overhang with Klenow fragment, and religating the plasmid. Both plasmids, ligated to pSW172, failed to complement the Δ*virB7* mutation, and the plasmid sustaining the frameshift mutation in *virB7* only partially complemented Δ*virB8* mutation. Therefore, as first indicated by our immunoblot studies (see above), these genetic complementation studies suggest that VirB7 and VirB8 interact and that this interaction somehow is facilitated by transcription of both genes from the same promoter.

Genetic complementation of Δ*virB9*. Figure 9 shows constructs tested for the ability to complement the Δ*virB9* muta-

TABLE 2. Summary of genetic complementation experiments

A348 Δ*virB*	VirB protein diminished[a]	Complementation by *Nde*I.*virB*[b]	Promoter requirement	Requirement for sequence(s)	
				Upstream	Downstream
Δ*virB1*	4, 10	+++	*virB*	*pvirB*	
Δ*virB2*	4, 10	+	*virB*	*pvirB*, *virB1*	ND[c]
Δ*virB3*	4	+++	*lacZ* or *virB*		
Δ*virB4*		+++	*lacZ* or *virB*		
Δ*virB5*		+++	*lacZ* or *virB*		
Δ*virB6*	4, 9, 10, 11	−	*lacZ* or *virB*	~55 bp	
Δ*virB7*	4, 9,[d] 10, 11	−	*lacZ* or *virB*		*virB8*
Δ*virB8*	4	++	*lacZ* or *virB*	*virB7*	
Δ*virB9*	4, 10, 11	−	*lacZ* or *virB*	~230 bp	
Δ*virB10*		+++	*lacZ* or *virB*		
Δ*virB11*		+++	*lacZ* or *virB*		

[a] Relative levels of proteins VirB4, VirB9, VirB10, and VirB11 were examined by immunoblot analysis.
[b] Greater numbers of pluses indicate higher levels of complementation; minus indicates absence of complementation.
[c] ND, the requirement for *virB3* coexpression was not determined.
[d] No detectable VirB9 protein by immunoblot analysis.

tion. Transcription from the *lacZ* promoter proved sufficient for complementation, as shown by the ability of constructs consisting of the *lacZ* promoter positioned upstream of the 3′ end of *virB8* and *virB9* (pJWB327 and pPCB992) to fully restore virulence of Δ*virB9* mutants (Fig. 9). However, transcription from the *lacZ* promoter of the *virB9* coding sequence alone (pPCB993) failed to complement the Δ*virB9* mutation (Fig. 9). These findings suggest either that important *cis*-acting sequences exist within the ~230 bp of the 3′ end of *virB8* or that *virB9* translation initiates upstream of the start site predicted by Ward et al. (41, 42). This upstream sequence potentially encodes the 5′ end of the *virB9* coding sequence, because no in-frame stop codon resides within 163 bp of the predicted start-site at bp 7095. However, this upstream region is devoid of obvious alternative start sites. Therefore, as proposed for *virB6*, we suggest that *cis*-acting sequences within this upstream region somehow modulate *virB9* gene expression.

DISCUSSION

With a long-term goal of elucidating structural and functional features of the *A. tumefaciens* T-complex transport system, genetic complementation studies were undertaken to assess the importance of each of the 11 genes constituting the *virB* operon of pTiA6NC. *virB* genes expressed in *trans* from a constitutive *lacZ* promoter or an inducible *virB* promoter were examined for the ability to restore virulence of nonpolar *virB* null mutants. As summarized in Table 2, these studies defined promoter and general sequence requirements for complementation of the 11 Δ*virB* mutations. These studies also demonstrated the importance of *virB2* through *virB11* for *A. tumefaciens* infection of dicot plants.

Currently, it is unknown whether each of the eight VirB homologs encoded by the *ptl* operon is essential for pertussis toxin secretion. However, a recent study by Lessl et al. (22) demonstrated the importance of three VirB homologs, TrbB (VirB11), TrbC (VirB2), and TrbE (VirB4), for conjugal transfer of RP4, mobilization of the IncQ plasmid RSF1010, and donor-specific phage growth. These authors also showed that the carboxyl terminus of TrbI, a VirB10 homolog, is dispensable for protein function (22). This also is the case for VirB10, as demonstrated by the combined studies of Stachel and Nester (33), who determined that the Tn3HoHo1 insertion mutant Mx41 is virulent on *K. daigremontiana*, and Ward et al. (44), who mapped transposon insertion 41 to a position 9 bp upstream of the VirB10 termination codon. Given the relatedness of these transport systems, an interesting question for future study is whether components of one system will substitute for related components of the heterologous systems.

Δ*virB1* mutants exhibited an attenuated virulence phenotype that could be restored to wild-type levels by *virB1* expression in *trans*. Interestingly, a *virB1* homolog is missing in the IncP (21) and IncW (18) conjugal transfer systems and the pertussis toxin secretion system (9, 46) but is present in the IncN (pKM101) conjugal transfer system (47a). Mutations in *virB1* and in the related pKM101 *traL* gene each result in a 10- to 100-fold reduction in DNA transfer efficiency, as shown by virulence assays with serially diluted *A. tumefaciens* cultures (data not shown) and by quantitating pKM101 conjugal transfer frequencies (47a, 48). Therefore, while this protein clearly is not an essential component of these macromolecular transport systems, it somehow enhances the efficiency of the transfer process. VirB1 possesses a characteristic signal sequence and, with two different fractionation protocols, was shown to be associated with one or both membranes (38). Given this localization, VirB1 may provide an accessory function during the assembly of the putative T-complex transport apparatus at the inner or outer membranes. If exposed extracellularly, VirB1 may also enhance the formation of mating contacts between *A. tumefaciens* and susceptible plant cells.

Our studies revealed another interesting feature of *virB1*, a requirement for this gene to be expressed from the *virB* promoter for complementation of Δ*virB1* mutations. A similar *virB* promoter specificity was essential for full complementation of Δ*virB2* mutations, although *lacZ* promoter-directed expression of *virB2* partially complemented these mutations. The *virB* promoter requirement may reflect a need for VirB1 and VirB2 to be synthesized concurrently with one or more of the other VirB proteins. As amply demonstrated for various pilus biogenesis systems (see reference 19), coordinate protein synthesis can serve to stabilize protein components via protein-protein interactions during assembly of a macromolecular structure. Notably, during P pilus assembly the periplasmic chaperone, PapD, interacts directly with and stabilizes the P pilin structural subunit, PapA, as well as the specialized subunits, PapE, PapF, PapG, and PapK, constituting the tip fibrillum (19). Alternatively, coordinate protein synthesis may promote protein stabilization if, for example, one protein is required for the efficient processing of a proprotein to a more stable form. The TraQ protein of the F plasmid is required for the processing of TraA, the F pilin subunit, to a stable, mature form. In the absence of TraQ processing activity, TraA is rapidly degraded (12, 23). By analogy, VirB1 and/or VirB2 may somehow stabilize, or, conversely, be stabilized, by other VirB proteins. Interestingly, VirB2 shares sequence similarities with TraA, and like TraA, VirB2 is proteolytically processed from a ~12- to a ~7-kDa form (28). Currently, it is unknown whether the processed form of VirB2 is more stable or represents the active form of the protein. If so, it will be of considerable interest to determine if VirB1 and/or another VirB protein participates in this processing activity.

Although we favor a model which explains the *virB1* and *virB2* complementation data in terms of protein-protein interactions, expression of one or both genes also may be subject to modulation at the transcriptional or translational level. For example, mRNA secondary structures encoded within *virB1* may enhance or diminish transcription or translation of *virB2*. Such modulation may preserve critical stoichiometric relationships between VirB2 and other VirB proteins. Alternatively, small proteins or RNA molecules encoded by open reading frames (ORFs) residing in the *virB* promoter region through *virB2* may modulate expression levels of *virB1* or *virB2*. We have identified a large ORF with the potential to code for a ~25-kDa protein on the strand opposite that which codes for the *virB* genes and in the region encompassed by the *virB1* and *virB2* genes (data not shown). Whether this ORF or other ORFs within the *virB* operon encode virulence factors or influence the expression of other *virB* genes remains to be determined.

Our genetic complementation data for the Δ*virB7* and Δ*virB8* mutations provided evidence for an interaction between the VirB7 and VirB8 proteins (Fig. 8). Furthermore, the findings that VirB9 protein content is enhanced when Δ*virB7* mutants express *virB7* in *trans* and is restored to wild-type levels when Δ*virB7* mutants coexpress *virB7* and *virB8* in *trans* suggest that VirB9 protein somehow is stabilized through interactions with VirB7 and VirB8. Previous sequence analyses (20, 30, 41, 42) led to a prediction, confirmed in this study (see Results), that VirB7 is a secreted protein. Sequence analyses also identified a consensus lipid modification sequence near the amino terminus of VirB7 (20, 30, 41, 42) and a region that

bears similarities to a family of lysis proteins that facilitate the secretion of colicins devoid of signal sequences across the bacterial envelope (30). VirB8 has been shown by immunoelectron microscopy to localize to the cytoplasmic membrane (39). VirB9 has been shown by sucrose density gradient centrifugation and Sarkosyl extraction to localize to both the cytoplasmic and the outer membrane fractions (28, 38). On the basis of these observations, we hypothesize that VirB7 is an outer membrane lipoprotein whose localization depends on the cytoplasmic-membrane-associated VirB8 protein. By analogy to the PapC protein (see reference 19), VirB7 may serve to anchor, and thereby stabilize, other VirB proteins such as VirB9 to the outer membrane. An alternative possibility is that VirB7 stabilizes VirB proteins such as VirB9 by facilitating their delivery to their final destinations within the cell envelope.

Previous studies of the *virB* operon showed the importance of *virB8* (10) and *virB9* (44) for virulence but failed to establish the importance of sequences residing upstream or downstream of these and additional *virB* genes for complementation. The identification of promoter and general sequence requirements in the present study highlights the importance of *cis*- or *trans*-regulatory mechanisms operating within the ~9.5-kb *virB* operon to modulate VirB protein content. The physiological importance of regulating specific VirB protein levels or activities is underscored by the studies of Ward et al. (43). These investigators discovered that the efficiency with which *A. tumefaciens* mobilizes DNA substrates to plant cells and, indeed, the substrate specificity of the T-complex transporter can be influenced by altering the expression levels of *virB9*, *virB10*, and *virB11*.

With the exception of VirB10, which may reside as a homotrimer within the cytoplasmic membrane (45), nothing is known about specific structural interactions among the VirB proteins. A *virB*-encoded T-complex transport apparatus may consist of a transmembrane channel dedicated to macromolecular transport, as proposed for the pertussis toxin secretion system (9, 46). The apparatus may also consist of a pilus structure which is important for establishing mating pair contacts between *A. tumefaciens* and plant cells, reminiscent of the sex pili encoded by the transfer operons of F, IncP, IncN, and IncW plasmids (see references 22 and 23). Definition of the T-complex transporter structure clearly is of central importance, not only to further our understanding of the *A. tumefaciens*-plant interaction but also to enhance our knowledge of how macromolecules such as large complexes of protein only or of DNA and protein are translocated across biological membranes. Elucidating the process by which this structure is assembled also offers the exciting possibility of enhancing, through targeted genetic manipulation, the efficiency of trans-kingdom DNA delivery and related macromolecular transport processes.

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REFERENCES

1. **Beijersbergen, A., A. D. Dulk-Ras, R. A. Schilperoort, and P. J. J. Hooykaas.** 1992. Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. Science **256:**1324–1327.
2. **Berger, B. R., and P. J. Christie.** 1993. The *Agrobacterium tumefaciens virB4* gene product is an essential virulence protein requiring an intact nucleoside triphosphate-binding domain. J. Bacteriol. **175:**1723–1734.
3. **Buchanan-Wollaston, V., J. E. Passiatore, and F. Cannon.** 1987. The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. Nature (London) **328:**172–175.
4. **Cangelosi, G. A., R. G. Ankenbauer, and E. W. Nester.** 1990. Sugars induce the *Agrobacterium* virulence genes through a periplasmic binding protein and a transmembrane signal protein. Proc. Natl. Acad. Sci. USA **87:**6708–6712.
5. **Chen, C.-Y., and S. C. Winans.** 1991. Controlled expression of the transcriptional activator gene *virG* in *Agrobacterium tumefaciens* by using the *Escherichia coli lac* promoter. J. Bacteriol. **173:**1139–1144.
6. **Christie, P. J., J. E. Ward, M. P. Gordon, and E. W. Nester.** 1989. A gene required for transfer of T-DNA to plants encodes an ATPase with autophosphorylating activity. Proc. Natl. Acad. Sci. USA **86:**9677–9681.
7. **Christie, P. J., J. E. Ward, S. C. Winans, and E. W. Nester.** 1988. The *Agrobacterium tumefaciens virE2* gene product is a single-stranded-DNA-binding protein that associates with T-DNA. J. Bacteriol. **170:**2659–2667.
8. **Citovsky, V., and P. Zambryski.** 1993. Transport of nucleic acids through membrane channels: snaking through small holes. Annu. Rev. Microbiol. **47:**167–197.
9. **Covacci, A., and R. Rappuoli.** 1993. Pertussis toxin export requires accessory genes located downstream from the pertussis toxin operon. Mol. Microbiol. **8:**429–434.
10. **Dale, E. M., A. N. Binns, and J. E. Ward, Jr.** 1993. Construction and characterization of Tn*5virB*, a transposon that generates nonpolar mutations, and its use to define *virB8* as an essential virulence gene in *Agrobacterium tumefaciens*. J. Bacteriol. **175:**887–891.
11. **Engstrom, P., P. Zambryski, M. Van Montagu, and S. Stachel.** 1987. Characterization of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. J. Mol. Biol. **197:**635–645.
12. **Frost, L. S., K. Ippen-Ihler, and R. A. Skurray.** 1994. An analysis of the sequence and gene products of the transfer region of the F sex factor. Microbiol. Rev. **58:**10–94.
13. **Gelvin, S. B., and L. L. Habeck.** 1990. *vir* genes influence conjugal transfer of the Ti plasmid of *Agrobacterium tumefaciens*. J. Bacteriol. **172:**1600–1608.
14. **Gheysen, G., R. Villarroel, and M. Van Montagu.** 1991. Illegitimate recombination in plants: a model for T-DNA integration. Genes Dev. **5:**287–297.
15. **Hobbs, M., and J. S. Mattick.** 1993. Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: a general system for the formation of surface-associated protein complexes. Mol. Microbiol. **10:**233–243.
16. **Horsch, R. B., H. J. Klee, S. Stachel, S. C. Winans, E. W. Nester, S. G. Rogers, and R. T. Fraley.** 1986. Analysis of *Agrobacterium tumefaciens* virulence mutants in leaf discs. Proc. Natl. Acad. Sci. USA **83:**2571–2575.
17. **Jin, S., T. Roitsch, P. J. Christie, and E. W. Nester.** 1990. The regulatory VirG protein specifically binds to a *cis*-acting regulatory sequence involved in transcriptional activation of *Agrobacterium tumefaciens* virulence genes. J. Bacteriol. **172:**531–537.
18. **Kado, C. I.** 1993. *Agrobacterium*-mediated transfer and stable incorporation of foreign genes in plants, p. 243–254. *In* D. B. Clewell (ed.), Bacterial conjugation. Plenum Press, New York.
19. **Kuehn, M. J., D. J. Ogg, J. Kihlberg, L. N. Slonim, K. Flemmer, T. Bergfors, and S. J. Hultgren.** 1993. Structural basis of pilus subunit recognition by the PapD chaperone. Science **262:**1234–1241.
20. **Kuldau, G. A., G. DeVos, J. Owen, G. McCaffrey, and P. Zambryski.** 1990. The *virB* operon of *Agrobacterium tumefaciens* pTiC58 encodes 11 open reading frames. Mol. Gen. Genet. **221:**256–266.
21. **Lessl, M., D. Balzer, W. Pansegrau, and E. Lanka.** 1992. Sequence similarities between the RP4 Tra2 and the Ti VirB region strongly support the conjugation model for T-DNA transfer. J. Biol. Chem. **267:**20471–20480.

22. **Lessl, M., D. Balzer, K. Weyrauch, and E. Lanka.** 1993. The mating pair formation system of plasmid RP4 defined by RSF1010 mobilization and donor-specific phage propagation. J. Bacteriol. **175**:6415–6425.
23. **Maneewannakul, K., S. Maneewannakul, and K. Ippen-Ihler.** 1993. Synthesis of F pilin. J. Bacteriol. **175**:1384–1391.
24. **Mantis, N. J., and S. C. Winans.** 1993. The chromosomal response regulatory gene *chvI* of *Agrobacterium tumefaciens* complements an *Escherichia coli phoB* mutation and is required for virulence. J. Bacteriol. **175**:6626–6636.
25. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57**:50–108.
26. **Shimoda, N., A. Toyoda-Yamamoto, J. Nagamine, S. Usami, M. Katayama, Y. Sakagami, and Y. Machida.** 1990. Control of expression of *Agrobacterium vir* genes by synergistic actions of phenolic signal molecules and monosaccharides. Proc. Natl. Acad. Sci. USA **87**:6684–6688.
27. **Shirasu, K., and C. I. Kado.** 1993. The *virB* operon of the *Agrobacterium tumefaciens* virulence regulon has sequence similarities to B, C and D open reading frames downstream of the pertussis toxin-operon and to the DNA transfer-operons of broad-host-range conjugative plasmids. Nucleic Acids Res. **21**:353–354.
28. **Shirasu, K., and C. I. Kado.** 1993. Membrane location of the Ti plasmid VirB proteins involved in the biosynthesis of a pilin-like conjugative structure on *Agrobacterium tumefaciens*. FEMS Microbiol. Lett. **111**:287–294.
29. **Shirasu, K., Z. Koukolíková-Nicola, B. Hohn, and C. I. Kado.** 1994. An inner-membrane-associated virulence protein essential for T-DNA transfer from *Agrobacterium tumefaciens* to plants exhibits ATPase activity and similarities to conjugative transfer genes. Mol. Microbiol. **11**:581–588.
30. **Shirasu, K., P. Morel, and C. I. Kado.** 1990. Characterization of the *virB* operon of an *Agrobacterium tumefaciens* Ti plasmid: nucleotide sequence and protein analysis. Mol. Microbiol. **4**:1153–1163.
31. **Shurvington, C. E., L. Hodges, and W. Ream.** 1992. A nuclear localization signal and the C-terminal omega sequence in the *Agrobacterium tumefaciens* VirD2 endonuclease are important for tumor formation. Proc. Natl. Acad. Sci. USA **89**:11837–11841.
32. **Stachel, S. E., E. Messens, M. Van Montagu, and P. Zambryski.** 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. Nature (London) **318**:624–629.
33. **Stachel, S. E., and E. W. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti-plasmid of *Agrobacterium tumefaciens*. EMBO J. **5**:1445–1454.
34. **Stachel, S. E., B. Timmerman, and P. Zambryski.** 1987. Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5′ *virD* gene products. EMBO J. **6**:857–863.
35. **Stachel, S. E., and P. C. Zambryski.** 1986. *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. Cell **47**:155–157.
36. **Steck, T. R., and C. I. Kado.** 1990. Virulence genes promote conjugative transfer of the Ti plasmid between *Agrobacterium* strains. J. Bacteriol. **172**:2191–2193.
37. **Thompson, D. V., L. S. Melchers, K. B. Idler, R. A. Schilperoort, and P. J. J. Hooykaas.** 1988. Analysis of the complete nucleotide sequence of the *Agrobacterium tumefaciens virB* operon. Nucleic Acids Res. **16**:4621–4636.
38. **Thorstenson, Y. R., G. A. Kuldau, and P. C. Zambryski.** 1993. Subcellular localization of seven VirB proteins of *Agrobacterium tumefaciens*: implications for the formation of a T-DNA transport structure. J. Bacteriol. **175**:5233–5241.
39. **Thorstenson, Y. R., and P. C. Zambryski.** 1994. The essential virulence protein VirB8 localizes to the inner membrane of *Agrobacterium tumefaciens*. J. Bacteriol. **176**:1711–1717.
40. **Varga, A. R., and S. Kaplan.** 1989. Construction, expression, and localization of a CycA::PhoA fusion protein in *Rhodobacter sphaeroides* and *Escherichia coli*. J. Bacteriol. **171**:5830–5839.
41. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1988. Characterization of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. **263**:5804–5814.
42. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. W. Nester.** 1990. Characterization of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. **265**:4768.
43. **Ward, J. E., Jr., E. M. Dale, and A. N. Binns.** 1991. Activity of the *Agrobacterium* T-DNA machinery is affected by *virB* gene products. Proc. Natl. Acad. Sci. USA **88**:9350–9354.
44. **Ward, J. E., Jr., E. M. Dale, P. J. Christie, E. W. Nester, and A. N. Binns.** 1990. Complementation analysis of *Agrobacterium tumefaciens* Ti plasmid *virB* genes by use of a *vir* promoter expression vector: *virB9*, *virB10*, and *virB11* are essential virulence genes. J. Bacteriol. **172**:5187–5199.
45. **Ward, J. E., Jr., E. M. Dale, E. W. Nester, and A. N. Binns.** 1990. Identification of a VirB10 protein aggregate in the inner membrane of *Agrobacterium tumefaciens*. J. Bacteriol. **172**:5200–5210.
46. **Weiss, A. A., F. D. Johnson, and D. L. Burns.** 1993. Molecular characterization of an operon required for pertussis toxin secretion. Proc. Natl. Acad. Sci. USA **90**:2970–2974.
47. **Winans, S. C.** 1992. Two-way chemical signaling in *Agrobacterium*-plant interactions. Microbiol. Rev. **56**:12–31.
47a. **Winans, S. C.** Personal communication.
48. **Winans, S. C., and G. C. Walker.** 1985. Conjugal transfer system of the IncN plasmid pKM101. J. Bacteriol. **161**:402–410.
49. **Zambryski, P. C.** 1992. Chronicles from the *Agrobacterium*-plant cell DNA transfer story. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43**:465–490.