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The *Agrobacterium tumefaciens virB4* Gene Product Is an Essential Virulence Protein Requiring an Intact Nucleoside Triphosphate-Binding Domain

BARBARA R. BERGER AND PETER J. CHRISTIE*

Department of Microbiology and Molecular Genetics, The University of Texas Health Science Center at Houston, Houston, Texas 77030

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Products of the ~9.5-kb *virB* operon are proposed to direct the export of T-DNA/protein complexes across the *Agrobacterium tumefaciens* envelope en route to plant cells. The presence of conserved nucleoside triphosphate (NTP)-binding domains in VirB4 and VirB11 suggests that one or both proteins couple energy, via NTP hydrolysis, to T-complex transport. To assess the importance of VirB4 for virulence, a nonpolar *virB4* null mutation was introduced into the pTiA6NC plasmid of strain A348. The 2.37-kb *virB4* coding sequence was deleted precisely by oligonucleotide-directed mutagenesis in vitro. The resulting Δ*virB4* mutation was exchanged for the wild-type allele by two sequential recombination events with the counterselectable *Bacillus subtilis sacB* gene. Two derivatives, A348ΔB4.4 and A348ΔB4.5, sustained a nonpolar deletion of the wild-type *virB4* allele, as judged by Southern blot hybridization and immunoblot analyses with antibodies specific for VirB4, VirB5, VirB10, and VirB11. Transcription of wild-type *virB4* from the *lac* promoter restored virulence to the nonpolar null mutants on a variety of dicotyledonous species, establishing *virB4* as an essential virulence gene. A substitution of glutamine for Lys-439 and a deletion of Gly-438, Lys-439, and Thr-440 within the glycine-rich NTP-binding domain (*Gly*-Pro-Iso-*Gly*-Arg-*Gly*-*Lys*-*Thr*) abolished complementation of A348ΔB4.4 or A348ΔB4.5, demonstrating that an intact NTP-binding domain is critical for VirB4 function. Merodiploids expressing both the mutant and wild-type *virB4* alleles exhibited lower virulence than A348, suggesting that VirB4, a cytoplasmic membrane protein, may contribute as a homo- or heteromultimer to *A. tumefaciens* virulence.

The distinctive feature of the *Agrobacterium tumefaciens* infection process is the natural ability of this phytopathogen to process and export oncogenes and other DNA to a wide range of dicotyledonous and monocotyledonous plant species (for reviews, see references 6, 38, 50, 78, and 82). Within the bacterium, the infection process is initiated by sensory recognition of wound-induced plant phenolics and sugar signal molecules by the VirA-VirG two-component regulatory system. Activated VirG, the response regulator, promotes the coordinate transcription of at least seven virulence operons, *virA*, *virB*, *virC*, *virD*, *virE*, *virF*, and *virG* (10, 35–37, 42, 52, 55, 58, 62, 79). Most of the products of the so-called *vir* regulon are required for processing a defined segment of the bacterial genome (T-DNA) from its position on the pTi (tumor-inducing) plasmid and exporting T-DNA to plant cells. Recent molecular studies have provided compelling evidence that the transfer-competent form of T-DNA actually consists of a T-DNA/protein complex (14, 15, 17, 19, 28, 31, 72, 81). To understand the T-DNA transfer process, therefore, it is critical to define the structure of the so-called T-complex as well as the specific interactions of T-complex protein subunits with both T-DNA and the dedicated T-complex transport machineries at the bacterial and plant membranes.

T-complexes are likely assembled during the processing reaction, which includes the following stages. (i) One or both of the *virC* gene products bind to enhancer sequences termed *overdrive* that flank the 25-bp imperfect direct repeats delimiting the T-DNA on octopine-type Ti plasmids (67, 68). VirC binding to *overdrive* has been proposed to facilitate recognition of the VirD endonuclease for the T-DNA border repeats; there is no evidence for direct interactions between VirC proteins and transfer-competent T-DNA. (ii) The VirD endonuclease, a multimeric protein consisting of VirD1 and VirD2 subunits, generates single-stranded (2, 34, 60, 61, 71, 80) or double-stranded (34, 64) scissions at T-DNA border sequences. (iii) Nicked T-DNA is released from the Ti plasmid by a strand displacement replication reaction that likely requires chromosomally encoded replication functions as well as the functions of several virulence proteins, including VirD1, a topoisomerase (26); VirE2, a single-stranded-DNA-binding protein (14, 15, 17); and VirE1, a small (~7 kDa) protein that has been proposed to interact with and stabilize the VirE2 single-stranded-DNA-binding protein (44).

A working model derived from the efforts of a number of laboratories presents the T-complex as a single-stranded T-DNA molecule with VirD2 covalently bound at the 5′ end and VirE2 single-stranded-DNA-binding protein associated tightly but noncovalently along the length of the molecule (14, 15, 17, 19, 28, 31, 72, 81). Both of these proteins, and, possibly other Vir proteins, may protect the single-stranded DNA from nucleolytic attack, provide transport signals for the dedicated export of the T-complex from the bacterium, and provide signals for directing T-complex transfer to and across the plant nuclear membrane. In fact, both VirD2 and VirE2 contain consensus eukaryotic nuclear localization sequences that target these proteins, when synthesized in plant protoplasts, to the plant nucleus (16, 32). A function for VirE2 in plants is further implicated by the finding that virulence is restored to an avirulent *virE2* mutant when this

* Corresponding author.

strain is inoculated onto transgenic plants expressing VirE2 protein (16).

As with transport of other nucleic acid-protein particles across prokaryotic (33) and eukaryotic (22, 53) membranes, transport of T-complexes is presumed to occur via dedicated heteromultimeric protein channels. A growing body of molecular genetic (41, 43, 57, 66, 73, 74–76) and biochemical (12, 21, 77) evidence suggests that the ~9.5-kb *virB* operon codes for some or all of the components of the T-complex transport system at the *A. tumefaciens* membrane. Early studies by Stachel et al. (59, 61) showed that although *virB* mutants retain the ability to sense plant signals, induce *vir* genes, and process T-DNA, they fail to incite tumor formation, suggesting that the *virB* gene products function at a late stage in the infection process. More recent studies by Ward et al. (75) showed that the *virB9*, *virB10*, and *virB11* gene products contribute to export not only of T-complexes but also of the broad-host-range IncQ plasmid RSF1010 to plant cells. Interestingly, these investigators demonstrated that for strains carrying both T-DNA and RSF1010-based plasmids, the relative transfer efficiencies of each molecule could be modulated by altering the expression levels of *virB9*, *virB10*, and *virB11* (75). These findings indicate, first, that different DNA substrates destined for export to plant cells utilize the same transporter and, second, that these substrates may compete for available transporter receptors. There also is evidence that *virB* gene products participate in the conjugative transfer of pTi (25) and IncQ (5) plasmids between bacteria. The recent discovery that the *tra2* operon of the IncP plasmid RP4 has extensive sequence similarity with the *virB* operon further supports a role for the *virB* proteins in a conjugal DNA transfer process (43). Consistent with their proposed transport functions, most if not all of the *virB* gene products associate with the bacterial envelope, as predicted first by computer analyses of the gene-derived protein sequences (41, 57, 66, 73, 74) and more recently by subcellular localization (12, 21, 77; this study) and VirB::PhoA protein fusion studies (9, 77).

Two VirB proteins, VirB4 and VirB11, possess putative nucleoside triphosphate (NTP)-binding domains (12, 66), suggesting that one or both proteins couple energy, via NTP hydrolysis, to T-complex transport. Initial biochemical studies demonstrated that purified VirB11 exhibits ATPase and autophosphorylating activities (12). This raises the interesting possibility that a VirB11 kinase functions to activate the T-complex transport process via phosphorylation (12). The potential importance of ATP binding or hydrolysis in T-complex transport is underscored by the discovery that VirB11 has features of ATP-binding subunits of the so-called ABC transporter superfamily (29, 70), including size, hydropathy profile, and predicted localization on the cytoplasmic face of the inner membrane (12). VirB11 also exhibits sequence similarities with proteins associated with DNA transport across the bacterial membrane (*Bacillus subtilis* ComGORF1 and KilB of the broad-host-range plasmid pRP4), pilin assembly (PilB), and protein secretion (PulE) (1, 18). The similarity with PilB, a morphogenesis protein, has led to speculation that VirB11 and related proteins function in chaperone-assisted assembly of multimeric transport machineries (18).

The studies reported here focused on VirB4 with the aim of establishing whether this protein is also important for virulence and, if so, whether the putative NTP-binding domain contributes to VirB4 function. We report the construction of a precise deletion of the *virB4* gene from the pTiA6NC plasmid by using the lethality gene *sacB* for marker exchange-eviction mutagenesis. Genetic complementation experiments with the nonpolar null mutants and the cloned wild-type gene established the importance of VirB4 for *A. tumefaciens* virulence. Two VirB4 derivatives sustaining mutations in the glycine-rich NTP-binding motif failed to complement the nonpolar null mutants, demonstrating that an intact NTP-binding domain is critical for VirB4 function. Finally, a merodiploid coexpressing the mutant and wild-type alleles exhibited diminished virulence relative to wild-type A348, suggesting that VirB4 functions as a homo- or heteromultimer or as a monomer that interacts with components of the T-complex substrate.

MATERIALS AND METHODS

Bacterial strains and plasmids. Table 1 lists the bacterial strains and plasmids used in this study. *Escherichia coli* and *A. tumefaciens* were transformed by electroporation with a Bio-Rad Laboratories (Richmond, Calif.) electroporator as described by Cangelosi et al. (9). The bacterial growth media, bacterial and phage growth conditions, and procedures for *vir* gene induction with acetosyringone have been described previously (12, 14).

Protein analysis, immunological techniques, and cell fractionation. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through 10.0 and 12.5% polyacrylamide gels and visualized by staining with Coomassie brilliant blue G in 10% methanol–10% glacial acetic acid (12). 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, as described by Bio-Rad Laboratories. Antibody specificities were previously documented for the VirB4 (76), VirB10 (76, 77), VirB11 (12), and VirE2 (14) proteins. Antibodies against VirB9 were kindly provided by J. Ward. Antibodies against VirB5 were produced as follows. Plasmid pJW275 was constructed by cloning the *Sal*I-12 fragment from pTiA6NC into pATH10 (73) (Table 1). The resulting *trpE*::*virB5* gene fusion transcribed from the *trp* promoter encodes a 49-kDa fusion protein consisting of TrpE joined to 127 amino acids at the carboxyl terminus of VirB5 (73). Lysates of 3-β-indole acrylic acid-induced *E. coli* cells carrying pJW275 were subjected to preparative-scale SDS-PAGE, and the overproduced fusion protein was isolated by gel elution as described previously for VirE2 (14). The TrpE::VirB5 fusion protein was injected into New Zealand White rabbits for antibody production. *A. tumefaciens* cells were separated into cytoplasmic, total membrane, cytoplasmic membrane, and outer membrane fractions, and the quality of fractionation was assessed as described previously (12, 14).

Construction of a Δ*virB4* mutation in vitro. A unique *Bam*HI site positioned 705 bp downstream of the *virB4* translational start site served as a junction site for reconstruction of the intact *virB4* coding sequence from two separate restriction fragments. A 2.38-kb *Bam*HI-*Sac*I fragment from pBB1, which contains *virB4* sequences 3′ of the *Bam*HI site, was introduced into pBB2, which contains *virB4* sequences 5′ of the *Bam*HI site, to yield pBB3 (Table 1). Next, a 3.71-kb *Eco*RV-*Sac*I fragment containing the intact *virB4* coding sequence along with 623 bp of upstream and 706 bp of downstream *virB* sequences was introduced into pBluescript II SK− to yield pBB4. To reduce the size of DNA to be deleted by site-directed mutagenesis, a 668-bp *Bgl*II-*Bam*HI fragment within the *virB4* coding sequence was removed by endonucleolytic cleavage and religation to gen-

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5α	F^- 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
SG935	F^- *lac trp pho msl htpR rpsL supC*(Ts)	S. Goff
A. tumefaciens		
A136	Strain C58 cured of pTi plasmid	Laboratory stock
A348	A136 containing pTiA6NC	Laboratory stock
LBA4404	Carries T-DNA deletion derivative of pTiA6NC	46
A348ΔB4.4	Carries *virB4* deletion derivative of pTiA6NC	This study
A348ΔB4.5	Carries *virB4* deletion derivative of pTiA6NC	This study
Plasmids		
pJW239	Carb^r, 5.15-kb *Bam*HI-14 fragment of pTiA6NC containing 3′ end of *virB4-virB9* cloned behind *lac* promoter in pTZ19R	J. Ward
pBB1	Carb^r, pJW239 deleted of 2.77-kb *Sac*I fragment; contains 3′ end of *virB4-virB5*	This study
pUC8Sal13a	Carb^r, 3.5-kb *Sal*I-13a fragment of pTiA6NC in pUC8	76
pBB2	Cam^r, 2.87-kb *Sal*I-*Bam*HI from pUC13A containing *virB1* through 5′ end of *virB4* cloned into pUC13CM	This study
pBB3	2.38-kb *Bam*HI-*Sac*I fragment from pBB1 inserted into pBB2 to reconstruct *virB1-virB5*	This study
pBB4	Carb^r, 3.71-kb *Eco*RV-*Sac*I fragment from pBB3 inserted into pBluescript II SK−	This study
pBB5	Carb^r, pBB4 deleted of a 668-bp *Bgl*II-*Bam*HI fragment within the *virB4* coding sequence	This study
pBB6	Carb^r, pBB5 deleted of the remaining ~1.7 kb of the *virB4* coding sequence	This study
pCF112	Kan^r Carb^r, *sacBR ori6K mobRk2*; mobilizable suicide vector	S. Winans
pBB7	Kan^r, ~1.5-kb *Pvu*II fragment from pBB6 containing Δ*virB4* with 623 bp of 5′- and 706 bp of 3′-flanking *virB* sequences inserted into *Xmn*I site in *bla* gene of pCF112	This study
pSW172	Tet^r, broad-host-range IncP plasmid containing *lac* promoter and polycloning region from pIC19	11
pBluescript II SK+	Carb^r, cloning vector	Stratagene
pBluescript II KS+	Carb^r, cloning vector	Stratagene
pBCSK+	Cam^r, cloning vector	Stratagene
pBCKS+	Cam^r, cloning vector	Stratagene
pZD1	Carb^r, 3.71-kb *Eco*RV-*Sac*I fragment from pBB3 inserted into pBluescript II KS+	This study
pZDM10	Carb^r, *virB4* overexpression plasmid; pZD1 deleted of 730 bp between the *lacZ* and *virB4* translational start sites	This study
pZDH10	Carb^r Tet^r, pZDM10 ligated at *Sac*I site to pSW172	This study
pZDH2	Carb^r Tet^r, pBluescript II KS+ ligated at *Sac*I site to pSW172	This study
pBB11	pZDM10 with a Glu codon substitution for Lys-439	This study
pBB12	pZDM10 with a Glu codon substitution for Lys-439	This study
pBB13	pZDM10 deleted of Gly-438, Lys-439, and Thr-440 codons	This study
pBB14	pZDM10 deleted of Gly-438, Lys-439, and Thr-440 codons	This study
pBB15	Carb^r Tet^r, pBB11 ligated at *Sac*I site to pSW172	This study
pBB16	Carb^r Tet^r, pBB12 ligated at *Sac*I site to pSW172	This study
pBB17	Carb^r Tet^r, pBB13 ligated at *Sac*I site to pSW172	This study
pBB18	Carb^r Tet^r, pBB14 ligated at *Sac*I site to pSW172	This study
pJW275	Carb^r, 3.97-kb *Sal*I-12 fragment of pTiA6NC in pATH10, resulting in *trpE*::*virB5* translational fusion	73
pMMB22	Carb^r, broad-host-range plasmid containing the *tac* promoter and *lacI*^{q}	3
pPC201	1.3-kb *Eco*RI-30 fragment of pTiA6NC in pMMB22 for *virB5* overexpression	This study
pGS1	Carb^r, pZDM10 deleted of the 1.4-kb *Eco*RI fragment within *virB4*	This study
pGS2	Carb^r Tet^r, pGS1 ligated at *Sac*I site to pSW172	This study

erate pBB5. The remaining ~1.7-kb *virB4* coding sequence was precisely deleted from pBB5 by oligonucleotide-directed mutagenesis as described previously (12). Briefly, uracil-containing template DNA was recovered from strain CJ236(pBB5) by infection with bacteriophage M13KO7. The oligonucleotide 5′-AAGTTGCGTCGTCTTCATTACACC ATTCCT-3′ was synthesized so that the 5′ half is complementary to 15 bases at the 5′ end of *virB5* and the 3′ half is complementary to 15 bases immediately preceding the *virB4* translational start site. The synthetic 30-mer was annealed to pBB5 template DNA to prime complementary-strand synthesis in vitro, and the resulting DNA was introduced into *E. coli* DH5α. Transformants were examined for deletion of the entire 2.37-kb *virB4* sequence by restriction enzyme digestion and then by DNA sequence analysis across the entire *Eco*RV-*Sac*I fragment. One plasmid, designated pBB6, among 12 plasmids analyzed had sustained the Δ*virB4* mutation, resulting in positioning of the 5′ end of *virB5* precisely at the former *virB4* translational start site. As shown in Fig. 1, pBB6 contains Δ*virB4* flanked by 623 bp of upstream and 706 bp of downstream wild-type *virB* sequences.

The Δ*virB4* mutation was subcloned into the *sacBR* suicide vector pCF112 (Table 1) for delivery into *A. tumefaciens* A348. This was achieved by digesting pBB6 at two *Pvu*II sites flanking the pBluescript II SK− multiple cloning site. The resulting 1.7-kb *Pvu*II fragment carrying Δ*virB4* and flanking *virB* DNA was introduced at the unique *Xmn*I site within the *bla* gene of pCF112 to yield pBB7. Plasmid

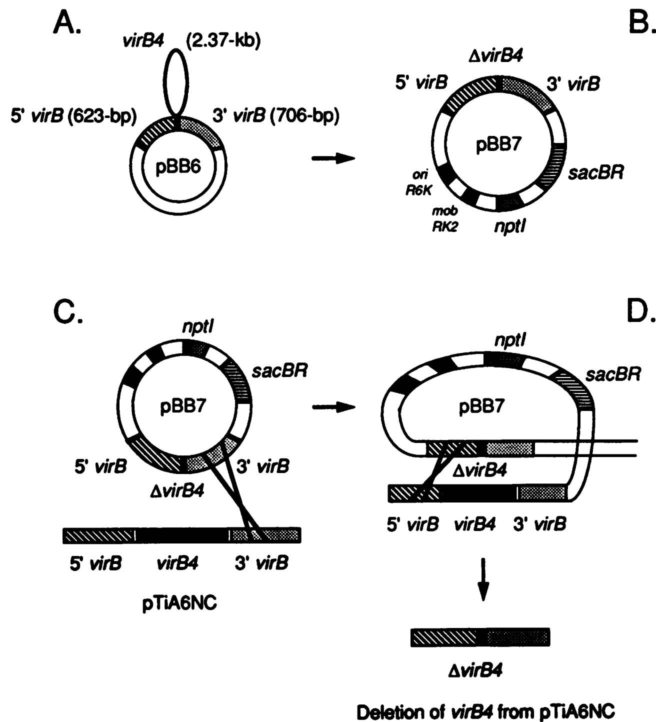

FIG. 1. Mutagenesis procedure used to delete *virB4* from pTiA6NC. (A) 1.7 kb of the *virB4* coding sequence was deleted from pBB5 by oligonucleotide-directed mutagenesis in vitro to generate pBB6. (B) The Δ*virB4* mutation with flanking *virB* sequences was cloned into the *sacB* suicide vector pCF112. (C) The Δ*virB4* mutation was recombined onto pTiA6NC by a single crossover event between homologous sequences on pBB7 and pTiA6NC. (D) Transformants were grown in the absence of antibiotic selection to allow excision of vector sequences and the wild-type *virB4* allele by a second crossover event. Cells were grown in sucrose-containing medium to counterselect for cells carrying the desired double recombination event. See text for details.

pCF112, constructed and kindly provided by C. Fuqua and S. Winans, contains the *pir* origin of replication of R6K (*ori6K*), which does not function in *A. tumefaciens*, the RK2 origin of transfer (*mobRK2*) for mobilization from *E. coli* to other gram-negative species, and *sacBR* from *Bacillus subtilis* (24). The *sacB* gene, regulated in *cis* by *sacR*, encodes levansucrase, an enzyme that catalyzes the hydrolysis of sucrose and the synthesis of levans, branched polymers of fructosyl residues. Levan toxicity has been demonstrated for a variety of gram-negative bacteria, making *sacB* an efficient counterselection marker for double recombination events (7, 39, 40, 45, 51, 54).

Cloning and expression of *virB4* in *E. coli* and *A. tumefaciens*. The *virB4* coding sequence was cloned and overexpressed in *E. coli* by introducing the 3.71-kb *Eco*RV-*Sac*I fragment from pBB4 into similarly digested pBluescript II KS+. The intact *virB4* gene in the resulting plasmid, pZD1, was oriented in the same direction as the *lac* promoter. Next, the 730 bp of DNA between the translational start sites of *lacZ* and *virB4* was deleted by the oligonucleotide mutagenesis procedure outlined above. The 30-mer 5′-ACTCGC GCCGAGCATAGCTGTTTCCTGTGT-3′ was synthesized so that the 5′ half is complementary to 15 bases at the 5′ end of the *virB4* gene and the 3′ half is complementary to 15 bases that immediately precede the *lacZ* translational start site on pBluescript II KS+. This 30-mer was annealed to uracil-containing pZD1 template to prime complementary-strand synthesis in vitro, and the resulting DNA was introduced into *E. coli* SG935 (Table 1). Transformed cells were screened for plasmids sustaining the 730-bp deletion and for overproduction of an ~85-kDa protein, the expected size of the VirB4 protein.

The precise fusion of the *virB4* coding sequence at the *lacZ* ATG was established for four plasmids, pZDM1, pZDM9, pZDM10, and pZDM12, by sequencing across the deletion junction. These plasmids were ligated at the unique *Sac*I site with the broad-host-range IncP plasmid pSW172 for *virB4* expression in *A. tumefaciens*. Each of these plasmids was analyzed for *virB4* expression in *E. coli* and *A. tumefaciens* and for the ability to complement *virB4* null mutants. Because all strains carrying these plasmids were phenotypically indistinguishable with respect to *virB4* expression and genetic complementation, we present results only for pZDM10 and its derivatives.

Construction of mutations in the NTP-binding domain of VirB4. Two mutations predicted to diminish or abolish ATP binding or hydrolysis were introduced into the glycine-rich NTP-binding domain (Gly-Pro-Iso-Gly-Arg-**Gly-Lys-Thr**), positioned between amino acid residues 433 and 440 of VirB4. The Lys-439 codon was changed to Glu with the oligonucleotide 5′-CATGAGCGTTGTCTGACCCCTCCC G-3′, and codons specifying Gly-438, Lys-439, and Thr-440 were deleted with the oligonucleotide 5′-CAAACATCATG AGCGTCCTCCCGATAGGCC-3′. CJ236(pZDM10) served as the source of template DNA for these experiments. Sequence analysis showed that two plasmids, designated pBB11 and pBB12, contained the substitution of Glu for Lys-439, and two plasmids, designated pBB13 and pBB14, contained the deletion of the Gly-438, Lys-439, and Thr-440 codons. All four plasmids were identical to each other and indistinguishable from the wild type throughout the 250 bp upstream and downstream of the site of mutation. As an added assurance that the NTP-binding mutations, and not mutations elsewhere in *virB4*, were responsible for the observed phenotypes, all four mutant plasmids (pBB11, pBB12, pBB13, and pBB14) were evaluated in these studies. Plasmids pBB11, pBB12, pBB13, and pBB14 were ligated at their unique *Sac*I sites with the broad-host-range IncP plasmid pSW172 to yield pBB15, pBB16, pBB17, and pBB18, respectively (Table 1).

Virulence assays. Virulence assays were performed by inoculating wound sites of *Kalanchoe diagremontiana* leaves with ~10^8 CFU of the various bacterial strains (12, 14). *Nicotiana xanthi* leaf disks were infected as described by Horsch et al. (30). Controls for the tumorigenesis assays included co-inoculating the same leaf with wild-type A348 (virulent); A136, a strain cured of the pTi plasmid (avirulent); and the various test strains. Each experiment was repeated at least four times, and tumor formation was monitored over a 3- to 4-month period.

RESULTS

Introduction of a nonpolar *virB4* null mutation into pTiA6NC. The wild-type *virB4* allele was deleted from pTiA6NC by a combination of site-directed (see Materials and Methods) and marker exchange-eviction mutageneses. Plasmid pBB7 was electroporated into A348, with selection for vector-encoded Kan^r. Because *ori6K* of pBB7 cannot replicate in *A. tumefaciens*, Kan^r transformants likely arise by plasmid integration into the bacterial genome by homol-

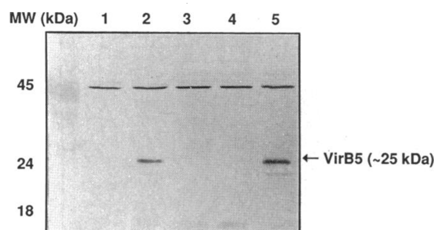

FIG. 2. Immunoblot showing expression of VirB5 protein in various *A. tumefaciens* strains. Total cell extracts were electrophoresed through SDS–12.5% polyacrylamide gels, proteins were transferred to nitrocellulose, and blots were developed with VirB5 antibodies. Uninduced and acetosyringone-induced A348 (lanes 1 and 2, respectively), induced A136 (lane 3), induced A136(pTi21) (51) (lane 4), and IPTG-induced A136(pPC201), which expresses *virB5* from the *lacZ* promoter (lane 5). Positions of molecular mass markers are indicated at the left. Immunoreactive VirB5 protein migrates with an apparent molecular size of ~25 kDa, as predicted from the gene-derived protein sequence (73, 74).

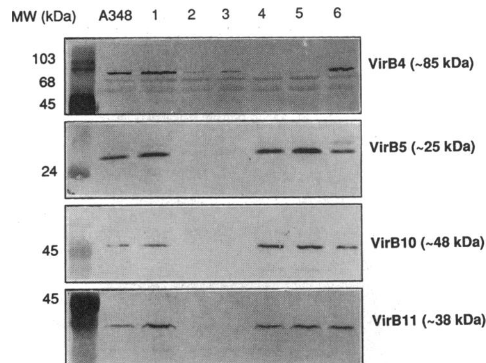

FIG. 3. Immunoblot analysis of putative *virB4* deletion derivatives of A348. Six independent Suc^r Kan^s isolates derived from the mutagenesis protocol outlined in Results and in Fig. 1 were analyzed for acetosyringone-inducible expression of *virB4*, *virB5*, *virB10*, and *virB11*. Antibodies specific for each of the corresponding gene products were used to develop immunoblots prepared from total-cell extracts. The immunoblot studies revealed the presence of VirB4, VirB5, VirB10, and VirB11 in cell extracts from A348 and mutants A348.B4.1 (lane 1) and A348.B4.6 (lane 6). Mutants A348B4.2 (lane 2) and A348B4.3 (lane 3) synthesized a low level of VirB4 and no detectable VirB5, VirB10, or VirB11. Mutants A348.B4.4 (lane 4) and A348B4.5 (lane 5) synthesized VirB5, VirB10, and VirB11, but no detectable VirB4; these strains were designated A348ΔB4.4 and A348ΔB4.5, respectively.

ogous recombination. Figure 1 shows that the desired single-crossover event between *virB* sequences flanking Δ*virB4* on pBB7 and homologous sequences on pTiA6NC generates a merodiploid exhibiting Kan^r and Suc^s. Ten isolates with these phenotypes were grown in MG/L broth for 16 h in the absence of kanamycin to allow the occurrence of a second crossing-over. Next, cells were diluted 1:1,000 in MG/L broth with added 5% sucrose as a counterselection for cells in which the *sacB* lethality gene had been excised. The desired double recombination event also excises additional vector sequences as well as the wild-type *virB4* allele. Overnight broth cultures were spread on MG/L plates with 5% sucrose, and 100 colonies derived from each of the 10 parental strains were screened for Kan^s and Suc^r. The frequency with which Kan^s Suc^r cells were recovered varied according to the parental strain from 0% for one strain, 10 to 40% for eight strains, to 87% for one strain. Similar frequencies of 7 to 39% were reported previously for plasmid eviction with *sacB* selection for double recombinants in *Yersinia enterocolitica* (40). The ability to recover progeny with the desired phenotypes appears to depend on a number of factors, including duration of exposure to sucrose, strain-dependent reversion to sucrose resistance, and the frequency of recombination, which itself is a function of the extent of DNA homology in the merodiploid (39, 40).

To determine whether the putative Δ*virB4* mutation abolished VirB4 synthesis and to assess the effects of the deletion on expression of *virB* genes residing 3′ of the deletion junction, we examined the ability of six putative *virB4* null mutants to synthesize VirB4, VirB10, and VirB11 by using antibodies specific for these proteins (see Materials and Methods). VirB5 synthesis was also examined to ensure that its synthesis was unaltered as a result of the marker exchange mutagenesis procedure. Figure 2 shows the specificity of antibodies raised against a TrpE::VirB5 fusion protein (see Materials and Methods) for VirB5. Antibodies reacted with a protein of ~25 kDa, the expected molecular size for VirB5, in extracts of acetosyringone-induced A348 (Fig. 2, lane 2) but not uninduced A348 (Fig. 2, lane 1), induced A136 (Fig. 2, lane 3), or A136(pTi21), which carries pTiA6NC with a Tn*3*HoHo1 insertion within or immediately upstream of the 5′ end of the *virB5* coding sequence (59) (Fig. 2, lane 4). Furthermore, immunoreactive material of the expected molecular size was detected in isopropylthiogalactopyranoside (IPTG)-induced A136(pPC201) (Fig. 2, lane 5). Plasmid pPC201 contains the *Eco*RI-30 fragment of pTiA6NC cloned into the *lacI*^{q} broad-host-range vector pMMB22 (3) so that the intact *virB5* coding sequence is placed under the transcriptional control of the *lac* promoter. These results are consistent with an earlier study showing that *E. coli* maxicells prepared from strain JC2926(pPC201) synthesized an ~25-kDa protein that is not present in maxicell extracts prepared from the vector-only strain JC2926(pMMB22) (13).

Figure 3 shows the results of immunoblot analyses of A348 and the six putative *virB4* deletion derivatives induced for *vir* gene expression. Extracts from two strains, A348.B4.4 and A348.B4.5, contained no detectable VirB4 protein in the absence (data not shown) or presence of acetosyringone (Fig. 3, lanes 4 and 5). Furthermore, subcellular fractions of these strains contained no detectable VirB4 protein, as determined by immunoblot analysis with the VirB4-specific antibodies (data not shown). In contrast, these strains synthesized wild-type levels of VirB5, VirB10, and VirB11 only after acetosyringone induction. Southern blot hybridization studies confirmed that A348.B4.4 and A348.B4.5 had sustained a deletion of the 2.37-kb *virB4* coding sequence; flanking *virB* sequences also were not detectably altered with respect to the wild type, and these strains were devoid of vector-related sequences (data not shown). These immunoblot and Southern hybridization data established that A348.B4.4 and A348.B4.5 were deleted of *virB4* and that the Δ*virB4* mutation had a nonpolar effect on expression of downstream *virB* genes. These strains were renamed A348ΔB4.4 and A348ΔB4.5.

Two strains, A348.B4.1 and A348.B4.6, exhibited wild-type levels of acetosyringone-inducible VirB4, VirB5, VirB10, and VirB11, as judged by immunoblot analysis (Fig. 3, lanes 1 and 6). The restriction endonuclease cleavage profiles of these strains were identical to those of A348, as judged by Southern hybridization analysis with *virB4*, flanking *virB* sequences, and vector sequences as DNA probes

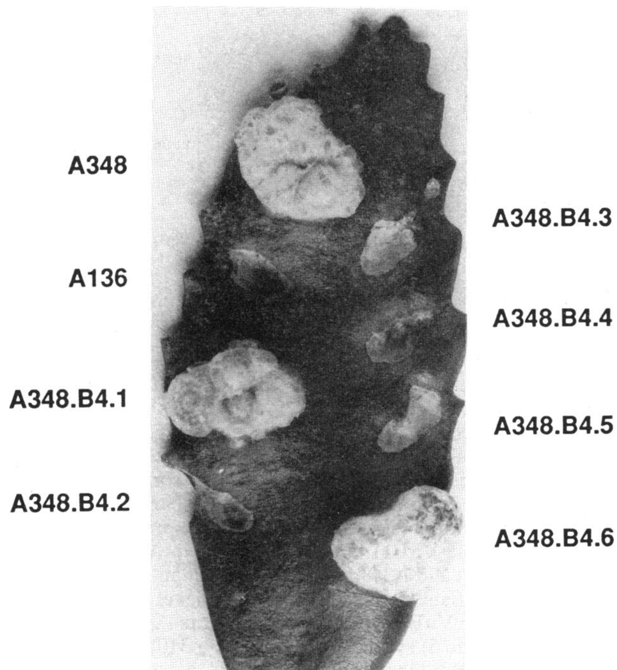

FIG. 4. Virulence of putative *virB4* deletion derivatives. *K. diagremontiana* leaves were wounded with a sterile toothpick and inoculated with A348, the wild-type strain; A136, an avirulent derivative lacking pTi; and the six putative Δ*virB4* mutants. Leaves were photographed at 2 weeks postinoculation, and tissues were observed over a 3-month period for development of tumors.

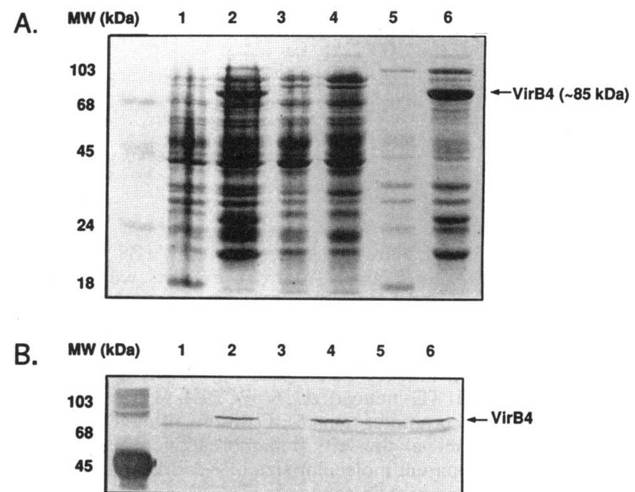

FIG. 5. Expression of *virB4* under control of the *lac* promoter in *E. coli* and *A. tumefaciens*. (A) Overproduction of VirB4 by *E. coli* SG935(pZDM10). DNA (703 bp) between the *lacZ* and *virB4* translational start sites was deleted by site-directed mutagenesis (see Materials and Methods). VirB4 production was visualized by SDS-PAGE and gel staining with Coomassie brilliant blue G. Total protein from uninduced (lane 1) and induced (lane 2) SG935 (pZDM10); total supernatants derived from low-speed (5,000 × *g*) centrifugation of uninduced (lane 3) and induced (lane 4) SG935 (pZDM10) cell extracts; corresponding cell pellets from uninduced (lane 5) and induced (lane 6) SG935(pZDM10). Material corresponding in size to VirB4 (~85 kDa) was detectable in abundance in total-cell extracts (lane 2) and in cell pellets (lane 6) from induced SG935(pZDM10). (B) Immunoblot analysis showing VirB4 production in *A. tumefaciens*. Total cellular protein (10 μg) from various *A. tumefaciens* strains was electrophoresed through SDS–12.5% polyacrylamide gels and transferred to nitrocellulose, and blots were developed with VirB4-specific antiserum. Uninduced (lane 1) and acetosyringone-induced (lane 2) A348; induced A348ΔB4.4(pZDH2) (lane 3); induced A348ΔB4.4(pZDM10) (lane 4); induced A348ΔB4.4 (pBB15) (lane 5); and induced A348ΔB4.4(pBB17) (lane 6). See Table 1 for descriptions of strains and plasmids.

(data not shown). These findings suggest that pBB7 excised precisely from pTiA6NC in these strains, resulting in abortive allelic exchange. Two strains, A348.B.2 and A348.B4.3, reproducibly exhibited reduced levels of VirB4 protein and no detectable VirB5, VirB10, or VirB11 proteins (Fig. 3, lanes 2 and 3). The Southern hybridization analyses revealed that vector-related sequences had been incompletely excised in these strains (data not shown). The presence of vector sequences probably resulted in RNA destabilization and/or premature transcription termination in these strains.

Figure 4 shows that *K. diagremontiana* tissues inoculated with the two presumptive nonpolar *virB4* null mutants, A348.ΔB4.4 and A348ΔB4.5, failed to develop tumors. Although *K. diagremontiana* leaves were photographed after 2 weeks, these plants and infected *N. xanthi* leaf disks (data not shown) exhibited no disease symptoms even after incubation for several months. In contrast, tissues inoculated with A348.B4.1 and A348.B4.6 developed tumors with the same kinetics and morphologies as tissues inoculated with A348 (Fig. 4), consistent with our interpretation that pBB7 integrated into and was then excised from the genome without altering the molecular architecture of these strains. Tissues inoculated with strains A348.B4.2 and A348.B4.3 failed to form tumors, as predicted from the immunoblot studies, which showed that these strains do not express the essential virulence genes *virB10* and *virB11*.

Expression of wild-type *virB4* in *E. coli* and *A. tumefaciens*. IPTG-induced DH5α(pZD1) cells synthesized a small amount of material immunoreactive with VirB4 antibodies that migrated in SDS-polyacrylamide gels as an ~85-kDa protein, the size predicted for VirB4 (see Materials and Methods). This material was not present in extracts from uninduced cells, cells carrying the vector only, or cells carrying pBB4, which contains *virB4* cloned in the opposite direction of transcription from the *lac* promoter (data not shown). To enhance *virB4* gene expression, 730 bp between the *lacZ* and *virB4* translation initiation codons was deleted by site-directed mutagenesis. The resulting plasmids, pZDM1, pZDM9, pZDM10, and pZDM12, each contain *virB4* fused precisely at the *lacZ* translation initiation site, as determined by restriction enzyme digestion and DNA sequence analyses (see Materials and Methods).

IPTG-induced SG935(pZDM10) significantly overproduced a protein of ~85 kDa (Fig. 5A, lane 2) after induction of the cells with IPTG. This protein was not detectable in extracts from uninduced SG935(pZDM10) (Fig. 5A, lane 1) or SG935 carrying pGS1, a pZDM10 derivative deleted of the 1.4-kb *Eco*RI fragment residing within the *virB4* sequence (data not shown). These results, together with the positioning of *virB4* immediately downstream of the *lacZ* translation initiation codon, the reactivity of the ~85-kDa material with VirB4-specific antibodies (see Fig. 5B), and the ability of the cloned gene to complement *virB4* null mutants (see below), establish the ~85-kDa protein as VirB4. IPTG-induced SG935(pZDM10) cells apparently sequester VirB4 into inclusion bodies, as deduced by our ability to detect light-

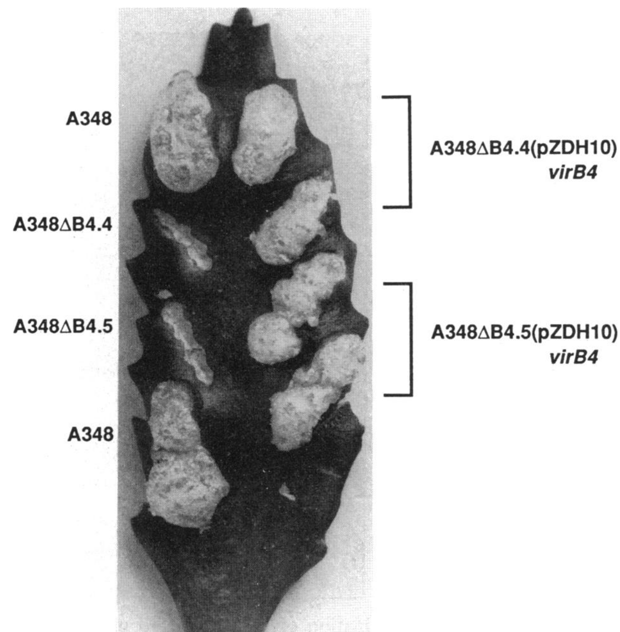

FIG. 6. Genetic complementation of Δ*virB4* mutants with pZDH10, which expresses *virB4* under control of the *lac* promoter. *K. diagremontiana* leaves were inoculated with the wild-type strain A348 and the Δ*virB4* mutants A348ΔB4.4 and A348ΔB4.5 on the left; corresponding mutants carrying pZDH10 were inoculated on the right.

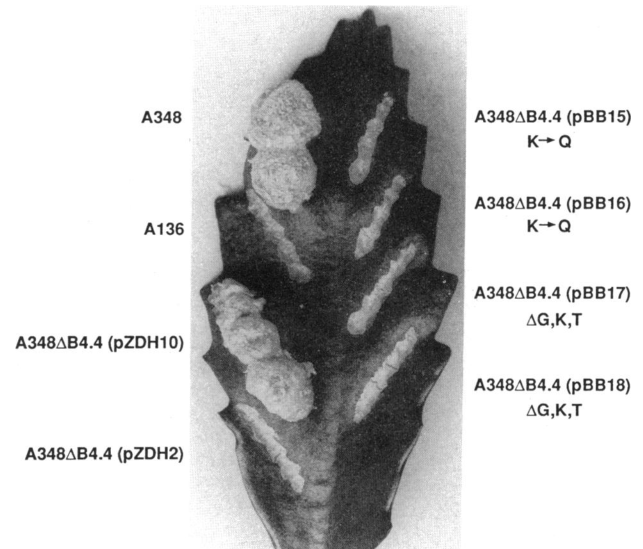

FIG. 7. Virulence assays of A348ΔB4.4 mutants expressing *virB4* alleles with mutations in the NTP-binding domain. Strains inoculated onto wounded *K. diagremontiana* include A348, the wild-type strain; A136, a derivative lacking pTi; A348ΔB4.4 carrying pZDH2, a vector-only control; A348ΔB4.4 carrying pBB15 and pBB16, derivatives of pZDM10 in which Lys-439 (K) within the NTP-binding domain of VirB4 was changed to glutamine (Q); and pBB17 and pBB18, derivatives of pZDM10 in which Gly-438 (G), Lys-439 (K), and Thr-440 (T) within the NTP-binding domain were deleted.

refractile bodies in these cells by microscopy and to pellet VirB4 protein by low-speed centrifugation (5,000 × *g*) of cell extracts (Fig. 5A, lane 6). VirB4 remained insoluble through repeated 1 M NaCl washes but solubilized when exposed to denaturants such as 8 M urea or 6 M guanidine-HCl.

To verify that *virB4* is expressed from the *lac* promoter in *A. tumefaciens* and to estimate VirB4 abundance in these cells, VirB4-specific antibodies were used to develop blots prepared with total cellular protein (10 μg) from acetosyringone-induced A348 (Fig. 5B, lane 2) and A348ΔB4.4 (pZDM10) (Fig. 5B, lane 4). Immunoreactive material was not detected in extracts from uninduced A348 (Fig. 5B, lane 1) or A348ΔB4.4 carrying pZDH2 (vector only; see Table 1) (Fig. 5B, lane 3). Similarly, transcription of two *virB4* ATP-binding mutant alleles (see below) from the *lac* promoter in A348ΔB4.4 resulted in synthesis of immunoreactive material identical in size to the wild-type protein (Fig. 5B, lanes 5 and 6). These studies showed that the products of the cloned wild-type gene as well as the mutant alleles were recognized by VirB4 antibodies and migrated to the same position as the wild-type protein synthesized by A348. Interestingly, strains expressing the wild-type and mutant alleles from the *lac* promoter on a multicopy plasmid possessed steady-state levels of VirB4 that were comparable to the levels of the wild-type protein synthesized from the native *virB* promoter. Whether VirB4 abundance is subject to posttranscriptional regulation remains to be examined.

VirB4 provides an essential virulence function. Figure 6 shows the results of genetic complementation tests demonstrating that expression of the cloned *virB4* gene restored virulence to A348ΔB4.4 and A348ΔB4.5. Furthermore, tumors incited by these strains resembled those incited by A348 in terms of both morphology and the time course of appearance. These experiments were repeated several times on several dicotyledonous species, including *N. xanthi* leaf disks, tomato stems, and potato tuber disks, with the same results (data not shown). To compare the relative transfer frequencies of A348 and the null mutants expressing *virB4* under control of the *lac* promoter, 10-fold serial dilutions of bacterial cultures were inoculated onto *K. diagremontiana* wound sites. Both A348 and the null mutants expressing *virB4* in *trans* incited tumors that were similar in appearance and in time course of appearance at dilutions ranging from 10^3 to 10^8 CFU/ml. Genetic complementation was also observed when plasmid DNA was isolated from A348ΔB4.4 (pZDH10) or A348ΔB4.5(pZDH10) and reintroduced into the null mutants. Isogenic strains carrying pZDH2 (vector only; Table 1) or pGS2, a pZDH10 derivative sustaining a 1.4-kb deletion of internal *virB4* sequence, were avirulent on all species and tissues tested. These findings confirm that *virB4* expression from the *lac* promoter on pZDH10 was responsible for the observed genetic complementation.

Intact NTP-binding domain is essential for VirB4 function. Virulence assays were performed to evaluate the importance of an intact NTP-binding domain for VirB4 function. A348ΔB4.4 cells carrying pZDM10 (wild-type *virB4*), pBB15 and pBB16 (Lys-439 to Glu substitution), and pBB17 and pBB18 (Gly-438, Lys-439, and Thr-440 deletion) were inoculated at 10^8 CFU onto wounded *K. diagremontiana* leaves. As shown in Fig. 7, the mutant alleles failed to restore virulence to these cells. In no instance have inoculated *K. diagremontiana* leaves or other dicotyledonous species, including tomato stems, potato tuber disks, and *N. xanthi* leaf sections, developed any tumors (data not shown). We can rule out the possibility that low steady-state levels of these proteins accounted for their inability to complement,

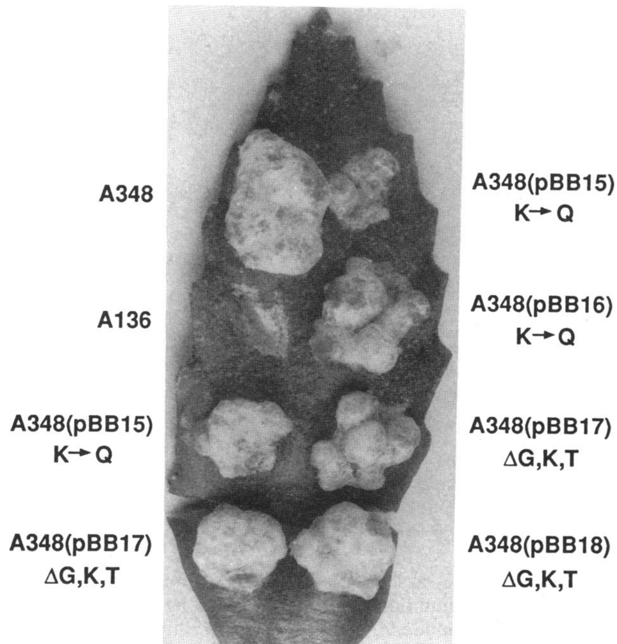

FIG. 8. Virulence assays of merodiploids coexpressing the *virB4* wild-type gene and mutant alleles sustaining mutations in the NTP-binding domain. Strain and plasmid designations are described in the legend to Fig. 7.

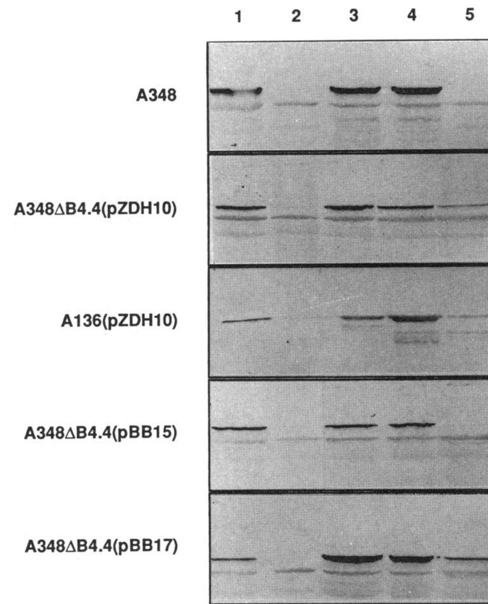

FIG. 9. Subcellular localization of wild-type and mutant VirB4 proteins in various *A. tumefaciens* strains, including A348, A348ΔB4.4, and A136. Proteins from the various cellular fractions were electrophoresed through SDS-polyacrylamide gels and transferred to nitrocellulose. The blots were probed with VirB4-specific antibodies. Total protein (lane 1); cytoplasmic protein (lane 2); total membrane protein (lane 3); cytoplasmic membrane protein (lane 4); outer membrane protein (lane 5). Strain designations are aligned adjacent to immunoreactive VirB4 proteins.

because the mutant proteins resembled the wild-type protein synthesized in A348 or in A348ΔB4.4(pZDM10) both in size and in steady-state abundance (Fig. 5B). These findings demonstrate the importance of an intact NTP-binding domain and, by extension, the ability of VirB4 to bind to and/or hydrolyze a mononucleotide for *A. tumefaciens* virulence. While these studies were in progress, Fullner and Nester (23) presented findings similar to ours at a recent meeting, and Shirasu et al. (56) showed the potential for VirB4 to hydrolyze ATP. Interestingly, at this meeting Kado and his colleagues (56) reported that *A. tumefaciens virB4* mutants were unable to agroinfect maize, establishing *virB4* as an essential virulence gene for a wide range of susceptible plant hosts.

The VirB proteins have been proposed to interact with other VirB proteins to form a multicomponent membrane apparatus to mediate T-complex transport and/or to stabilize bacteria-plant cell contacts (38, 73, 77, 82). If VirB4 constitutes one of the transporter subunits, or if the protein functions as a multimer, then a merodiploid expressing both the wild-type and mutant alleles may differ phenotypically from wild-type A348. To test this possibility, we inoculated wounded *K. diagremontiana* with 10^8 CFU each of A348 carrying pBB15, pBB16, pBB17, or pBB18. Figure 8 shows that although coexpression of the mutant and wild-type alleles did not abolish tumor formation, the merodiploids consistently incited the formation of smaller and morphologically aberrant tumors compared with A348. Tumors incited by the merodiploids typically consisted of multiple, isolated foci of undifferentiated cells, whereas tumors formed by infection with A348 characteristically developed as smooth mounds. These phenotypes likely reflect a proliferation of different numbers of transformed cells, since inoculations with serially diluted cultures revealed that A348 at a reduced inoculum size of 10^4 to 10^5 CFU incited the formation of tumors that morphologically resembled the tumors incited by the merodiploid inoculated at 10^8 CFU (data not shown). These data, combined with results of our immunoblot analyses (Fig. 5B), suggest that when cells produce comparable levels of the wild-type and mutant VirB4 proteins, the mutant protein somehow interferes with wild-type protein function.

Subcellular localization of VirB4 in different *A. tumefaciens* strains. VirB4 lacks an amino-terminal signal sequence and a hydrophobicity profile reminiscent of integral membrane or secreted proteins (73, 74, 83). However, VirB4 localizes almost exclusively to the cytoplasmic membrane, as determined by immunoblot analyses of subcellular fractions of A348 and of A348ΔB4.4(pZDH10) (Fig. 9). Occasionally, a small amount of VirB4 was detected in outer membrane preparations, but this was not consistently reproducible. These data, together with the results of the merodiploid analysis described above, raise the intriguing possibility that VirB4 interacts with the membrane via an association with one or more of the integral membrane components of the T-complex transporter. If so, VirB4 partitioning may be altered when it is synthesized in A136, a strain cured of the pTi plasmid. Subcellular fractionation of A136(pZDM10) and immunoblot analysis showed that VirB4 remains associated with the cytoplasmic membrane despite the absence of *vir*- or pTi-encoded functions (Fig. 9). A parallel study of A136(pJW353), which expresses the *virB11* gene from the *lacZ* promoter (12), revealed that VirB11 also partitions in this strain, as in wild-type A348, primarily to the cytoplasmic membrane (11a). Thus, both VirB4 and VirB11 integrate directly into the membrane or associate tightly with chromosomally encoded membrane proteins.

To evaluate whether mutations in the NTP-binding domain affect VirB4 partitioning, subcellular fractions of A348ΔB4.4 cells carrying pBB15 (Lys→Glu) or pBB17 (Gly, Lys, and Thr deletion) were examined for the presence of the mutant proteins. Figure 9 shows that the mutant proteins, as with wild-type VirB4, partitioned almost exclusively with the cytoplasmic membrane. These findings suggest that the association of VirB4 with the cytoplasmic membrane occurs independently of possible conformational changes induced by NTP binding and/or hydrolysis.

DISCUSSION

This study focused first on establishing the importance of VirB4 for *A. tumefaciens* tumorigenicity and second on evaluating the functional significance of NTP binding or hydrolysis by this protein. The first objective was achieved in part by constructing a precise deletion of the *virB4* coding sequence from the pTiA6NC plasmid. To date, there are two other reports in which the functional importance of *A. tumefaciens* Vir proteins encoded by multigenic operons was tested by constructing deletions in the pTi plasmid. McBride and Knauf (44) deleted both open reading frames of the *virE* operon from pTiA6NC and then expressed one or both genes on multicopy plasmids to establish the requirement for the product of the upstream open reading frame, *virE1*, for virulence. Similarly, Vogel and Das (69) deleted the *virD* operon from pTiA6NC and, by combinatorial expression of the *virD* genes in *trans*, established that the *virD3* gene product is not required for tumor formation.

Alternative approaches have been devised to evaluate the importance of the *virB* gene products for tumorigenicity. In a recent study, *virB9*, *virB10*, and *virB11* were shown to encode essential virulence functions by their combinatorial expression in *A. tumefaciens* mutants sustaining Tn3HoHo1 insertions in the corresponding resident genes on pTiA6NC (76). For genes located near the proximal end of the operon, several groups have begun to test antibiotic resistance "cassettes" or transposons with outward-reading promoters for transcribing *virB* genes residing downstream of the insertion site (23, 56). Our desire to retain the transcriptional fidelity of the *virB* operon from its native promoter, without addition of antibiotic resistance genes or vector-borne sequences that could complicate subsequent genetic studies, prompted us to test the combined in vitro and in vivo mutagenesis strategy described in this report for constructing a nonpolar *virB4* null mutation.

Restriction endonuclease cleavage and religation, to remove a ~670-bp *Bgl*II-*Bam*HI fragment, and site-directed mutagenesis, to remove the remaining ~1.7-kb of the *virB4* coding sequence, resulted in positioning of the 5' end of *virB5* precisely at the former *virB4* translational start site. Next, the Δ*virB4* mutation was exchanged for the wild-type allele on pTiA6NC by two sequential recombination events (Fig. 1). A suicide vector carrying Δ*virB4* was recombined onto pTiA6NC by a single crossover event, and then the *sacB* lethality gene, isolated from *B. subtilis* by Gay et al. (24), was used to select cells in which vector sequences and the wild-type *virB4* allele had been excised by a second crossover event. Among six Kan^s Suc^r strains analyzed, A348ΔB4.4 and A348ΔB4.5 carried the Δ*virB4* mutation, as judged by Southern blot hybridization analysis. Two lines of evidence established that the null mutation was nonpolar on expression of downstream *virB* genes. First, A348ΔB4.4 and A348ΔB4.5 expressed *virB5*, *virB10*, and *virB11* after acetosyringone induction, as determined by immunoblot analysis. Second, expression of the *virB4* gene under control of the *lac* promoter restored virulence to the deletion mutants.

The utility of the *sacB* lethality gene as a counterselection for generating unmarked mutations has been demonstrated for a variety of gram-negative bacteria (7, 39, 40, 45, 54). The novel contribution of the present study is the demonstration that this method, combined with site-directed mutagenesis in vitro, can be used to precisely delete any gene within a multigenic operon without affecting expression of the remaining genes. Our ability to recover a ~1.7-kb deletion of *virB4* sequence among 12 plasmids analyzed demonstrates that large gene deletions can be obtained at reasonable frequencies with this "loop-out" mutagenesis technique. The deletions can then be introduced into the bacterial genome quite efficiently by using the *sacB* counterselection system, as demonstrated in the present report and in previous studies (7, 39, 40, 45, 54). The overall design of the mutagenesis strategy may need to be modified when characterizing multigenic operons encoding overlapping genes or operons carrying internal regulatory sequences or internal secondary promoters.

The avirulence of A348ΔB4.4 and A348ΔB4.5, together with the ability of the wild-type gene to complement the null mutations, demonstrated that *virB4* is an essential virulence gene. Although it is not yet possible to assign a precise biological function to VirB4 or to the other essential *virB* gene products, VirB9, VirB10, and VirB11, studies by Stachel et al. (61, 62) suggested that the products of the *virB* operon are not required for *vir* gene activation or T-complex synthesis. We have confirmed this specifically for VirB4 by demonstrating that the phenolic inducer acetosyringone activates expression of the *vir* genes in A348ΔB4.4 cells. Furthermore, A348ΔB4.4 cells synthesize wild-type levels of the putative single-stranded T-DNA intermediate, as determined by probing nondenaturing Southern blots with T-DNA-specific probes (11a). These findings, the localization of VirB4 to the cytoplasmic membrane (Fig. 9), and the diminished virulence of a merodiploid expressing mutant and wild-type *virB4* alleles (Fig. 8) together support our hypothesis that VirB4 functions as a component of a multimeric T-complex transport system. At present, however, we cannot exclude the possibility that VirB4 acts at an undefined stage in T-complex transport.

The availability of a nonpolar *virB4* null mutant and the cloned wild-type gene enabled us to examine whether VirB4 contributes an enzymological function to *A. tumefaciens* virulence. Results from several laboratories suggest that the invariant lysine within the glycine-rich, type A motif, as defined by Walker et al. (70), interacts with ATP or GTP and contributes to the NTP-dependent functions of these proteins (4, 20, 27, 65). For example, a Lys-to-Glu substitution at the corresponding position of the *E. coli* FhuC protein abolished ATP hydrolysis as well as the ability of the associated transport system to import iron(III) hydroxamates (4). To evaluate the functional significance of the NTP-binding domain of VirB4, we constructed a similar Lys-439 to Glu substitution. In addition, because previous studies of related ATPases have shown that single amino acid substitutions within this glycine-rich motif may diminish and not abolish NTP-binding and/or hydrolysis (27, 65), we deleted Gly-438, Lys-439, and Thr-440 with the aim of constructing a mutant protein with absolutely no residual NTP-binding or hydrolysis activity. The two mutant proteins were indistinguishable from the wild-type protein in mobility in one-dimensional SDS-polyacrylamide gels, abundance when expressed from the *lac* promoter in *A. tumefaciens* (Fig.

5B), and localization to the cytoplasmic membrane (Fig. 9). However, their synthesis failed to restore virulence to strains A348ΔB4.4 and A348ΔB4.5, demonstrating that an intact NTP-binding domain is critical for VirB4 to contribute to *A. tumefaciens* virulence. Current biochemical studies in our laboratory are aimed at testing our prediction that the mutant proteins are altered in their ability to bind or hydrolyze NTPs.

The diminished virulence of merodiploids coexpressing mutant and wild-type *virB4* alleles provides genetic evidence that the mutant proteins may interfere with wild-type VirB4 function. We envisage several possible molecular mechanisms to account for this interference. The mutant proteins may compete with the wild-type protein during T-complex transporter assembly, resulting in the formation of functional but inefficient transporters or a reduced number of functionally wild-type transporters. Alternatively, VirB4 may function as a homomultimer, in which case interactions between wild-type and mutant monomers may diminish or abolish biochemical function. A third possibility is that VirB4 contributes to virulence as a monomer that interacts directly with the protein or DNA components of the T-complex substrate. In this case, the mutant proteins may diminish the virulence of merodiploids indirectly by titrating available substrate.

If VirB4 is an integral component of the T-complex transporter, its deletion or mutation may affect protein-protein interactions among the remaining constituents. An initial comparative study of fractionated A348 and A348ΔB4.4 cells has failed to reveal any differences in the subcellular partitioning of VirB5, VirB9, VirB10, VirB11, VirC1, VirC2, VirD2, VirD4, VirE1, and VirE2 (11a). More-refined genetic studies, such as the isolation of dominant negative mutations that completely suppress the virulence of merodiploids coexpressing the mutant and wild-type alleles, would provide additional, strong genetic evidence for VirB4 multimerization. Further definition of the T-complex transporter composition and structure will require additional genetic approaches, such as intra- and intergenic allelic suppression, as well as biochemical approaches, such as a chemical crosslinking technique recently used by Ward et al. (77) to demonstrate the oligomerization of VirB10.

Similarities between *A. tumefaciens* transformation of plant cells and interbacterial conjugation (43, 63) suggest that studies of other DNA transport systems may offer insights into VirB4 structure and function. Although the IncP and IncW interbacterial plasmid transfer systems contain Tra functions analogous to at least some of the VirB functions (43), little genetic or biochemical information exists about these proteins. In contrast, the conjugal transfer system of the *E. coli* F plasmid, the bacterial paradigm for DNA translocation, requires the activities of two well-characterized membrane-localized ATPases, TraI, a helicase, and TraD (33, 48). While TraD has been shown to exhibit limited sequence similarity with VirD4 (78), it also has several striking biochemical and genetic properties in common with VirB4. The ability of mutants to complete all other conjugation stages suggests that TraD and VirB4 function late in the transfer process (33, 61, 62; this study). Furthermore, although each protein lacks a signal sequence and extensive hydrophobic regions reminiscent of membrane or secreted proteins, each associates exclusively with the inner membrane, as determined by immunoblot analysis of subcellular fractions (Fig. 8) (48). TraD and VirB4 are present in abundance in membrane fractions, suggestive of a structural role in DNA transport (21, 48, 83). Indeed, genetic studies have demonstrated that TraD is not plasmid specific, leading to speculation that this protein forms a nonspecific pore for DNA export (33). A similar property for VirB4 would account for the ability of *A. tumefaciens* to export not only T-complexes but also RSF1010-based plasmids to plant cells.

One or both transport systems may also be capable of exporting protein only. For example, the ability of the T-complex transporter to export VirE2 protein either to the extracellular milieu or between bacteria might account for the ability of a T-DNA deletion strain, LBA4404, to restore virulence to a *virE2* mutant in mixed infections (14, 46, 47). VirB4 is required for this so-called phenomenon of extracellular complementation, as demonstrated by the inability of A348ΔB4.4 or A348ΔB4.5 to incite tumors when mixed with LBA4404 (11a). TraD also has been proposed to energize F plasmid transport, based on the ability of the purified protein to hydrolyze ATP (48). Our results showing that an intact NTP-binding domain is critical for VirB4 to contribute to virulence suggest that VirB4 supplies an analogous enzymatic function for T-complex transport. Finally, purified TraD (48) and VirB4 in total-cell extracts (11a) bind to DNA-cellulose columns. One or both proteins may bind DNA directly or, as proposed previously for VirB11 (12), indirectly via associations with protein components of the transfer-competent DNA-protein particle. Given their similarities, TraD and VirB4 may supply analogous functions for their respective transport systems, both as structural components, i.e., channel proteins, and as activators of the transport process, i.e., through ATP binding and hydrolysis or phosphorylation. TraD fails to complement the nonpolar *virB4* null mutants (11a), which may not be surprising given the general lack of sequence similarities between *A. tumefaciens virB* genes and *E. coli* F *tra* genes (73). However, other bacterial conjugation systems, including those encoded by the broad-host-range IncP, IncQ, and IncW plasmids, may possess a functional equivalent of VirB4 with the ability to complement the *virB4* null mutants. Evidence for genetic complementation in a heterologous DNA transport system would greatly facilitate future structure-function studies of VirB4 and related Tra proteins.

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