JOURNAL OF BACTERIOLOGY, June 2001, p. 3636–3641 Vol. 183, No. 12
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.12.3636–3641.2001

Functional Analysis of the *Agrobacterium tumefaciens* T-DNA Transport Pore Protein VirB8

RENU B. KUMAR[1] AND ANATH DAS[1,2]*

Department of Biochemistry, Molecular Biology, and Biophysics[1] and Plant Molecular Genetics Institute,[2] University of Minnesota, St. Paul, Minnesota 55108

Received 15 December 2000/Accepted 15 March 2001

The VirB8 protein of *Agrobacterium tumefaciens* is essential for DNA transfer to plants. VirB8, a 237-residue polypeptide, is an integral membrane protein with a short N-terminal cytoplasmic domain. It interacts with two transport pore proteins, VirB9 and VirB10, in addition to itself. To study the role of these interactions in DNA transfer and to identify essential amino acids of VirB8, we introduced random mutations in *virB8* by the mutagenic PCR method. The putative mutants were tested for VirB8 function by the ability to complement a *virB8* deletion mutant in tumor formation assays. After multiple rounds of screening 13 mutants that failed to complement the *virB8* deletion mutation were identified. Analysis of the mutant strains by DNA sequence analysis, Western blot assays, and reconstruction of new point mutations led to the identification of five amino acid residues that are essential for VirB8 function. The substitution of glycine-78 to serine, serine-87 to leucine, alanine-100 to valine, arginine-107 to proline or alanine, and threonine-192 to methionine led to the loss of VirB8 activity. When introduced into the wild-type strain, $virB8_{S87L}$ partially suppressed the tumor forming ability of the wild-type protein. Analysis of protein-protein interaction by the yeast two-hybrid assay indicated that $VirB8_{R107P}$ is defective in interactions with both VirB9 and VirB10. A second mutant $VirB8_{S87L}$ is defective in interaction with VirB9.

DNA transfer from *Agrobacterium tumefaciens* to plants results in crown gall tumor disease. Tumor formation requires the presence of the tumor-inducing (Ti)-plasmid in the infecting bacterium. The transferred (T)-DNA is stably integrated into the plant nuclear genome and direct constitutive expression of the phytohormone biosynthetic genes in the transformed plant. The altered hormone level leads to the loss of cell division control, yielding a tumorous phenotype (8, 30). The virulence (*vir*) region of the Ti-plasmid is essential for DNA transfer. The *vir* region, a 35-kb DNA segment, is composed of five major loci, *virA, virB, virD, virE*, and *virG* (23). Proteins encoded in the *vir* region process the Ti-plasmid to produce a single-stranded T-strand DNA comprised of the bottom strand of the T-DNA (1, 24). The T-strand DNA is postulated to cross the bacterial membrane through a transport pore composed primarily of the proteins encoded in the *virB* operon (6, 15, 28). The *virB* operon encodes 11 proteins, VirB1 to VirB11 (15, 28). All except VirB1 are essential for DNA transfer (5). VirB1 is required for a high efficiency of DNA transfer.

Molecular characterization of the *virB* operon led to the hypothesis that the VirB proteins function in the biogenesis of a transport pore through which the T-strand DNA moves from the bacterium to the plant cell (15, 28). The subsequent discovery of the presence of homologs of the VirB proteins in other bacterial systems supports this hypothesis (7). Proteins essential for the conjugal transfer of *Escherichia coli* plasmids, the secretion of the *Bordetella pertussis* toxin protein, and the pathogenicity of *Helicobacter pylori* exhibit significant homology to the VirB proteins. Homologs of the VirB proteins in *Brucella suis, Rickettsia prowazekii, Legionella pneumophila*, and *Bartonella henselae* have also been identified. The conservation of these proteins and their role in various biological processes suggest that the VirB family of proteins function in the export of macromolecules to both prokaryotic and eukaryotic hosts.

The structure of the transport pore is not known. We proposed that VirB6, VirB7, VirB8, VirB9, and VirB10 are the primary constituents of the T-DNA transport pore (9). VirB7, a lipoprotein, is anchored to the outer membrane (13), while VirB8 and VirB10 are inner membrane proteins (9, 25, 29). VirB7 forms a disulfide-linked complex with VirB9 (2, 3, 22). We recently demonstrated that VirB8, VirB9, and VirB10 interact with one another (10). Chemical cross-linking and immunoprecipitation studies indicated that VirB7, VirB9, and VirB10 participate in the formation of oligomeric complexes (2–4, 22, 29). These studies support the proposed role of the VirB7 to VirB10 proteins in transporter assembly.

In a recent study we reported that VirB8, VirB9, and VirB10 are present in a protein complex (16). The subcellular location of two of the proteins, VirB9 and VirB10, changed dramatically in the presence of the other VirB proteins. Immunofluorescence and immunoelectron microscopy studies showed that the two proteins localized to a few sites on the membrane in the presence of the other VirB proteins. In immunoelectron microscopy, gold particles representing the two proteins were found in clusters in the presence of the VirB proteins. In contrast, gold particles were found mostly as a single particle all along the cell periphery in the absence of the other VirB proteins. The reorganization of cellular location of VirB9 and VirB10 was dependent on VirB8 since a deletion in *virB8* abolished the reorganization. The important role of VirB8 in the assembly of the transporter complex led us to study this

* Corresponding author. Mailing address: Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, 1479 Gortner Ave., St. Paul, MN 55108. Phone: (612) 624-3239. Fax: (612) 625-5780. E-mail: anath@cbs.umn.edu.

protein in detail. In the present study we report the identification of amino acids essential for VirB8 function and the role of interactions of VirB8 with the other VirB proteins in T-DNA transfer to plants.

MATERIALS AND METHODS

Strains and plasmids. *A. tumefaciens* A348 contains the octopine Ti-plasmid pTiA6. PC1008 is a derivative of *A. tumefaciens* A348 with a nonpolar in-frame deletion in *virB8* (5). *A. tumefaciens* A136 lacks a Ti-plasmid. The *E. coli* strains used in this study were DH5αF′ and CJ236 (relevant genotype: *dut ung*).

Plasmid pAD1420 contains a chimeric *virD*p-*virB7-virB8* gene. It was constructed by cloning the *Alw*NI-*Sph*I fragment (nucleotides 6131 to 7197) into plasmid pAD1416 digested with *Sal*I and *Sph*I. The *Alw*NI and *Sal*I ends were blunt ended by treatment with T4 DNA polymerase prior to cloning. Plasmid pAD1416 is a pUC118 derivative containing the *virD* promoter (−384 to +7 [12]) in the polylinker region. Plasmid pAD1423 contains the *virDp-virB7-virB8* gene in pUC119 (26) and was obtained by cloning the gene as a 1.5-kb *Sst*I-*Hin*dIII fragment from pAD1420. Plasmid pAD1433 was constructed by cloning plasmid pAD1423 as a *Hin*dIII fragment into the *Hin*dIII site of the wide-host-range plasmid pTJS75 (21).

Mutagenesis of *virB8*. Random mutations in *virB8* were introduced by PCR mutagenesis (27). The *virB8* coding region of plasmid pAD1423C15S (2) was amplified with primers B7C15S (dCGCTTTGAGCGGATCCCAGACAAATG AC) and the m13 reverse sequencing primer using *Taq* DNA polymerase. The C15S mutation in VirB7 is not present in the *virB8* plasmids used in this study and was used here for convenience. The 0.94-kb amplified fragment was purified with QIAquick PCR purification kit (Qiagen, Inc.), digested with *Bgl*II and *Sph*I, and cloned into similarly digested pAD1433. A mutant library was constructed by isolating plasmid DNA from a pool of *E. coli* transformants. The *Bgl*II-*Sph*I fragment (nucleotides 6353 to 7197) encodes *virB8* in its entirety and the C-terminal 14 residues of *virB7*.

Targeted mutagenesis. Mutations at a specific site in *virB8* were introduced by deoxyoligonucleotide-directed site-specific mutagenesis using uracil containing single-stranded pAD1420 DNA as a template for second-strand synthesis (17). The mutation and the mutagenic primers (complementary strand, with mutation underlined) were as follows: valine-52 to isoleucine, dCTTGAGCAAT<u>A</u>TTCC CCAAAA; serine-87 to leucine, dGGCAATCGC<u>A</u>AGATAGACAC; arginine-107 to alanine, dCTCTCACGCAGG<u>GCT</u>ACGTACTCCCAC; valine-189 to methionine, dGTACTCACCA<u>T</u>AGGCATTTTG; and threonine-192 to methionine, dGCGGTCCAC<u>A</u>TACTCACCAC. The mutations were identified by DNA sequence analysis (20). After linearization, the mutant plasmids pAD1420-V52I, -S87L, -R107A, -V189M, and -T192M were cloned into the *Hin*dIII site of the wide-host-range plasmid pTJS75 to construct plasmids pAD1630, -1631, -1688, -1632, and -1633, respectively.

Interactions of the VirB proteins. Interactions of VirB8 and its mutants with the VirB proteins were monitored by the two-hybrid assay in yeast as described earlier (10, 11). Plasmids that express LexA-VirB and activator-VirB fusions were introduced into the yeast strain AD842 by transformation, and the transformants were tested for the expression of the reporter *lacZ* gene on solid medium containing the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). A blue colony color indicates a *lacZ*$^+$ phenotype and a positive interaction.

The LexA-VirB8 and the activator-VirB8, -VirB9, and -VirB10 fusion plasmids pAD1529, -1516, -1517, and -1493, respectively, have been described previously (10). For the construction of plasmids expressing the LexA-VirB8 mutant fusions, sequences encoding residues 60 to 237 (the periplasmic domain) were amplified by PCR and cloned as an *Eco*RI-*Xho*I fragment into plasmid pJK202. All fusions were confirmed by DNA sequence analysis (20).

Other methods. Plasmid DNA was introduced into *A. tumefaciens* by electroporation (18). The virulence of *A. tumefaciens* was monitored by tumor formation assays on *Kalanchöe daigremontiana* leaves (11). The DNA sequence of the *virB8* mutants and the gene fusions was determined by the dideoxy chain termination method using double-stranded DNA as a template and Sequenase (20; U.S. Biochemical Corp.). The level of VirB8 and its mutants was monitored by Western blot assays with purified anti-VirB8 antibodies (16).

RESULTS

Random mutagenesis of *virB8*. Random mutations in *virB8* were introduced by error-prone PCR amplification with *Taq* DNA polymerase (27). The mutant *virB8* genes were cloned into the unmutagenized wide-host-range vector pAD1433 as a *Bgl*II-*Sph*I fragment. Plasmid pAD1433 is a wide-host-range derivative of pAD1423 (2) that contains a chimeric *virD*$_P$-*virB7-virB8* gene. The *virB7* sequences were included in the clone because the expression of *virB8* requires upstream sequences (5). A *virB8* mutant library was constructed by isolating plasmid DNA from approximately 2,600 independent *E. coli* transformants. The library DNA was introduced into the *A. tumefaciens* PC1008, a strain with a nonpolar deletion in *virB8*, by electroporation (18). Four hundred independent transformants were purified and tested for *virB8* function by tumor formation assays on *Kalanchöe* leaves (11). A mutation that abolishes *virB8* function will not form tumors at the site of infection. After several rounds of screening, we identified 13 mutations that failed to form tumors and were avirulent (Fig. 1 and Table 1).

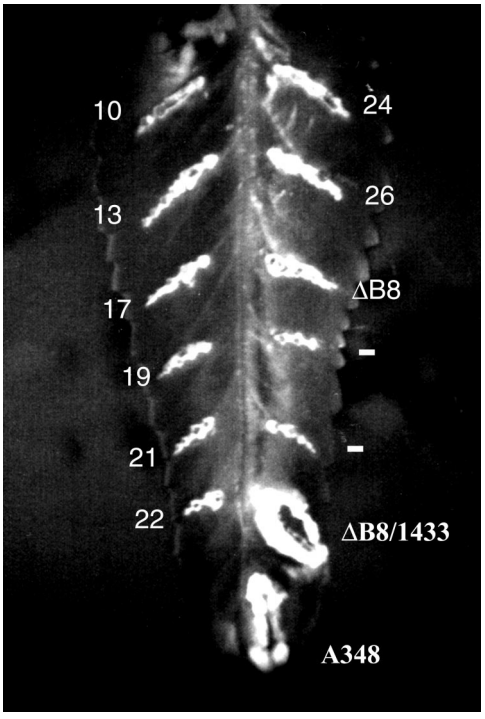

FIG. 1. Phenotype of the *virB8* mutants. The *virB8* mutants were tested for the ability to complement a deletion in *virB8*. The mutants in *A. tumefaciens* PC1008 (ΔB8) were used to infect *K. daigremontiana* leaves and scored for tumor formation 3 weeks after infection. A subset of the mutants listed on Table 1 is shown. The numbers indicate the mutant number. Plasmid pAD1433 or its derivative harbors *virB8* or the mutant. A348, wild-type strain; −, uninfected wound site.

Identification of the *virB8* mutants. DNA sequences of the entire mutagenized region of the 13 mutants were determined to identify the site(s) of mutation. Ten mutants had a single-base-pair change that led to a change in the amino acid sequence (Table 1). The remaining three mutants (mutants 19, 21, and 26) had changes in two positions that led to alterations in two amino acids. The collection of mutants contained five unique point mutations that led to the change of arginine-34 to a stop codon (R34X), glycine-78 to serine (G78S), alanine-100 to valine (A100V), arginine-107 to proline (R107P), and tryptophan-193 to a stop codon (W193X). Two mutants, G78S and

TABLE 1. Identification of the avirulent *virB8* mutants

Mutant(s)	Nucleotide change at position[a]	Amino acid change at residue
10, 11, 12, 23, and 25	G 6616 → A	Glycine-78 → serine
13	G 6962 → A	Tryptophan-193 → stop
17 and 18	C 6683 → T	Alanine-100 → valine
19	C 6644 → T and G 6949 → A	Serine-87 → leucine and valine-189 → methionine
21	G 6613 → A and C 6703 → A	Aspartic acid-77 → asparagine and arginine-107 → serine
22	C 6484 → T	Arginine-34 → stop
24	G 6704 → C	Arginine-107 → proline
26	G 6538 → A and C 6959 → T	Valine-52 → isoleucine and threonine-192 → methionine

[a] Residue number is assigned according to Ward et al. (28).

A100V, were isolated more than once. One double mutant, mutant 21, contains a mutation in arginine-107, a residue identified as an essential one from the analysis of mutant 24. Consequently, this mutant was not characterized further. All *virB8* genes were found to contain a C→G change in position 6425 of Ward et al. (28). The change alters amino acid 14 from threonine to serine.

To study whether a mutation destabilized the VirB8 protein, we performed Western blot assays (Fig. 2). All mutants, except mutants 13 and 24, accumulated VirB8 at a level comparable to that produced by the wild-type gene, indicating that in most cases a mutation did not affect protein stability (lanes 3 to 9 and 13). Mutant 24 accumulated a low level of the mutant protein; however, the level is similar to that in the wild-type strain A348 (compare lanes 2 and 8). This result suggests that the loss of function in mutant 24 is due to the effect of the change in amino acid sequence on protein function and not on protein stability. A double mutant, mutant 21, that contains a different substitution of arginine-107 expresses a stable protein, suggesting that all substitutions of residue 107 may not have a strong negative effect on protein stability. To confirm the requirement of arginine-107 in VirB8 function, we introduced an arginine-to-alanine change at position 107 by site-specific mutagenesis (17) and studied the effect of the mutation on VirB8 function and stability. The mutation abolished VirB8 function and was avirulent in a DNA transfer assay (Fig. 3). The mutation had no effect on protein stability (Fig. 2, lane 12).

Identification of the mutation(s) conferring phenotype to the double mutants. The avirulent phenotype of the other two double mutants, mutant 19 and mutant 26, can be due to a single-amino-acid change or both mutations. To identify the mutation(s) responsible for the phenotype, we introduced the individual mutations into *virB8* by site-directed mutagenesis. Each *virB8* mutant was introduced into the *virB8* deletion mutant PC1008, and the effect of the mutation on *virB8* function was tested by complementation assays (Fig. 4). Two mutations, the alteration of serine-87 to leucine (S87L) and threonine-192 to methionine (T192M), abolished *virB8* function, indicating that a single-amino-acid change in both cases is sufficient to confer an avirulent phenotype. The other two mutations, valine-52 to isoleucine and valine-189 to methionine, had no effect on *virB8* function. The loss of *virB8* function in $virB8_{S87L}$ and $virB8_{T192M}$ is not due to protein stability because both mutant proteins were stable (Fig. 2, lanes 10 and 11). The phenotype of each of the double mutants is therefore due to a single amino acid change.

***virB8S87L* is a semidominant mutant.** To study whether the presence of a mutant protein affects the function of the wild-type protein, we introduced the five mutants into *A. tumefaciens* A348 and studied the effect of the mutation on VirB8 function by virulence assays. One mutant, $virB8_{S87L}$, had a semidominant phenotype. Three weeks after infection, *A tumefaciens* A348 $virB8_{S87L}$ consistently exhibited an avirulence phenotype (Fig. 5). After a prolonged infection (3 to 4 months), the mutant showed an attenuated phenotype, indicating that the mutant does not have a fully dominant phenotype. Three mutants, $virB8_{G78S}$, $virB8_{A100V}$, and $virB8_{R107P}$, had no effect on the virulence of the wild-type strain. The phenotype of the $virB8_{T192M}$ mutant was variable from experiment to experiment. *A tumefaciens* A348 $virB8_{T192M}$ exhibited an attenuated phenotype, suggesting that this mutant may have a semidominant phenotype.

Mutations in *virB8* affect its interaction with other VirB proteins. VirB8 interacts with VirB9, VirB10, and itself (10). All or a subset of these interactions are likely to be essential for

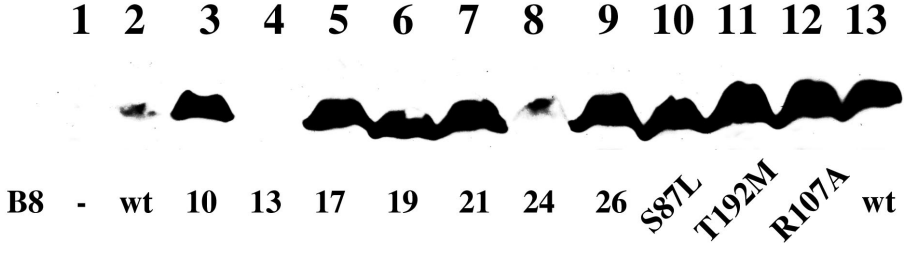

FIG. 2. Effect of the *virB8* mutations on protein stability. The level of VirB8 and its mutants were monitored by Western blot assays using purified VirB8 antibodies (16). Lanes 1, 2, and 13, uninduced A348, induced A348, and PC1008/pAD1433, respectively; lanes 3 to 12, *virB8* mutants 10, 13, 17, 19, 21, 24, 26, $virB8_{S87L}$, $virB8_{T192M}$, and $virB8_{R107A}$, respectively.

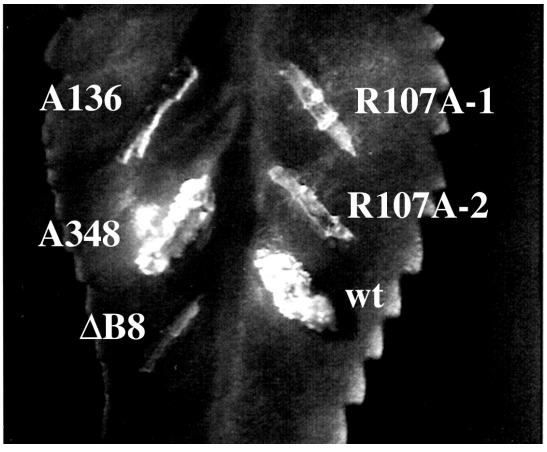

FIG. 3. Role of VirB8 arginine-107 in DNA transfer to plants. An arginine at position 107 of VirB8 was changed to alanine by site-specific mutagenesis (17). The mutants were tested by complementation assays. wt, wild-type *virB8*; R107A, $virB8_{R107A}$.

the assembly and function of the T-DNA transporter. A corollary to the hypothesis is that a mutant defective in an essential interaction will have an avirulent phenotype. We therefore tested whether an avirulent mutation identified in our study is

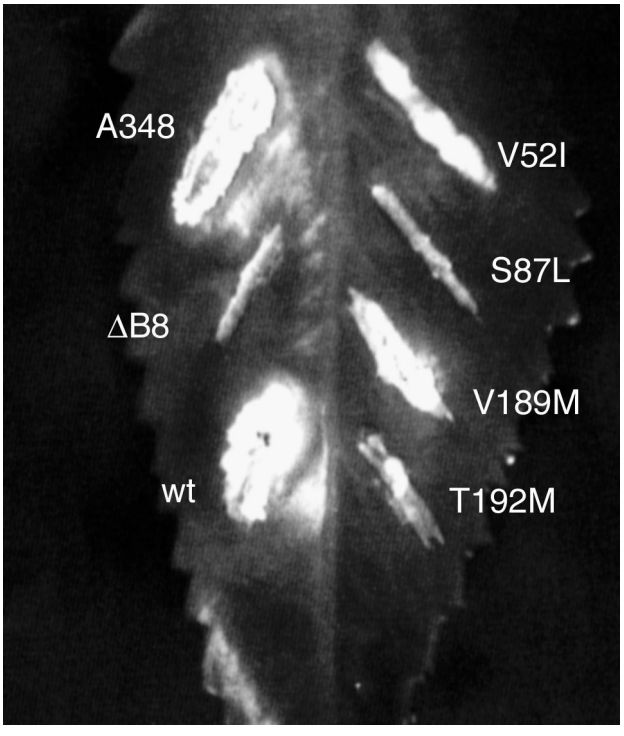

FIG. 4. Identification of amino acids responsible for the avirulent phenotype of the double mutants. Mutations that led to single-amino-acid substitutions in *virB8* were introduced by site-specific mutagenesis, and the mutants were introduced into *A. tumefaciens* PC1008. The resultant strains were used to infect *K. daigremontiana* leaves. The strains used for infection were *A. tumefaciens* A348 (A348), PC1008 (ΔB8), and PC1008 harboring a plasmid that expresses wild-type *virB8* (wt), $virB8_{V52I}$ (V52I), $virB8_{S87L}$ (S87L), $virB8_{V189M}$ (V189M), or $virB_{T192M}$ (T192M).

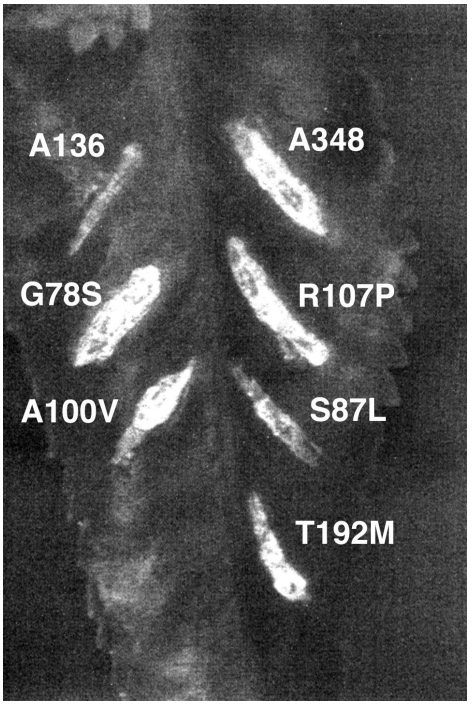

FIG. 5. Dominant-recessive phenotype of the *virB8* mutants. A plasmid expressing *virB8* or its mutant was introduced into *A. tumefaciens* A348, and the resultant strains were used to infect *K. daigremontiana* leaves.

defective in interactions of VirB8. The two-hybrid assay in yeast was used to study the interactions of the VirB8 mutants (10, 11). The activator-VirB8, -VirB9, or -VirB10 fusion was introduced into a yeast strain containing the LexA-VirB8 mutant fusion. Interaction between the two fusion proteins was determined by monitoring expression of the reporter *lacZ* gene. A positive interaction results in a blue colony color phenotype of the yeast strain when grown on solid medium containing the chromogenic substrate X-Gal. All mutants were proficient in interaction with VirB8 (Fig. 6, top row). These results indicate that the mutant fusion proteins are expressed in yeast, stable and properly targeted to the yeast cell nucleus. Two of the mutants were found to be defective in the VirB8-VirBx interactions. One, $VirB8_{R107P}$, failed to interact with

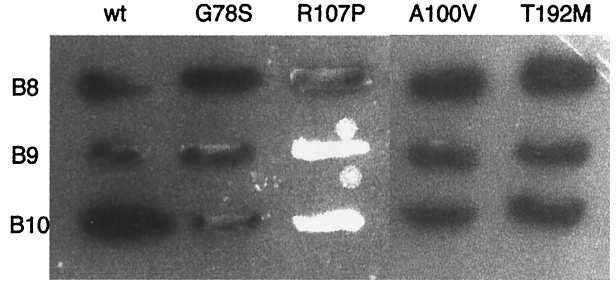

FIG. 6. Interaction of the VirB8 mutants with VirB8, VirB9, and VirB10. The interaction of VirB8 (wt) and its mutant with VirB8 (B8), VirB9 (B9), and VirB10 (B10) was monitored by the two-hybrid assay in yeast as described previously (10). A blue colony color indicates a positive interaction.

A. tum.	71	YLWI-RAD	**G**	TV	D-SEVSISRLP	ATQEEAVVN**A**	S	L	WE	YV**R**LRE	SYD	ADTAQYA
R. etli	81	....-.P.	.	..	.-...V.....	....K.....	.	.	..	...M..	..T	....
B. pert.	114	LIEVEKSS	.	AA	S-VVTQFEPRD	F.PDTLMNQY	W	.	TR	..AA..	R..	WH.I.HD
B. henselae	42	VIRVDNST	.	II	E-TVSALKET.	NDYD..ITRY	F	A	SK	...A..	GFQ	LSEAEHN
B. suis	75	LVRVNAQT	.	AP	.-ILT.LDEKS	VSYDTVMDKY	W	.	SQ	..IA..	T..	WY.L.KD
L. pneu.	62	LVHH-YDN	.	VT	T-V.PMENK.T	PINR-.QIES	D	I	AR	.IQY..	...	.SSYRAQ
pKM101	71	H.LT-LNE	A	.H	EVQQ.KLT.DQ	TSYGDEIDKF	W	.	TQ	..IH..	...	FYSV.VD
R. prow.	84	..IK-DDS	E	KQ	ATITNTKHSTL	.NPYI..A.I	M	.	QN	..KQ..	K.N	Y..LKEQ
H.pylori	84	FVDFLNQD	K	HY	A--IIQRADKS	ISSNEALARS	L	I	GA	..LN..	.IN	RIDDKSR

FIG. 7. Conservation of glycine-78 and sequences around arginine-107 in the VirB8 homologs. The amino acid sequence of a segment of *A. tumefaciens* VirB8 and its homologs is shown. Residues identical to the *A. tumefaciens* VirB8 sequence are shown as dots. Sequences that exhibited a high degree of conservation are boxed. Glycine-78, alanine-100, and arginine-107 are shown in boldface. The numbers on the left indicate the position of the first amino acid residue shown in the figure. The gaps were introduced to achieve maximum homology.

both VirB9 and VirB10, indicating that arginine-107 is essential for interactions of VirB8. A second mutant, $VirB8_{S87L}$, is defective in interaction with VirB9 but not with VirB8 and VirB10 (10). Mutations at the other three sites, i.e., amino acids 78, 100, and 192, did not affect the ability of VirB8 to interact with VirB9 and VirB10.

DISCUSSION

VirB8 is a bitopic membrane protein with a short cytoplasmic segment at its N terminus (9, 25). It is an essential virulence protein and is postulated to be a primary constituent of the T-DNA transporter (5, 16). The C-terminal periplasmic segment encodes domains essential for interaction with VirB9, VirB10, and itself (10). Random mutagenesis of *virB8* led to the identification of five essential amino acids: glycine-78, serine-87, alanine-100, arginine-107, and threonine-192. All of the residues mapped to the large periplasmic domain. Two sets of mutations mapped within 10 or fewer residues of each other, and the four mutations lie within a 30-residue segment, i.e., amino acids 78 to 107. The mutations, however, probably affect different functions. Two mutations abolished interaction of VirB8 with another VirB protein in yeast two-hybrid assays. The substitution of arginine at position 107 with proline led to the loss of interactions with both VirB9 and VirB10. A mutation at residue 100, however, did not affect its interaction with VirB9, VirB10, or itself. Similarly, a serine-to-leucine change at residue 87 led to the loss of interaction with VirB9, but a mutation at residue 78 had no effect on the interaction. Therefore, the four mutations probably affect different functions of VirB8. Since two avirulent mutations are defective in the VirB8-VirB9 interaction, these results indicate that the VirB8-VirB9 interaction is essential for DNA transfer. The role of the VirB8-VirB10 interaction cannot be predicted from this study because of the pleiotropic nature of the $virB8_{R107P}$ mutation.

The loss of function of $VirB8_{G78S}$, $VirB8_{A100V}$, and $VirB8_{T192M}$ indicates that VirB8 has other functions in addition to its interaction with the three VirB proteins. These functions may include, among others, interaction with other transport pore protein(s) essential for the assembly of the transporter and/or interaction with a transported substrate. One *virB8* mutant, $virB8_{S87L}$, exhibited a semidominant phenotype. Semidominance is probably the result of partial activity of a VirB8-VirB8 mutant oligomer in DNA transfer. This property of the mutant protein suggests that VirB8 forms an oligomer, and oligomerization of VirB8 is required for its function. Studies using the two-hybrid assay and that on the subcellular localization of VirB8 presented here and previous studies (Fig. 6; see also references 10 and 16) support the hypothesis that VirB8 forms an oligomer.

VirB8 is found conserved in the family of type IV transport system proteins. Homologs of VirB8 are found in *B. suis, B. henselae, B. pertussis, E. coli* conjugal plasmids, *H. pylori, L. pneumophila, Rhizobium etli*, and *R. prowazekii*. One amino acid, the alteration of which led to an avirulent phenotype, glycine-78, is conserved in all of these proteins; however, the sequences around it show very little conservation (Fig. 7). This residue probably has an important role in the tertiary structure of the protein. Two mutations, R107P and T192M, mapped to areas that are found conserved in all homologs. One, arginine-107, falls within a nine-residue region that contains four invariant residues. This region (residues 105 to 113) has the consensus sequence YVnnREт/sYᴅ/ɴ (n, any residue; invariant residues are in large capitals, and highly conserved residues are in small capitals). The high degree of conservation of these sequences and the phenotype of the $virB8_{R107P}$ mutant suggest that this region in all homologs functions in interaction with other components of the transporter complex. A region with a significantly high homology among the VirB8 proteins is the C-terminal end. The C-terminal 18 residues of all but one homolog share a minimum of 50% identity.

We postulated that VirB8 identifies the site of transport pore assembly (16). This protein has the unique property of localizing to a few sites on the bacterial membrane. The interaction of VirB8 will target the other proteins to this site for the assembly of the transport pore. Two mutants, $virB8_{S87L}$ and $virB8_{R107P}$, are expected to be defective in the assembly of the transporter because of their failure to interact with at least one pore constituent. The effect of the other mutations on transport pore cannot be predicted at this time. A mutant can form a nonfunctional transport pore, a transport pore that has no defect in assembly but cannot transport a substrate. Alternatively, a mutant can fail to assemble the transport pore. While a mutant is proficient in interaction with several constituents, it

can fail to interact with another unidentified component of the pore or is defective in a higher-order interaction that is essential for pore assembly.

ACKNOWLEDGMENTS

We thank Peter Christie for the *Agrobacterium* sp. strain PC1008, Roger Brent for the plasmids and strains for the yeast two-hybrid assays, Paul Judd for discussions, and Alison Pirro and Xiao-Chao Ruan for excellent technical assistance.

This work was supported by grants from the University of Minnesota Agricultural Experiment Station and the University of Minnesota Graduate School.

REFERENCES

1. **Albright, L. M., M. F. Yanofsky, B. Leroux, D. Q. Ma, and E. W. Nester.** 1987. Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. J. Bacteriol. **169:**1046–1055.
2. **Anderson, L. B., A. V. Hertzel, and A. Das.** 1996. *Agrobacterium tumefaciens* VirB7 and VirB9 form a disulfide-linked protein complex. Proc. Natl. Acad. Sci. USA **93:**8889–8894.
3. **Baron, C., Y. R. Thorstenson, and P. C. Zambryski.** 1997. The lipoprotein VirB7 interacts with VirB9 in the membranes of *Agrobacterium tumefaciens*. J. Bacteriol. **179:**1211–1218.
4. **Beaupre, C. E., J. Bohne, E. M. Dale, and A. N. Binns.** 1997. Interactions between VirB9 and VirB10 membrane proteins involved in movement of DNA from *Agrobacterium tumefaciens* into plant cells. J. Bacteriol. **179:**78–89.
5. **Berger, B., and P. Christie.** 1994. Genetic complementation analysis of the *Agrobacterium tumefaciens virB* operon: *virB2* through *virB11* are essential virulence genes. J. Bacteriol. **176:**3646–3660.
6. **Christie, P.** 1997. *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. J. Bacteriol. **179:**3085–3094.
7. **Covacci, A., J. L. Telford, G. Del Giudice, J. Parsonnet, and R. Rappuoli.** 1999. *Helicobacter pylori* virulence and genetic geography. Science **284:**1328–1333.
8. **Das, A.** 1998. DNA transfer from *Agrobacterium* to plant cells in crown gall tumor disease, p. 343–363. *In* B. B. Biswas and H. K. Das (ed.), Subcellular biochemistry: plant microbe interactions. Plenum Publishing Corp, London, England.
9. **Das, A., and Y.-H. Xie.** 1998. Construction of transposon Tn*3phoA*: its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins. Mol. Microbiol. **27:**405–414.
10. **Das, A., and Y.-H. Xie.** 2000. *Agrobacterium tumefaciens* T-DNA transport pore proteins VirB8, VirB9, and VirB10 interact with one another. J. Bacteriol. **182:**758–763.
11. **Das, A., L. Anderson, and Y.-H. Xie.** 1997. Delineation of the interaction domains of *Agrobacterium tumefaciens* VirB7 and VirB9 by use of the yeast two-hybrid assay. J. Bacteriol. **179:**3404–3409.
12. **Das, A., S. Stachel, P. Ebert, P. Allenza, A. Montoya, and E. Nester.** 1986. Promoters of *Agrobacterium tumefaciens* Ti-plasmid virulence genes. Nucleic Acids Res. **14:**1355–1364.
13. **Fernandez, D., T. A. Dang, G. Spudich, X.-R. Zhou, B. Berger, and P. Christie.** 1996. The *Agrobacterium tumefaciens virB7* gene product, a proposed component of the T-complex transport apparatus, is a membrane-associated lipoprotein exposed at the periplasmic surface. J. Bacteriol. **178:**3156–3167.
14. **Finley, R., and R. Brent.** 1995. Interaction trap cloning in yeast, p. 169–203. *In* B. Hames and D. Glover (ed.), DNA cloning, expression systems: a practical approach. Oxford University Press, Oxford, England.
15. **Kuldau, G. A., G. De Vos, 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.
16. **Kumar, R., Y.-H. Xie, and A. Das.** 2000. Subcellular localization of the *Agrobacterium tumefaciens* T-DNA transport pore proteins: VirB8 is essential for the assembly of the transport pore. Mol. Microbiol. **36:**608–617.
17. **Kunkel, T.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
18. **Mersereau, M., G. Pazour, and A. Das.** 1990. Efficient transformation of *Agrobacterium tumefaciens* by electroporation. Gene **90:**149–151.
19. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Sanger, F., S. Nicklen, and A. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
21. **Schmidhauser, T., and D. Helinski.** 1985. Regions of broad-host-range plasmid RK2 involved in replication and stable maintenance in nine species of gram-negative bacteria. J. Bacteriol. **164:**446–455.
22. **Spudich, G., D. Fernandez, X.-R. Zhou, and P. Christie.** 1996. Intermolecular disulfide bonds stabilize VirB7 homodimers and VirB7/VirB9 heterodimers during biogenesis of the *Agrobacterium tumefaciens* T-complex transport pore. Proc. Natl. Acad. Sci. USA **93:**7512–7517.
23. **Stachel, S., and E. Nester.** 1986. The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **5:**1445–1454.
24. **Stachel, S., B. Timmerman, and P. Zambryski.** 1986. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. Nature **322:**706–712.
25. **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.
26. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153:**3–11.
27. **Vogel, A., and A. Das.** 1994. Mutational analysis of *Agrobacterium tumefaciens* pTiA6 *vir*D1: Identification of functionally important residues. Mol. Microbiol. **12:**811–817.
28. **Ward, J. E., D. E. Akiyoshi, D. Regier, A. Datta, M. P. Gordon, and E. Nester.** 1988. Characterization of the *virB* operon from *Agrobacterium tumefaciens* Ti plasmid. J. Biol. Chem. **263:**5804–5814.
29. **Ward, J. E., 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.
30. **Zupan, J., T. Muth, O. Draper, and P. Zambryski.** 2000. The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. Plant J. **23:**11–28.