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Agrobacterium tumefaciens VirB7 and VirB9 form a disulfide-linked protein complex

(plant tumor/virulence genes/VirB proteins/VirB7–VirB9 complex/disulfide bond)

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ABSTRACT **_Agrobacterium tumefaciens_ VirB proteins are essential for gene transfer from bacteria to plants. These proteins are postulated to form a transport pore to allow transfer of the T-strand DNA intermediate. To study the function of the VirB proteins in DNA transfer, we developed an expression system in _A. tumefaciens_. Analysis of one VirB protein, VirB9, by Western blot assays showed that under nonreducing conditions VirB9, when expressed alone, migrates as a ~31-kDa band but that it migrates as a ~36-kDa band when expressed with all other VirB proteins. The 36-kDa band is converted to the 31-kDa band by the reducing agent 2-mercaptoethanol. Using strains that contain a deletion in a defined _virB_ gene and strains that express specific VirB proteins, we demonstrate that the 36-kDa band is composed of VirB9 and VirB7 that are linked to each other by a disulfide bond. Mutational studies demonstrate that cysteine residues at positions 24 of VirB7 and 262 of VirB9 participate in the formation of this complex.**

The transformation of a susceptible plant cell by the soil bacterium *Agrobacterium tumefaciens* results from the transfer and integration of a segment of the tumor-inducing (T_i) plasmid DNA into the plant nuclear genome. The virulence (*vir*) genes of the T_i plasmid play an essential role in the DNA transfer and integration processes. The portion of the T_i plasmid that is transferred to plant cells (T-DNA) is defined by a 24-bp direct repeat sequence known as the border sequence. Two proteins of the *virD* operon, VirD1 and VirD2, initiate the processing of the T-DNA by introducing a site- and strand-specific nick at the T-DNA borders, leading to the formation of a single-stranded T-strand DNA composed of the bottom strand of the T-DNA. The T-strand DNA contains a VirD2 molecule covalently attached to its 5′-end and is believed to be an intermediate in DNA transfer to plants (for reviews, see refs. 1 and 2).

Little is known about the mechanism of DNA transfer from bacteria to plants. It is postulated that DNA transfer occurs through a transport pore primarily composed of the VirB proteins. Of the 11 VirB proteins, 10 (VirB2–VirB11) are essential for DNA transfer. The other protein (VirB1) is required for a high efficiency of DNA transfer (3). The observations that the VirB proteins have no role in T-strand DNA synthesis and that most of the VirB proteins are membrane associated led to their proposed role in DNA transfer (4–10). Subsequently, DNA sequence analysis of the *trb* genes of the conjugative plasmid pRP4 and that of the *ptl* operon of the pathogenic bacteria *Bordetella pertussis* revealed that these operons encode homologs of the VirB proteins (11, 12). The requirement of the *trb* genes in conjugal transfer of plasmid DNA, that of the *ptl* genes in the secretion of the *Bordetella* toxin protein, and that of the *virB* genes in T-DNA transfer suggests the existence of a common transport pathway for macromolecule export. The requirement of the *virB* genes in tumor formation, in DNA transfer to plants in a transient DNA transfer assay, in conjugal transfer of an IncQ plasmid RSF1010 between two *Agrobacterium* strains, and most recently in DNA transfer from *Agrobacterium* to yeast provides experimental support for the transport pore model (13–17). In addition to the VirB proteins, the *vir*-mediated DNA transfer processes require VirD4, an essential virulence protein (13, 16, 18).

While the requirement for VirB proteins and VirD4 in DNA transfer is well-established, the role of each individual protein in this process is not clear. Two VirB proteins, VirB4 and VirB11, contain a nucleotide-binding motif that is essential for tumor formation (19, 20). These proteins are postulated to participate in an energy-requiring step in DNA transfer. VirB4 is also essential for the localization of VirB3 into the outer membrane (21). VirB7 stabilizes VirB9 and probably a few other VirB proteins, and VirB9, VirB10, and VirB11 are required in a stoichiometric ratio since coordinated expression of all three proteins are essential for the relief of RSF1010-mediated inhibition of T-DNA transfer (3, 22). To understand how these proteins carry out their function and to define the constituents and the assembly of the putative transport pore, it is necessary to define the interactions among the various VirB proteins. To simplify these studies, we developed a system that allows overproduction of a protein or a set of proteins in *Agrobacterium*. Using this system, we demonstrate that migration of VirB9 in SDS/polyacrylamide gels under a nonreducing environment is affected by the presence of other VirB proteins. We demonstrate that this aberrant migration results from the formation of a complex between VirB9 and VirB7, and a disulfide linkage mediates the formation of this complex. The cysteine residues involved in this process were identified by site-specific mutagenesis.

METHODS

Construction of a VirB Deletion Plasmid. To introduce a deletion in the *virB* locus of the octopine T_i plasmid pT_iA6, we constructed a plasmid pLA9 that contains an ~1.5 kb segment of the 3′ end of *virA* (a *Sac*I–*Kpn*I fragment) followed by the kanamycin-resistance gene of the plasmid pUC4K (23) and a 1.3-kb *Bgl*II–*Pst*I fragment encompassing the pT_iA6 *virG* in the plasmid vector pTJS75 (24). Plasmid pLA9 was introduced into *A. tumefaciens* A348 by triparental mating (25) and colonies resistant to tetracycline, kanamycin, and carbenicillin were selected. Plasmid pPH1JI (which is resistant to gentamycin) was then introduced into *A. tumefaciens* A348/pLA9 and colonies resistant to gentamycin and kanamycin were selected. The resistant colonies were screened for sensitivity to carbenicillin. The carbenicillin-sensitive colonies are expected to contain a substitution of the *virB* locus with the kanamycin-

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Abbreviations: T_i, tumor-inducing; T-DNA, the portion of the T_i plasmid that is transferred to plant cells; β-ME, 2-mercaptoethanol.

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resistance gene resulting from homologous recombination at the *virA* and the *virG* sequences. Several such colonies were analyzed by Southern blot hybridization to confirm that these cells had lost *virB* and one was designated *A. tumefaciens* A348ΔB.

Construction of *A. tumefaciens* A348ΔB2, A348ΔB3, and A348ΔB7. The pT_iA6 *virB* operon was cloned into the plasmid vector pBlueScriptII KS+ (Stratagene) as a 10.5-kb *NdeI–XhoI* fragment to construct pAD1287. The 5′ end of the *virB* operon was subcloned by digesting pAD1287 with *SstI* followed by ligation generating pAV128. The 3′ end of the *virB* operon was subcloned as a 5.3-kb *SstI* fragment into pBlueScriptII gent (a gentamycin resistant derivative of pBlueScriptII) to construct pAV134R. A deletion of the ORF for *virB2*, *virB3*, or *virB7* in plasmids pAV128 or pAV134R was introduced by oligonucleotide-directed site-specific mutagenesis (26). The mutants, pAV128ΔB2, pAV128ΔB3, and pAV134RΔB7, were confirmed by DNA sequence analysis (27). The entire *virB* operon was reconstructed by ligating the 3′ *SstI* fragment of pAV134R into pAV128ΔB2 and pAV128ΔB3 creating pAD1287ΔB2 and pAD1287ΔB3, respectively. The *SstI* fragment from pAV134RΔB7 was subcloned back into the *SstI* site of pAV128 constructing pAD1287ΔB7. Plasmids pAD1287ΔB2, -ΔB3, and -ΔB7 were fused to the wide-host-range plasmid pAV140, a derivative of pTJS75 (24), at a unique *KpnI* site. The plasmids were then introduced into *A. tumefaciens* A348ΔB to construct *A. tumefaciens* A348ΔB2, A348ΔB3, and A348ΔB7, respectively.

Construction of VirB Overproducing Plasmids. Plasmid pAD1369 overproduces all of the VirB proteins in *A. tumefaciens*. This plasmid contains the entire *virB* locus of pT_iA6, the *virG* constitutive mutant *virGN54D* (28), and the high copy mutant of the wide-host-range plasmid pTJS75, 75Δ2 (29). pAD1369 was obtained by cloning the 10.5-kb *virB* locus of pAD1287 into plasmid pAD1307. When introduced into the T_i-plasmidless strain *A. tumefaciens* A136, pAD1369 supports overproduction of the VirB proteins (data not shown).

For overproduction of VirB9, plasmid pAD1388 was constructed by cloning the *virB9* coding region as a 1.13-kb *Bst*EII–*Bam*HI fragment downstream from the *virD* promoter of plasmid pAD1336. Plasmid pAD1336 is a pUC118 derivative containing a 391-bp *virD* promoter fragment (−384 to +7; ref. 30). The chimeric *virB9* gene in pAD1388 contains a 130 residue 5′ nontranslated sequence. A 7.5-kb *Eco*RI fragment containing *virGN54D* and 75Δ2 was then cloned into the unique *Eco*RI site of pAD1388 to construct plasmid pAD1389. To overproduce both VirB9 and VirB7, a 398-bp *NaeI–PvuII* fragment containing the *virB7* ORF was cloned into a unique *Eco*ICRI site of plasmid pAD1388 to construct pAD1399. This cloning places *virB7* downstream of *virB9* and both genes are under the control of the *virD* promoter. The spacer between *virB9* and *virB7*, a 155 nucleotide segment, has a 44 amino acid ORF that contains the first 17 amino acids of VirB10. This chimeric ORF terminates 43 nucleotides upstream of the *virB7* translation initiation codon. The wide-host-range high copy derivative of this plasmid, pAD1400, was constructed by cloning the 7.5-kb *Eco*RI fragment mentioned earlier into pAD1399. Plasmids were introduced into *A. tumefaciens* A136 by electroporation (31). The desired mutations in *virB7* and *virB9* were introduced by oligonucleotide directed site-specific mutagenesis of single-stranded pAD1399 DNA. (26). All mutations were confirmed by DNA sequence analysis (27).

Cross-Linking Studies, Protein Analysis, and Other Procedures. Cross-linking experiments with the membrane permeable cross-linker dimethyl 3,3′-dithiobispropionimidate (DTBP) were performed essentially according to Ward *et al.* (9) after minor modifications. Briefly, *A. tumefaciens* A136 containing the appropriate plasmid was grown in 25 ml AB medium, pH 6.5 (1× AB salts/25 mM sodium phosphate, pH 6.5/0.2% glucose) to an A_{600} of ~0.6. Cells were collected by centrifugation at 6000 × *g* for 10 min, washed three times with 10 ml phosphate buffer (20 mM sodium phosphate, pH 7.6; 20 mM sodium chloride), and resuspended in the same buffer at a concentration of 4 A_{600} unit/ml. DTBP was added to the cell suspension at a final concentration of 5 mM and the mixture was incubated at room temperature for 30 min. The reaction was terminated by the addition of 1 M Tris·HCl (pH 7.6) to a final concentration of 20 mM. After an additional 15-min incubation, the cells were collected by centrifugation, washed with 1 ml of 50 mM Tris·HCl (pH 7.6), and resuspended in 150 μl of buffer (100 mM Tris·HCl, pH 8.0/1 mM EDTA). The cell suspension was aliquoted into two tubes, 0.5 vol 3× Laemmli protein denaturation buffer [with or without 2-mercaptoethanol (β-ME); ref. 32] was added, and samples were denatured at 37°C for 30 min. Proteins were analyzed by SDS/PAGE, followed by Western blot analysis using anti-VirB9 antibody (33).

Anti-VirB9 antibody was raised in rabbits using a TrpE′–′VirB9 fusion protein as an antigen. A *trpE′–′virB9* fusion was constructed by cloning a 2.8-kb *BglII* fragment (encodes the C-terminal 219 residues of VirB9) into the *Bam*H1 site of plasmid pATH2 (34). The fusion protein was purified by SDS/PAGE and used as an antigen for the production of polyclonal antibody in rabbits.

RESULTS

Overproduction of VirB Proteins. To study the expression, stability, interaction, and function of the VirB proteins, it is essential to express them individually and as a defined subset both in the absence and in the presence of other components necessary for tumor formation. In previous studies on the VirB proteins, an *A. tumefaciens* strain containing a T_i-plasmid was used (8–10, 19–21). While such a system is closest to the natural one, the use of a T_i plasmid-containing strain will reflect the effect of not only the other VirB proteins but also additional Vir proteins, the T-complex, and any interaction between these components. A multitude of interactions can make it difficult to interpret some results. To avoid undesired complications as well as to define the interactions among the VirB proteins, we developed a system to overproduce only the VirB proteins. This system will also be useful for the overproduction of any protein in *A. tumefaciens*.

For the overproduction of the VirB proteins, we combined two of our previous observations; (*i*) a *virG* mutant, *virGN54D*, supports constitutive expression of the *vir* genes (28), and (*ii*) a deletion within the *oriV* of the wide-host-range plasmid pTJS75 leads to a substantial increase in plasmid copy number in *A. tumefaciens* (29). A plasmid pAD1369 that combines these two features and contains the entire *virB* locus was constructed and introduced into *A. tumefaciens* A136. Analysis of whole cell extracts by Western blot assays using several anti-VirB antibodies showed that in comparison to induced *A. tumefaciens* A348, this strain produces a substantially higher level of many VirB proteins (data not shown). A similar approach was used for the overproduction of individual VirB proteins except that the coding sequence(s) of interest was placed under the control of the *virD* promoter. While both *virB* and *virD* are strong promoters (35), we chose the *virD* promoter because of the ease in its cloning.

Aberrant Migration of VirB9 in SDS/PAGE. The postulate that the VirB proteins participate in the formation of a membrane pore for T-strand DNA transfer from bacteria to plants suggests that protein–protein interactions play an essential role in the assembly and stability of the putative pore complex. To understand the pore structure, it is necessary to identify the components of this structure and to define the interactions between them. Cross-linking studies have been widely used to analyze macromolecular complexes. In this study, we used a similar approach to determine whether VirB9 interacts with another VirB protein(s).

Two strains, *A. tumefaciens* AD1369 (that overproduces all of the VirB proteins) and *A. tumefaciens* AD1389 (that overproduces only VirB9), were used for cross-linking studies using the cleavable, membrane-permeable cross-linker DTBP (Fig. 1). These strains do not contain any other T_i-plasmid gene except for the mutant *vir* gene regulator *virGN54D*. A Western blot assay using anti-VirB9 antibodies showed that several cross-reacting bands are present in *A. tumefaciens* AD1389 whether the cells were treated with DTBP or not (lanes 1 and 2). An estimate of the molecular mass of the bands indicates that the fastest migrating 31-kDa band (labeled B9) probably represents the VirB9 monomer [predicted molecular mass of the precursor, 32.2 kDa (6)]. Of the other bands, the 60-kDa band is probably VirB9 homodimer. The strong signal at the top of the gel (lane 1) is probably due to nonspecific cross-linking of VirB9 with cellular proteins. This signal was not observed in the presence of other VirB proteins (lane 3). A similar analysis of *A. tumefaciens* AD1369, a strain that overproduces all of the VirB proteins, yielded a totally different band pattern (lanes 3 and 4). In the absence of the cross-linker, a major band of ~36 kDa (labeled B9′) and two minor bands of ~23 kDa were observed (lane 4). The 36-kDa band probably contains the full-length VirB9, whereas the smaller ones are likely to represent proteolytic degradation products. The 36-kDa band, however, is migrating slower than the 31-kDa band of *A. tumefaciens* AD1389 (discussed below). Two additional bands of ~72 kDa and 150 kDa were also observed in the presence of the cross-linker (lane 3). The 72-kDa band probably represents a dimer of the 36-kDa band.

The protein samples analyzed in these experiments (lanes 1–4) were denatured in the absence of a reducing agent (β-ME) because the cross-linker DTBP is sensitive to it. To ensure that all bands observed in these experiments are specific for VirB9, one-half of the samples were denatured in the presence of β-ME and analyzed similarly. A single major band of 31 kDa was observed in all samples irrespective of whether the host cell expressed only VirB9 or all of the VirB proteins and whether or not the cells were treated with DTBP (lanes 5–8). This band comigrates with the 31-kDa band

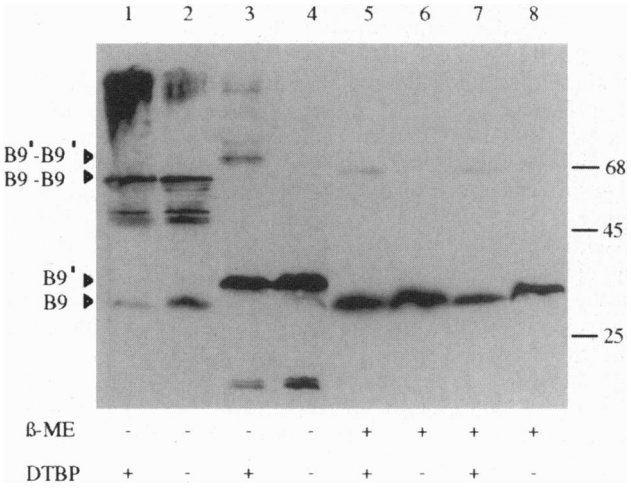

FIG. 1. Analysis of VirB9 protein of *A. tumefaciens* expressing *virB9* or *virB*. Total proteins from *A. tumefaciens* AD1389 (overproduces VirB9) and AD1369 (overproduces all VirB proteins) were analyzed by Western blot using anti-VirB9 antibody. Proteins were electrophoresed on an SDS/15% polyacrylamide gel. Where indicated, cells were treated with the cross-linker DTBP according to Ward *et al.* (9). Lanes: 1, 2, 5, 6, AD1389; 3, 4, 7, 8, AD1369. No β-ME was present in the protein denaturation buffer used for samples in lanes 1–4. The position of VirB9 (B9), VirB9 complex (B9′), and the putative dimers (B9–B9, B9′–B9′) are indicated by arrowheads. Numbers on the right indicate the position of the marker proteins.

observed in *A. tumefaciens* AD1389 (lane 2), indicating that it represents the VirB9 monomer. The 36-kDa band observed in *A. tumefaciens* AD1369 (lanes 3 and 4) must therefore represent a protein complex of VirB9 and another VirB protein. This hypothesis is further supported by the observation that the 36-kDa band is converted to the 31-kDa band upon treatment with β-ME (lanes 7 and 8). Therefore, the 36-kDa band must include VirB9 and a second protein linked to it by a disulfide linkage.

Under nonreducing conditions, *A. tumefaciens* AD1389 shows three additional VirB9 specific bands of approximately 60, 53, and 43 kDa. The 60-kDa band can be a VirB9 dimer but the other two, because of their size, must be composed of VirB9 and a cellular protein. These complexes are sensitive to β-ME, indicating that they are held together by a disulfide linkage (lanes 5 and 6). Since these complexes result from interaction of VirB9 with itself or with other cellular proteins, these results suggest that VirB9 contains a reactive sulfhydryl group. None of these bands were observed in cells that express all of the VirB proteins (lane 4), indicating that the affinity of VirB9 for its partner (to form the 36-kDa complex) is much higher than that for itself and for other cellular proteins. Since the 36-kDa complex forms only in the presence of other VirB proteins and is converted to the 31-kDa band upon treatment with β-ME, this complex must consist of VirB9 and a second VirB protein(s) linked through a disulfide linkage. To ensure that the formation of the disulfide linkage is not an artifact of cell lysis, iodoacetamide, an inhibitor of disulfide bond formation, was added to the protein denaturation buffer. Addition of iodoacetamide had no effect on the formation of the 36-kDa band indicating that the VirB9 complex is a naturally occurring one and does form *in vivo* (data not shown).

Identification of the Protein That Interacts with VirB9. VirB9 when expressed alone migrates as a 31-kDa band in SDS/PAGE under nonreducing conditions. However, it migrates as a 36-kDa band when all of the VirB proteins are expressed together (Fig. 1). The 36-kDa band is a protein complex composed of VirB9 and another VirB protein. The small difference in the sizes of the VirB9 complex and VirB9 (36 kDa versus 31 kDa) suggests that the interacting protein is small in size. The *virB* operon can encode three small proteins, namely, VirB2, VirB3, and VirB7 [M_r of precursors, 12.4, 11.6, and 5.9 kDa, respectively (6)]. The observation that the 36-kDa complex is dissociated by β-ME indicates that the interaction is mediated by a sulfhydryl group and, therefore, complex formation requires the participation of a cysteine residue. Of the three small VirB proteins, VirB3 does not contain a cysteine. Therefore, VirB2 and VirB7 are the most likely candidates for participating in the formation of the VirB9 complex.

To identify the partner for VirB9, we determined whether a deletion in VirB2, VirB3, or VirB7 affects the formation of the 36-kDa complex (Fig. 2). As evident from the results, a deletion of VirB2 or VirB3 did not abolish the formation of the 36-kDa band (lanes 3 and 4), indicating that these proteins are not essential for the formation of the VirB9 complex. The 36-kDa band of *A. tumefaciens* A348ΔB2 and *A. tumefaciens* A348ΔB3 was converted to the 31-kDa band upon treatment with β-ME as well (lanes 8 and 9). *A. tumefaciens* A348ΔB7, a strain with a deletion of *vir*B7, however, showed no VirB9 specific band (lanes 5 and 10). This result is similar to that reported by Berger and Christie (3) who proposed that VirB7 is essential for the stability of VirB9. However, the overproduction system we used allowed easy detection of VirB9 in the absence of VirB7 (lanes 1 and 6; also Fig. 1), indicating that an increase in the expression level of VirB9 can compensate for its lability.

Since VirB2 and VirB3 do not participate in complex formation with VirB9 and the interacting protein is small in size, the partner for VirB9 must be VirB7. To test this hypothesis and to determine whether VirB9 and VirB7 are sufficient for complex formation, we constructed *A. tumefa-*

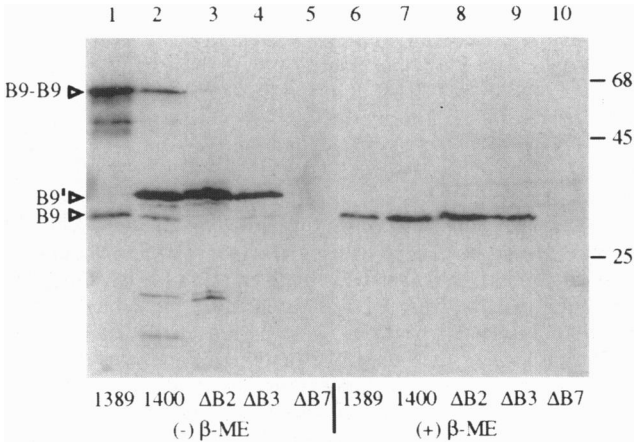

FIG. 2. Identification of the protein that interacts with VirB9. Total proteins of *A. tumefaciens* strains expressing different subset of VirB proteins were analyzed by Western blot assays as described in the legend to Fig. 1. ΔB2, ΔB3, and ΔB7 indicate *A. tumefaciens* strains that express all VirB proteins except for VirB2, VirB3, and VirB7, respectively. *A. tumefaciens* AD1389 and AD1400 express VirB9, and VirB7 and VirB9, respectively.

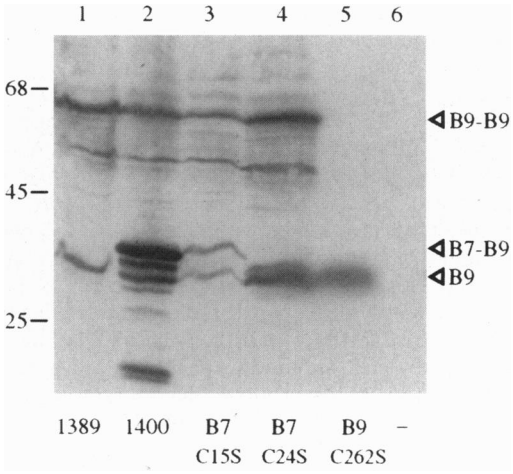

FIG. 3. Effect of cysteine to serine substitutions in VirB7 and VirB9 on VirB7–VirB9 complex formation. The cysteine residues of VirB7 and VirB9 that are candidates for participation in the VirB7–VirB9 complex formation were altered to serine by site-specific mutagenesis. The effect of a mutation on VirB7–VirB9 complex formation was analyzed by Western blot assays as described in the legend to Fig. 1. VirB proteins expressed in the various strains are as follows: lane 1, VirB9; lane 2, VirB7 and VirB9; lane 3, VirB7C15S and VirB9; lane 4, VirB7C24S and VirB9; lane 5, VirB7 and VirB9C262S; lane 6, none. CnS indicates cysteine to serine substitutions at the *n* position of the protein. The protein denaturation buffer did not contain β-ME.

ciens AD1400 (A136/pAD1400), which overproduces both VirB7 and VirB9 from a synthetic operon P_{virD}-*vir*B9-*vir*B7. An analysis of VirB9 from this strain showed that under nonreducing conditions, the majority of the VirB9 protein migrated as a 36-kDa band. A small amount of the protein migrated as 31- and 60-kDa bands (Fig. 2, lane 2). Since the presence of VirB7 allowed the formation of the 36-kDa band and a reducing agent converts it to a 31-kDa band (lane 7), these results demonstrate that the 36-kDa band is a complex of VirB7 and VirB9. No other VirB protein is necessary for the formation of this complex.

Identification of the Amino Acids Involved in VirB7–VirB9 Complex Formation. The dissociation of the VirB7–VirB9 complex by β-ME, a sulfhydryl reducing agent, indicates that the interaction between the two proteins is mediated by cysteines. The pT_iA6 VirB9, a 293 amino acid polypeptide (precursor), contains two cysteines at positions 11 and 262. Of these, residue 11 lies in the putative signal peptide region. Therefore, the most likely residue for interaction with VirB7 is residue 262. The pT_iA6 VirB7, a 55 amino acid polypeptide (precursor), contains four cysteines at positions 4, 7, 15, and 24. Of these, residues 4 and 7 are likely to lie in the signal sequence leaving residue 15 or 24 to be the candidate for interaction with VirB9. To identify the functional cysteines in VirB7 and VirB9, we introduced a cysteine to serine substitution of residues 15 and 24 of VirB7 (VirB7C15S and VirB7C24S) and that of residue 262 of VirB9 (VirB9C262S). The mutant plasmids were introduced into *A. tumefaciens* A136 and the VirB9 complex was analyzed by Western blot assays (Fig. 3). As discussed earlier, VirB9 from *A. tumefaciens* AD1389, a VirB9 overproducing strain, migrates as a 31-kDa band (lane 1) and that from *A. tumefaciens* AD1400, which overproduces both VirB7 and VirB9, migrates as a 36-kDa band (lane 2). In the latter strain, both the 36-kDa and the 31-kDa bands are observed, indicating the presence of both VirB7–VirB9 complex and free VirB9. The additional low molecular mass bands probably represent proteolytic degradation products. Analysis of the strains bearing the cysteine to serine substitutions in VirB7 and in VirB9 show that the 36-kDa band is present in *virB7C15S* mutant (lane 3) but not in the *virB7C24S* and *virB9C262S* mutants (lanes 4 and 5). Therefore, cysteine 24 of VirB7 and cysteine 262 of VirB9 must participate in the formation of the VirB7–VirB9 complex. The presence of high molecular mass bands in a strain harboring a *virB9* plasmid (lanes 1–4) and their absence in the *virB9C262S* mutant confirm our previous conclusion that these bands result from disulfide bond formation by the reactive −SH group of VirB9 with itself and with other cellular proteins. In all cases, only a single major band of 31 kDa was observed in the presence of β-ME (data not shown). The *virB7C15S* mutant consistently showed a lower level of VirB9, suggesting that this mutation affects the stability of VirB9.

Cross-Linking of VirB7 and VirB9. The preceding studies established that VirB9 interacts with VirB7 and this interaction is mediated by cysteine 262 of VirB9 and cysteine 24 of VirB7. To determine whether these cysteine residues are essential for VirB7–VirB9 interaction, we performed a cross-linking experiment with the VirB7 and VirB9 mutant proteins (Fig. 4). The expectations were that if the cysteine residues of the two proteins are the only elements that bring them in close proximity of each other, the loss of these residues will have a significant effect on VirB7–VirB9 complex formation. On the other hand, if the cysteine residues are necessary but not essential for complex formation, mutant proteins that lack the cysteines will remain in close proximity to allow cross-linking of the components of the complex. Analysis of mutant strains with cysteine to serine substitutions in VirB7 or VirB9 showed that the VirB7C15S protein forms the 36-kDa complex both in the absence and presence of the cross-linker (lanes 5 and 6). The VirB7C24S protein, however, forms a low amount of this complex only in the presence of the cross-linker and VirB9C262S fails to form any detectable level of this complex (lanes 7–10). In all samples, except that in lane 9, the putative VirB9 dimer of 60 kDa was also present indicating that these strains produce excess VirB9.

The 36-kDa band is the predominant VirB9 specific band in *A. tumefaciens* AD1400, a strain that expresses wild-type VirB7 and VirB9 (lane 3). In the presence of the cross-linker, an additional band of 72 kDa was also observed (lane 4). A similar band was also observed when the *virB* overproducing strain *A. tumefaciens* AD1369 was used in cross-linking studies (Fig. 1). The presence of this band in a strain overproducing only VirB7 and VirB9 indicates that the formation of this complex does

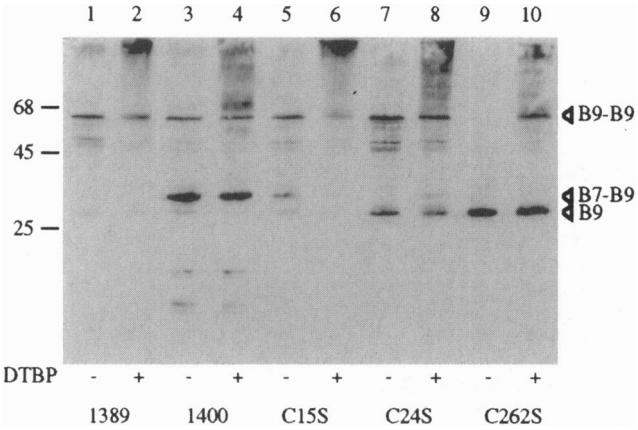

FIG. 4. Effect of mutations in *virB7* and *virB9* on the formation of a cross-linked VirB7–VirB9 complex. Complex formation was monitored by Western blot assays as described in the legend to Fig. 1 except that proteins were separated on an SDS/12.5% polyacrylamide gel. VirB proteins expressed in the different strains are: 1389, VirB9; 1400, VirB7 and VirB9; C15S, VirB7C15S and VirB9; C24S, VirB7C24S and VirB9; C262S, VirB7 and VirB9C262S. Where indicated, cells were treated with the cross-linker DTBP.

not depend on other VirB proteins. Based on the size, it is probable that this complex is a tetramer of two VirB7 and two VirB9. This can result from the interaction of one VirB7 dimer with a VirB9 dimer or from the dimerization of a VirB7–VirB9 complex. The former probably is the case because the results presented in this study suggest that VirB9 can form a homodimer.

Studies on the mutants show that VirB7C24S can form a low level of cross-linked VirB7–VirB9 complex but VirB9C262S can form almost no such complex. The lack or a reduced ability of complex formation of these two mutants suggests that the mutations affect the tertiary/quaternary structure of the proteins such that they are no longer in an optimal spatial arrangement for this particular cross-linker to be highly effective. The altered structure of the mutant proteins may affect their function. The cysteine 262 to serine substitution of VirB9, however, did not destroy its dimerization domain because in the presence of the cross-linker the VirB9 homodimer was easily detectable (lane 10). Results similar to that presented in this study were independently obtained by P. Christie and coworkers (see ref. 38). Their study also showed that cysteine 24 of VirB7 and cysteine 262 of VirB9 are essential for tumor formation.

DISCUSSION

The transfer of the T-strand DNA from *Agrobacterium* to plants requires VirD4 and the VirB proteins. The VirB proteins are postulated to form a transport pore that allows transfer of the T-strand DNA across the various bacterial membranes (for review, see ref. 2). To identify the constituents of the transport pore and to understand the assembly of this structure, it is necessary to define the interactions among the various VirB proteins. This process in turn requires functional analysis of each of the proteins. To facilitate these studies, we developed an expression system that allows overproduction of a protein in a T_i-plasmidless *Agrobacterium* strain. This approach allows us to use a defined system and to avoid complex interactions among the various components of the T-DNA transfer machinery of a T_i-plasmid-containing strain.

Studies on the VirB proteins demonstrate that the presence of a VirB protein can affect the properties of a second VirB protein. For example, a deletion of the VirB7 coding region led to a significant decrease in the steady-state level of VirB9 and a few other VirB proteins (3). VirB4 is necessary for the localization of VirB3 to the outer membrane (21) and coexpression of three VirB proteins, VirB9, VirB10, and VirB11, is essential to relieve the RSF1010-mediated inhibition of T-DNA transfer to plants (22). These results strongly suggest that interactions among the VirB proteins are involved in steps leading to DNA transfer. To understand some of these interactions, we reasoned that a comparison of the properties of a VirB protein when expressed alone and when expressed with other VirB proteins will provide an alternate approach to identify proteins that interact with one another and to define these interactions. We tested this approach by using the VirB9 protein because we speculated that this protein is a good candidate for participating in a protein complex based on the requirement of VirB9, VirB10, and VirB11 for the relief of the RSF1010 mediated inhibition of T-DNA transfer and on the potential translational coupling of VirB8, VirB9, and VirB10 (7, 22).

An analysis of strains producing VirB9 showed that under nonreducing conditions the presence of other VirB proteins affects the migration of VirB9 in an SDS/polyacrylamide gel (Fig. 1). This anomalous behavior results from the formation of a protein complex between VirB7 and VirB9. No other Vir protein is necessary for the formation of this complex (Fig. 2). Sensitivity of the complex to the reducing agent β-ME indicates that a disulfide bond participates in complex formation. Mutational analysis indicated that cysteine 24 of VirB7 and cysteine 262 of VirB9 are involved in the disulfide bond formation (Fig. 3). The disulfide bond must be important in the spatial arrangement of the two proteins because mutations that prevented disulfide bond formation abolished or severely attenuated the formation of the cross-linked complex (Fig. 4). It is expected that the formation of the cross-linked complex would not be negatively affected unless a mutation affects the structure of a protein.

Accumulation of VirB9 requires the presence of VirB7, indicating that VirB7 stabilizes VirB9 (Fig. 2). An earlier study of Berger and Christie (3) made a similar observation. When *virB9* was cloned into a high copy plasmid, the presence of VirB7 was not necessary for the detection of VirB9 by Western blot assays. However, the presence of *virB7* on the same plasmid led to an increase in the level of VirB9. The *virB7C15S* mutant strain on the other hand consistently exhibited a low level of VirB9. Cysteine 15 of VirB7 is the N-terminal residue of the mature peptide and participates in an interaction with a lipid (39). The latter interaction is presumably important for anchoring VirB7 to the outer membrane. VirB7C15S would be expected to lose this interaction and may not be targeted to its proper cellular location. Whether this leads to the loss in its ability to stabilize VirB9 remains to be seen.

What role does the VirB7–VirB9 complex play, if any, in T-DNA transfer? A comparison of the *A. tumefaciens* VirB proteins and its homologs in the bacterial conjugal plasmids as well as in the pathogenic bacterium *B. pertussis* shows that these two proteins are not conserved in all the systems. While VirB7 and VirB9 are highly conserved in both the octopine and nopaline T_i plasmids, the *B. pertussis ptl* operon does not encode a VirB7 homolog (6, 7, 12). Similarly, the conjugal plasmid RP4 lacks a VirB7 homolog but another conjugal plasmid pKM101 appears to contain one (11, 36). However, the homology between VirB7 and its pKM101 homolog, TraN, is rather limited (nine identities and three conservative substitutions in a 41 residue segment). pKM101 TraN lacks a cysteine at a position equivalent to residue 24 of VirB7 but contains one at the first position of the mature peptide. The presence of the N-terminal cysteine suggests that TraN, like VirB7, is a lipoprotein.

A homolog of VirB9 is found in the *ptl* operon (PtlF) and in the conjugal plasmid pKM101 (TraO) but not in the conjugal plasmid RP4. Pohlman *et al.* (36) indicated that RP4 TrbC is homologous to VirB9. However, most of the similarities between these two proteins lie in the hydrophobic regions, and

RP4 TrbC exhibits a comparable level of similarity to *Agrobacterium* VirB2. Based on the size, sequence similarity, and gene organization, TrbC is probably a homolog of VirB2. It therefore appears that only pKM101 contains both VirB7 and VirB9 homologs. The cysteine residues involved in VirB7–VirB9 interaction are not conserved in the pKM101 Tra proteins. If TraN and TraO, like VirB7 and VirB9, are involved in a complex formation, this complex must involve other interactions.

The observation that VirB7 and VirB9 form a complex is the first demonstration of a direct interaction between two VirB proteins. These results allow us to build a basic framework for the assembly of the transport pore. The VirB7 protein is a lipoprotein anchored to the outer membrane of the bacterium (39). The VirB7–VirB9 complex probably represents a part of a subcomplex that allows T-DNA to exit through the outer membrane. This type of an arrangement predicts that both VirB7 and VirB9 proteins would face the periplasmic space. Studies on membrane topology using chimeric proteins containing the *Escherichia coli* alkaline phosphatase support this hypothesis (refs. 3 and 37; unpublished results). A second subset of proteins would form a subcomplex on the inner membrane allowing the exit of the T-DNA through this membrane. VirB6, an integral membrane protein with multiple transmembrane domains, is likely to be the pore protein in this subcomplex. Finally, the two subcomplexes would link to form the transport pore. The components of these complexes as well as the proteins essential for the assembly of the entire structure remain to be defined.

Note. This paper was scheduled to appear with that of Spudich *et al.* (38). The *Proceedings* office failed to delay publication of the paper by Spudich *et al.* as requested by the authors of both papers.

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