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Agrobacterium tumefaciens T-Complex Transport Apparatus: a Paradigm for a New Family of Multifunctional Transporters in Eubacteria†

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INTRODUCTION

Bacterial conjugation has long served as a model system for developing a mechanistic understanding of how nucleic acids translocate across biological membranes. Early studies of the *Escherichia coli* F-plasmid conjugation system led to the view that conjugation is a contact-dependent process which, at least among the gram-negative bacteria, requires two cell surface structures in the donor cell that are probably joined. One structure is an extracellular filament termed the sex pilus for initiating the physical coupling of donor and recipient cells. The second is a DNA conductance channel or conjugal pore for transmission of DNA substrates across the donor cell envelope (34, 46). Classically, conjugation systems have been considered to operate on principles that are mechanistically quite different from systems dedicated to protein transport. However, recent studies have identified at least three similarities among conjugation and protein transport processes. First, sequence-based studies have identified homologies among subunits of a variety of eubacterial transport systems, including those dedicated to DNA transfer, selective protein secretion, assembly of type IV fimbriae and flagella, and extrusion of filamentous phage (44). Second, there is an accumulating body of experimental evidence supporting the notion that the conjugal transfer of DNA proceeds via recognition of sequences or motifs associated not with the DNA per se but with proteins bound to the DNA (34 and this minireview). Finally, some members of the type III protein secretion family (see below) recently identified in bacterial pathogens of plants and humans directly "inject" protein substrates into eukaryotic cells by a process requiring cell contact and, in some cases, the elaboration of extracellular filaments; in principle these structures could functionally resemble conjugative sex pili (39, 64).

This minireview will focus on recent investigations of the *Agrobacterium tumefaciens* transport system responsible for delivery of oncogenic DNA (T-complex) across the bacterial envelope. The T-complex transporter belongs to a growing family of transporters whose subunits share extensive sequence similarities. For the present, this secretion family is referred to as a type IV secretion system, as originally proposed by Salmond (72). This classification distinguishes the type IV secretion system from other dedicated secretion pathways, including the type I secretion system exemplified by *E. coli* hemolysin export, the type II secretion system exemplified by *Klebsiella oxytoca* pullulanase export, and the type III secretion system exemplified by *Yersinia pestis* Yop export (for detailed information about these protein export systems, see references 30, 39, 68, and 72). However, as illustrated above, a taxonomic classification based on sequence similarities of transporter subunits is likely to prove obsolete as additional structural or functional similarities are identified among various macromolecular transport systems. By definition, members of the type IV system involved in conjugation transmit DNA to recipient cells by a contact-dependent process. Beyond requiring cell-to-cell contact, however, until very recently almost nothing was known about the biochemical reactions that govern the processing of DNA into a transfer-competent substrate or the transmission of substrate from donor to recipient cells. The first aim of this minireview is to highlight recent work demonstrating sequence and functional similarities between the T-complex transport system and related conjugation and protein export systems. The second aim is to summarize new results from structural studies that define, for the first time, early stages in the assembly of a bacterial conjugation apparatus.

OVERVIEW OF THE *AGROBACTERIUM* INFECTION PROCESS

A. tumefaciens incites crown gall disease in a wide range of dicotyledonous plant species. The disease, characterized by neoplastic transformation at the site of infection, results from the transfer and expression of oncogenes from the bacterium to susceptible plant cells. Infection is initiated when bacteria sense and respond to an array of signals, including specific classes of plant phenolic compounds and monosaccharides, acidic pH, and low phosphate level, that are present at a plant wound site. Signal perception is mediated by the VirA-VirG two-component transduction system together with ChvE, a periplasmic sugar-binding protein. The activated phosphorylated form of VirG coordinates expression of a set of at least six virulence operons, *virA*, *-B*, *-C*, *-D*, *-E*, and *-G* residing within a ~35-kb region of a large Ti (tumor-inducing) plasmid. These *vir* gene products largely contribute to two stages in the infection process: (i) processing of a second region of the Ti plasmid designated T-DNA into a translocation-competent substrate (T-complex) and (ii) delivery of T-complexes to plant nuclei. Additional chromosomal virulence loci are also required for successful T-complex transfer to plant cells. Once inside the nucleus, the T-DNA integrates into the plant genome, where subsequent expression of transferred oncogenes results in loss of cell division control and, ultimately, the formation of crown gall tumors. An autoinduction system that promotes the spread of the Ti plasmid and associated *vir* determinants among bacteria at the plant wound site has also been described. Detailed information about these aspects of the *A. tumefaciens* infection process can be found in several excellent reviews (38, 45, 97, 101, 102).

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SIMILARITIES BETWEEN T-DNA TRANSFER AND PLASMID CONJUGATION SYSTEMS

Translocation-competent substrate. A decade ago, it was shown that (i) the right T-DNA border sequence is essential for and determines the direction of DNA transfer from *A. tumefaciens* to plant cells, (ii) *virD1* and *virD2* encode an endonuclease that generates single-stranded nicks at specific sites within T-DNA border sequences, and (iii) the predominant product of this nicking reaction is a free single-stranded T-DNA molecule that corresponds to one strand of T-DNA. Noting similarities to the *E. coli* F-plasmid conjugation system, it was postulated that a mechanistic relationship exists between T-DNA transfer and plasmid conjugation (97, 101). In the last 10 years, a large body of evidence has accumulated supporting the notion that DNA processing reactions associated with interkingdom T-DNA transfer and bacterial conjugation are analogous, if not equivalent. The most extensive similarities have been documented for the processing of T-DNA and the broad-host-range (BHR) IncP plasmid RP4. The substrates for the nicking enzymes of both systems, T-DNA border sequences and the RP4 origin of transfer (*oriT*), exhibit a high degree of sequence similarity (52, 95). The nicking enzymes, VirD2 of pTi and TraI of RP4, possess conserved active-site motifs that are located within the N-terminal halves of these proteins (63). Purified forms of both proteins cleave at the nick sites within T-DNA borders and the RP4 *oriT*, respectively, although for unknown reasons VirD2 also cleaves at *oriT* while TraI fails to cleave at T-DNA borders (60, 61, 74). Both VirD2 and TraI remain covalently bound to the 5′ phosphoryl end of the nicked DNA via conserved tyrosine residues Tyr-29 (60, 89) and Tyr-22 (61), respectively. Both proteins also catalyze a rejoining activity reminiscent of type I topoisomerases (60, 61). VirD1 is dispensable for VirD2-mediated cleavage of single-stranded substrate in vitro, but it is required for cleavage of supercoiled double-stranded substrate in vitro (74) and for generation of the free T-DNA complex in vivo (97). These properties are consistent with the proposal that VirD1 supplies a function analogous to that of TraJ of plasmid RP4, which is thought to interact with *oriT* as a prerequisite for TraI binding to an *oriT* DNA-protein complex. Thus, according to the current model, processing of conjugal plasmids and T-DNA involves sequence-specific recognition and strand-specific cleavage by dedicated nicking enzymes followed by a strand displacement reaction, which generates a free single-stranded transfer intermediate. A simultaneous replication reaction is invoked for replacement of the displaced strand of T-DNA or plasmid in the donor cell (52, 97, 102).

The T-DNA and plasmid conjugation systems also encode single-stranded DNA binding proteins (SSBs) which are thought to coat the length of the transferred single-stranded molecule (20, 21, 26). The translocation-competent form of DNA therefore is depicted as a single-stranded DNA molecule (86, 100) covalently bound at its 5′ end by the nicking enzyme and coated along its length with an SSB (20, 97). It should be noted, however, that for reasons summarized below an alternative model has emerged for the translocation-competent form of T-DNA. According to this model, the T-DNA is delivered to plant cells as a single-stranded T-strand covalently bound at its 5′ end with VirD2 but free of VirE2 SSB. Both the VirD2–T-strand and VirE2 SSB are independently exported via the T-complex transport apparatus to the same plant cell. Once inside the plant cell, the naked T-strand is coated along its length with VirE2 SSB, thus forming the T-complex (13, 82). Although the end result of both models is the same, an assembled T-complex in the plant cell, identifying the form of the transferred substrate is important for understanding the requirements for substrate recognition and interaction by the transport machinery. According to the former model, the T-strand is completely coated with protein and thus the transport machinery presumably would not interact directly with DNA. According to the latter model, the T-strand is naked except for an association with VirD2 protein at the 5′ end and thus the transport machinery would interact both with protein and with naked DNA.

At this juncture, it is also worth noting that while T-DNA and RP4 plasmid processing reactions resemble each other, several observations suggest that the corresponding transfer intermediates are endowed with different properties. First, *A. tumefaciens* induced for synthesis of the Vir proteins accumulate detectable levels of free T-strands in the bacterium (97). By contrast, donor cells have not been shown to accumulate free RP4 plasmid transfer intermediates. These findings could indicate that processing and transport reactions are temporally uncoupled for T-complexes but coupled for conjugative plasmids. Second, T-complexes must translocate across membranes of diverse cell types, including the cytoplasmic and outer membranes of the *A. tumefaciens* envelope, the plant plasma membrane, and the nuclear membrane. Moreover, at least these latter two membranes are spatially separated. Interbacterial transmission of conjugative plasmids is a comparatively simpler process, involving movement of the transfer intermediate across two bacterial cell envelopes that are physically attached and possibly even fused together. Third, T-DNA integrates by illegitimate recombination into the plant genome (97), whereas RP4 plasmid transfer intermediates generally recircularize.

Experimental evidence suggests protein components of the transfer intermediates contribute to the different fates of transferred T-strands and single-stranded RP4 DNA in recipient cells. In the case of the RP4 transfer intermediate, the in vitro rejoining activity of TraI relaxase is consistent with a role in promoting plasmid recircularization, and a primase activity associated with the TraC1 SSB is consistent with a role in second-strand synthesis in the bacterial recipient (61). In the case of the T-complex, both the VirD2 protein and the VirE2 SSB possess functional nuclear localization sequences for delivery of T-complexes across the eukaryotic nuclear membrane (22, 102). Interestingly, VirD2 and a form of VirE2 containing a single amino acid change in its nuclear localization sequences localize to the nuclei when microinjected into *Drosophila* embryos and *Xenopus* oocytes (40). Furthermore, the modified VirE2 binds and actively transports DNA into the nuclei of *Xenopus* oocytes, showing conservation of function of these proteins in animal and plant species (40). Both VirD2 and VirE2 also appear to contribute to the efficient integration of T-DNA into the eukaryotic genome (71, 87), although a recent analysis by Bundock and Hooykaas (17) indicates that host proteins also play important roles in the integration step.

Transport machinery. Besides similarities in T-DNA and BHR plasmid processing reactions, there are now several lines of evidence supporting the notion that *A. tumefaciens* evolved as a phytopathogen in part by coopting a set of conjugal *tra* genes for use in delivery of T-DNA across the *A. tumefaciens* envelope (98). Early genetic studies suggested that products of the ~9.5-kb *virB* operon are the most likely candidates for assembling into a cell surface structure for translocation of T-DNA across the *A. tumefaciens* envelope (49, 50, 98, 101). Sequence analyses of the *virB* operon have supported this prediction by showing that the hydropathy patterns of most of the deduced products are characteristic of membrane-associated proteins (51, 76, 83, 90, 91). Furthermore, as depicted in

TABLE 1. Similarities among putative components of type IV transport machineries and properties of the VirB proteins

Homologous proteins from transfer systems[a]						Interkingdom T-DNA transport of VirB proteins			
Protein export[b]		Conjugation[c]							
cag	*ptl*	IncF *tra*	IncW *trw*	IncP *trb*	IncN *tra*	*virB*[d]	Localization[e]	Biochemical property and/or proposed function[f]	Reference(s)[g]
		Orf 169		TrbN	TraL	**VirB1**	Exported	Transglycosylase	4, 6, 12, 57
						VirB1*	Cell exterior	Cell-cell contact	4
	PtlA	TraA	TrwL	TrbC	TraM	**VirB2**	Exported	Pilin subunit, cell contact	12, 48, 50, 75
	PtlB	TraL	TrwM	TrbD	TraA	**VirB3**	Exported	Unknown	12, 75
CagE, PicB	PtlC	TraC	TrwK	TrbE	TraB	**VirB4**	Transmembrane	ATPase, transport activation	11, 12, 25, 35, 47, 78
		TraE	TrwJ	TrbF	TraC	**VirB5**	Exported	Unknown	12, 25, 33
	PtlD		TrwI	TrbL	TraD	**VirB6**	Transmembrane	Candidate pore former	12, 101
	PtlI		TrwH		TraN	**VirB7**	Outer membrane	Lipoprotein, nucleation center as a covalent VirB7-VirB9 dimer)	1, 5, 7, 12, 27, 31, 32, 79, 80
	PtlE		TrwG		TraE	**VirB8**	Periplasmic face of inner membrane	Unknown	12, 24, 32, 85
	PtlF		TrwF		TraO	**VirB9**	Outer membrane	Nucleation center as a covalent VirB7-VirB9 dimer	1, 5, 7, 12, 27, 31, 32, 33, 79, 80, 92, 93
	PtlG	TraB	TrwE	TrbI	TraF	**VirB10**	Transmembrane	Unknown	7, 12, 31–33, 92–94
	PtlH		TrwD	TrbB	TraG	**VirB11**	Cytoplasm or inner membrane	ATPase, transport activation	12, 19, 32, 33, 69, 81, 92, 93
		TraD	TrwB	TraG		**VirD4**	Transmembrane	ATPase, coupler of DNA processing and transport systems	54, 58

[a] Proteins encoded by the genes indicated in boxheads are listed in table. Proteins that are homologous to other proteins from other transfer systems, including VirB proteins, are shown in horizontal rows. The portion of the table showing protein homologies is adapted from Firth et al. (34) and Kado (50).

[b] Genes encoding CagE (18) and PicB (88) are identical. The *H. pylori cag* pathogenicity island codes for additional VirB homologs (10). The *B. pertussis ptl* operon codes for the Ptl protein homologs shown (23, 29, 77, 96, 98).

[c] Proteins shown are encoded by transfer regions of the gram-negative bacterial plasmids F (IncF) (34, 50), R388 (IncW) (14, 50), RP4α (IncP) (53), and pKM101 (IncN) (67).

[d] VirB proteins shown are encoded by the *vir* region of the *A. tumefaciens* pTi plasmid (51, 76, 83, 90, 91).

[e] Subcellular localization studies are cited in the text. Exported means at least some protein crosses across one or both membranes. Transmembrane means the protein spans the cytoplasmic membrane at least once.

[f] Based on the presence of recognizable motifs and results of genetic and biochemical studies.

[g] Cites studies establishing the importance of each VirB protein for DNA transfer and studies addressing possible functions of the VirB proteins.

Table 1, recent sequence analyses of the genetically defined transfer regions of the IncN (67), IncP (53), and IncW (14) BHR plasmids have revealed that many of the deduced Tra proteins are highly similar to the VirB proteins and that the genes encoding homologous proteins are almost always located at similar positions in the respective transfer operons. Several of the VirB and BHR Tra proteins are also similar to Tra proteins of *E. coli* F plasmid (34), suggesting that these conjugation systems may all have evolved from a common ancestral transfer apparatus.

T-DNA transport requires VirB and VirD4 proteins (Table 1). A VirD4-type activity appears to be of general importance for conjugation, given that VirD4 homologs are required for transmission of BHR plasmids and F plasmid. VirD4 and its homologs localize predominantly at the cytoplasmic face of the cytoplasmic membrane and thus may assemble as components of the cognate transport machinery (2, 58). The VirD4 homologs possess Walker A nucleotide binding motifs, and mutagenesis of this motif in TraG of RP4 abolishes protein function (2). Interestingly, *E. coli* carrying F *traD* or RP4 *traG* mutant plasmids elaborate pili and form mating aggregates yet fail to transfer DNA (2, 34). On the basis of these observations, it has been postulated that the VirD4 family of proteins utilize the energy of nucleoside triphosphate hydrolysis to link protein complexes required for DNA processing and DNA translocation (34).

In addition to similarities in subunit composition, two lines of study have shown that the T-complex and BHR conjugal plasmid transfer systems are functionally related. First, both systems are capable of delivering non-self-transmissible IncQ plasmids to recipient cells (8, 13, 15, 41). In fact, the T-DNA transport system delivers IncQ plasmids to both plant and bacterial recipient cells, although interbacterial transfer of IncQ plasmids by this system occurs at appreciably lower frequencies (10^{-3} to 10^{-4} transconjugants per donor cell) than occurs by the BHR plasmid transfer systems (10^{-1} per donor) (36, 41). Thus, IncQ transfer intermediates carry the requisite sequence or structural information for recognition as a translocation-competent substrate by the T-DNA and BHR plasmid transport systems. Of further interest, *A. tumefaciens* cells carrying both T-DNA and an IncQ plasmid efficiently transfer the IncQ plasmid to plant cells but are greatly inhibited in their ability to incite tumor formation (92). This oncogenic suppression phenotype is relieved by the coordinate overexpression of *virB9*, *virB10*, and *virB11* (92). Together, these findings are suggestive of competition among T-complex and IncQ substrates for the transport machinery. The data raise the intriguing possibilities that subunit stoichiometries of VirB9, VirB10, and VirB11 influence the affinity of a given transport apparatus for a particular substrate or that these proteins are rate limiting for transporter assembly (13). No corresponding IncQ interference effect has been documented for conjugal transfer of BHR plasmids. However, IncQ suppression of T-DNA transfer does have a parallel in plasmid conjugation in that certain plasmid fertility inhibition systems appear to act posttranslationally by influencing assembly or function of transport systems encoded by coresident conjugative plasmids (34).

Second, both the T-complex and BHR plasmid transfer systems are capable of delivering DNA across kingdom boundaries to *Saccharomyces cerevisiae* (16, 17, 43, 65). Transfer of the narrow-host-range (NHR) plasmid F to yeast has also been demonstrated (17). One interpretation of these findings is that

at least certain conjugation systems are nonselective with respect to the recipient cell type. However, this idea remains to be rigorously examined, since to date there has been no demonstration of the transfer of conjugative NHR or BHR plasmids to plant cells or conjugal transmission of bacterial DNA to animal cells. The successful transfer of DNA to yeast is dependent on the presence of stabilizing sequences, such as a yeast autonomously replicating sequence or a telomere, or regions of homology between the transferred DNA and the yeast genome for integration by homologous recombination (16, 17, 43, 65). Recently, Bundock and Hooykaas (17) reported that when the T-DNA lacks any extensive regions of homology with the *S. cerevisiae* genome, it integrates at random positions by illegitimate recombination reminiscent of T-DNA integration in plants. Thus, despite demonstrations of DNA transfer from bacteria to yeast via plasmid conjugation systems, the T-DNA transfer system still appears to be uniquely evolved for transfer to plants and for integration by illegitimate recombination into eukaryotic genomes.

Conjugation, whether within or across kingdom boundaries, requires direct physical contact between donor and recipient cells and thus represents the first identified contact-dependent transport system. There is strong genetic evidence that the BHR plasmids elaborate pili; by analogy to the F-plasmid paradigm, these structures are postulated to initiate cell-to-cell contact during conjugation (34, 41, 46). Early studies established roles for several *A. tumefaciens* chromosomal genes such as *chvA*, *chvB*, *exoC*, and *att* in mediating attachment to plant cells (97). Although chromosome-encoded attachment mechanisms could obviate a need for pilus-dependent contact, two groups recently reported that *A. tumefaciens* cells elaborate extracellular filaments resembling sex pili. Kado (49) has reported detection of short, rigid structures as well as long, flexible structures on the surfaces of flagellum-free agrobacteria. The short filaments are reminiscent of pili elaborated by IncP, IncN, and IncW plasmids, whereas the long structures are more reminiscent of pili produced by F plasmid (46). Fullner et al. (37) documented the presence of long, flexible structures exclusively on cells expressing each of the *virB* and *virD4* genes. Furthermore, these investigators discovered that cells incubated at the usual laboratory growth temperature of 28°C rarely possess pili, whereas cells grown at ~19 to 22°C possess these structures in abundance (37). This observation correlates nicely with previous findings that low temperature stimulates the *virB*-dependent transfer of IncQ plasmids to bacterial recipients and T-DNA transfer to plants (36). Taken together, these observations are compatible with a role for extracellular filaments in mediating contact between agrobacterial donors and bacterial, yeast, and plant recipient cells. However, further studies are needed to elucidate the precise contributions of chromosomal and pilus-mediated attachment functions. In addition, the assembly, composition, and structure of the putative sex pili, the influence of temperature on pilus formation, and the nature of pilus-mediated contact with recipient cells are important areas of future investigation.

SIMILARITIES BETWEEN DNA TRANSFER AND PROTEIN EXPORT SYSTEMS

Sequence studies have also revealed that most of the VirB and BHR plasmid Tra proteins are highly similar to the Ptl proteins of *Bordetella pertussis* (Table 1) (29, 96). This is a very exciting discovery because the Ptl proteins direct the export not of DNA but of a protein substrate, the six-subunit pertussis toxin (PT), across the *B. pertussis* envelope. Very recent sequence studies also identified a second set of homologs of VirB4 and several other VirB proteins synthesized from the *cag* pathogenicity island of *Helicobacter pylori* (Table 1) (10, 18, 88). These proteins are postulated to function in the export of a factor that stimulates induction of interleukin-8 secretion in gastric epithelial cells (10, 18, 88). Together, these findings establish for the first time an evolutionary link between transport systems dedicated to conjugal DNA transfer and protein export. Indeed, a common ancestry for these transport systems is not surprising in view of the proposed structures of T-DNA and BHR plasmid transfer intermediates as single-stranded DNA molecules covalently attached at their 5′ ends to their cognate relaxase proteins and coated by SSB. This structure, coupled with the observed 5′→3′ polarity of T-DNA and BHR plasmid transfer (97), supports the notion that the bound relaxases carry the requisite substrate signals and thus serve as pilot proteins to guide DNA translocation.

If operationally defined DNA transfer systems are in fact dedicated protein export systems that only coincidentally translocate DNA via its association with protein substrates, conjugation systems should be capable of translocating protein only. Indeed, this appears to be the case for the T-complex transporter. Otten et al. (59) made the early discovery that mixed infections between two avirulent mutants, one with a deletion of T-DNA and one with a *virE2* mutation, resulted in tumorigenesis. To explain this observation, it was postulated that the *virE2* mutant exports uncoated T-DNA and the strain lacking T-DNA exports VirE2 protein only. Once exported, these molecules then assemble into a nucleoprotein particle for transmission to the plant nucleus. A recent demonstration that both strains in the mixed infection require VirB proteins for restoration of virulence strongly suggests that VirD2–T-strands and VirE2 substrates are exported via the T-complex transport machinery (13). Upon export, the cellular compartment in which T-DNA–VirE2 complexes form—the bacterial cytoplasm or periplasm, the extracellular milieu, or the plant cell—has been the subject of much debate. At present, the favored model is that VirE2 and VirD2–T-strands are exported into the same plant cell and that T-complex assembly takes place there. Perhaps the most compelling evidence in support of this model is that a *virE* mutant incites the formation of wild-type tumors on transgenic plants expressing *virE2*, suggesting that VirE2 functions exclusively in the plant (22). Interestingly, VirE1, a second product of the *virE* operon, appears to be important for translocation of VirE2 but is nonessential for translocation of VirD2–T-strand molecules (82).

Parallel findings indicate that the T-complex transport machinery also exports VirF, a virulence protein which is important for infection of certain dicot species: (i) mixed infections between an avirulent strain lacking T-DNA and avirulent strains with mutations in *virF* incite tumorigenesis, (ii) VirF transport is dependent on the VirB proteins, and (iii) *virF* mutants incite wild-type tumor formation on transgenic plants expressing *virF*, suggestive of a role for VirF in the plant (70). Taken together, the T-complex transporter appears capable of directing the transfer of at least four proteins, VirD2 (possibly only as VirD2–T-strands), RSF1010 MobA nicking enzyme (possibly only as MobA–single-stranded RSF1010), VirE2, and VirF. The ability to transport protein, coupled with the demonstration that the T-complex transporter indiscriminantly transfers any DNA of interest (including the entire ~170-kb Ti plasmid [56]) as long as a recognition site for the VirD2 nicking enzyme is present, strongly supports the notion that transferred proteins, not transferred DNA, possess the necessary sequence or structural information for translocation.

Information about the routes by which T-complex and *B. pertussis* PT substrates are translocated across the gram-nega-

tive bacterial envelope is just beginning to emerge. The PT subunits have typical N-terminal signal sequences. In addition, the S1 subunit is required for efficient secretion of toxin, suggesting that the assembled molecule is transported across at least one membrane (66). On the basis of these observations, it seems likely that PT export occurs by a two-step transport process whereby a general secretion machinery analogous to the *sec* system of *E. coli* directs subunit export across the cytoplasmic membrane and then the Ptl proteins promote export of assembled toxin across the outer membrane. In contrast, at least two lines of evidence support a one-step model for translocation of T-complexes and BHR plasmids. First, protein components of T-complexes, VirD2 and VirE2, are devoid of characteristic N-terminal signal sequences, suggesting that these proteins carry novel transport signals for export by a dedicated machinery. Second, as discussed in more detail below, the observed stabilizing activities of certain VirB proteins suggest that the VirB proteins assemble as a single multimeric complex that spans the *A. tumefaciens* envelope. In its simplest form, this complex of VirB proteins would correspond to a transenvelope translocation channel, possibly located at membrane fusion junctions. Other data, however, may support a two-step transfer model (99). Finally, it should also be kept in mind that a conjugal pore is only postulated to exist given that the bacterial cell envelope represents a significant barrier to the passage of macromolecules such as negatively charged nucleic acid polymers. It is formally possible that the physical coupling of donor and recipient cells during mating induces significant rearrangements in cell envelope components. In principle, such rearrangements could enable the transport of T-DNA by a process or structure that no longer involves all of the VirB proteins. In accordance with such a model, a DNA transport machinery could minimally consist of a protein complex at the cytoplasmic membrane which functions to (i) select appropriate substrates for transfer and (ii) supply a source of energy to drive translocation.

STRUCTURE-FUNCTION STUDIES OF THE T-COMPLEX TRANSPORT MACHINERY

Localization of VirB proteins at the *A. tumefaciens* envelope. The general organization of the VirB proteins at the *A. tumefaciens* cell surface has been characterized by subcellular fractionation, proteinase K susceptibility, and reporter protein fusion experiments (Table 1). Most of the VirB proteins display the property of fractionating with both membranes, in line with the view that these proteins assemble as a membrane-spanning protein channel (11, 19, 25, 31, 33, 47, 69, 75, 81, 84, 94). All of the VirB proteins except for VirB11 possess periplasmic domains, as shown by protease susceptibility and PhoA fusion experiments (9, 19, 25, 31, 94). VirB6 possesses six potential membrane-spanning domains and thus could be a candidate for assembling at the cytoplasmic membrane as a component of the conjugal pore (101). As described below in more detail, VirB1 is processed and a C-terminal truncation product, VirB1*, accumulates at the cell exterior and in the extracellular milieu (4). To date, VirB1* is the only *vir* gene product that has been shown experimentally to localize extracellularly. However, it should be noted that certain VirB proteins may be redistributed to the cell exterior or even be transferred to another cell upon contact with recipient cells. The F-plasmid transfer system establishes a precedent for protein redistribution during conjugation. In response to a mating signal, F pili are assembled from a reservoir of cytoplasmic membrane-associated TraA monomers (34). It is therefore of considerable interest that VirB2 exhibits sequence similarity to TraA (75), and like TraA, VirB2 is proteolytically processed from a 12-kDa propeptide to a 7-kDa mature product which fractionates with the cytoplasmic membrane (48, 75). Thus, at this juncture, candidate VirB proteins with an extracellular function include VirB1* and VirB2. Figure 1 depicts a hypothetical transporter complex developed from predicted locations of the VirB proteins and protein-protein interaction data described below.

The membrane topologies of VirB4 and VirB11, two generally hydrophilic ATPases required for DNA transfer, have been examined in some detail. Despite the notable absence of contiguous hydrophobic residues of sufficient length for formation of a membrane-spanning domain, only ~30% of VirB11 partitions with the soluble fraction (19, 69). The rest of the protein associates with the cytoplasmic membrane (19, 33, 69, 81, 84). Approximately one-half of this membrane-associated form completely resists extraction with chemical treatments, while the remaining half is released with urea (33, 69). These biochemical properties are atypical of peripheral or integral membrane proteins and suggest that VirB11 may exist in different states in vivo. In support of this view, mutations in the consensus Walker A nucleotide binding motif cause a shift in distribution almost entirely to the membrane-associated form, suggesting that VirB11 may have a nucleotide-dependent, dynamic interaction with the cytoplasmic membrane possibly analogous to *E. coli* SecA translocase (69). In contrast to VirB11, VirB4 associates tightly with the cytoplasmic membrane (11, 25). VirB4 appears to possess two exported domains in its N-terminal half, as deduced from reporter protein fusion and protease susceptibility experiments (25). Although the functional significance of these putative extracytoplasmic domains is unknown, one interesting possibility is that the transmembrane topology of VirB4 facilitates activation of the translocation process via ATP-induced conformational changes in the transenvelope mating channel (25).

Roles of *virB1* and related genes in transmembrane transport. The importance of individual *virB* genes for DNA transfer has been assessed by construction of nonpolar null mutations (11, 12, 24, 35, 78). Studies of a defined set of nonpolar mutants with precise *virB* gene deletions showed that *virB2* through *virB11* encode factors that are absolutely essential for DNA transfer to plants as well as to yeast and bacterial recipients (11, 12, 37, 65). By contrast, strains in which *virB1* had been deleted are still conjugation proficient, but DNA transfer efficiencies typically are reduced by 1 to 2 orders of magnitude (12). On the basis of these findings, it was suggested that VirB2 to VirB11 are essential core components of the T-complex transfer machinery and VirB1 plays an auxiliary role in transport (12). Recent studies have provided insights into how VirB1 might contribute to T-DNA transfer. VirB1 possesses motifs that are conserved among eukaryotic lysozymes and bacterial and bacteriophage lytic transglycosylases (6, 28, 57). These proteins belong to a larger family of hydrolytic enzymes that catalyze the cleavage of glycosidic bonds in peptidoglycan polymers (28). Such peptidoglycan-metabolizing enzymes have been found to be associated with other DNA and protein transport machineries and with cell surface structures including flagella and fimbriae (28).

As observed for *virB1*, null mutations of other transport-associated transglycosylase genes decrease the efficiency of substrate transfer (6, 28). On the basis of the widespread association of hydrolases with diverse systems that promote movement of molecules across the bacterial envelope, it is tempting to speculate that these enzymes function as morphogenetic determinants. Indeed, three phenotypes of *virB1* mutants support a role for VirB1 in assembly of the T-complex transfer machinery. First, a nonpolar *virB1* deletion mutant

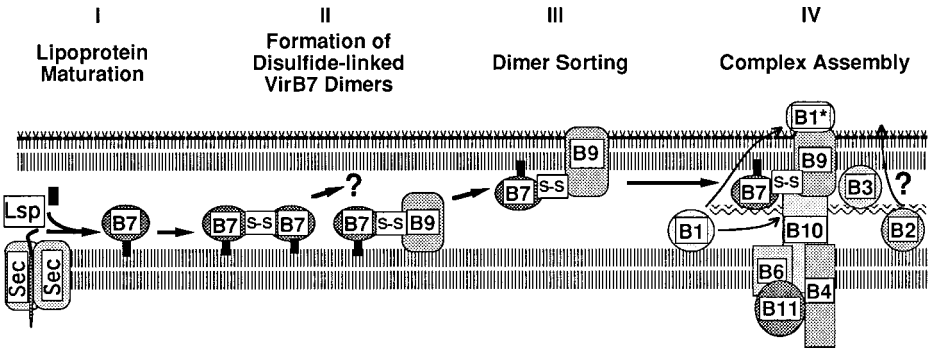

FIG. 1. A hypothetical model depicting early stages in the assembly of the T-complex transport apparatus. The model emphasizes the importance of a disulfide cross-linked VirB7-VirB9 (B7 s=s B9) heterodimer positioned at the outer membrane for recruitment and stabilization of VirB proteins. The black rectangle denotes fatty acid modification of the VirB7 (B7) lipoprotein, and Lsp is signal peptidase II. The dual role of VirB1 (B1) as a transglycosylase for local degradation of peptidoglycan (wavy lines) and as an extracellular factor (VirB1* [B1*]) for establishing contact with recipient cells is shown (thin arrows from VirB1). A proposed recruitment of VirB2 (B2) from a cytoplasmic membrane reservoir for pilus polymerization is indicated (thin arrow). Only the VirB proteins for which there is experimental evidence of close proximity based on chemical cross-linking data or on stabilizing protein interactions are shown in contact with one another. VirB5 (B5) and VirB8 (B8) are also likely components of this transport apparatus. Although the positioning of proteins is in accordance with predicted subcellular localizations, the model is not intended to portray spatial or stoichiometric relationships. Only the disulfide cross-linking of VirB7 and VirB9 has been conclusively demonstrated (1, 5, 7, 32, 79, 80).

accumulates aberrantly low levels of the VirB4 and VirB10 proteins (12). This could result from the inefficient assembly of a stabilized DNA conductance channel across the peptidoglycan layer. Second, as might be expected of cells deficient in transporter assembly, Δ*virB1* mutants do not elaborate the extracellular filaments detected on wild-type cells (37). Finally, *virB1* mutants harboring substitutions in the predicted active-site residues exhibit an attenuated virulence phenotype, supporting the notion that VirB1 functions as a transglycosylase (57). A VirB1 homolog, protein 19 of plasmid R1, also has been shown to be important for efficient conjugation as well as entry of bacteriophage R17, implicating a role for this protein in conductance of nucleic acids across the bacterial envelope (6).

VirB1 is also processed at residue 173, and the C-terminal 73 residue product, VirB1*, is found in the supernatant or in loose association with the outer membrane (4). VirB1* may be a component of the transport apparatus, as deduced from results of chemical cross-linking and immunoprecipitation experiments (4; see below). Thus, the N- and C-terminal portions of VirB1 are postulated to contribute two distinct functions for T-DNA transfer. An N-terminal transglycosylase activity may augment the efficiency of transporter assembly, and a secreted C-terminal product may contribute, possibly as a pilus subunit, to the formation or stabilization of contacts between *A. tumefaciens* and plant recipient cells (4).

Studies of the VirB4 and VirB11 ATPases. In addition to defining membrane configurations, studies of VirB4 and VirB11 have begun to examine possible enzymatic contributions of these proteins to T-DNA transport. The importance of intact Walker A nucleotide triphosphate binding motifs for function of both proteins was established by mutational analysis (11, 35, 69, 81). These findings, combined with demonstrations that purified forms of both proteins exhibit weak ATPase activities (19, 78), indicate that VirB4 and VirB11 couple the energy of ATP hydrolysis to transport. Both of these putative ATPases appear to contribute functions of general importance for macromolecular transport, since homologs have been identified among a large number of DNA and protein transport systems (Table 1) (52). Of further possible significance, VirB11 (19) and two homologs, TrbB of IncP RP4 (62) and EpsE of *Vibrio cholerae* (73) have also been reported to autophosphorylate. VirB4 and VirB11 may contribute to transport in at least two ways. By analogy to the ATPase subunits of the ATP binding cassette (ABC) transporter superfamily (30), these proteins may convert cognate transport machineries from inactive to active forms by inducing ATP-dependent conformational shifts or by stabilizing shifts that result from substrate binding. Alternatively, these proteins may function as chaperones to assist in morphogenesis of the transport apparatus.

Merodiploid studies have provided genetic evidence that VirB4 and VirB11 function as homo- or heteromultimers. Strains carrying wild-type *virB4* and alleles with mutations in the region that codes for the Walker A nucleotide triphosphate binding motif exhibit attenuated virulence on plants (11) and diminished transfer of IncQ plasmids to bacterial recipients (35). This transdominant phenotype is likely due to the interference of the VirB4 mutants on assembly of functional homo- or heteromultimers. Although no specific protein contacts have been identified, it is interesting to note that VirB4 synthesis is correlated both with the accumulation of VirB3 and its pattern of distribution during cellular fractionation (47). In contrast to these results, merodiploids carrying wild-type *virB11* and alleles with mutations in the region that codes for the Walker A motif exhibit wild-type virulence (69, 81). This lack of transdominance raises the possibility that an activity associated with ATP binding plays a role in VirB11 multimerization (81). Recently, it was shown that merodiploids expressing wild-type *virB11* and an allele composed of the 3′ half of the gene exhibits attenuated virulence. These findings suggest that VirB11 functions as a homo- or heteromultimer and, furthermore, the C-terminal half of VirB11 possesses a protein interaction domain (69). The Walker A motif is located in the C-terminal half of VirB11, and it is not known whether the C-terminal truncation derivative binds nucleotide. Thus, the hypothesis that nucleotide binding or hydrolysis modulates the oligomeric state of VirB11 still remains to be tested.

VirB7 lipoprotein and formation of stabilizing intermolecular disulfide bridges. Recent studies indicate that VirB7 is critical for assembly of a functional T-complex transport machinery. VirB7 possesses a characteristic signal sequence that ends with a consensus peptidase II cleavage site characteristic of bacterial lipoproteins. Evidence for maturation of VirB7 by a pathway elucidated for the major outer membrane lipopro-

tein of *E. coli* was supplied by in vivo labeling of VirB7 or VirB7::PhoA41 with [^{3}H]palmitate (5, 31) and processing inhibition of the proprotein with globomycin, a known inhibitor of signal peptidase II (31). Maturation of VirB7 as a lipoprotein is critical for its proposed role in T-complex transporter biogenesis (31 and see below). In agreement with sequence-based predictions, VirB7 fractionates predominantly with the outer membrane independently of the presence of other Ti plasmid-encoded proteins (31).

The VirB7 lipoprotein has been shown to interact directly with VirB9. The first hint of a possible interaction between these proteins was supplied by the demonstration that VirB9 accumulation is strongly dependent on cosynthesis of VirB7, suggestive of a possible stabilizing effect of VirB7 on VirB9 (12). Fernandez et al. (31) further showed that levels of VirB9 are modulated through the regulated synthesis of VirB7 in cells devoid of other VirB proteins. Immunoprecipitation (5, 79), two-dimensional gel electrophoresis (5, 80), and mutational studies (see below) conclusively established that the observed stabilizing activity is mediated through a direct interaction between VirB7 and VirB9. Interestingly, VirB7 assembles not only as VirB7-VirB9 heterodimers but also as homodimers (5, 79). There is also evidence that VirB9 assembles into higher-order multimeric complexes (1).

It is of particular interest that all homo- and heteromultimeric complexes identified in these studies resisted solubilization with heat and sodium dodecyl sulfate treatments but were readily dissociated upon exposure to reducing agents, indicating that the monomeric subunits are joined by disulfide bridges (1, 5, 7, 32, 79, 80). A covalent association was confirmed by the demonstration that mutant proteins with Ser substitutions for VirB7 Cys-24 and VirB9 Cys-262, the only internal Cys residues in these proteins, failed to form dimers (1, 79). Moreover, these mutant derivatives did not accumulate to high steady-state levels, indicating that disulfide bridge formation serves to stabilize both VirB7 and VirB9 monomers (79). As described below, the VirB7-VirB9 heterodimer is postulated to play a role in stabilizing other VirB proteins during assembly of a functional transporter (32, 79).

Whether the disulfide bridge is essential for dimer formation was the subject of recent investigation. Studies using the yeast two-hybrid screen supplied evidence for dimerization of VirB7 and VirB9 independently of disulfide bridge formation (5, 27). Furthermore, cooverproduction of Cys substitution mutants of VirB7 or VirB9 and the interactive native forms of VirB9 or VirB7, respectively, partially restores virulence of nonpolar Δ*virB7* or Δ*virB9* mutants (27). These findings suggest that VirB7 and VirB9 are capable of dimerizing independently of disulfide bond formation. However, protein stabilization data strongly suggest that disulfide cross-linking plays an important role in stabilizing the VirB7-VirB9 heterodimer (32, 79). It is therefore probable that, under the nonphysiological conditions of protein overproduction in *A. tumefaciens*, enough noncovalent VirB7-VirB9 heterodimers are formed to allow at least for the assembly of partially functional transporters. Once properly assembled, the transporter appears to be inherently stable (12, 32); at this point, there probably is no longer a requirement for disulfide-mediated stabilization of VirB7 and VirB9.

A recent report demonstrated that the VirB7 homolog PtlI in the PT secretion system forms a disulfide cross-link with the VirB9 homolog PtlF (29). In parallel to findings for VirB7 and VirB9, the formation of complexes is important for stabilization of PtlF (29). Thus, even at this early stage of structural analysis of these two transport systems, a common mechanism has been identified for stabilization of interactive subunits. Although there are numerous examples in which intramolecular disulfide bonds stabilize and activate secreted proteins, at least in eubacteria the formation of intermolecular disulfide bonds is an unusual mechanism for stabilizing interactive proteins. The novelty of this reaction mechanism is further highlighted by the finding that although VirB7 and VirB9 assemble as covalent dimers in *A. tumefaciens* and related species, dimer formation is not observed in *E. coli* (79). This observation raises the intriguing possibility that *A. tumefaciens* possesses novel factors not found in *E. coli* that are critical for formation of these intermolecular bridges (79).

What is the physiological significance of dimerization with respect to T-complex transport? Analysis of nonpolar null mutants showed that the absence of VirB7 or VirB9 synthesis is correlated with a reduction in steady-state levels of several other VirB proteins (12, 32). Furthermore, differential expression assays have shown that several VirB proteins accumulate in proportion to the abundance of the VirB7-VirB9 heterodimer (32). These findings suggest that the heterodimer plays an important role in stabilizing other VirB proteins during transporter biogenesis. If this hypothesis is correct, direct protein interactions should be demonstrable. Indeed, recent cross-linking studies have provided evidence for interactions between the heterodimer and two VirB proteins. A complex of VirB9 and VirB1* was recovered by chemical cross-linking of whole cells followed by cell lysis and immunoprecipitation with VirB9 antibodies (4). The importance of VirB7 for this interaction was not shown but may be assumed, since VirB9 accumulates to wild-type levels only in the context of the VirB7-VirB9 heterodimer. Given that VirB1* is proposed to localize at the exterior face of the outer membrane, these results suggest that VirB7-VirB9 heterodimer is positioned in such a way that an interactive surface of VirB9 protrudes through the outer membrane.

Two lines of study indicate that the heterodimer interacts with VirB10. First, a differential expression assay supplied evidence that the VirB7-VirB9 heterodimer stabilizes VirB10 in the absence of most other VirB proteins (32). Second, higher-order VirB10 complexes, obtained as a result of chemical cross-linking of whole cells or isolated membranes (94), are formed only in cells which synthesize VirB7 and VirB9 (7). The heterodimer is not a component of these higher-order complexes, but this could be due to inappropriate spacing of ε-amino groups of lysine residues for reaction with the cross-linking reagent used in these studies. Interestingly, point mutations clustered between residues 52 and 91 of VirB9 prevent cross-linking of VirB10, suggesting that the effect of the heterodimer on VirB10 complex formation involves a protein interaction domain localized within the first quarter of VirB9 (7).

AN ASSEMBLY PATHWAY FOR THE T-COMPLEX TRANSPORTER

These recent studies have identified several early reactions of an assembly pathway for the T-complex transport apparatus. According to the proposed pathway shown in Fig. 1, VirB7 and VirB9 monomers are exported and processed into mature polypeptides via a general secretion machinery analogous to the *E. coli sec* system. Monomers then assemble, most probably during protein folding, as covalently cross-linked dimers. Cross-linking serves to stabilize VirB7 or VirB9 homodimers or VirB7-VirB9 heterodimers. While each of these dimers ultimately may contribute to delivery of T-complexes to plant cells, the available data argue strongly that only the heterodimer is required for transporter biogenesis (32, 79). Next, the VirB7-VirB9 heterodimer is sorted to the outer membrane by an undefined mechanism. Once the heterodimer is properly po-

sitioned at the outer membrane, the next stage of assembly involves recruitment of other VirB proteins. Presumably, nucleation of a functional transport machinery is aided by the action of a VirB1 transglycosylase. The evidence for an interaction between VirB9 and VirB1* suggests that VirB9 spans the outer membrane and may provide an anchoring function for an extracellular structure. Similarly, the observed stabilizing interaction between the VirB7-VirB9 heterodimer and VirB10 highlights the importance of the proper positioning of an outer membrane-associated VirB complex for nucleation of a transenvelope translocation channel.

Even at this early stage of analysis, it is evident that several biochemical reactions postulated to guide assembly of the T-complex transport apparatus are also critical reactions in the assembly of other cell surface structures. First, lipoproteins are almost ubiquitously found as subunits of macromolecular surface structures. Thus, lipoprotein biosynthesis and sorting reactions must occur early during the associated assembly processes. Lipoproteins may simply represent structural components of these surface structures or, as shown for VirB7, play more fundamental roles as morphogenetic determinants. At least two outer membrane lipoproteins have been shown to function as nucleating or stabilizing proteins: (i) PulS lipoprotein, which positions and stabilizes PulD, a proposed outer membrane "gatekeeper" subunit of the general secretory pathway involved in pullulanase secretion (42), and (ii) FlgH, which assembles at the outer membrane as the basal body L-ring component of *E. coli* flagella (55). In addition, the observed stabilizing effect of PtlI on PtlF (29) suggests that the PtlI-PtlF complex may function similarly to the VirB7-VirB9 heterodimer as a nucleation center for assembly of the PT transporter. Second, as for VirB7 and VirB9, disulfide bridges stabilize secreted subunits of several macromolecular surface structures. In *E. coli*, a disulfide bond formation pathway comprised of the Dsb proteins catalyzes formation of such disulfide bridges (3). Roles for Dsb proteins or Dsb homologs have been documented in assembly of various protein secretion systems, *Haemophilus influenzae* competence machinery, *E. coli* flagella, *Vibrio cholera* toxin-coregulated colonization pilus, and *Rhodobacter capsulatus c*-type cytochromes (3). Third, Dijsktra and Keck (28) have presented a strong case for the general importance of peptidoglycan-metabolizing enzymes such as the VirB1 transglycosylase for assembly of numerous transenvelope structures. Finally, as postulated for VirB4 and VirB11, ATPase subunits of transport systems likely supply energetic requirements for assembly or function of cognate surface structures (44). In addition to these conserved reaction mechanisms, sequence relationships among machinery components continue to be unveiled, underscoring the notion that bacteria have appropriated a limited set of biochemical strategies and structural building blocks for assembly of a vast array of functionally diverse surface structures.

CONCLUSIONS AND FUTURE DIRECTIONS

Significant contributions by numerous laboratories working in the area of *A. tumefaciens* pathogenicity have begun to shed light on molecular details of the T-DNA translocation process. Additional insights will be gained by testing and refining the proposed assembly pathway described above. Future studies also need to address the following questions. (i) What is the nature of the substrate recognition signal(s)? (ii) What is the role of ATP hydrolysis in transporter assembly or function? (iii) What is the translocation route across the cell envelope? (iv) What is the role of the sex pilus in conjugation? (v) What features contribute to the apparent promiscuity of the T-complex transport system? A recent resurgence of interest in conjugation processes, fueled in part by the enormous biotechnological importance of the *A. tumefaciens* plant transformation system and in part by a heightened concern over the widespread dissemination of antibiotic resistance determinants among bacterial pathogens, should provide the necessary momentum for obtaining answers to these questions.

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