Published in final edited form as:

Curr Top Microbiol Immunol. 2018 ; 418: 233–260. doi:10.1007/82_2018_94.

The *Agrobacterium* VirB/VirD4 T4SS: Mechanism and Architecture Defined Through In Vivo Mutagenesis and Chimeric Systems

Yang Grace Li, Peter J Christie

Department of Microbiology and Molecular Genetics, McGovern Medical School, 6431 Fannin St, Houston, TX 77030, USA

Abstract

The *Agrobacterium tumefaciens* VirB/VirD4 translocation machine is a member of a superfamily of translocators designated as type IV secretion systems (T4SSs) that function in many species of gram-negative and gram-positive bacteria. T4SSs evolved from ancestral conjugation systems for specialized purposes relating to bacterial colonization or infection. *A. tumefaciens* employs the VirB/VirD4 T4SS to deliver oncogenic DNA (T-DNA) and effector proteins to plant cells, causing the tumorous disease called crown gall. This T4SS elaborates both a cell-envelope-spanning channel and an extracellular pilus for establishing target cell contacts. Recent mechanistic and structural studies of the VirB/VirD4 T4SS and related conjugation systems in *Escherichia coli* have defined T4SS architectures, bases for substrate recruitment, the translocation route for DNA substrates, and steps in the pilus biogenesis pathway. In this review, we provide a brief history of *A. tumefaciens* VirB/VirD4 T4SS from its discovery in the 1980s to its current status as a paradigm for the T4SS superfamily. We discuss key advancements in defining VirB/VirD4 T4SS function and structure, and we highlight the power of in vivo mutational analyses and chimeric systems for identifying mechanistic themes and specialized adaptations of this fascinating nanomachine.

1 Introduction: A VirB/VirD4 T4SS Primer

Agrobacterium tumefaciens delivers oncogenic T-DNA and several effector proteins to plant target cells via a type IV secretion system (T4SS) (Alvarez-Martinez and Christie 2009). This nanomachine, elaborated by the 11 VirB proteins (VirB1–VirB11) and the VirD4 subunit, is termed the VirB/VirD4 T4SS. Evidence for the existence of an envelope-spanning T-DNA transfer system, and clues that this system functions by a conjugation-like mechanism, arose through discoveries resulting from sequencing of pTi plasmids nearly 30 years ago (Beijersbergen et al. 1994; Kulda et al. 1990; Shirasu et al. 1990; Ward et al. 1988). Sequencing of the *virB* locus revealed similarities between the predicted VirB proteins and subunits of conjugation or mating-pair-formation (Mpf) systems. Further sequence analyses revealed similarities between the VirD subunits and the DNA transfer and replication (Dtr) factors involved in processing at origin-of-transfer (*oriT*) sequences

associated with conjugative plasmids (Stachel and Zambryski 1986). In quick succession, studies demonstrated nicking activities of VirD1 and VirD2 at T-DNA border repeats in *A. tumefaciens* and *Escherichia coli* and then in vitro using purified proteins (Albright et al. 1987; Filichkin and Gelvin 1993; Stachel et al. 1987; Veluthambi et al. 1988; Yanofsky et al. 1986; Young and Nester 1988). The VirB/VirD4 system also was shown to transfer a conjugative element, the IncQ plasmid RSF1010, which lacks genes for its own T4SS but codes for Dtr factors that both process the plasmid and mobilize its conjugative transfer through various T4SSs. The *A. tumefaciens* system was first shown to deliver RSF1010 to plant cells and subsequently to other agrobacterial cells (Beijersbergen et al. 1992; Buchanan-Wollaston et al. 1987; Fullner 1998; Ward et al. 1991). These findings established that the VirB system functions as a *bona fide* conjugation system in mediating both interbacterial and interkingdom DNA transfer.

The next major conceptual advance in our understanding of the VirB system in a broader biological context resulted from the discovery in the mid-1990s that the 11 VirB subunits, and homologous Tra subunits encoded by conjugation systems, are also related to subunits of the pertussis toxin (PT) export system of *Bordetella pertussis* (Shirasu and Kado 1993; Winans et al. 1996). This observation expanded our view of the versatility of conjugation-like machines; over evolutionary time, ancestral conjugation machines acquired novel functions enabling interkingdom trafficking of DNA and protein substrates during infection processes. Systems assembled from homologs of VirB and Tra subunits were then grouped as a new secretion superfamily, the type IV secretion systems or T4SSs (Christie 1997; Salmond 1994). With the explosion of sequenced bacterial genomes and mechanistic studies deciphering infection processes during the 1990s and 2000s, new T4SSs were discovered so that now this superfamily includes translocators functioning in most if not all bacterial species (Bhatty et al. 2013). All systems bear at least a few signature subunits enabling their classification, but in fact the T4SSs are a highly compositionally, structurally, and functionally diverse superfamily. This is evidenced at a functional level by the ability of these systems to (i) deliver mobile genetic elements (MGEs) to other bacteria and to eukaryotic cells, (ii) deliver protein substrates (termed ‘effectors’) to eukaryotic target cells, (iii) inject a protein toxin to bacterial recipients, or (iv) export or import DNA or protein substrates to or from the extracellular milieu (Grohmann et al. 2018). Indeed, the *A. tumefaciens* VirB/VirD4 system itself exhibits striking versatility in its ability to translocate DNA and various protein effectors to plant cells during infection, as well as DNA to other agrobacteria, fungi, and even human cells (Beijersbergen et al. 1992; Bundock et al. 1995; Kunik et al. 2001; Piers et al. 1996). From the early discovery of the *A. tumefaciens* VirB/VirD4 T4SS in the 1980s through today, contributions by many laboratories have helped shape this system as an important paradigm for the T4SS superfamily. In this chapter, we summarize recent progress toward defining the structure and function of the VirB/VirD4 T4SS, with a focus on insights gained through mutational analyses and substitutions of domains, subunits, or entire machine subassemblies with related systems.

2 Overview of the VirB/VirD4 Machine Assembly and Architecture

The *virB* gene arrangements and functions of their products are depicted in Fig. 1a. The *virB* genes comprise an operon inducibly expressed from an upstream *virB* promoter upon

sensing by *A. tumefaciens* of plant-derived phenolic compounds, although evidence exists for at least one or two additional internal promoters (Berger and Christie 1994; Stachel and Nester 1986). *virD4* is coexpressed with *virD* genes encoding the Dtr processing factors (Stachel and Nester 1986). Studies defining subcellular localizations and membrane topologies of VirB and VirD4 subunits, coupled with definition of subunit interaction networks by two-hybrid and pull-down assays, provided an early view of the architectural arrangement of the VirB/VirD4 T4SS across the *A. tumefaciens* cell envelope (Christie 1997). Importantly, this body of work included the discovery that the outer membrane (OM) lipoprotein VirB7 interacts via a disulfide cross-link with VirB9, and this heterodimer in turn forms a stabilizing interaction with the cell-envelope-spanning subunit VirB10 (Anderson et al. 1996; Baron et al. 1997; Fernandez et al. 1996; Spudich et al. 1996). The VirB7/VirB9/VirB10 complex was originally designated as the ‘core’ complex and now is termed the outer membrane core complex or OMCC (Christie 1997; Grohmann et al. 2018). Once assembled, the OMCC stabilizes other VirB subunits localized in the periplasm or inner membrane (IM) (Beaupre et al. 1997; Fernandez et al. 1996). These early findings led to a proposal that the VirB/VirD4 machine is built by the ordered assembly of the OMCC followed by recruitment and assembly of an inner membrane complex (the IMC) (Christie 1997). The VirB/VirD4 system also is composed of three ATPases (VirB4, VirB11, and VirD4) localized in the cytoplasm or at the cytoplasmic face of the IM. These ATPases are not required for assembly of the OMCC, but rather form part of the IMC either stably or transiently (see below).

In addition to the envelope-spanning translocation channel, the VirB subunits elaborate an extracellular pilus termed the T-pilus (Fullner et al. 1996; Kado 2000). Mutational studies identified interesting classes of mutations that selectively prevent assembly of a functional translocation channel but not the T-pilus (Tra⁻, Pil⁺) or, conversely, inhibit assembly of the T-pilus production but not the translocation channel (Tra⁺, Pil⁻). These ‘uncoupling’ mutations have proven highly informative for assigning VirB and VirD4 building blocks of the channel vs T-pilus. Cells lacking VirD4, for example, are Tra⁻ but elaborate wild-type T-pili. By contrast, cells lacking the VirB1 lytic transglycosylase efficiently transfer substrates but fail to elaborate T-pili. Further studies identified ‘uncoupling’ mutations in the VirB11 ATPase, the IMC subunit VirB6, and the OMCC subunits VirB9 and VirB10 (Christie et al. 2005; Jakubowski et al. 2005; Sagulenko et al. 2001; Zhou and Christie 1997). These findings provided strong evidence that the VirB/VirD4 system alternatively assembles as the translocation channel or T-pilus. In an infection setting, it makes sense that upon activation of the *vir* regulon by plant-derived signals, the VirB system would elaborate T-pili for bacterial attachment and colonization of plant tissues. Then, once productive bacterial-plant cell contacts are established, a second signal would stimulate a transition from the T-pilus assembly mode to formation of the translocation channel, in part through recruitment of the VirD4 ATPase for interkingdom transfer of T-DNA and effector proteins (Christie et al. 2005). From a more mechanistic perspective, the ability to isolate Tra⁺, Pil⁻ ‘uncoupling’ mutations also strongly indicates that substrates are not translocated through the pilus, which is consistent with early genetic and structural findings that *E. coli* conjugation systems mediate the formation of tight donor–target cell junctions as a prerequisite for DNA transfer (see Durrenberger et al. 1991; Samuels et al. 2000).

3 Defining the Route of DNA Transfer Through the VirB/VirD4 T4SS

Although a general architecture of the VirB/VirD4 T4SS was inferred from fractionation and topology studies (See Christie 1997), direct evidence that the VirB subunits form a translocation channel was lacking until a modified chromatin immunoprecipitation assay was employed to identify subunit contacts with the translocating T-DNA (Fig. 1b) (Cascales and Christie 2004b). In this assay, termed transfer DNA immunoprecipitation (or TrIP), *A. tumefaciens* cells were induced for assembly of the VirB/VirD4 T4SS and treated with formaldehyde (FA) to cross-link channel subunits to the translocating DNA substrate. Following detergent solubilization of the cell envelope, antibodies to the VirB and VirD4 subunits were used to immunoprecipitate the respective machine component and the precipitated material was analyzed for the presence of T-DNA by PCR amplification. In a semiquantitative variation of this assay, termed QTrIP, labeled radionucleotide was added in the logarithmic phase of PCR product amplification. By TrIP, six of the 12 VirB/VirD4 subunits formed FA-crosslinkable contacts with the T-DNA transfer intermediate as well as that of the RSF1010 plasmid substrate. These proteins included the VirD4 and VirB11 ATPases residing at the cytoplasmic face of the IM, the integral IM subunits VirB6 and VirB8, the VirB2 pilin, and the OM-associated VirB9 (Fig. 1b). TrIP studies with various mutant strains and strains producing subsets of VirB and VirD4 proteins confirmed this sequence of DNA–VirB/VirD4 subunit contacts and further identified contributions of the remaining VirB subunits to substrate transfer across the cell envelope (Atmakuri et al. 2004; Jakubowski et al. 2004, 2005).

The DNA substrate first contacts the VirD4 ATPase, which can occur even in the absence of other VirB proteins (Fig. 1b, c) (Cascales and Christie 2004b). Mutations in the nucleotide-binding site of VirD4 did not abrogate DNA binding, indicating that ATP energy does not drive the VirD4–DNA substrate interaction (Atmakuri et al. 2004). In a second step of the transfer pathway, VirD4 delivers the DNA to the VirB11 ATPase (Cascales and Christie 2004b). This transfer reaction requires VirD4 and VirB11, but proceeds independently of other IM-associated subunits. Mutations in the nucleotide sites of both proteins also did not affect DNA binding, further indicating that ATP energy also does not drive DNA substrate transfer from VirD4 to VirB11. Interestingly, in reconstitution experiments, this transfer step proceeds only if the core subunits (VirB7–VirB10) are coproduced, which led to a proposal that VirD4 and/or VirB11 physically interact with one or more components of the core complex for substrate transfer (Atmakuri et al. 2004). In a third reaction, VirD4 and VirB11 coordinate with the third ATPase, VirB4, to deliver the DNA substrate to two integral IM proteins, VirB6 and VirB8 (Cascales and Christie 2004b). This is an ATP energy-driven reaction, as evidenced by a lack of substrate transfer in strains bearing NTP-binding site mutant forms of any of the three ATPases (Atmakuri et al. 2004). The reconstitution experiments further showed that the core complex also is essential for DNA transfer to VirB6 and VirB8, suggesting a coordination of function between the IM- and the OM-associated subassemblies. Finally, VirB6 and VirB8 deliver the DNA substrate to the VirB2 pilin and the core subunit VirB9. Mutational analyses established that the N- and C-terminal regions of polytopic VirB6 contribute to the delivery of DNA to VirB2 and VirB9, which was consistent with evidence that the IM subassembly interacts directly with the OM-

associated channel (Jakubowski et al. 2003). Based on results of these TrIP studies, it was postulated that the VirB2 pilin forms a part of the cell-envelope-spanning channel (Jakubowski et al. 2005). However, efforts to detect substrate interactions with the extended T-pilus were unsuccessful, further arguing against a role for the T-pilus in mediating T-DNA transfer to plant cells (Cascales and Christie 2004b).

Results of the TrIP studies provided the first direct evidence that MGEs are translocated across the cell envelope through a conjugative machine. Furthermore, these studies identified presumptive components of the ‘mating’ channel as VirD4, VirB11, VirB6, VirB8, VirB2, and VirB9. Other VirB subunits, e.g., VirB3, VirB4, VirB5, VirB7, VirB10, did not cross-link with the substrate, but null mutations blocked formation of specific substrate–channel subunit contacts in the transfer pathway, establishing their importance for specific stages of substrate translocation (Cascales and Christie 2004b). Gratifyingly, the route of DNA transfer defined using the TrIP assay is compatible with results of biochemical studies defining subcellular locations and interactions among the VirB and VirD4 subunits, as well as more recent ultrastructural findings (Fig. 1b, c). With respect to the latter, the *E. coli* conjugative plasmid R388 codes for a T4SS, termed Trw, which is closely related phylogenetically to the VirB/VirD4 T4SS. A large substructure of the Trw T4SS was purified and structurally analyzed by single-particle negative-stain transmission electron microscopy (NS-EM) (Low et al. 2014). The complex was termed the VirB₃₋₁₀ substructure for the fact it is composed of homologs of the *A. tumefaciens* VirB3 through VirB10 subunits. Noteworthy features of the VirB₃₋₁₀ substructure include: (i) a barrel-shaped OMCC of a size and architecture similar to that of the OMCCs associated with the pKM101 Tra and *A. tumefaciens* VirB/VirD4 T4SSs (see below), (ii) a thin stalk structure connecting the OMCC to the IMC, and (iii) a highly asymmetric IMC composed of an inner membrane platform and two side-by-side hexamers of the VirB4-like ATPase extending into the cytoplasm. In the most recent structure, a dimer of VirD4-like TrwB was associated with the base of the IMC, sandwiched between the two VirB4 hexamers (Redzej et al. 2017). In view of sequence and compositional similarities, it is reasonable to speculate that the VirB₃₋₁₀/VirD4 complex is a structural archetype for the VirB/VirD4 system and other closely related T4SSs. By combining results of the TrIP and ultrastructural studies, possible transfer routes for DNA substrates across the VirB/VirD4 T4SS can be envisioned (see Fig. 1b, c & below).

4 Processing of T-DNA and Effector Protein Substrates and Substrate Recognition Signals for Transfer

Several steps of the DNA transfer pathway delineated by the TrIP studies have been analyzed in molecular and structural detail. The initiating step of T-DNA transfer involves its recruitment to the VirB/VirD4 T4SS. In conjugation systems, prevailing models depict the relaxosome as the set of processing factors bound as a preinitiation complex at *oriT* (de la Cruz et al. 2010). This quiescent complex is activated upon binding to the VirD4 substrate receptor, which results in a switch in the relaxase to a translocation mode (Lang et al. 2011). The active relaxase cleaves the T-strand, the T-strand is unwound from its template strand, and the relaxase pilots the T-strand through the transfer channel (de la Cruz et al. 2010). Among the conjugation systems, therefore, processing of the transferred strand is

spatiotemporally linked to translocation through the conjugation machine. For T-DNA, the process is formally similar in the sense that the VirD2 relaxase is guided to the *oriT*-like T-DNA border sequence by the VirD1 accessory factor to catalyze nicking of the T-strand for transfer (Yanofsky et al. 1986; Albright et al. 1987; Stachel et al. 1987). However, there are also important differences in the requirements for formation of the catalytically active relaxosome (see Fig. 2). For example, efficient recruitment of VirD2 to the T-DNA border sequences requires not only VirD1 but also two other Vir proteins, VirC1 and VirC2 (Atmakuri et al. 2007; Lu et al. 2009). These latter proteins bind a sequence termed *overdrive* located immediately adjacent to the right border repeat sequences of octopine-type Ti plasmids (Toro et al. 1989). Binding of the VirD and VirC accessory factors stimulates T-DNA processing and also leads to the generation of many copies (~30 or more) of free VirD2-T-strand transfer intermediates in a cell (Atmakuri et al. 2007; Veluthambi et al. 1988). VirC1 is a member of the ParA family of ATPases that mediate partitioning of chromosomes and plasmids during cell division. VirC1 localizes at *A. tumefaciens* cell poles to recruit the VirD2-T-strand complex to the cell poles and to interact with VirD4, the substrate receptor for the VirB/D4 T4SS (Atmakuri et al. 2007). *A. tumefaciens* therefore adapted an ancestral Par-like function for the novel purposes of stimulating a conjugative DNA-processing reaction and promoting DNA substrate docking with a cognate T4SS receptor. Both activities potentially mediate transfer of many copies of T-DNA from each bacterium to susceptible plant host cells, presumably for enhanced probability of infection. At a molecular level, this implies that the relaxosomal complex is activated in the absence of binding to VirD4, and also that unwinding of the T-strand from its template is temporally and spatially uncoupled from its engagement with VirD4 and translocation through the VirB/VirD4 channel (Fig. 2).

How the complex of VirC and VirD proteins bound to the T-strand engage with the VirB/VirD4 T4SS is presently not known, but some general features of this interaction have been identified. For example, VirD2 carries a translocation sequence at its C terminus that mediates its translocation, as demonstrated with the Cre recombinase reporter assay for translocation (CRAFT) (Vergunst et al. 2000, 2005). In this assay, full-length or fragments of protein substrates, e.g., VirD2, are fused at their N-termini to Cre recombinase and translocation is monitored to a bacterial or plant target cell engineered to carry *lox* sites arrayed so that Cre-mediated excision yields a reporter activity, e.g., restoration of antibiotic resistance or GFP fluorescence. By use of the CRAFT assay, VirD2's C-terminal translocation signal was shown to consist of a cluster of positively charged Arg residues, leading to a proposal that the relaxase engages with VirD4 at least in part through ionic interactions. The VirD2 part through ionic interactions. The VirD2-T-strand-VirD4 interaction is considerably more complex than this, however, as evidenced by recent work showing that VirD2 additionally carries an internal signal(s) conferring recognition by the VirB/VirD4 machinery (van Kregten et al. 2009). Furthermore, as noted above, the VirC and VirD1 accessory factors form a ternary complex with VirD2 and VirD4, pointing to factors other than just VirD2 for coupling of the VirD2-T-strand substrate with the receptor. Indeed, chromosomal factors termed VirD2-binding proteins (VBPs) also promote recruitment of the VirD2-T-strand complex to VirD4 (Guo et al. 2007; Padavannil et al. 2014). How the VBPs and VirD1/C1/C2 accessory factors coordinate with each other and the VirD2-T-strand

transfer intermediate to control reiterative rounds of substrate–VirD4 docking temporally and spatially remains to be defined (Fig. 2).

The VirB/VirD4 T4SS also translocates several effector proteins, including VirD5, VirE2, VirE3, and VirF (from *A. tumefaciens*), and GALLS-CT and GALLS-FL (from *Agrobacterium rhizogenes*) to target cells (Fig. 2) (Hodges et al. 2006; Schrammeijer et al. 2003; Simone et al. 2001; Vergunst et al. 2000). These proteins are translocated independently of the VirD2–T-strand substrate through the VirB/VirD4 T4SS. Where characterized, these effectors carry positively charged C-terminal domains that are required for engagement with VirD4. Although several are translocated independently of a requirement for a secretion chaperone, the single-stranded binding protein VirE2 must bind its cognate chaperone, VirE1, in *A. tumefaciens* to prevent self-aggregation and premature binding to the T-DNA substrate prior to translocation (Atmakuri et al. 2003; Vergunst et al. 2003; Zhao et al. 2001).

5 VirD4 Receptors

The VirD4 receptors are alternatively termed type IV coupling proteins or T4CPs because they link substrates to the T4SS (Cabezón et al. 1997). VirD4-like ATPases are associated with nearly all T4SSs, and the presence of a *virD4*-like gene in sequenced bacterial genomes has served as a marker for a possible T4SS gene cluster (Bhatty et al. 2013). A role for VirD4 subunits in substrate reception was first suggested in early genetic studies showing that VirD4 subunits from different T4SSs can sometimes be exchanged (Cabezón et al. 1997). Specifically, the VirD4 homologs TraG and TrwB, encoded respectively by the RP4 and R388 conjugation systems, substituted for each other in mediating transfer of the promiscuous IncQ plasmid RSF1010 through the heterologous R388 and RP4 mating channels. Similarly, *A. tumefaciens* VirD4 functionally substituted for its homolog pTiC58-encoded TraG in supporting transfer of RSF1010 through the pTiC58 channel (Hamilton et al. 2000). These early receptor-swapping studies highlighted two features of the VirD4 subunits. First, these receptors have evolved to recognize specific repertoires of substrates, although promiscuous IncQ plasmids also have evolved mechanisms to bypass certain substrate specificity checkpoints (see below). Second, VirD4 subunits can functionally engage with heterologous translocation channels. This latter finding is in line with recent results of biochemical fractionation and ultrastructural studies indicating that the VirD4 receptors associate only peripherally, and possibly transiently, with the translocation channel (Larrea et al. 2013; Redzej et al. 2017). Further studies defining the ‘coupling’ activities of VirD4 receptors in substrate recruitment and channel association are described below.

VirD4 subunits are composed of three or four domains that contribute in distinct ways to receptor or ‘coupling’ functions (Fig. 3) (Alvarez-Martinez and Christie 2009; Gomis-Ruth et al. 2001). Broadly, their N-terminal transmembrane domains (NTDs) are implicated in forming contacts with T4SS channel subunits, and the C-proximal cytosolic moieties mediate substrate docking and energize substrate transfer reactions (Atmakuri et al. 2004; Hormaeche et al. 2004, 2006; Llosa et al. 2003). An X-ray structure of the soluble, ~50-kDa cytoplasmic domain of the TrwB receptor encoded by the conjugative plasmid R388, revealed a globular hexameric assembly in which each subunit is composed of two distinct

domains, a nucleotide-binding domain (NBD) and a 7-helix motif called the all- α -domain (AAD) that faces the cytoplasm. The six TrwB protomers assemble to form a globular ring that is ~110 Å in diameter and 90 Å in height, with a ~20-Å-wide channel in the center that constricts to 8 Å at the cytoplasmic pole (Gomis-Ruth et al. 2001). While lacking among homologs associated with well-characterized *E. coli* conjugation systems (e.g., pKM101, R388), many subunits including *A. tumefaciens* VirD4 also possess sequence-variable C-terminal domains (CTDs) that are typically enriched in acidic residues (Alvarez-Martinez and Christie 2009; Kwak et al. 2017).

5.1 VirD4's NTD: Spatial Positioning and Interaction with the IMC

The N-terminal 87 residues of VirD4 are composed of two membrane-spanning α -helices and an intervening periplasmic loop (Fig. 3) (Das and Xie 1998). Deletion of this NTD does not abolish VirD4's ability to bind the T-strand or VirE2 substrates, but does block substrate transfer to the VirB11 ATPase (Atmakuri et al. 2004; Cascales et al. 2013). These findings suggest that integration of VirD4 into the membrane or NTD-mediated interactions with VirB channel subunits is required for a productive interaction with VirB11. Interestingly, deletion of the entire 30-residue periplasmic loop similarly abolishes substrate transfer to VirB11, whereas smaller deletions of this loop are permissive for this transfer step (Cascales et al. 2013). Early studies showed that VirD4 localizes at *A. tumefaciens* cell poles even in the absence of other VirB channel components. Deletion of the NTD blocks polar localization, establishing the importance of the N-terminal motif for spatial positioning (Atmakuri et al. 2003; Kumar and Das 2002). VirD4's NTD also interacts with VirB10, which as discussed below is required for transduction of intracellular signals and gating of the distal portion of the VirB channel (see Sect. 6.4).

5.2 VirD4's AAD: Substrate Specificity

The AADs of VirD4 subunits were proposed to play a role in substrate binding, based on structural evidence that the AAD is positioned at the base of the TrwB homohexamer in the crystal structure (Fig. 3) (Gomis-Ruth et al. 2001). These studies further established that the AAD structurally resembles the DNA-binding domain of XerD recombinase, suggestive of a DNA-binding activity (Gomis-Ruth et al. 2001). Mutational studies defined the importance of TrwB's AAD for substrate trafficking, and in the *A. tumefaciens* system a deletion of VirD4's AAD also blocked substrate transfer (de Paz et al. 2010; Whitaker et al. 2015). Further evidence that the VirD4's AAD contributes to substrate recruitment was gained from AAD swapping studies, which showed that VirD4 bearing a heterologous AAD did not support transfer of T-DNA and the VirE2 effector protein to plant cells (Whitaker et al. 2015). Strikingly, however, this VirD4::AAD chimera supported transfer of the mobilizable IncQ plasmid RSF1010 between agrobacterial cells. Furthermore, a purified form of VirD4's AAD bound VirD2 but not VirD1 or heterologous processing factors, and also bound DNA although without sequence or strand specificity. Finally, deletion of VirD2's C-terminal translocation signal did not affect VirD2's relaxase activity but did abolish binding to VirD4 in vitro and correspondingly blocked *A. tumefaciens*-mediated T-DNA transfer to plants (Whitaker et al. 2015). Taken together, these findings support a model in which VirD4's AAD recruits the VirD2 relaxase and covalently associated T-strand through binding of VirD2's C-terminal translocation signal. Interestingly, however, the promiscuous IncQ

plasmid RSF1010 retains the ability to bind VirD4 by a mechanism that bypasses the AAD substrate specificity checkpoint (Whitaker et al. 2015).

5.3 VirD4's CTD: Regulator of Substrate Transfer

VirD4 also has a long (104-residue) CTD enriched in acidic residues, which is required for T-strand transfer to plants. Studies exploring the function of VirD4's CTD exploited a discovery that the 'conjugation' system encoded by the *E. coli* plasmid pKM101 can be reconfigured to deliver protein substrates to bacterial recipients by use of engineered chimeric receptors (Fig. 3) (Whitaker et al. 2016). These chimeras consist of the NTD of the VirD4-like TraJ receptor of the pKM101 system joined to the cytosolic moieties of VirD4 homologs associated with effector translocator systems. In the case of the *A. tumefaciens* VirB/VirD4 system, the chimeric receptor is composed of TraJ's NTD fused to VirD4's NBD/AAD/CTD domains. Intriguingly, this chimeric receptor functionally interacted with the pKM101 translocation channel to support conjugative transfer to *E. coli* recipients of the IncQ plasmid RSF1010, as well as several effector proteins (VirE2, VirF, VirE3) as monitored with the CRAFT assay (Fig. 3). Deletion of VirD4's CTD from the chimeric receptor conferred differential effects on protein trafficking (Whitaker et al. 2016). The

CTD variant failed to support VirE2 translocation, but conferred elevated transfer of VirE3 and VirF through the pKM101 channel. Corresponding studies with chimeric receptors composed of receptor domains from VirD4 subunits from *Anaplasma phagocytophilum* and *Wolbachia pipientis* also supported transfer of known or candidate effector proteins from these species through the pKM101 channel. These VirD4 subunits also have long, acidic CTDs, and deletion of the CTDs from the chimeric receptors also attenuated or enhanced transfer frequencies of different protein effectors (Alvarez-Martinez and Christie 2009; Whitaker et al. 2016). Taken together, these findings support the notion that the CTDs of VirD4 receptors have evolved both for expansion of the effector repertoire and as a regulatory domain to exert spatiotemporal control of effector presentation to the T4SS (Fig. 3).

6 The Envelope-Spanning VirB/VirD4 T4SS

6.1 Cytoplasmic Entrance: The ATPase Energy Center

In *A. tumefaciens*, VirD4 physically and functionally interacts with the VirB4 and VirB11 ATPases to drive substrate transfer through the T4SS (Atmakuri et al. 2004; Cascales and Christie 2004b; Pena et al. 2012; Ripoll-Rozada et al. 2013; Savvides et al. 2003). The VirB₃₋₁₀/VirD4 substructure presents a view of the spatial arrangement of VirB4 hexamers and VirD4 dimers, but at this time it is thought that the VirD4 receptors function in vivo as homohexamers. This, coupled with the lack of a VirB11 subunit in the R388-encoded structure, limits our knowledge of how the three ATPases physically and functionally interact to drive early-stage substrate processing reactions. Whereas VirD4 and VirB4 subunits are phylogenetically related and likely adopt similar hexameric structures, VirB11 is by contrast a member of a large family of ATPases termed 'traffic ATPases' that are associated with the types II, III, and IV secretion systems (Kato et al. 2015; Pena and Arechaga 2013; Savvides 2007). VirB11-like ATPases cofractionate with the cytoplasm and IM, suggestive of a dynamic association with the membrane or the VirB/VirD4 T4SS

(Rashkova et al. 1997). VirB11 homologs form stable hexameric rings of ~100–120 Å in diameter (Savvides et al. 2003; Yeo et al. 2000). The N- and C-terminal halves of the six protomers each form rings, giving rise to a double-stacked structure wherein the nucleotide-binding site is at the interface between the two domains. EM studies have shown that the VirB11 hexamers undergo dynamic structural changes upon ATP binding and hydrolysis, although the functional importance of these transitions is not known at this time (Savvides et al. 2003). VirD4 delivers the recruited DNA substrate to the VirB11 ATPase (Fig. 2). As mentioned earlier, this transfer step does not require ATP hydrolysis by either subunit, suggestive of a direct pass-off from one to the other subunits independently of any energy-driven conformational changes (Atmakuri et al. 2004). Mutational studies have further defined contributions of the VirD4 and VirB11 ATPases to early and late stages of substrate transfer. With regard to the early-stage transfer reactions, VirD4 deleted of its NTD retained the ability to bind the T-DNA substrate, but failed to transfer the substrate to VirB11, suggesting that VirD4 must be docked at the IM to productively engage with VirB11 (Cascales et al. 2013). Also as noted earlier, transfer of T-DNA from VirD4 to VirB11 requires cosynthesis of VirB7, VirB9, and VirB10 (Atmakuri et al. 2004). VirB10 is likely responsible for communicating directly with VirD4 to activate this first substrate transfer step, as suggested by evidence that VirD4 interacts with VirB10 in the *A. tumefaciens* VirB/VirD4 T4SS as well as the related *E. coli* plasmid R388 transfer system (Atmakuri et al. 2004; de Paz et al. 2005; Llosa et al. 2003).

Mutational analyses of VirB11 have been informative in deciphering the role of VirB11 in substrate transfer. For example, one large class of VirB11 mutations imposes blocks in T-DNA transfer across the T4SS at different points by preventing substrate transfer from (i) VirD4 to VirB11, (ii) VirB11 to VirB6/VirB8, or (iii) VirB6/VirB8 to VirB2/VirB9 (Cascales et al. 2013). Mutations conferring these stage-specific blocks are predicted to affect VirB11's interaction with VirD4 in distinct ways or disrupt ATP-binding or hydrolysis activities required for latter-stage transfer reactions (see also Sect. 7). A second broad class of VirB11 mutations was designated as substrate discrimination mutations because they selectively impair transfer of one but not other substrates through the VirB/VirD4 T4SS. Mutations in the ATP-binding pocket block all substrate transfer, but the substrate discrimination mutations map elsewhere and block T-DNA transfer without affecting transfer of the RSF1010 plasmid or VirE2 effector substrates (Cascales et al. 2013; Sagulenko et al. 2001; Zhou and Christie 1997). In a type III secretion system, the VirB11-like subunit InvC dissociates chaperones and unfolds effectors prior to translocation (Akeda and Galan 2005). If VirB11 catalyzes similar reactions, the substrate discrimination mutations might selectively impair an activity required for processing of T-DNA but not the RSF1010 or VirE2 substrates.

6.2 The IMC

Based on the VirB₃₋₁₀ structure, the IMC of the *A. tumefaciens* VirB/VirD4 T4SS is composed of the integral membrane subunits VirB3, VirB6, and VirB8, the N terminus of VirB10, two VirB4 hexamers, and a VirD4 dimer or hexamer (see Fig. 1c) (Low et al. 2014). The IMC is postulated to form a channel across the membrane, but there remain several possible routes by which substrates transit the IM: (1) the lumen of the VirD4 hexamer, (2)

the lumen of the VirB4 hexamer, or (3) a channel formed by the VirB6 and VirB8 subunits (Fig. 1c). Favoring the latter pathway, DNA substrates form close contacts with both VirB6 and VirB8 in the TriP assay (Cascales and Christie 2004b). Mutations in VirB6 were also shown to permit DNA contacts with VirB6 but block contacts with VirB8, suggesting that the DNA substrate sequentially associates with VirB6 and then VirB8 (Jakubowski et al. 2004). Finally, as mentioned above, the ATP-binding/hydrolysis activities of all three ATPases (VirD4, VirB4, VirB11) are necessary for establishment of the DNA substrate contacts with VirB6 and VirB8, suggesting that ATP energy drives substrate transfer through the IM channel (Atmakuri et al. 2004; Cascales and Christie 2004b).

6.3 The OMCC

Recently, the VirB7/VirB9/VirB10 subassembly was purified from *A. tumefaciens* and shown to adopt a ring-shaped complex resembling equivalent subassemblies of T4SSs encoded by the conjugative plasmids pKM101 and R388 (Fig. 4) (Gordon et al. 2017). Overall, these OMCCs present as large (~1-MDa) double-walled barrels composed of an IM-associated or I layer and an OM-associated or O layer. The I layer is composed of the VirB9 and VirB10 subunits and narrows to form a ring of ~55–85 Å diameter at the IM. The O layer is composed of VirB7, VirB9, and VirB10 subunits that form a main body and narrow cap with a hole of 20–30 Å that is presumed to span the outer membrane. Structural analyses further showed that the OMCC is composed of 14 copies of each of the three subunits (Chandran et al. 2009; Fronzes et al. 2009). The distal end of the OMCC consists of a cap, which is built from 14 copies of an α -helical domain of the VirB10 subunit termed ‘the antennae projection’ or ‘AP.’ This cap is thought to span the OM and form a pore through which substrates are conveyed and the T-pilus projects (Banta et al. 2011; Chandran et al. 2009). Interestingly, despite extensive contacts among the VirB7, VirB9, and VirB10 proteins in the OMCC, each of these subunits is tolerant of substitution and small deletion and insertion mutations (Gordon et al. 2017; Jakubowski et al. 2005, 2009). Indeed, certain structural motifs, such as the OM-spanning cap or a short α -helical arm at the base of the OMCC, are completely dispensable for substrate transfer through the T4SS (Gordon et al. 2017). Perhaps the most extreme example of the compositional flexibility of the OMCC was demonstrated through the construction of chimeric T4SSs in *E. coli*. These chimeric T4SSs are composed of the IMC from the pKM101-encoded T4SS joined to OMCCs derived from heterologous T4SSs (Fig. 4). The heterologous OMCCs were from the *E. coli* Trw_{R388} conjugation machine, the *A. tumefaciens* VirB/VirD4 T4SS, and the *Bordetella pertussis* Ptl system, respectively. In their native contexts, these OMCCs mediate transfer of a DNA substrate (Trw_{R388}), a combination of DNA and protein substrates (*A. tumefaciens* VirB/VirD4), and the multi-subunit pertussis toxin (Ptl). Yet, in the context of chimeric T4SSs, the heterologous OMCCs supported transfer of the pKM101 DNA substrate to *E. coli* recipient cells. The functionality of these chimeric T4SSs underscores the highly structural and functional conservation of OMCCs among the T4SSs (Fig. 4) (Gordon et al. 2017).

6.4 Signal Activation of the Translocation Channel

The functionality of these chimeric machines is particularly intriguing in view of early findings that *A. tumefaciens* VirB10 undergoes a structural transition that is required for substrate transfer through the distal region of the VirB/VirD4 T4SS (Cascales and Christie

2004a). This structural transition was detected as a change in susceptibility to the *S. griseus* protease upon treatment of spheroplasts and occurs in response to a combination of ATP energy utilization as well as DNA substrate binding by the VirD4 and VirB11 ATPases (Cascales et al. 2013; Cascales and Christie 2004a). VirB10 thus serves not only as a structural scaffold for the translocation channel, but also to transduce intracellular signals to the distal portion of the channel. Signal activation is thought to induce a conformational change in the channel allowing for substrate passage. Consistent with this mode of action, a mutation in a region of VirB10 located near the OM locks the channel in an activated conformation, resulting in leakage of the VirE2 substrate to the cell surface independently of target cell contact (Banta et al. 2011).

7 The T-Pilus: Assembly and Function

7.1 T-Pilus Subunits

The VirB/VirD4 T4SS and related conjugation systems elaborate conjugative pili in addition to the translocation channel (Fig. 5). The VirB-encoded pilus, termed the T-pilus, is composed of the major pilin subunit VirB2 and a minor subunit VirB5 (Aly and Baron 2007; Kado 2000; Lai and Kado 1998). VirB2 is synthesized as a pro-pilin of ~12.3-kDa in molecular size, which is cleaved of its relatively long (~5-kDa) signal sequence to yield a mature protein of ~7-kDa that inserts into the IM via two hydrophobic domains. The N- and C-termini of the mature protein then undergo a novel cyclization reaction, yielding a covalently joined cyclic peptide (Eisenbrandt et al. 1999). The cyclized pilin monomers form a pool in the IM for recruitment to build the pilus polymer in response to a presently undefined signal. Pilus nucleation is thought to require VirB5, which binds the tip of the T-pilus and likely plays an important role in mediating attachment of *A. tumefaciens* with plant or other agrobacterial target cells (Aly and Baron 2007; Yuan et al. 2005). Early studies established that T-pilus assembly requires most of the same subunits needed to build the translocation channel (Berger and Christie 1994; Fullner et al. 1996). Two important exceptions are VirB1, which is essential for T-pilus production but not a functional translocation channel, and VirD4, which is required for substrate transfer but not T-pilus production (Berger and Christie 1994; Zupan et al. 2007). The dispensability of VirB1 for channel formation is of interest in view of its function as a lytic transglycosylase, which presumably should be necessary for extension of the channel across cell wall. However, the channel might form during periods of active cell wall remodeling or through recruitment of another host-encoded hydrolase, dispensing with the need for a dedicated hydrolase.

For pilus assembly, VirB1 might be required for successful recruitment of VirB2 and/or VirB5 to the T4SS, or it may be necessary for pilus extension beyond the cell surface. Favoring this latter idea, VirB1 undergoes proteolysis to yield separate N-terminal transglycosylase and C-terminal domains (Zupan et al. 2007). The C-terminal domain, designated VirB1*, is required for T-pilus production and, interestingly, is exported across the OM where it associates with the T-pilus. VirB1 thus is postulated to contribute in at least two ways to pilus production. Its N-terminal transglycosylase domain provides localized lysis of the cell wall for extension of the pilus through the periplasm, and its C-terminal VirB1* domain promotes T-pilus assembly through protein-protein interactions with T-pilus

subunits (Fig. 5) (Zupan et al. 2007). In line with this proposal, certain VirB1 homologs are capable of functionally substituting for VirB1 in supporting T-pilus production in *A. tumefaciens*. For example, homologs associated with T4SSs encoded by *Brucella suis* and the *E. coli* conjugative plasmid pKM101, but not those associated with T4SSs encoded by the *Helicobacter pylori* Cag T4SS and the F plasmid, substitute for *A. tumefaciens* VirB1. Interestingly, the complementing homologs carry C-terminal domains whereas those from *H. pylori* and the F plasmid do not have such domains. These findings underscore the importance of the C-terminal domains of VirB1-like subunits for pilus production (Hoppner et al. 2004).

VirB5 is a minor pilin subunit that associates with the tip of the T-pilus (Fig. 5) (Aly and Baron 2007). VirB5 is implicated in nucleation of the T-pilus from either an inner or outer membrane platform, and also might play a role in binding of plant cell receptors (Yuan et al. 2005). Indeed, such receptor-binding activity was shown for VirB5-like CagL, which is associated with the *H. pylori* Cag T4SS. CagL binds β -integrin receptors present on the surface of mammalian epithelial cells to promote *H. pylori* binding and infection (Backert et al. 2008) (Barden et al. 2013). In *A. tumefaciens*, a corresponding function is supported by the findings that *A. tumefaciens* cells engineered to overproduce VirB5, or the addition of exogenous VirB5 to an infection site, enhances T-DNA transfer as monitored by a transient expression assay (Lacroix and Citovsky 2011). VirB5 therefore appears to function both in nucleation of the T-pilus and in mediating attachment to plant cell receptors to facilitate establishment of productive *A. tumefaciens*–plant cell mating junctions. Interestingly, the VirB5 homolog, TraC, from the pKM101 T4SS weakly substitutes for VirB5 with respect to T-pilus production, although not for T-DNA transfer (Schmidt-Eisenlohr et al. 1999). It is not surprising that *A. tumefaciens* VirB5 has evolved a specialized attachment motif(s) for binding of plant cells that is missing in homologs associated with dedicated bacterial conjugation systems. VirB5 also binds the *trans*-zeatin enzyme Tzs, which is involved in biosynthesis of cytokinin and mediates its localization to the *A. tumefaciens* cell surface (Aly et al. 2008). Cytokinin induces production of At14a, a plant protein that links plant cell walls to the plasma membrane and the cytoskeleton (Sardesai et al. 2013). A recent study further presented evidence that the plant cytoskeleton may be a conduit for translocation of the VirE2 effector through the plant cell (Yang et al. 2017). Thus, VirB5-mediated localization of Tzs to the cell surface could stimulate the localized synthesis of cytokinins, which in turn would promote localized transfer of T-DNA and effector proteins.

7.2 T-Pilus Biogenesis Pathway

Several features of the T-pilus assembly pathway have been defined. In a Cys-accessibility study, the topology of membrane-integrated, cyclic form of VirB2 was mapped by determining the accessibility of engineered Cys residues to membrane-impermeable thiol reactive reagents. These studies established a topology in which the loop formed by cyclization of the N- and C-termini is located in the periplasm and a small loop between the two hydrophobic sequences is in the cytoplasm (Kerr and Christie 2010). Interestingly, Cys residues in the cytoplasmic loop that are inaccessible to thiol modification in the absence of the VirB T4SS became accessible in its presence. Further studies established that coproduction of the VirB4 ATPase and Cys-substituted VirB2 pilins sufficed to promote

accessibility of the cytoplasmic Cys residues to thiol modification. VirB4 also was shown to release VirB2 into the periplasm in osmotic shock experiments, and to form an immunoprecipitable complex with VirB2, suggestive of a direct effect of this ATPase on VirB2's membrane topology. Mutation of the Walker A nucleotide triphosphate-binding site abolished all of the observed VirB4-mediated activities. Further, production of VirB11 together with VirB4 affected VirB2's membrane topology, suggesting a coordination of function between the two ATPases. Together, these data support a model in which VirB4, with an unspecified contribution by VirB11, functions as a dislocase to extract membrane-inserted VirB2 as a prerequisite for T-pilus assembly (Fig. 5) (Kerr and Christie 2010). VirB4 subunits are signatures of all bacterial conjugation machines and thus might function generally as pilin dislocases.

Once pilins are extracted from the membrane, how and where on the cell envelope do they polymerize? These questions are not fully answered, but protein–protein interaction studies have provided some clues. For example, VirB2 interacts with the VirB5 pilus-tip protein and with the IMC subunit VirB8. VirB5 also interacts with VirB8, but additionally with the OMCC subunit VirB10 (Yuan et al. 2005). Based on this interaction network, a VirB2–VirB5 complex might engage with VirB8, which in turn could form a nucleation platform for extension of the pilus through the OMCC to the cell exterior. The chamber of the *A. tumefaciens* OMCC is approximately 100 Å in diameter, sufficiently large to house the T-pilus (~10 nm in width). However, the OM pore formed by the cap domain is at most only 20–30 Å and clearly not large enough to accommodate the pilus (Chandran et al. 2009; Gordon et al. 2017). Thus, if the T-pilus assembles from an IM platform, pilus extension must induce gross structural changes in the distal region of the OMCC.

In the above model, the OMCC could be considered a passive structural scaffold for the extending T-pilus. However, two observations favor a more active role for the OMCC in pilus polymerization. First, mutations in VirB10 that do not affect elaboration of a functional translocation channel were shown to selectively block pilus biogenesis (Jakubowski et al. 2009). The most noteworthy of these pilus-blocking mutations are deletions of part or all of the AP domains comprising the distal cap (Gordon et al. 2017). The essentiality of the cap domain suggests it could play an active role in pilus assembly. Second, although the chimeric T4SSs described above support DNA transfer (See Fig. 4), the heterologous OMCCs associated with pKM101's IMC did not support pilus biogenesis (Gordon et al. 2017). These findings suggest that the OMCC has evolved a specific role in assembly of cognate pili. Accordingly, following recruitment of VirB2–VirB5 complexes to VirB8, the pilin subunits might be shunted within the OMCC's chamber to the distal cap, which in turn mediates pilus nucleation. Another scenario depicted in Fig. 5 is that VirB2 begins to polymerize from an IM platform, but as a thin fiber that extends to the cell surface. Upon contacting the OMCC and, specifically, the distal cap, the fiber transitions to form the helically arrayed mature T-pilus.

7.3 Other Attachment Mechanisms

Whether pilus nucleation initiates from IM or OM platforms remains one of the central questions surrounding T-pilus biogenesis. A related question is whether the T-pilus

contributes directly to substrate transfer. Although assembly of the channel and T-pilus require nearly the same subunits, mutational studies have identified classes of mutations that selectively block substrate transfer without affecting pilus production (Tra⁻, Pil⁺) or conversely, permit substrate transfer but block pilus production (Tra⁺, Pil⁻) (Jakubowski et al. 2005, 2009; Sagulenko et al. 2001). It is possible that the latter mutations simply block pilus outgrowth and that *A. tumefaciens* cells harboring such mutations form short pili that cannot be detected by current assays. However, studies of the *E. coli* pKM101 system also have identified ‘uncoupling’ mutations that completely block pilus biogenesis as monitored by resistance to filamentous phages, which are known to bind the pilus tip (Gordon et al. 2017). The identification of ‘uncoupling’ mutations clearly argues against a direct role for the T-pilus in substrate transfer, suggesting instead that the T-pilus functions predominantly or exclusively as an attachment organelle. It is interesting to note that VirB2 pilin is required in Pil⁻, Tra⁺ ‘uncoupling’ mutants for successful T-DNA transfer. Furthermore, VirB2 interacts with various plant cytosolic proteins of established importance for efficient T-DNA transfer (Hwang and Gelvin 2004; Huang et al. 2018). These findings raise the possibility that in addition to their roles in T-pilus polymerization pilin monomers might alternatively be delivered via the T4SS into plant cells where contacts with certain plant proteins stimulate VirB/VirD4-mediated transformation. *A. tumefaciens* also elaborates several adhesive organelles or molecules on its cell surface, including unipolar polysaccharide (UPP), other exopolysaccharides (cyclic- β -1,2-D-glucan, succinoglycan, lipopolysaccharide), cellulose, calcium-binding surface proteins (rhicadhesin, RapA1), and type IV pili (Bash and Matthysse 2002; Heindl et al. 2014; Li et al. 2012; Matthysse 2014; Matthysse et al. 2005; Wang et al. 2014; Xu et al. 2013). It can be predicted that a combination of T-pili, possibly the VirB2 pilin and VirB5/Tzs, and other surface adhesins coordinate productive binding and colonization of *A. tumefaciens* on plant tissues. Importantly, even in the absence of T-pilus production, these alternative surface adhesins suffice to establish contacts necessary for VirB/VirD4-mediated interkingdom translocation.

8 Conclusions

Studies of the *A. tumefaciens* VirB/VirD4 T4SS have supplied key mechanistic and structural insights into the T4SS secretion superfamily. Compositionally, this is one of the simplest T4SSs, especially among systems elaborating both a trans-envelope translocation channel and an extracellular pilus. Early work defined the contributions and subcellular distributions of individual VirB and VirD4 subunits to machine and pilus assembly. Next, the development of creative in vivo assays, such as CRAfT and TrIP, refined our understanding of substrate recruitment and transfer reactions. Most recently, structural characterization of purified T4SS subassemblies, including OMCCs from the *A. tumefaciens* VirB/VirD4 system and several related conjugation machines, as well as the larger VirB₃₋₁₀/VirD4 substructure has provided an architectural context for envisioning how these machines mediate substrate transfer across the cell envelope.

Strikingly, certain machine subassemblies, such as the VirD4 substrate receptor and the OMCC, are exchangeable between the VirB/VirD4 and closely related conjugation systems. Studies of such chimeric systems have been highly informative in two ways. First, investigations have supplied new mechanistic detail regarding substrate recruitment and

pilus biogenesis pathways. Second, these studies have supplied a proof-of-principle for the engineering T4SSs with novel functions. Engineering of the *E. coli* pKM101 Tra T4SS, once considered a 'dedicated' conjugation machine, allowed for diversification of the substrate repertoire of this highly efficient nanomachine to include a potentially wide array of effector proteins. By extension, this conjugation system or other T4SSs normally functioning in infection processes, e.g., *A. tumefaciens* VirB/VirD4, *H. pylori* Cag, *Legionella* Dot/Icm, might be engineered to recruit and translocate novel substrates of potential therapeutic benefit to different eukaryotic target cells of interest. Indeed, as a first step in the application of T4SSs as substrate delivery platforms, T4SSs functioning in *Bartonella henselae*, *Legionella pneumophila*, and *Coxiella burnetii* were recently retailored to deliver DNA substrates to distinct mammalian cells (Guzman-Herrador et al. 2017).

Despite the remarkable progress in our studies of the VirB/VirD4 T4SS, there remain many questions: (1) How do the VirD4, VirB4, and VirB11 subunits coordinate their ATPase activities to recruit substrates and drive their translocation; (2) What is the architecture of the envelope-spanning channel, how is it gated at its entrance and exit points, and what signals regulate channel activity? (3) What is the structural and functional relationship of the translocation channel and the T-pilus? and (4) How does the T4SS establish productive pilus-dependent and -independent contacts with target cells? Answers to these questions will require continued commitment to basic studies of the paradigmatic VirB/VirD4 and related conjugation systems. Clearly, however, the pace of research on the T4SSs is accelerating rapidly at the ultrastructural level due to the implementation of high-resolution microscopy approaches. When combined with creative new in vivo approaches enabling structure–function assignments and definition of machine dynamics in real time, the incipient discoveries promise to refine and possibly completely reshape our views of these fascinatingly complex nanomachines.

Acknowledgements

Work in the Christie laboratory is supported by NIH grant R01GM48476.

References

- Akeda Y, Galan JE (2005) Chaperone release and unfolding of substrates in type III secretion. *Nature* 437:911–915. 10.1038/nature03992 [PubMed: 16208377]
- Albright LM, Yanofsky MF, Leroux B, Ma DQ, Nester EW (1987) Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single-stranded T-DNA. *J Bacteriol* 169:1046–1055 [PubMed: 3029014]
- Alvarez-Martinez CE, Christie PJ (2009) Biological diversity of prokaryotic type IV secretion systems. *Microbiol Mol Biol Rev* 73:775–808 [PubMed: 19946141]
- Aly KA, Baron C (2007) The VirB5 protein localizes to the T-pilus tips in *Agrobacterium tumefaciens*. *Microbiology* 153:3766–3775 [PubMed: 17975085]
- Aly KA, Krall L, Lottspeich F, Baron C (2008) The type IV secretion component VirB5 binds to the trans-zeatin biosynthetic enzyme Tzs and enables its translocation to the cell surface of *Agrobacterium tumefaciens*. *J Bacteriol* 190:1595–1604 [PubMed: 18165307]
- Anderson LB, Hertzler AV, Das A (1996) *Agrobacterium tumefaciens* VirB7 and VirB9 form a disulfide-linked protein complex. *Proc Natl Acad Sci USA* 93:8889–8894 [PubMed: 8799123]

- Atmakuri K, Cascales E, Burton OT, Banta LM, Christie PJ (2007) *Agrobacterium* ParA/MinD-like VirC1 spatially coordinates early conjugative DNA transfer reactions. *EMBO J* 26:2540–2551 [PubMed: 17505518]
- Atmakuri K, Cascales E, Christie PJ (2004) Energetic components VirD4, VirB11 and VirB4 mediate early DNA transfer reactions required for bacterial type IV secretion. *Mol Microbiol* 54:1199–1211 [PubMed: 15554962]
- Atmakuri K, Ding Z, Christie PJ (2003) VirE2, a type IV secretion substrate, interacts with the VirD4 transfer protein at cell poles of *Agrobacterium tumefaciens*. *Mol Microbiol* 49:1699–1713 [PubMed: 12950931]
- Backert S, Fronzes R, Waksman G (2008) VirB2 and VirB5 proteins: specialized adhesins in bacterial type-IV secretion systems? *Trends Microbiol* 16:409–413 [PubMed: 18706815]
- Banta LM et al. (2011) An *Agrobacterium* VirB10 mutation conferring a type IV secretion system gating defect. *J Bacteriol* 193:2566–2574 [PubMed: 21421757]
- Barden S, Lange S, Tegtmeyer N, Conradi J, Sewald N, Backert S, Niemann HH (2013) A helical RGD motif promoting cell adhesion: crystal structures of the *Helicobacter pylori* type IV secretion system pilus protein CagL. *Structure* 21:1931–1941 [PubMed: 24076404]
- Baron C, Thorstenson YR, Zambryski PC (1997) The lipoprotein VirB7 interacts with VirB9 in the membranes of *Agrobacterium tumefaciens*. *J Bacteriol* 179:1211–1218 [PubMed: 9023204]
- Bash R, Matthysse A (2002) Attachment to roots and virulence of a *chvB* mutant of *Agrobacterium tumefaciens* are temperature sensitive. *Mol Plant Microbe Interact* 15:160–163 [PubMed: 11876426]
- Beaupre CE, Bohne J, Dale EM, Binns AN (1997) Interactions between VirB9 and VirB10 proteins involved in movement of DNA from *Agrobacterium tumefaciens* into plant cells. *J Bacteriol* 179:78–89 [PubMed: 8981983]
- Beijersbergen A, Dulk-Ras AD, Schilperoort RA, Hooykas PJJ (1992) Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. *Science* 256:1324–1327 [PubMed: 17736763]
- Beijersbergen A, Smith SJ, Hooykaas PJ (1994) Localization and topology of VirB proteins of *Agrobacterium tumefaciens*. *Plasmid* 32:212–218 [PubMed: 7846145]
- Berger BR, Christie PJ (1994) Genetic complementation analysis of the *Agrobacterium tumefaciens* *virB* operon: *virB2* through *virB11* are essential virulence genes. *J Bacteriol* 176:3646–3660 [PubMed: 8206843]
- Bhatty M, Laverde Gomez JA, Christie PJ (2013) The expanding bacterial type IV secretion lexicon. *Res Microbiol* 164:620–639. 10.1016/j.resmic.2013.03.012 [PubMed: 23542405]
- Buchanan-Wollaston V, Passiatore JE, Cannon F (1987) The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. *Nature* 328:172–175
- Bundock P, den DRA, Beijersbergen A, Hooykaas PJ (1995) Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens*. *EMBO J* 14:3206–3214 [PubMed: 7621833]
- Cabezón E, Sastre JJ, de la Cruz F (1997) Genetic evidence of a coupling role for the TraG protein family in bacterial conjugation. *Mol Gen Genet* 254:400–406 [PubMed: 9180693]
- Cameron TA, Roper M, Zambryski PC (2012) Quantitative image analysis and modeling indicate the *Agrobacterium tumefaciens* type IV secretion system is organized in a periodic pattern of foci. *PLoS ONE* 7:e42219 10.1371/journal.pone.0042219 [PubMed: 22860087]
- Cascales E, Atmakuri K, Sarkar MK, Christie PJ (2013) DNA substrate-induced activation of the *Agrobacterium* VirB/VirD4 type IV secretion system. *J Bacteriol* 195:2691–2704. 10.1128/JB.00114-13 [PubMed: 23564169]
- Cascales E, Christie PJ (2004a) *Agrobacterium* VirB10, an ATP energy sensor required for type IV secretion. *Proc Natl Acad Sci U S A* 101:17228–17233 [PubMed: 15569944]
- Cascales E, Christie PJ (2004b) Definition of a bacterial type IV secretion pathway for a DNA substrate. *Science* 304:1170–1173 [PubMed: 15155952]
- Chandran V, Fronzes R, Duquerroy S, Cronin N, Navaza J, Waksman G (2009) Structure of the outer membrane complex of a type IV secretion system. *Nature* 462:1011–1015 [PubMed: 19946264]
- Christie PJ (1997) *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in eubacteria. *J Bacteriol* 179:3085–3094 [PubMed: 9150199]

- Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowski S, Cascales E (2005) Biogenesis, architecture, and function of bacterial type IV secretion systems. *Annu Rev Microbiol* 59: 451–485 [PubMed: 16153176]
- Das A, Xie YH (1998) Construction of transposon Tn3phoA: its application in defining the membrane topology of the *Agrobacterium tumefaciens* DNA transfer proteins. *Mol Microbiol* 27:405–414 [PubMed: 9484895]
- de la Cruz F, Frost LS, Meyer RJ, Zechner EL (2010) Conjugative DNA metabolism in Gram-negative bacteria. *FEMS Micro Rev* 34:18–40. 10.1111/j.1574-6976.2009.00195.x
- de Paz HD, Larrea D, Zunzunegui S, Dehio C, de la Cruz F, Llosa M (2010) Functional dissection of the conjugative coupling protein TrwB. *J Bacteriol* 192:2655–2669 [PubMed: 20363945]
- de Paz HD, Sangari FJ, Bolland S, Garcia-Lobo JM, Dehio C, de la Cruz F, Llosa M (2005) Functional interactions between type IV secretion systems involved in DNA transfer and virulence. *Microbiology* 151:3505–3516 [PubMed: 16272374]
- Durrenberger MB, Villiger W, Bachi TH (1991) Conjugational junctions: morphology of specific contacts in conjugating *Escherichia coli* bacteria. *J Struct Biol* 107:146–156 [PubMed: 1807350]
- Eisenbrandt R, Kalkum M, Lai EM, Lurz R, Kado CI, Lanka E (1999) Conjugative pili of IncP plasmids, and the Ti plasmid T pilus are composed of cyclic subunits. *J Biol Chem* 274:22548–22555 [PubMed: 10428832]
- Fernandez D, Spudich GM, Zhou XR, Christie PJ (1996) The *Agrobacterium tumefaciens* VirB7 lipoprotein is required for stabilization of VirB proteins during assembly of the T-complex transport apparatus. *J Bacteriol* 178:3168–3176 [PubMed: 8655495]
- Filichkin SA, Gelvin SB (1993) Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* Vir D1, D2 endonuclease. *Mol Microbiol* 8:915–926
- Fronzes R, Schafer E, Wang L, Saibil HR, Orlova EV, Waksman G (2009) Structure of a type IV secretion system core complex. *Science* 323:266–268 [PubMed: 19131631]
- Fullner KJ (1998) Role of *Agrobacterium virB* genes in transfer of T complexes and RSF1010. *J Bacteriol* 180:430–434 [PubMed: 9440537]
- Fullner KJ, Lara JC, Nester EW (1996) Pilus assembly by *Agrobacterium* T-DNA transfer genes. *Science* 273:1107–1109 [PubMed: 8688097]
- Gomis-Ruth FX, Moncalian G, Perez-Luque R, Gonzalez A, Cabezon E, de la Cruz F, Coll M (2001) The bacterial conjugation protein TrwB resembles ring helicases and F1-ATPase. *Nature* 409:637–641 [PubMed: 11214325]
- Gordon JE et al. (2017) Use of chimeric type IV secretion systems to define contributions of outer membrane subassemblies for contact-dependent translocation. *Mol Microbiol* 105:273–293. 10.1111/mmi.13700 [PubMed: 28452085]
- Grohmann E, Christie PJ, Waksman G, Backert S (2018) Type IV secretion in gram-negative and gram-positive bacteria. *Mol Microbiol* 107:455–471 [PubMed: 29235173]
- Guo M, Hou Q, Hew CL, Pan SQ (2007) *Agrobacterium* VirD2-binding protein is involved in tumorigenesis and redundantly encoded in conjugative transfer gene clusters. *Mol Plant Microbe Interact* 20:1201–1212 [PubMed: 17918622]
- Guzman-Herrador DL, Steiner S, Alperi A, Gonzalez-Preito C, Roy CR, Llosa M (2017) DNA delivery and genomic integration into mammalian target cells through type IV A and B secretion systems of human pathogens. *Front Microbiol* 8:1503 10.3389/fmicb.2017.01503 [PubMed: 28878740]
- Hamilton CM et al. (2000) TraG from RP4 and TraG and VirD4 from Ti plasmids confer relaxosome specificity to the conjugal transfer system of pTiC58. *J Bacteriol* 182:1541–1548 [PubMed: 10692358]
- Heindl JE, Wang Y, Heckel BC, Mohari B, Feirer N, Fuqua C (2014) Mechanisms and regulation of surface interactions and biofilm formation in *Agrobacterium*. *Front Plant Sci* 5:176 10.3389/fpls.2014.00176 [PubMed: 24834068]
- Hodges LD, Vergunst AC, Neal-McKinney J, den Dulk-Ras A, Moyer DM, Hooykaas PJ, Ream W (2006) *Agrobacterium rhizogenes* GALLS protein contains domains for ATP binding, nuclear localization, and Type IV secretion. *J Bacteriol* 188:8222–8230 [PubMed: 17012398]

- Hoppner C, Liu Z, Domke N, Binns AN, Baron C (2004) VirB1 orthologs from *Brucella suis* and pKM101 complement defects of the lytic transglycosylase required for efficient type IV secretion from *Agrobacterium tumefaciens*. J Bacteriol 186:1415–1422 [PubMed: 14973016]
- Hormaeche I, Iloro I, Arrondo JL, Goni FM, de la Cruz F, Alkorta I (2004) Role of the transmembrane domain in the stability of TrwB, an integral protein involved in bacterial conjugation. J Biol Chem 279:10955–10961 [PubMed: 14699106]
- Hormaeche I, Segura RL, Vecino AJ, Goni FM, de la Cruz F, Alkorta I (2006) The transmembrane domain provides nucleotide binding specificity to the bacterial conjugation protein TrwB. FEBS Lett 580:3075–3308 [PubMed: 16678163]
- Hwang HH, Gelvin S (2004) Plant proteins that interact with VirB2, the *Agrobacterium tumefaciens* pilin protein, mediate plant transformation. Plant Cell 16:3148–3167 [PubMed: 15494553]
- Huang FC, Fu BJ, Liu YT, Chang YR, Chi SF, Chien PR, Huang SC, Hwang HH (2018) *Arabidopsis* RETICULON-LIKE3 (RTNLB3) and RTNLB8 participate in *Agrobacterium*-mediated plant transformation. Int J Mol Sci 19(2). pii: E638 10.3390/ijms19020638 [PubMed: 29495267]
- Jakubowski SJ, Cascales E, Krishnamoorthy V, Christie PJ (2005) *Agrobacterium tumefaciens* VirB9, an outer-membrane-associated component of a type IV secretion system, regulates substrate selection and T-pilus biogenesis. J Bacteriol 187:3486–3495 [PubMed: 15866936]
- Jakubowski SJ, Kerr JE, Garza I, Krishnamoorthy V, Bayliss R, Waksman G, Christie PJ (2009) *Agrobacterium* VirB10 domain requirements for type IV secretion and T pilus biogenesis. Mol Microbiol 71:779–794 [PubMed: 19054325]
- Jakubowski SJ, Krishnamoorthy V, Cascales E, Christie PJ (2004) *Agrobacterium tumefaciens* VirB6 domains direct the ordered export of a DNA substrate through a type IV secretion system. J Mol Biol 341:961–977 [PubMed: 15328612]
- Jakubowski SJ, Krishnamoorthy V, Christie PJ (2003) *Agrobacterium tumefaciens* VirB6 protein participates in formation of VirB7 and VirB9 complexes required for type IV secretion. J Bacteriol 185:2867–2878 [PubMed: 12700266]
- Judd PK, Kumar RB, Das A (2005a) Spatial location and requirements for the assembly of the *Agrobacterium tumefaciens* type IV secretion apparatus. Proc Natl Acad Sci U S A 102:11498–11503 [PubMed: 16076948]
- Judd PK, Kumar RB, Das A (2005b) The type IV secretion apparatus protein VirB6 of *Agrobacterium tumefaciens* localizes to a cell pole. Mol Microbiol 55:115–124 [PubMed: 15612921]
- Kado CI (2000) The role of the T-pilus in horizontal gene transfer and tumorigenesis. Curr Opin Microbiol 3:643–648 [PubMed: 11121787]
- Kato J, Lefebvre M, Galan JE (2015) Structural features reminiscent of ATP-driven protein translocases are essential for the function of a Type III secretion-associated ATPase. J Bacteriol 197:3007–3014. 10.1128/JB.00434-15 [PubMed: 26170413]
- Kerr JE, Christie PJ (2010) Evidence for VirB4-mediated dislocation of membrane-integrated VirB2 pilin during biogenesis of the *Agrobacterium* VirB/VirD4 type IV secretion system. J Bacteriol 192:4923–4934 [PubMed: 20656905]
- Kuldau GA, De VG, Owen J, McCaffrey G, Zambryski P (1990) The *virB* operon of *Agrobacterium tumefaciens* pTiC58 encodes 11 open reading frames. Mol Gen Genetics 221:256–266
- Kumar RB, Das A (2002) Polar location and functional domains of the *Agrobacterium tumefaciens* DNA transfer protein. VirD4. Mol Microbiol 43:1523–1532 [PubMed: 11952902]
- Kunik T, Tzfira T, Kapulnik Y, Gafni Y, Dingwall C, Citovsky V (2001) Genetic transformation of HeLa cells by *Agrobacterium*. Proc Natl Acad Sci USA 98:1871–1876 [PubMed: 11172043]
- Kwak MJ et al. (2017) Architecture of the type IV coupling protein complex of *Legionella pneumophila*. Nat Microbiol 2:17114 10.1038/nmicrobiol.2017.114 [PubMed: 28714967]
- Lacroix B, Citovsky V (2011) Extracellular VirB5 enhances T-DNA transfer from *Agrobacterium* to the host plant. PLoS ONE 6:e25578 10.1371/journal.pone.0025578 [PubMed: 22028781]
- Lai EM, Kado CI (1998) Processed VirB2 is the major subunit of the promiscuous pilus of *Agrobacterium tumefaciens*. J Bacteriol 180:2711–2717 [PubMed: 9573157]
- Lang S et al. (2011) An activation domain of plasmid R1 TraI protein delineates stages of gene transfer initiation. Mol Microbiol 82:1071–1085. 10.1111/j.1365-2958.2011.07872.x [PubMed: 22066957]

- Larrea D, de Paz HD, Arechaga I, de la Cruz F, Llosa M (2013) Structural independence of conjugative coupling protein TrwB from its Type IV secretion machinery. *Plasmid* 70:146–153. 10.1016/j.plasmid.2013.03.006 [PubMed: 23583564]
- Li G, Brown PJ, Tang JX, Xu J, Quardokus EM, Fuqua C, Brun YV (2012) Surface contact stimulates the just-in-time deployment of bacterial adhesins. *Mol Microbiol* 83:41–51. 10.1111/j.1365-2958.2011.07909.x [PubMed: 22053824]
- Llosa M, Zunzunegui S, de la Cruz F (2003) Conjugative coupling proteins interact with cognate and heterologous VirB10-like proteins while exhibiting specificity for cognate relaxosomes. *Proc Natl Acad Sci U S A* 100:10465–10470 [PubMed: 12925737]
- Low HH et al. (2014) Structure of a type IV secretion system. *Nature* 508:550–553. 10.1038/nature13081 [PubMed: 24670658]
- Lu J, den Dulk-Ras A, Hooykaas PJJ, Glover JNM, Glover (2009) *Agrobacterium tumefaciens* VirC2 enhances T-DNA transfer and virulence through its C-terminal ribbon-helix-helix DNA-binding fold. *Proc Natl Acad Sci USA* 106:9643–9648 [PubMed: 19482939]
- Matthysse AG (2014) Attachment of *Agrobacterium* to plant surfaces. *Front Plant Sci* 5:252 10.3389/fpls.2014.00252 [PubMed: 24926300]
- Matthysse AG, Marry M, Krall L, Kaye M, Ramey BE, Fuqua C, White AR (2005) The effect of cellulose overproduction on binding and biofilm formation on roots by *Agrobacterium tumefaciens*. *Mol Plant Microbe Interact* 18:1002–1010. 10.1094/MPMI-18-1002 [PubMed: 16167770]
- Padavannil A et al. (2014) Dimerization of VirD2 binding protein is essential for *Agrobacterium* induced tumor formation in plants. *PLoS Pathog* 10:e1003948 10.1371/journal.ppat.1003948 [PubMed: 24626239]
- Pena A, Arechaga I (2013) Molecular motors in bacterial secretion. *J Mol Microbiol Biotechnol* 23:357–369. 10.1159/000351360 [PubMed: 23920498]
- Pena A et al. (2012) The hexameric structure of a conjugative VirB4 protein ATPase provides new insights for a functional and phylogenetic relationship with DNA translocases. *J Biol Chem* 287:39925–39932 [PubMed: 23035111]
- Piers KL, Heath JD, Liang X, Stephens KM, Nester EW (1996) *Agrobacterium tumefaciens*-mediated transformation of yeast. *Proc Natl Acad Sci U S A* 93:1613–1618 [PubMed: 8643679]
- Rashkova S, Spudich GM, Christie PJ (1997) Characterization of membrane and protein interaction determinants of the *Agrobacterium tumefaciens* VirB11 ATPase. *J Bacteriol* 179:583–591 [PubMed: 9006008]
- Redzej A et al. (2017) Structure of a VirD4 coupling protein bound to a VirB type IV secretion machinery. *EMBO J* 36:3080–3095 [PubMed: 28923826]
- Ripoll-Rozada J, Zunzunegui S, de la Cruz F, Arechaga I, Cabezon E (2013) Functional interactions of VirB11 traffic ATPases with VirB4 and VirD4 molecular motors in type IV secretion systems. *J Bacteriol* 195:4195–4201. 10.1128/JB.00437-13 [PubMed: 23852869]
- Sagulenko E, Sagulenkov V, Chen J, Christie PJ (2001) Role of *Agrobacterium* VirB11 ATPase in T-pilus assembly and substrate selection. *J Bacteriol* 183:5813–5825. 10.1128/JB.183.20.5813-5825.2001 [PubMed: 11566978]
- Salmond GPC (1994) Secretion of extracellular virulence factors by plant pathogenic bacteria. *Ann Rev Phytopathol* 32:181–200
- Samuels AL, Lanka E, Davies JE (2000) Conjugative junctions in RP4-mediated mating of *Escherichia coli*. *J Bacteriol* 182:2709–2715 [PubMed: 10781537]
- Sardesai N, Lee LY, Chen Y, Yi H, Olbricht GR, Stirnberg A, Jeffries J, Xiong K, Doerge RW, Gelvin SB (2013) Cytokinins secreted by *Agrobacterium* promote transformation by repressing a plant myb transcription factor. *Sci Signal* 6:ra100 10.1126/scisignal.2004518 [PubMed: 24255177]
- Savvides SN (2007) Secretion superfamily ATPases swing big. *Structure* 15:255–257 [PubMed: 17355860]
- Savvides SN et al. (2003) VirB11 ATPases are dynamic hexameric assemblies: new insights into bacterial type IV secretion. *EMBO J* 22:1969–1980 [PubMed: 12727865]

- Schmidt-Eisenlohr H, Domke N, Baron C (1999) TraC of IncN plasmid pKM101 associates with membranes and extracellular high-molecular-weight structures in *Escherichia coli*. *J Bacteriol* 181:5563–5571 [PubMed: 10482495]
- Schrammeijer B, Dulk-Ras AdA, Vergunst AC, Jurado Jacome E, Hooykaas PJ (2003) Analysis of Vir protein translocation from *Agrobacterium tumefaciens* using *Saccharomyces cerevisiae* as a model: evidence for transport of a novel effector protein VirE3. *Nucl Acids Res* 31:860–868 [PubMed: 12560481]
- Shirasu K, Kado CI (1993) The *virB* operon of the *Agrobacterium tumefaciens* virulence regulon has sequence similarities to B, C and D open reading frames downstream of the pertussis toxin-operon and to the DNA transfer-operons of broad-host-range conjugative plasmids. *Nucleic Acids Res* 21:353–354 [PubMed: 8441642]
- Shirasu K, Morel P, Kado CI (1990) Characterization of the *virB* operon of an *Agrobacterium tumefaciens* Ti plasmid: nucleotide sequence and protein analysis. *Mol Microbiol* 4:1153–1163 [PubMed: 2233252]
- Simone M, McCullen CA, Stahl LE, Binns AN (2001) The carboxy-terminus of VirE2 from *Agrobacterium tumefaciens* is required for its transport to host cells by the virB-encoded type IV transport system. *Mol Microbiol* 41:1283–1293 [PubMed: 11580834]
- Spudich GM, Fernandez D, Zhou XR, Christie PJ (1996) Intermolecular disulfide bonds stabilize VirB7 homodimers and VirB7/VirB9 heterodimers during biogenesis of the *Agrobacterium tumefaciens* T-complex transport apparatus. *Proc Natl Acad Sci U S A* 93:7512–7517 [PubMed: 8755505]
- Stachel SE, Nester EW (1986) The genetic and transcriptional organization of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. *EMBO J* 5:1445–1454 [PubMed: 3017694]
- Stachel SE, Timmerman B, Zambryski P (1987) Activation of *Agrobacterium tumefaciens vir* gene expression generates multiple single-stranded T-strand molecules from the pTiA6 T-region: requirement for 5' virD gene products. *EMBO J* 6:857–863 [PubMed: 3595560]
- Stachel SE, Zambryski PC (1986) *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. *Cell* 47:155–157 [PubMed: 3768954]
- Toro N, Datta A, Carmi OA, Young C, Prusti RK, Nester EW (1989) The *Agrobacterium tumefaciens* virC1 gene product binds to overdrive, a T-DNA transfer enhancer. *J Bacteriol* 171:6845–6849 [PubMed: 2592351]
- van Kregten M, Lindhout BI, Hooykaas PJ, van der Zaal BJ (2009) *Agrobacterium*-mediated T-DNA transfer and integration by minimal VirD2 consisting of the relaxase domain and a type IV secretion system translocation signal. *Mol Plant-Microbe Inter: MPMI* 22:1356–1365
- Veluthambi K, Ream W, Gelvin SB (1988) Virulence genes, borders, and overdrive generate single-stranded T-DNA molecules from the A6 Ti plasmid of *Agrobacterium tumefaciens*. *J Bacteriol* 170:1523–1532 [PubMed: 2832367]
- Vergunst AC, Schrammeijer B, den Dulk-Ras A, de Vlaam CM, Regensburg-Tuink TJ, Hooykaas PJ (2000) VirB/D4-dependent protein translocation from *Agrobacterium* into plant cells. *Science* 290:979–982 [PubMed: 11062129]
- Vergunst AC, van Lier MC, den Dulk-Ras A, Grosse Stuve TA, Ouwehand A, Hooykaas PJ (2005) Positive charge is an important feature of the C-terminal transport signal of the VirB/D4-translocated proteins of *Agrobacterium*. *Proc Natl Acad Sci USA* 102:832–837 [PubMed: 15644442]
- Vergunst AC, Van Lier MC, Den Dulk-Ras A, Hooykaas PJ (2003) Recognition of the *Agrobacterium tumefaciens* VirE2 translocation signal by the VirB/D4 transport system does not require VirE1. *Plant Physiol* 133:978–988 [PubMed: 14551327]
- Wang Y, Haitjema CH, Fuqua C (2014) The Ctp type IVb pilus locus of *Agrobacterium tumefaciens* directs formation of the common pili and contributes to reversible surface attachment. *J Bacteriol* 196:2979–2988. 10.1128/JB.01670-14 [PubMed: 24914181]
- Ward JE, Akiyoshi DE, Reiger D, Datta A, Gordon MP, Nester EW (1988) Characterization of the *virB* operon from an *Agrobacterium tumefaciens* Ti plasmid. *J Biol Chem* 263:5804–5814 [PubMed: 3281947]

- Ward JE Jr, Dale EM, Binns AN (1991) Activity of the *Agrobacterium* T-DNA transfer machinery is affected by *virB* gene products. *Proc Natl Acad Sci U S A* 88:9350–9354 [PubMed: 11607226]
- Whitaker N et al. (2016) Chimeric coupling proteins mediate transfer of heterologous type IV effectors through the *Escherichia coli* pKM101-encoded conjugation machine. *J Bacteriol* 198:2701–2718. 10.1128/JB.00378-16 [PubMed: 27432829]
- Whitaker N, Chen Y, Jakubowski SJ, Sarkar MK, Li F, Christie PJ (2015) The all-alpha domains of coupling proteins from the *Agrobacterium tumefaciens* VirB/VirD4 and *Enterococcus faecalis* pCF10-encoded type IV secretion systems confer specificity to binding of cognate DNA substrates. *J Bacteriol* 197:2335–2349. 10.1128/JB.00189-15 [PubMed: 25939830]
- Winans SC, Burns DL, Christie PJ (1996) Adaptation of a conjugal transfer system for the export of pathogenic macromolecules. *Trends Microbiol* 4:64–68 [PubMed: 8820569]
- Xu J, Kim J, Koestler BJ, Choi JH, Waters CM, Fuqua C (2013) Genetic analysis of *Agrobacterium tumefaciens* unipolar polysaccharide production reveals complex integrated control of the motile-to-sessile switch. *Mol Microbiol* 89:929–948. 10.1111/mmi.12321 [PubMed: 23829710]
- Yang Q, Li X, Tu H, Pan SQ (2017) *Agrobacterium*-delivered virulence protein VirE2 is trafficked inside host cells via a myosin XI-K-powered ER/actin network. *Proc Natl Acad Sci USA* 114:2982–2987 [PubMed: 28242680]
- Yanofsky MF, Porter SG, Young C, Albright LM, Gordon MP, Nester EW (1986) The *virD* operon of *Agrobacterium tumefaciens* encodes a site-specific endonuclease. *Cell* 47:471–477 [PubMed: 3021341]
- Yeo HJ, Savvides SN, Herr AB, Lanka E, Waksman G (2000) Crystal structure of the hexameric traffic ATPase of the *Helicobacter pylori* type IV secretion system. *Mol Cell* 6:1461–1472 [PubMed: 11163218]
- Young C, Nester EW (1988) Association of the VirD2 protein with the 5' end of T strands in *Agrobacterium tumefaciens*. *J Bacteriol* 170:3367–3374 [PubMed: 3403506]
- Yuan Q et al. (2005) Identification of the VirB4-VirB8-VirB5-VirB2 pilus assembly sequence of type IV secretion systems. *J Biol Chem* 280:26349–26359 [PubMed: 15901731]
- Zhao Z, Sagulenko E, Ding Z, Christie PJ (2001) Activities of *virE1* and the VirE1 secretion chaperone in export of the multifunctional VirE2 effector via an *Agrobacterium* type IV secretion pathway. *J Bacteriol* 183:3855–3865 [PubMed: 11395448]
- Zhou XR, Christie PJ (1997) Suppression of mutant phenotypes of the *Agrobacterium tumefaciens* VirB11 ATPase by overproduction of VirB proteins. *J Bacteriol* 179:5835–5842 [PubMed: 9294442]
- Zupan J, Hackworth CA, Aguilar J, Ward D, Zambryski P (2007) VirB1* promotes T-pilus formation in the vir-Type IV secretion system of *Agrobacterium tumefaciens*. *J Bacteriol* 189:6551–6563 [PubMed: 17631630]

Fig. 1.

Composition and architecture of the *A. tumefaciens* VirB/VirD4 T4SS. **a** Order of the *virB* genes in the *virB* operon; *virD4* is encoded by the separate *virD* operon (hashed lines). Functions, localization, or subassembly associations of the encoded products are listed. PG, peptidoglycan; IMC, inner membrane complex; OMCC, outer membrane core complex; T4CP, type IV coupling protein. **b** Summary of transfer DNA immunoprecipitation (TrIP) studies showing that the T-DNA forms formaldehyde (FA)-crosslinkable contacts with the VirD4 and VirB proteins listed at top. The corresponding deletion mutations permit (+) or prevent (–) FA cross-linking of T-DNA with VirB/VirD4 subunits listed, indicative of stage-specific blocks in the transfer pathway denoted at the right. **c** Architecture of the VirB/VirD4 T4SS, with positions of subunits identified. Components of the pilus, OMCC, IMC, and ATPase energy center are listed. The overall architecture and subunit stoichiometries (in parentheses at left) are based on the R388-encoded VirB_{3–10} solved by single-particle, negative-stain electron microscopy (Low et al. 2014). The model shows predicted locations of VirB1, VirB1*, VirB2, VirD4, VirB11, and the T-pilus, which were missing from the VirB_{3–10} substructure

Fig. 2.

Substrate processing and recruitment to the VirB/VirD4 T4SS. The VirD2 relaxase and accessory factors (VirD1, VirC1, VirC2) bind the *oriT*-like T-DNA border repeats (red rectangles) of the T-DNA on pTi plasmid, forming the catalytically active relaxosome. VirD2 (green oval) nicks and remains covalently associated with the strand T-DNA destined for transfer (T-strand). In vir-induced cells, the transfer intermediate accumulates in as many as 30 copies per cell. VirD2's translocation signal (TS; + charged C terminus), ParA-like VirC1, and VirD2-binding proteins (VBPs) target the transfer intermediate to the VirB/VirD4 T4SS for delivery to the plant cell. Protein effectors are recruited by charged C-terminal TSs by a chaperone-independent (VirE3, VirF, GALLS) or -dependent (VirE2 effector::VirE1 chaperone) mechanism. The VirB/VirD4 T4SS was reported to localize at the *A. tumefaciens* cell poles (Atmakuri et al. 2007; Judd et al. 2005a, b; Kumar and Das 2002), although more recently the VirB subunits were shown to assemble as non-random foci around the cell (Cameron et al. 2012)

Fig. 3.

VirD4 domain composition and functionality of chimeric receptors. Left: VirD4 domains and experimentally derived functions. Right: A VirD4 chimera composed of the N-terminal transmembrane domain (NTD) of the pKM101-encoded TraJ (red) and the cytosolic (NBD/AAD/CTD) moiety of *A. tumefaciens* VirD4 supports transfer of *A. tumefaciens* effector proteins (VirE2, VirE3, VirF) through the 'dedicated' DNA conjugation system encoded by *E. coli* pKM101 (red) (Whitaker et al. 2016)

Fig. 4.

Structural conservation of T4SS outer membrane subassemblies. Upper: Structures of outer membrane core complexes (OMCCs) associated with the *E. coli* pKM101 Tra and *A. tumefaciens* VirB/VirD4 T4SSs, as determined by single-particle, negative-stain electron microscopy (NS-EM). Dimensions of the overall subassemblies and openings at proximal and distal ends are indicated. Structures are reproduced with permission from (Gordon et al. 2017). Lower: Functionality of chimeric T4SSs composed of the pKM101-encoded Tra IMC joined to heterologous OMCCs from the *E. coli* R388-encoded Trw, *A. tumefaciens* VirB/VirD4, and *B. pertussis* Ptl T4SSs. Transfer frequencies of pKM101 are presented as transconjugants per donor resulting from 2 h filter matings between *E. coli* donors and recipients. Data are from (Gordon et al. 2017)

Fig. 5.

Postulated T-pilus assembly pathway. Pilus-associated subunits are exported to the periplasm (P) or integrated into the inner membrane (IM) by the general secretory pathway (GSP). The pilus-tip protein VirB5 is recruited to the VirB T4SS where it nucleates pilus assembly from an IM platform composed in part of VirB8 (brown lollipop). VirB1 is proteolytically cleaved in the periplasm, and its N-terminal transglycosylase domain is recruited to the T4SS where it facilitates pilus assembly through localized degradation of the cell wall. The C-terminal domain, VirB1* (B1*), is delivered to the T-pilus tip where it facilitates pilus polymerization and extension. The VirB2 pilin is integrated into the IM, cyclized by covalent linkage of its N- and C-termini, and recruited upon receipt of an unknown signal to the T4SS for pilus assembly. The VirB4 ATPase, with a contribution by the VirB11 ATPase, catalyzes dislocation of the pilin monomers from the IM. The model depicts the IM as the nucleation platform for a thin pilus fiber that extends through the OMCC and across the outer membrane (OM). Once the fiber contacts the distal region of the OMCC, further polymerization leads to extension of a thicker (~10 nm) helically arrayed filament from the cell surface