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## BBA Review Revised Mechanism and Structure of the Bacterial Type IV Secretion Systems

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### Abstract

The bacterial type IV secretion systems (T4SSs) translocate DNA and protein substrates to bacterial or eukaryotic target cells generally by a mechanism dependent on direct cell-to-cell contact. The T4SSs encompass two large subfamilies, the conjugation systems and the effector translocators. The conjugation systems mediate interbacterial DNA transfer and are responsible for the rapid dissemination of antibiotic resistance genes and virulence determinants in clinical settings. The effector translocators are used by many Gram-negative bacterial pathogens for delivery of potentially hundreds of virulence proteins to eukaryotic cells for modulation of different physiological processes during infection. Recently, there has been considerable progress in defining the structures of T4SS machine subunits and large machine subassemblies. Additionally, the nature of substrate translocation sequences and the contributions of accessory proteins to substrate docking with the translocation channel have been elucidated. A DNA translocation route through the *Agrobacterium tumefaciens* VirB/VirD4 system was defined, and both intracellular (DNA ligand, ATP energy) and extracellular (phage binding) signals were shown to activate type IV-dependent translocation. Finally, phylogenetic studies have shed light on the evolution and distribution of T4SSs, and complementary structure-function studies of diverse systems have identified adaptations tailored for novel functions in pathogenic settings. This review summarizes the recent progress in our understanding of the architecture and mechanism of action of these fascinating machines, with emphasis on the ‘archetypal’ *A. tumefaciens* VirB/VirD4 T4SS and related conjugation systems.

### Keywords

Conjugation; DNA transfer; pathogenesis; protein translocation; pilus; coupling protein; traffic ATPases; core complex

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## 1. Introduction

The type IV secretion systems (T4SSs) are a diverse group of translocation systems present in nearly all bacterial and some archaeal species [1–6]. These systems translocate DNA and monomeric and multimeric protein substrates to bacterial or eukaryotic target cells. The two main T4SS subfamilies are the conjugation machines and the effector translocator systems, both of which deliver substrates intercellularly by a mechanism requiring establishment of direct cell-to-cell contact [7]. The conjugation machines translocate single-stranded DNA substrates within and across many bacterial species. These systems are responsible for shaping of genomes on an evolutionary time-scale and dissemination of antibiotic resistance genes and virulence determinants in clinical settings [1, 8]. The effector translocators deliver protein substrates directly to eukaryotic cells. These systems are central to infection processes of Gram-negative bacterial pathogens including *Agrobacterium tumefaciens*, *Helicobacter pylori*, *Brucella spp.*, *Bartonella spp.*, *Rickettsial spp.*, and *Legionella pneumophila* [1–6]. A third smaller group of T4SSs is represented by the DNA release system of the *Neisseria gonorrhoeae* GGI system, the *H. pylori* ComB competence system and the *Bordetella pertussis* pertussis toxin export (Ptl) system [9–11]. These systems take up DNA from the milieu or release DNA or protein substrates into the milieu independently of contact with another cell.

The *A. tumefaciens* VirB/VirD4 system is one of the best-characterized T4SSs in Gram-negative bacteria and here will serve as a reference point for discussion of these fascinating machines. This system is composed of 11 VirB proteins synthesized from the *virB* operon and the VirD4 subunit from the separate *virD* operon [12]. It functions to deliver a fragment of the *A. tumefaciens* genome, oncogenic T-DNA, as well as several effector proteins, e.g., VirE2, VirE3, VirF, to susceptible plant cells, resulting in the tumorous Crown Gall disease [13]. Many T4SSs of Gram-negative bacteria are composed of homologs of most or all of the VirB and VirD4 subunits, reflecting a common ancestry and likely conservation of machine architecture [14, 15]. However, many T4SSs also display striking differences in subunit composition and number compared to the ‘archetypal’ VirB/VirD4 T4SS and related systems. Many Gram-positive conjugation systems, for example, are composed of only a subset of the VirB/VirD4 homologs [15, 16], whereas other systems such as the *L. pneumophila* Dot/Icm [4] and *H. pylori* Cag T4SSs [3, 5, 17] are built from nearly 30 subunits of which only a few bear discernible homology to the VirB/VirD4 subunits. These T4SSs almost certainly possess important variations in structure and mechanism of action in comparison to the VirB/VirD4-like systems.

Since publication of a review on the *A. tumefaciens* VirB/VirD4 system in this journal in 2004 [18], there has been considerable progress on several fronts in structure-function studies of T4SSs. X-ray structures have been solved for homologs of many of the VirB/VirD4 subunits and high-resolution X-ray or cryoelectron microscopy images now exist for several T4SS machine subassemblies. *In vivo* studies have identified substrate translocation sequences and defined a general translocation route for substrate passage through the VirB/VirD4 T4SS. Intra- and extracellular signals have been shown to activate T4SS channels for secretion of cognate substrates or uptake of bacteriophage that utilize T4SSs as receptors. Finally, phylogenetic studies have shed light on the evolution and distribution of T4SSs in

bacteria and archaea, and complementary structure-function studies have unveiled adaptations tailored for assembly of T4SSs across diverse cell envelopes and for a variety of novel applications in pathogenic settings.

This review is intended to update the reader about the structure and function of T4SSs, with an emphasis on systems closely related to the *A. tumefaciens* VirB/VirD4 system. Due to limitations in space, we will not attempt a comprehensive overview of the literature but instead highlight recent data from studies exploring fundamental mechanisms. We refer the reader to a number of excellent, specialized reviews on topics such as T4SS gene regulation [19–22], the biological and functional diversity of T4SSs [1, 2, 7, 15, 16, 23], and the physiological consequences of T4SS-mediated effector translocation during infection [3, 24–28].

## 2. General architectural/functional features of paradigmatic VirB/VirD4 T4SSs

There is accumulating evidence that the T4SSs of Gram-negative bacteria evolved through consolidation of four functionally-distinct machine subassemblies: i) the type IV coupling protein (T4CP), a hexameric ATPase related to the SpoIIIE/FtsK DNA translocases that physically couples early DNA and protein substrate processing reactions to the translocation machinery, ii) an inner membrane complex (IMC) responsible for substrate transfer across the inner membrane, iii) an envelope spanning outer membrane complex (OMC) required for substrate passage across the periplasm and outer membrane, and iv) the conjugative pilus, an extracellular organelle that initiates contact with potential recipient cells (Fig. 1). The first three subassemblies interact to form the translocation system. Surprisingly, the physical relationship between the translocation channel and conjugative pilus is not yet specified, nor is it known whether pilus polymerization initiates from a platform at the inner or outer membrane.

The *A. tumefaciens* VirB/VirD4 subunits and subassemblies are depicted in Fig. 1. VirD4 is the T4CP, also termed the substrate receptor (where necessary for clarity, we will identify the species or plasmid origin of a protein in subscript, e.g., VirD4<sub>At</sub>). VirD4-like T4CPs possess canonical Walker A and B nucleoside triphosphate binding motifs [29] and family members have been shown to bind and/or hydrolyze ATP *in vitro* [30–32]. The IMC is composed of the ATPases VirB4 and VirB11, polytopic membrane proteins VirB3 and VirB6, and bitopic protein VirB8. The OMC consists of a structure termed the core complex and a translocation channel presumptively located within the central chamber of the core complex. The *A. tumefaciens* core complex is composed of outer membrane-associated lipoprotein VirB7 and VirB9 and the cell-envelope-spanning subunit VirB10, and the channel itself is likely composed of the pilin subunit VirB2 and domains of VirB8 and VirB9 [12, 33]. The core complex of the closely related *E. coli* pKM101-encoded T4SS is configured as a large barrel composed of 14 copies each of homologs of VirB7, VirB9, and VirB10 that extends across the entire cell envelope [33, 34]. The *A. tumefaciens* VirB-encoded pilus is composed of the pilin subunit VirB2 [35] and the pilus-tip adhesin VirB5 [36]. The lytic transglycosylase VirB1 is required for pilus biogenesis, but not for assembly of the translocation channel [37].

A formaldehyde (FA)-crosslinking assay termed transfer DNA immunoprecipitation (TrIP) was used to identify close contacts between the *A. tumefaciens* T-DNA substrate and subunits of the VirB/VirD4 T4SS during translocation [38]. Contacts were detected with the VirD4 T4CP, VirB11 ATPase, inner membrane proteins VirB6 and VirB8, and periplasmic/outer membrane subunits VirB2 and VirB9 (Fig. 1). Further TrIP analyses with *virB* and *virD4* null mutants supplied evidence that DNA substrate - channel subunit contacts are ordered such that the VirD4 T4CP receptor first engages the DNA substrate and then transfers it to VirB11. VirB11 in turn delivers the substrate to a presumptive channel composed of VirB6 and VirB8 for translocation across the inner membrane. The substrate then passes through the periplasm and across the outer membrane via a channel minimally composed of VirB2 and VirB9 [38]. By combining the available structural and biochemical data, it can be postulated that T4SS substrates dock with the VirD4 T4CP and then are transferred to the IMC for further processing and translocation across the inner membrane via a channel composed minimally of VirB6 and VirB8. The IMC sits within or at the base of the OMC/core, forming specific contacts with the N-terminal region of VirB10 and, possibly, the N-terminal region of VirB9. The C-terminal region of VirB8, one or more domains of VirB9, and the VirB2 pilin form the translocation channel within the central chamber of the VirB7/VirB9/VirB10 core complex for conveyance of substrates to the cell surface [14, 39].

### 2.1. Structural variations/adaptations among T4SSs

The structural features described to date for the *A. tumefaciens* VirB/VirD4 T4SS and closely related systems are probably conserved among many T4SSs, yet as a whole this transporter superfamily has evolved considerable mosaicism in terms of subunit composition, and thus overall architecture and function. Variations exist in each of the four T4SS machine subassemblies, as illustrated in Fig. 2 for some of the better-characterized T4SSs. Some T4SSs, including the *B. pertussis* Ptl and *Brucella spp.* VirB systems lack a VirD4-like T4CP and therefore utilize alternative mechanisms for substrate recognition. Some systems, including the *E. coli* F plasmid transfer system and Gram-positive bacterial conjugation systems lack a VirB11 homolog [1, 11, 15, 16, 40, 41]. Indeed, the Gram-positive conjugation machines lack several VirB homologs, including the OMC/core and pilin subunits. These ‘minimized’ systems are composed only of the IMC/T4CP complex for delivery of substrates across their single membranes and one or two extracytoplasmic proteins that presumptively form a channel across the thick cell wall [15, 16]. By contrast, IMCs of the *L. pneumophila* Dot/Icm (Fig. 2) and *H. pylori* Cag (not shown) systems are composed of many more subunits than the *A. tumefaciens* VirB/VirD4 system [4, 5, 17, 42].

With respect to the OMC/core, although homologs of the *A. tumefaciens* VirB7, VirB9, and VirB10 subunits are widespread among Gram-negative systems, the core subunits often possess additional novel domains (Fig. 2). In the *H. pylori* Cag T4SS, for example, only a small C-terminal region of CagY is similar to VirB10. Both the N- and C-terminal regions possess transmembrane domains, and a large central region composed of multiple repeats is surface-displayed and associates with a pilus structure. Similarly, CagT is a predicted VirB7-like lipoprotein, but also possesses a novel, surface-exposed variable region [5, 17]. Structural variation in the OMC composition can also occur through cooption of novel

proteins or protein complexes, as exemplified with the *E. coli* F transfer system (Fig. 2). In addition to the TraV/K/B core complex, the F plasmid-encoded OMC consists of a set of proteins, TraF, -H, -U, -W and TrbB, -I that form an interaction network distinct from the core complex [43–46]. The TraF/H/U/W-TrbB/I complex is implicated in the unique capacity of F-type conjugative pili to dynamically extend and retract and confer efficient mating in liquid media. Finally, many structural variations exist among T4SS surface components and pili. T4SSs including all Gram-positive conjugation systems and the *B. pertussis* Ptl system lack extracellular pili altogether [15, 16]. Conversely, many effector translocators including the *Bartonella* spp. Trw and *H. pylori* Cag systems elaborate antigenically-variable pili or other fibrous or sheathed structures (See [1]). Although such structures are generally considered to mediate target cell attachment and immune evasion, in the *H. pylori* Cag system, the sole substrate CagA has been shown to associate with the Cag T4SS-encoded pilus (Fig. 2), pointing to a direct contribution of the pilus to substrate delivery to mammalian target cells [17, 47, 48].

### 3. Substrates/translocation signals/Substrate docking reactions

The type IV secretion substrates typically rely on the T4CP receptor for translocation and, accordingly, carry translocation signals required for substrate-T4CP docking. A small number of substrates carry classical N-terminal signal sequences for translocation across the inner membrane via the general secretory (Sec) pathway (Fig. 3).

#### 3.1. T4CP-dependent translocation

Conjugation systems, most effector translocator systems, and the *N. gonorrhoeae* DNA release system use the T4CP-dependent pathway [1, 23]. Substrates translocated by this pathway bind the T4CP receptor via one or both of the following translocation signals: i) signals located at an unstructured C terminus consisting of clusters of positively-charged or hydrophobic residues or ii) one or more internal signals. Docking of both types of substrates also often requires chaperones or adaptor proteins (Fig. 3).

Studies exploring the nature of type IV secretion signals capitalized on findings that T4SSs can translocate reporter proteins, e.g., Cre recombinase, adenylate cyclase (CyaA), TEM  $\beta$ -lactamase, intercellularly when fused to an effector. In early studies, the *A. tumefaciens* VirB/VirD4 system was shown to translocate fusion proteins composed of Cre joined to the N termini of VirE2 or VirF effectors to plant and yeast cells [49]. Similarly, the *L. pneumophila* Dot/Icm T4SS translocated a CyaA-RalF effector to mammalian cells [50]. For both systems, the translocation signals were localized within unstructured C termini of the effectors, and consisted of clusters of positively charged Arg residues (for *A. tumefaciens* VirB/VirD4 substrates) or hydrophobic residues (for *L. pneumophila* Dot/Icm substrates) [51, 52].

Many T4SS effectors (or candidates) have now been identified with intercellular translocation assays [53–56]. Effectors typically possess C-terminal translocation signals, but there is also accumulating evidence for the importance of additional motifs for T4CP docking. These signals are located terminally or within the protein (Fig. 2). In *Bartonella* spp., for example, the *Bartonella*-translocated effector proteins (Beps) are translocated

through a VirB/VirD4-like system. The Beps display a modular architecture with a bipartite secretion signal composed of a positively charged C terminus and at least one internal domain termed a Bep-intracellular delivery domain (BID) [50]. Interestingly, BIDs are also present in relaxases associated with conjugative plasmids carried by various  $\alpha$ -proteobacterial species. The TraA relaxase encoded by *A. tumefaciens* plasmid pATC58 carries both a positively-charged C terminus and BIDs and, strikingly, TraA fragments carrying both types of recognition signals mediate translocation of Cre through a *Bartonella henselae* VirB/VirD4 system [50]. Thus, an effector translocator system and an ancestrally related conjugation system recognize common substrate signals. In *H. pylori*, a positively charged C-terminal tail is important for translocation of the CagA substrate through the Cag T4SS, and this tail can even be exchanged with similar motifs from other effectors to yield CagA translocation [57]. However, CagA translocation additionally requires an intact N-terminal motif of unspecified sequence composition [57] as well as internal motifs required for interaction with the specialized secretion chaperone CagF [58–60].

In the conjugation systems, the relaxase component of the DNA transfer intermediate confers DNA substrate specificity (Fig. 3). Several relaxases (MobA<sub>R1162</sub>, VirD2<sub>At</sub>, TraA<sub>pATC58</sub>, TrwC<sub>R388</sub>, TraI<sub>R1</sub>) were shown to mediate transfer of N-terminally-fused Cre to target cells [50, 51, 61, 62], and domain-mapping studies have identified two or more translocation signals (Fig. 3). MobA<sub>R1162</sub> is composed of two domains, an N-terminal relaxase domain and a C-terminal primase domain, and each domain can separately mediate translocation of Cre through the plasmid R751-encoded T4SS. Mutation of the positively charged C terminus of the primase domain or of the catalytic sites of the relaxase or primase domains abolishes translocation, suggesting that MobA<sub>R1162</sub> translocation requires a C-terminal signal as well as motifs exposed only in the folded protein [62].

More recently, the F-like relaxase TraI<sub>R1</sub> was found to carry two internal translocation signals (TSs), designated TSA and TSB, that mapped within ~300 residue regions located in each half of the protein (Fig. 3) [63]. Further studies identified key conserved residues conforming to a consensus sequence (G[E/D]R[L/M]R[V/F]T) in both TS motifs which, interestingly, were also identified in the MobA<sub>R1162</sub> translocation signals. A crystal structure of the TSA region of TraI<sub>R1</sub> was solved. It consists of three domains, each with structural homologies to SF1B helicase family domains, and a putative interaction surface was mapped to one of the helicase domains through mutational analysis [64]. These findings lend support to early models whereby helicase-mediated unwinding of the DNA substrate from its template strand is physically coupled with the T4CP and proceeds concomitantly with substrate transfer through the mating channel. These results also establish for the first time that a structural fold can serve as a signal for translocation through the T4CP-dependent pathway.

Substrates also can have distinct translocation signals for docking with different T4SSs (Fig. 3). Translocation of MobA<sub>R1162</sub> relaxase through the R751-encoded T4SS channel requires two internal translocation signals, but a positively-charged C-terminal motif suffices for translocation through the *A. tumefaciens* VirB/VirD4 T4SS [65]. Similarly, translocation of the TrwC<sub>R388</sub> relaxase through R388-encoded Trw T4SS requires two internal translocation sequences with consensus motifs resembling those in TraI<sub>R1</sub> and MobA<sub>R1162</sub>, but

translocation through the *B. henselae* VirB/VirD4 T4SS requires only an intact C terminus [66].

### 3.2 Contributions of other factors to substrate-T4CP docking

In addition to the intrinsic translocation signals discussed above, various accessory factors, adaptor proteins, or chaperones are often required for type IV secretion (Fig. 3).

**3.2.1. Dtr accessory factors**—In conjugation systems, Dtr (DNA transfer and replication) accessory factors play important roles as components of the relaxosome to stimulate relaxase nicking at *oriT*. In *A. tumefaciens*, VirC1 stimulates the nicking activity of the relaxase VirD2<sub>At</sub> through binding to a DNA sequence termed *overdrive* located adjacent to the *oriT*-like T-DNA border sequence [67]. Interestingly, VirC1 was identified as a member of the ParA/Soj/MinD ATPase superfamily [68], and further work established that VirC1 localizes at cell poles and interacts with a second Dtr accessory factor VirC2, the VirD2 relaxase, and the VirD4 T4CP, which is also polarly localized. Through these interactions, VirC1 is postulated to spatially couple the T-DNA substrate and the T-DNA processing reaction at the site of the VirB/VirD4 T4SS (Fig. 3) [69]. ParA homologs are associated with other conjugative plasmids and integrative and conjugative elements (ICEs), and further studies examining their contributions to the substrate-T4CP docking reaction are warranted. Spatial positioning of the MobA<sub>R1162</sub> relaxase near a T4SS is also important for R1162 plasmid transfer. Recall that MobA<sub>R1162</sub> has two functionally distinct domains, each with a translocation signal. The accessory factor MobB<sub>R1162</sub> was found to stimulate translocation efficiencies of both MobA domains by a mechanism dependent on binding of MobB<sub>R1162</sub> to the membrane [62]. MobB<sub>R1162</sub> thus can be postulated to function analogously to VirC1 by tethering the R1162 substrate at or near a T4CP (Fig. 3). R1162 plasmids are non-selftransmissible plasmids that promiscuously translocate through many different T4SSs, and conceivably MobB<sub>R1162</sub> functions to stabilize inherently weak interactions between MobA and different T4CPs [62].

**3.2.2. Effector protein chaperones/adaptors**—Effector proteins are translocated through their cognate T4SSs by alternative pathways distinguishable by the requirement of an adaptor protein or chaperone (Fig. 3). In *A. tumefaciens*, translocation of VirF through the VirB/VirD4 T4SS proceeds independently of other known factors, whereas translocation of VirE2 requires cosynthesis of the VirE1 chaperone [70]. VirE1 shares several features of the specialized chaperones associated with type III secretion systems (T3SSs), including a small size, acidic pI, and an amphipathic helix. An X-ray structure solved for a VirE1 chaperone/VirE2 effector complex identified an unusual structural arrangement of N- and C-terminal domains of VirE2 wrapped around the VirE1 chaperone [71] (Fig. 2). VirE1 does not participate directly in mediating the VirE2 interaction with the VirD4 T4CP [72, 73]. Rather, chaperone binding prevents self-aggregation of VirE2 and binding of DNA within the bacterium. Formation of the complex also ensures that VirE2's disordered C-terminal tail is solvent exposed and accessible for docking with the T4CP [71].

In *L. pneumophila*, the Dot/Icm T4SS translocates more than ~250 effectors of which a subset are dependent on the IcmSW chaperone complex [74]. A complex of 4 proteins

including DotM, DotN, IcmS, and IcmW has been shown to interact with the DotL T4CP. DotM and DotN are membrane associated and stabilize DotL, whereas IcmS and IcmW are small, acidic proteins that appear to function similarly to VirE1 and the T3SS specialized secretion chaperones [42]. IcmS and IcmW heterodimerize and the IcmSW complex appears to promote conformations in cognate secretion substrates required for productive engagement with the DotL T4CP (Fig. 3) [75, 76]. A recent study supplied evidence that a C-terminal domain of DotL directly binds the IcmSW complex and that this interaction is necessary for delivery of IcmSW-dependent effectors through the Dot/Icm channel [77]. The findings prompted the interesting model that IcmSW binds an effector in the cytosol and, like VirE1, maintains it in a translocation-competent state. The effector docks via a C-terminal translocation signal with the DotL T4CP, whereupon IcmSW is released and sequestered through interaction with the DotL C terminus. This permits effector translocation through the Dot/Icm T4SS and, ultimately, recycling of the IcmSW chaperone to the cytosol for another round of substrate binding and translocation [77].

In *H. pylori*, CagA is the sole known protein substrate of the Cag T4SS [5, 78]. Crystal structures of CagA show that this protein is comprised of 5 domains that contribute in distinct ways to modulation of eukaryotic cell function. In addition to its N- and C-terminal signals, CagA must interact with its chaperone CagF for translocation [58, 59]. CagF has features reminiscent of VirE1 and the IcmS and IcmW chaperones, although it is much larger (~35-kDa) than these chaperones. Recently, it was shown that distinct domains of CagF bind to the five domains of CagA, each with  $\mu\text{M}$  affinity. The findings led to a proposal that multiple copies of CagF form CagA domain contacts necessary for maintaining the highly labile CagA effector in a translocation-competent, protease-resistant conformation [60]. CagF is also membrane-associated and has been proposed to mediate delivery of CagA to the Cag T4SS [58].

Why do such a diverse array of intrinsic motifs and extrinsic accessory factors exist for substrate docking with the T4CP receptor? A reasonable hypothesis is that this complexity evolved for coordination of type IV secretion with other cellular activities. Conjugation must be tightly coordinated with the cell cycle to avoid the competing activities of conjugative DNA metabolism and chromosome replication and segregation. Similarly, the effector translocators must precisely time delivery of potentially many different effectors to the eukaryotic host cell during the infection process. While translocation signals carried by relaxases and effector proteins confer substrate specificity by mediating T4CP docking, these or other intrinsic motifs also mediate interactions with accessory factors, adaptors, or chaperones. It can be speculated that complex regulatory circuitries regulate the synthesis or activity of the accessory factors for spatiotemporal control of substrate presentation to the transfer apparatus.

### 3.3. T4CP-independent translocation

Two effector translocator systems have been shown to translocate secretion substrates via a T4CP-independent pathway. The *B. pertussis* Ptl system translocates its only substrate, the pertussis toxin (PT), exclusively across the outer membrane (Fig. 3) [11, 79]. The 5 subunits (S1–S5) comprising this A/B toxin each carry canonical N-terminal *sec* signal sequences



and they are exported across the inner membrane via the Sec system. In the periplasm, the S1–S5 subunits fold and oligomerize to form active PT, which then docks with the Ptl machine for translocation across the outer membrane. The Ptl system is unique among the known T4SSs in its capacity to translocate a large, multisubunit toxin across the outer membrane by a cell-contact-independent mechanism.

The *Brucella spp.* VirB T4SS also lacks a T4CP, but interestingly a number of translocated effector proteins have now been identified and shown to carry translocation signals at either terminus [53, 55, 80]. Effectors with N-terminal *sec* signals, e.g., BPE123, most probably are exported via the Sec system to the periplasm, where they then engage with the T4SS channel for transit across the outer membrane [55]. However, effectors with C-terminal signals, e.g., VceA and VceC, presumptively are not exported through the Sec system [53]. Interestingly, VceC was shown to translocate via its C-terminal translocation signal through the T4CP-dependent Dot/Icm system of *L. pneumophila* [53]. This likely represents another example of a substrate showing promiscuity for docking with different T4SSs, but the findings still do not answer the interesting question of how type IV substrates bearing C-terminal signals dock with the *Brucella spp.* VirB T4SS.

#### 4. The T4CP substrate receptor

The role of T4CPs as substrate receptors is now well supported by genetic and biochemical findings. T4CPs can functionally substitute for one another, in some cases conferring substrate specificity switching [66, 81, 82]. In *A. tumefaciens*, the TrIP studies showed that the VirD4 T4CP binds the T-DNA substrate independently of the VirB subunits and also by a mechanism dependent on processing of the T-DNA by the VirD2 relaxase [38]. *Bacillus fragilis* plasmid pLV22a also was shown to form a formaldehyde-crosslinkable contact with the Tra<sub>GRP4</sub> T4CP [83], and *Enterococcus faecalis* pCF10 with the PcfC<sub>pCF10</sub> T4CP [31]. Complementary *in vitro* studies have identified interactions between T4CPs and relaxosome components or protein substrates, and also demonstrated stimulatory effects of purified T4CPs on relaxase cleavage reactions [1, 23, 84].

T4CPs characteristically possess an N-terminal transmembrane domain and a large C-terminal cytoplasmic region comprised of a nucleotide-binding domain (NBD), an all- $\alpha$ -domain (AAD), and in some cases a C-terminal extension (CTE) [1, 85, 86]. An X-ray structure of the 50-kDa soluble domain of TrwB<sub>R388</sub> revealed a globular hexameric assembly in which each subunit is composed of an NBD and an AAD (Fig. 4). The NBD is composed of a central twisted  $\beta$ -sheet flanked by several helices on both sides, and the AAD contains seven helices with a structure similar to the N-terminal domain of XerD recombinase. The six TrwB protomers assemble to form a globular ring that is  $\sim 110$  Å in diameter and 90 Å in height, with a  $\sim 20$  Å-wide channel in the center. This channel narrows to 8 Å at the cytoplasmic pole of the hexamer. Nucleotide binding pockets are located at the interface between the subunits. The N-terminal domain of TrwB<sub>R388</sub> is predicted to project across the inner membrane on the basis of electron microscopy imaging [87]. The full-length protein is thus configured as a ball-stem, F<sub>1</sub>-F<sub>0</sub>-like structure (Fig. 4) [85, 86]. In view of noted sequence and structural similarities with the FtsK and SpoIIIE DNA translocases and findings that duplex DNA threads through the annulus of the FtsK hexamer [88, 89], it has

been suggested that the T4CP might function similarly to drive ssDNA substrate transfer across the inner membrane (see Section 9).

Interesting structural variations exist in T4CP domain architecture [1, 90]. The well-characterized T4CPs from Gram-negative systems, e.g., TraD<sub>F</sub>, TraG<sub>RP4</sub>, TrwB<sub>R388</sub>, VirD4<sub>At</sub>, typically range in molecular sizes of ~600 – 750 residues and possess a minimum of two predicted N-terminal TM domains with an intervening periplasmic loop of ~30–50 residues. TraG encoded by *Salmonella typhi* plasmid R27A-encoded TraG and related T4CPs comprise a distinct subfamily of T4CPs with much smaller (~4 residues) periplasmic domains and TraJ interaction partners. TraJ subunits are hydrophobic, multi-pass membrane proteins with sequence relatedness to the N terminus of FtsK translocase. Thus, it was proposed that TraJ and TraG cumulatively represent the domain architecture of the larger FtsK/SpoIIIE DNA translocases [91]. Members of another T4CP subfamily, represented by *S. agalactiae* pIP501 and *B. cenecapacia* AU1054, appear to lack N-terminal TM domains entirely, but upstream of the genes for these T4CPs are genes coding for small proteins (~150–200 residues) with 2 – 4 predicted TM domains. These subunit pairs might interact to form novel two-partner T4CPs [1].

Some T4CPs also carry CTEs [1]. The contributions of CTEs to substrate transfer are largely unspecified, with the exception that the CTE of TraD<sub>F</sub> plays a critical role in the F plasmid docking reaction. The CTE is necessary for efficient F transfer but hinders RSF1010 transfer [92–94]. This region of TraD was shown to bind TraM, which is a component of the relaxosome. An X-ray structure for the TraD CTE bound to the TraM tetramerization domain shows that the TraD peptide forms extensive contacts with each TraM monomer, indicating that as many as four TraD CTEs could simultaneously bind a single TraM tetramer. These findings suggest that a TraD hexamer might establish extensive contacts with TraM *in vivo*, forming the basis of a highly specific relaxosome - T4CP interaction [95].

The T4CP interacts with the IMC, although as mentioned above substitution of a T4CP with a closely related homolog can yield functional conjugation systems. There is some evidence for T4CP interactions with VirB10-like subunits of the core complex, and the interaction surfaces generally map within the N-terminal regions of both subunits [96]. Results of two-hybrid studies and mutational analyses have pointed to a contribution of the transmembrane helices of both subunits to this interaction [97]. In *A. tumefaciens*, however, the transmembrane domain (TMD) of VirB10 can be substituted with heterologous TM domains, including a nondimerizing poly-Leu/Ala helix, without loss of channel function, and further mutational analyses implicated the N-terminal periplasmic loop of VirD4 and both cytoplasmic and periplasmic domains of VirB10 in this interaction [98–100]. Given the subunit complexity of the IMC and OMC/core, it is reasonable to predict that the T4CP forms multiple, possibly dynamic, interactions with both subassemblies.

## 5. The inner membrane complex (IMC)

The *A. tumefaciens* IMC is composed of the energy ATPases VirB4 and VirB11 in association with two polytopic membrane proteins VirB3 and VirB6 and the bitopic subunit VirB8.

### 5.1. The ATP energy center

Besides the T4CP, the VirB4 and VirB11 ATPases are required for elaboration of most T4SSs. VirB4 ATPases are ubiquitous components of T4SSs, whereas the VirB11 subunits are missing from some Gram-negative systems, e.g., the F plasmid T4SS, and all Gram-positive systems [1, 90]. VirB4 subunits are integral or tightly-associated membrane proteins [101, 102], and the VirB11 ATPases bind the membrane peripherally and typically co-fractionate with the membrane and cytosol, suggestive of a possible dynamic association with the IMC [103, 104].

The VirB4 ATPases are large (>70-kDa) proteins with consensus Walker A and B NTP-binding domains and a variable number of predicted TMDs. VirB4 ATPases share a common ancestry with the T4CPs [90], and there is now structural evidence by cryoelectron microscopy that VirB4-like TrwK<sub>R388</sub> assembles as a hexamer (Fig. 4) [105]. By small-angle X-ray scattering, low-resolution structures were solved for a membrane-extracted dimeric form of pKM101 TraB and a soluble monomeric form of LvhB4 from *L. pneumophila* [106]. These structural data, and earlier biochemical findings [29], suggest that the VirB4 ATPases might exist in different oligomeric states, possibly transitioning between lower- and higher-order complexes to fulfill distinct activities within the cell. VirB4 homologs form multiple contacts with other IMC subunits as well as the VirB10 component of the core complex [107–109].

Despite the structural advances, it remains unknown how VirB4 subunits catalyze substrate transfer. In *A. tumefaciens*, VirB4 did not form detectable FA-crosslinks with the T-DNA substrate, but catalytically-active VirB4 was necessary for detection of substrate crosslinks with the VirB6 and VirB8 channel components [38, 107]. These findings led to a proposal that VirB4 mediates substrate transfer indirectly through coordination of its ATP binding/hydrolysis activities with those of VirD4 and VirB11 [107]. However, more recent studies of the *E. faecalis* pCF10-encoded T4SS showed that VirB4-like PrgJ forms FA-crosslinks with pCF10 in a reaction requiring the pCF10-encoded Dtr factors and PfcC T4CP [31]. Additionally, purified VirB4 homologs encoded by *E. coli* plasmids pKM101 and R388 recently were shown to bind DNA substrates *in vitro* [105, 110]. These findings, coupled with recent structural evidence for localization of VirB4 hexamers at the base of the IMC, suggest the possibility that VirB4 participates more directly in substrate transfer than previously envisioned.

VirB11 ATPases are structurally related to a large family of AAA<sup>+</sup> ATPases that also include the GspE ATPases associated with type II secretion systems (T2SSs) and type IV pilus assembly systems [111]. Crystal structures were solved for *H. pylori* HP0525 (now called Cag $\beta$ ; [5]) and *B. suis* VirB11, and both present as hexameric, double-stacked rings in which the N-terminal domains (NTDs) interact to form one ring and the C-terminal domains

(CTDs) form the second (Fig. 4) [111, 112]. For Cag $\beta$ , the CTD adopts a RecA fold, whereas the N-terminal domain is unique. The *B. suis* VirB11 monomer differs dramatically from that of Cag $\beta$  by a large domain swap caused by the insertion of additional sequences into the linker between the NTD and the CTD. The overall assembly of the VirB11 hexamer remains intact compared to Cag $\beta$  but the domain organization profoundly modifies the nucleotide-binding site and the interface between subunits. Based on sequence comparisons, it is likely that most VirB11 homologs display a hexameric structure closely resembling *B. suis* VirB11 [112].

VirB11 subunits are required for substrate transfer and pilus biogenesis. In *A. tumefaciens*, the T-DNA substrate was shown to crosslink with VirB11, and this contact was dependent on VirD2 nicking at the T-DNA borders and production of the VirD4 T4CP. These findings led to the proposal that the T4CP receptor delivers the T-DNA substrate to VirB11 for further processing [38, 107]. The VirB11 subunits might use the mechanical leverage generated by nucleotide-dependent conformational changes to unfold relaxases and protein substrates and dissociate bound accessory factors, e.g., relaxosome subunits or secretion chaperones [113]. Such an activity would be reminiscent of that described for InvC, a VirB11-like subunit associated with a type III secretion system in *Salmonella enterica* [114]. VirD4, VirB11, and VirB4 physically and functionally interact to energize substrate transfer [107, 115], but further biochemical studies are needed to decipher the molecular details of ATP-dependent reactions mediating substrate processing and translocation through the IMC.

## 5.2. The inner membrane channel

In addition to the ATPases, the postulated subunits of the IMC and, specifically, the inner membrane translocon include VirB3, VirB6, and VirB8. *A. tumefaciens* VirB3 is a small, two-pass membrane protein that binds to VirB4. VirB3 subunits invariably are cosynthesized with VirB4-like ATPases and in some systems the two are fused as a single protein, suggestive of a 1:1 stoichiometry and coordination of function [1]. VirB6 subunits have evolved as two distinct subtypes. Those resembling *A. tumefaciens* VirB6 are ~300 residues with 5 or more membrane-spanning domains and a large central, periplasmic domain. The ‘extended-VirB6’ subunits are larger (>500 residues) with an N-terminal polytopic membrane domain and a large C-terminal hydrophilic domain [1]. The role of this C-terminal domain is unspecified, although there is evidence in the *E. coli* F plasmid [116] and *Vibrio cholerae* SXT integrative and conjugative element (ICE) [117, 118] transfer systems for protrusion or release of this domain to the cell surface and into target cells. In *A. tumefaciens*, FA-crosslinking of VirB6 with the T-DNA substrate depended on synthesis of catalytically active forms of VirD4 and the VirB4 and VirB11 ATPases, supporting the notion that ATP energy is necessary for substrate engagement with the VirB6 component of the translocon [107, 119].

VirB8 subunits are bitopic proteins with a short cytoplasmic N-terminal domain, TM domain, and large C-terminal periplasmic domain [120]. X-ray structures have been solved for the periplasmic fragments of *B. suis* [121, 122] and *A. tumefaciens* [121, 122] VirB8 subunits (Fig. 4). Recent studies have identified VirB8-like subunits as common components of Gram-positive systems, and crystal structures now exist for the periplasmic domains of

two VirB8-like subunits, *Clostridium perfringens* pCW3-encoded TcpC [123] and *Streptococcus agalactiae* pIP501-encoded TraM [124]. VirB8 domains present as large extended  $\beta$ -sheets with five  $\alpha$ -helices, giving rise to an overall globular fold. These subunits packed as dimers or trimers in the crystal structures, and results of mutational analyses suggest that oligomerization is physiologically relevant [125, 126]. This dimer interface has even served as a target for a high-throughput screen aimed at identifying small molecule inhibitors of T4SSs [127]. In *A. tumefaciens*, VirB8 formed an FA-crosslink with the T-DNA, pointing to a direct role for this subunit in substrate translocation [38]. In the TRIP assays, some VirB6 mutations were shown not to affect substrate crosslinking with VirB6, but did block substrate crosslinking with VirB8 [119]. VirB6 and VirB8 thus appear to participate sequentially in substrate transfer, reflecting a possible architectural arrangement for this portion of the translocation channel.

## 6. The outer membrane complex (OMC) and core

Early studies of the *A. tumefaciens* VirB/VirD4 system showed that cosynthesis of the VirB7 lipoprotein, VirB9, and VirB10 were required for stabilization of the other two subunits, as well as most of the other VirB subunits (see [12]). Results of these studies led to the concept of the ‘core complex’, an intrinsically stable VirB7/B9/B10 subassembly that serves as a structural scaffold during machine biogenesis. Existence of this core complex has now been validated by detection of ring-shaped core complexes from *A. tumefaciens* by transmission electron microscopy [128] and high resolution structural analysis of the related *E. coli* pKM101-encoded TraN/O/F core complex by cryoelectron microscopy and X-ray crystallography (Fig. 4) [33, 34].

The pKM101 core complex, composed of 14 copies of each subunit, is a 1.05 MDa structure of 185 Å in width and height [33]. It is composed of two layers (I- and O-layers) that form a double-walled ring-like structure. The I-layer, composed of the N-terminal domains of TraO and TraF, is anchored in the inner membrane with a 55 Å diameter hole formed by the TM helices of TraF. The O-layer, composed of TraN and the C-terminal domains of TraO and TraF, has a main body and a narrower cap on the outermost side of the complex that is thought to insert in the outer membrane. A narrow hole (10 Å) exists in the cap, resulting in a channel extending through the entire cylindrical structure. A crystal structure of the O-layer revealed that the 14 copies of a TraF/VirB10 domain comprising two  $\alpha$ -helices separated by a loop, termed the antennae projection (AP), form the cap structure (Fig. 4) [34, 98]. In view of biochemical evidence that the N-terminal region of VirB10 stably inserts into the inner membrane [34, 98], it is now evident that the VirB10/TraF subunits are entirely unique bacterial proteins in their capacity to span the entire cell envelope.

The transenvelope topology of the core complex lends itself well to a role as a structural scaffold for the T4SS [14, 98]. The inner membrane ring formed by the TM helices of VirB10 is predicted to encircle or sit on top of the IMC. The central chamber of the core complex is approximately 100 Å in diameter, sufficiently large to house VirB subunits postulated on the basis of the TRIP studies and other findings to include the C-terminal domain of VirB8, the pilin subunit VirB2, and a portion of the N-terminal domain of VirB9 [14, 39]. The entire OMC, consisting of the core complex encircling the central translocation

channel, is predicted to interact with the IMC and mediate passage of substrates through the periplasm and outer membrane.

## 7. Activation of substrate transfer through the distal portion of the translocation channel

Most T4SSs translocate their cargo substrates only in response to establishment of productive contact with target cells. These T4SSs therefore must assemble in a closed state and require specific signals for channel activation. Studies have now shown that a combination of intracellular and extracellular signals activates substrate transfer. These studies also have provided evidence that VirB10 subunits function not only as components of the core structural scaffold but also play a central role in signal perception.

*A. tumefaciens* VirB10 undergoes a conformational switch, detectable as a change in protease susceptibility, in response to two intracellular signals: i) DNA substrate binding to the VirD4 and VirB11 ATPases and ii) ATP energy use by the VirD4 and VirB11 ATPases [99, 129]. Further work established that this conformational switch is required for passage of the DNA substrate through the distal portion of the translocation channel [38]. The nature of this switch is not yet known, but coupling of these intracellular signals to gating of the outer membrane pore is suggested by isolation of a mutation near the VirB10 AP pore that ‘locks’ VirB10 in the energized conformation and allows for release of secretion substrates to the cell surface independently of target cell contact [130]. Interestingly, it has also been reported that a DNA substrate and a Dtr accessory factor stimulate the ATPase activity of TrwB<sub>R388</sub> T4CP *in vitro* [131]. In a generalized model, therefore, one can postulate a sequence of reactions whereby docking of the DNA substrate with the T4CP and subsequent transfer to VirB11 stimulates the catalytic activities of one or both ATPases. ATP-dependent conformational changes in the ATPases induces a VirB10 conformational switch that is transduced to the C-terminal region of the core complex near or at the cap structure, resulting in channel opening and substrate passage. This overall energy-coupled transfer reaction is reminiscent of that described for the TonB energy coupling system, which also induces channel gating at the outer membrane in response to energy sensing through contacts with an inner membrane energy harvesting complex [129]. Notably, VirB10 senses ligand and ATP energy signals and is a component of the supramolecular core complex, whereas TonB senses the proton motive force and functions as a monomer or dimer [132]. Deciphering the molecular details of energy transduction along the core complex and the mechanism of OM gating remain intriguing areas for future investigation.

In early studies of the *E. coli* F plasmid transfer system, genetic evidence was presented for the existence of a ‘mating signal’ that was propagated by recipient cells upon contact with donor cells [133]. The nature of this mating signal remains unknown, but recent work showed that an external signal is transduced across the T4SS to the T4CP [134]. These studies explored the requirements for transduction by bacteriophage R17, whose receptor is the conjugative pilus encoded by the IncF plasmid R1. The findings established that R17 infects only piliated cells in which the plasmid R1 relaxosome is docked with the TraD<sub>R1</sub> T4CP. Nicking activity of the R1 relaxase is also required, establishing that the relaxosome must be catalytically active. In this system, the TraD T4CP does not display detectable

ATPase activity even when bound by relaxosome. However, binding of bacteriophage R17 to the conjugative pilus stimulates TraD activity and, correspondingly, phage uptake. Activation of the R1 T4SS therefore requires relaxosome docking at the T4CP as well as phage binding to the R1-encoded pilus. Although the findings highlight the role of phage binding to the pilus receptor for T4SS activation, the authors suggest that phage binding mimics the natural mating signal propagated upon pilus-mediated contact with the recipient cell [134]. In light of these findings and the transenvelope topology of VirB10 and its demonstrated role in sensing of intracellular ligand and energy signals, it is enticing to suggest that extracellular signals are also transduced along VirB10 and the core complex to the cell interior.

## 8. The translocation route: one or two steps?

In the T4SS translocation pathway presented here, the T-DNA substrate is delivered in one step through a channel that spans the entire cell envelope. This one-step pathway is supported by considerable biochemical and structural data for conjugation machines. However, as described above (Section 3.3, Fig. 3), there is evidence that the *B. pertussis* Ptl T4SS and, possibly, the *Brucella spp.* VirB system, recruit substrates from the periplasm, implying a two-step translocation pathway. In this pathway, substrates are translocated first across the inner membrane to the periplasm and then engage with the OMC for translocation across the outer membrane [135].

The *B. pertussis* Ptl T4SS is composed of close homologs of all of the VirB proteins except for the VirB5 pilus-tip adhesin. This system does not elaborate detectable pili, but it likely assembles a composite IMC/OMC structure that is architecturally similar to structures visualized for the archetypal conjugation systems [11]. This raises a fundamental question of where the multisubunit PT engages with the Ptl translocation channel. The toxin may enter the core's chamber through a lateral gate, but this seems unlikely given the extensive network of contacts observed between the TraO/VirB9 and TraF/VirB10 subunits [33, 34, 136]. Upon Sec-mediated secretion, the PT subunits associate with the outer membrane where they undergo disulfide crosslinking and oligomerization. This suggests that the outer membrane might be the site of substrate engagement with the T4SS; if so, PT might dock with the distal end of the OMC at or near the core's cap structure. This is an appealing model because it dispenses with the need for the large multisubunit toxin to pass through a narrow translocation channel within the core chamber and, instead, simply dock at the distal end of the T4SS. For PT extrusion across the outer membrane, a mechanism analogous to that envisioned for type II secretion systems (T2SSs) might be employed [137, 138]. For type II secretion, substrates delivered to the periplasm via the Sec system are thought to engage with the tip of a pseudopilus structure elaborated by the T2SS. Upon substrate docking, the pseudopilus extends and pushes the substrate through an outer membrane secretin. Substrate is released into the milieu, and the pseudopilus retracts for another round of substrate docking and extrusion. It is interesting to note that *B. pertussis* PT is a member of the A/B toxin superfamily and that all other multisubunit A/B toxins are exported via T2SSs. It seems reasonable that the type II and Ptl systems have evolved similar extrusion mechanisms for secretion of these large, multisubunit toxins.

Do other T4SSs use a two-step mechanism for translocation? In *A. tumefaciens*, there is some evidence for accumulation of protein substrates in the periplasm [139, 140], although results of the TrIP studies did not support the notion that the T-DNA transfer intermediate accumulates in this compartment [38]. Presently, there is no structural information about the interface of the T4CP/IMC, and OMC/core complex, and it is conceivable that a lateral gate exists at the junction of the inner membrane and periplasm. The notion that T4SSs employ a two-step translocation route has largely been driven by the demonstrated phylogenetic and structural similarities between the hexameric NBDs of T4CPs and the SpoIIIE and FtsK DNA motor proteins [141]. FtsK and SpoIIIE translocate directionally along DNA [142–144], and T4CPs might similarly thread the ssDNA substrate in a 5' to 3' direction through NBD hexamer and transmembrane stem to the periplasm [135, 145]. Whether all T4SS substrates transiently enter the periplasm, via either a T4CP or IMC translocon, before entering the OMC/core remains an intriguing question for further study.

## 9. The conjugative pilus

Many Gram-negative bacterial T4SSs elaborate extracellular pili. For the VirB/VirD4 and related conjugation systems, the genetic requirements are nearly the same for assembly of these two structures, with the exception that the T4CP is not required for pilus production and the VirB1 transglycosylase is not required for assembly of the translocation channel. It is tempting to depict the T4SS channel and T-pilus as a single, supramolecular organelle, but at this time there is no structural evidence for this association. Nevertheless, the genetic findings suggest that the transenvelope IMC/OMC structure functions as a scaffold both for the channel and pilus (Fig. 5).

There are two well-characterized groups of conjugative pili, F-pili elaborated by the *E. coli* F plasmid and other plasmids of the IncF, IncH1, and IncH2 incompatibility (Inc) groups and P-pili produced by *E. coli* plasmids of the P, N, or W Inc groups and the *A. tumefaciens* VirB/VirD4 T4SS [41, 146]. Studies of these pilus systems have identified physical properties of pilins and pili as well as early-stage reactions required for polymerization (Fig. 4). The F-type pili are typically ~90 Å and flexible, and range in length up to 1 micron. These pili dynamically extend and retract, enabling donor cells to bind and draw recipient cells into physical contact for establishment of the mating junction. The P-type pili are thicker (90–110 Å), more rigid, and much shorter than F-pili although length measurements are complicated by the fact that isolated pili are typically broken [147–149]. These pili do not appear to undergo cycles of extension/retraction, but instead accumulate in the milieu, either through breakage or an active sloughing mechanism. They tend to aggregate as a mesh of polymers, which is thought to promote nonspecific clumping of donor and recipient cells and subsequent formation of mating junctions.

The F- and P-pili are synthesized as pro-proteins with unusually long (~30–50 residues) leader peptides that are cleaved upon insertion into the inner membrane (Fig. 5) [45, 150]. Upon membrane insertion, the pro-pilins are processed further by LepB signal peptidase and additional proteases. The N termini of both F- and P-type pilins are modified, the F-type pilin by *N*-acetylation and the P-type pilin by covalent linkage to the C terminus in a head-to-tail cyclization reaction [41, 151, 152] Cyclization stabilizes the P-type pilins in the



membrane and is essential for pilus assembly. Mature F- and P-type pilins accumulate in the inner membrane, the former as a pool estimated at ~100,000 monomers [153]. Upon receipt of an unknown signal, the pilin monomers are extracted from this pool to build the pilus. A recent study explored the mechanism by which pilins are extracted from the hydrophobic membrane environment by assaying for accessibility of Cys-residues engineered along the length of *A. tumefaciens* VirB2 to a membrane impermeable thiol-reactive reagent [154]. This study defined the inner membrane topology of VirB2 and also presented evidence that the VirB4 ATPase modulates the structural state of membrane-integrated pilin. Catalytically-active VirB4 induced changes in the thiol accessibility of engineered Cys residues in VirB2 that were consistent with a role for the ATPase in extraction of the pilin from the membrane [154]. Independently, evidence was presented that VirB4 stabilizes subunits required for pilus assembly and is essential for interaction of VirB2 with the pilus-tip protein VirB5, a reaction thought to be essential for pilus nucleation [108, 155]. Taken together, these findings support a model in which VirB4 dislocates mature pilins from the inner membrane and mediates formation of the VirB2-VirB5 nucleation complex for subsequent pilus polymerization (Fig. 5).

Where in the cell envelope do pili polymerize? As mentioned above, the recent structural studies place VirB4 monomers or hexamers at the base of the IMC, in physical association with the core complex [106]. This association might facilitate the lateral translocation of pilin monomers through the membrane and into the chamber of the core complex. Once in the chamber, pilins would then undergo polymerization to build the conjugative pilus (Fig. 5) [1]. The central chamber of the pKM101-encoded core complex is approximately 100 Å in diameter, sufficiently large to house the pilus (90–110 Å). However, the outer membrane ring formed by TraF's AP domain is at most only 32 Å and clearly not large enough to accommodate pili [14]. If pili assemble from a platform of subunits located at the inner membrane, pilus extension through the chamber and across the outer membrane must induce gross structural changes in the distal portion of the core complex. A model for pilus assembly from an inner membrane platform is reminiscent of that described for type IV pili [156]. For F-pilus biogenesis, this is an appealing model because F-pili, like type IV pili, successively recycle membrane-integrated pilins during extension and retraction. Alternatively, pili might assemble at the outer membrane, on a platform formed by the AP domain of the core complex [1, 108]. Accordingly, upon extraction from the inner membrane the hydrophobic pilins would be chaperoned within the core's chamber or directly across the periplasm to the outer membrane for nucleation. Such an assembly pathway is reminiscent of that described for P or Type I fimbriae [157]. Clearly, answering the long-standing question of whether conjugative pili nucleate from an inner or outer membrane platform remains a central challenge to this field.

## 10. Summary and Future Directions

In the last decade, there has been striking progress in definition of structural and mechanistic features of type IV secretion. Results with the TrIP assay supplied the first direct evidence that these machines indeed assemble as DNA transfer channels, and the data generated the first architectural view of the VirB/VirD4 channel. The CryoEM structure of the pKM101-encoded TraN/TraO/TraF core complex generated further excitement in the field, followed

quickly by an X-ray structure of the outer portion of this complex. Higher resolution studies generated a refined structure of the core complex and allowed the fitting of atomic models. In view of this remarkable progress, we can anticipate a near-atomic resolution structure of an entire T4SS in the very near future. The mechanistic studies also have generated a more detailed understanding of substrate translocation signals and contributions of accessory factors to substrate engagement with the T4CP receptor. *In vivo* biochemical approaches have enabled formulation of topological models for all the conserved T4SS subunits, and mutant analyses and domain swapping experiments continue to provide insights into specific contributions of individual subunits and domains to channel and pilus assembly and function.

There remain, however, many “black boxes” about type IV secretion. Major gaps in our knowledge exist across the entire machine. At the cytoplasmic entrance to the channel, how do the ATPases coordinate their activities and how does ATP binding and hydrolysis contribute to assembly of the translocation channel and substrate processing or transfer, or biogenesis of the conjugative pilus or other surface structures? At the inner membrane, what constitutes the channel, and in what form – folded or unfolded - is the substrate translocated? In the periplasm, what is the nature of the junction between the IMC and the core complex and does a channel extend from one to the next? Or is a two-step translocation model generally applicable for T4SSs; if so, where does the substrate gain access to the translocation channel for delivery across the outer membrane? Where does the pilus initiate polymerization and what is its physical relationship with the IMC/core complex? Finally, at the outer membrane, what constitutes the outer membrane pore and how is the pore gated by intracellular and extracellular signals? And perhaps the blackest of the boxes pertains to the donor-target cell junction. How do donor cells establish productive contacts with target cells, and what is the basis for the extremely broad host range of some systems such as the *A. tumefaciens* VirB/VirD4 T4SS which can deliver substrates to many different bacterial, plant, fungal, and human cell types?

Answers to these and other outstanding questions will continue to require structural analyses of machine subassemblies *in vitro* as well as high-resolution imaging of machines in their native membrane environments. The forthcoming images will provide us with an architectural blueprint, but a comprehensive mechanistic understanding of how these machines actually work will require a combination of creative *in vivo* genetic and state-of-the-art cell biological approaches together with *in vitro* reconstitution assays. Finally, to fully appreciate the biological diversity of T4SSs and to gain a better understanding of the mechanistic themes and variations, it is imperative that we invest comparable effort in studies of systems very distantly related to the *A. tumefaciens* VirB/VirD4 T4SS, e.g., effector translocators employed by intracellular pathogens and Gram-positive and archaeal conjugation systems [1].

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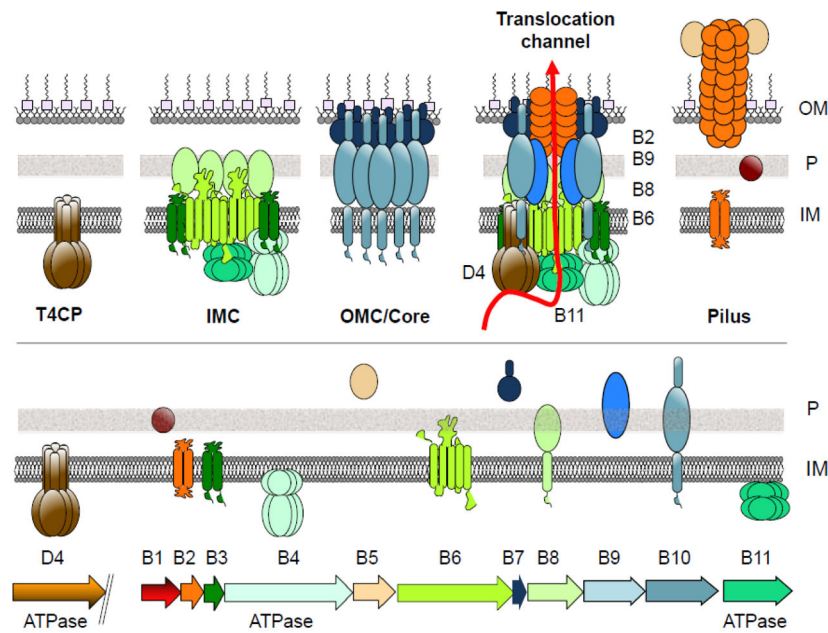
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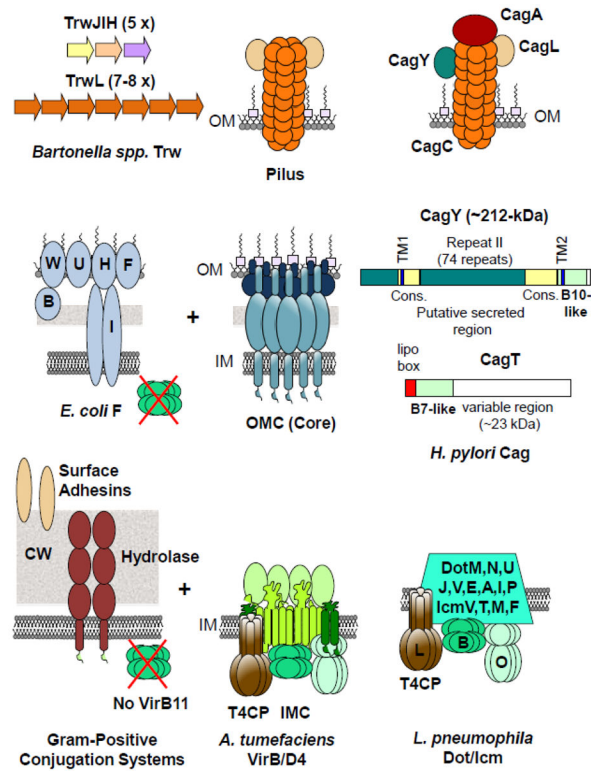
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### Highlights

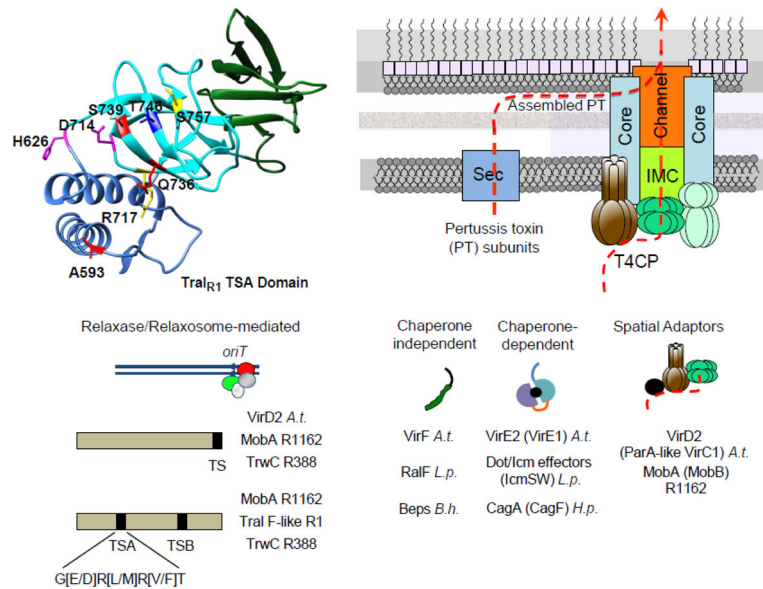
- We review structural information about bacterial type IV secretion systems (T4SSs).
- We discuss adaptations to type IV machines that evolved for specialized functions.
- We summarize functions of subunits and subassemblies of archetypal T4SSs.
- We review mechanisms for substrate presentation to the bacterial T4SSs.
- We present models depicting the substrate translocation route through T4SSs.



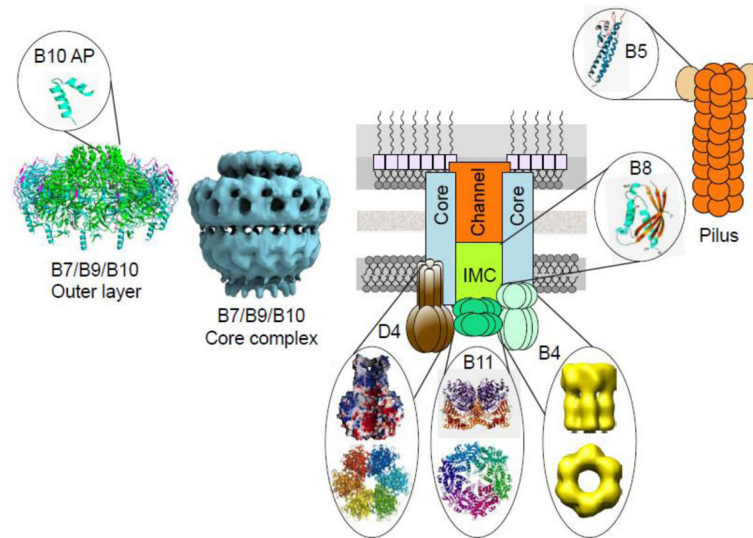
**Fig. 1.** Schematic depicting subunits and subassemblies of the *A. tumefaciens* VirB/VirD4 type IV secretion system (T4SS). Bottom: The 11 VirB subunits are synthesized from the *virB* operon and VirD4 from the separate *virD* operon. Lower: The VirB/VirD4 subunits are inserted in the inner membrane (IM) or delivered to the periplasm (P) with general topologies/locations as indicated. Upper: The subunits form a network of interactions resulting in four functional subassemblies: i) T4CP substrate receptor, ii) inner membrane translocase (IMC), iii) outer membrane complex (OMC)/core complex, and iv) extracellular pilus. The T4CP, IMC, and OMC interact to form the substrate translocation channel (red arrow). During translocation, the T-DNA forms formaldehyde-crosslinkable contacts with the 6 VirB/VirD4 subunits listed [38]. The IMC and OMC, together with the VirB1 transglycosylase, mediate assembly of the conjugative pilus.

**Fig. 2.**

Examples of T4SS structural adaptations. Bottom: Gram-positive conjugation systems are composed mainly of homologs of the *A. tumefaciens* T4CP (VirD4) and IMC (VirB3, VirB4, VirB6, VirB8), plus a cell wall hydrolase presumptively for elaboration of a channel across the cell wall. These ‘minimized’ systems lack a VirB11 homolog, the OMC/core, and conjugative pili, and may or may have associated surface adhesins. The T4CP/IMC of the *L. pneumophila* Dot/Icm system has three channel ATPases, but the membrane channel is compositionally much more complex than that of *A. tumefaciens*. Middle: The OMC/core can vary in structure/composition. The *E. coli* F plasmid-encoded OMC consists of a core complex (TraV/K/B), as well as the complex of Tra/Trb proteins shown whose function is implicated in pilus extension and retraction. In the *H. pylori* Cag system, the presumptive core subunits are much larger than their *A. tumefaciens* counterparts and possess novel domains that extend beyond the cell surface and associate with extracellular pili. Top: T4SS-encoded surface organelles can vary in composition and structure in comparison to the *A. tumefaciens* VirB-encoded pilus. The *Bartonella* spp. Trw system elaborates antigenically-variable pili through use of different pilins, and the *H. pylori* Cag system elaborates pili that are composed of several different subunits. The sole substrate of the Cag T4SS, CagA, also associates with the Cag pilus. Some T4SSs do not elaborate pili, e.g., Gram-positive conjugation systems, or instead produce novel fibrous or sheathed structures or surface adhesins.

**Fig. 3.**

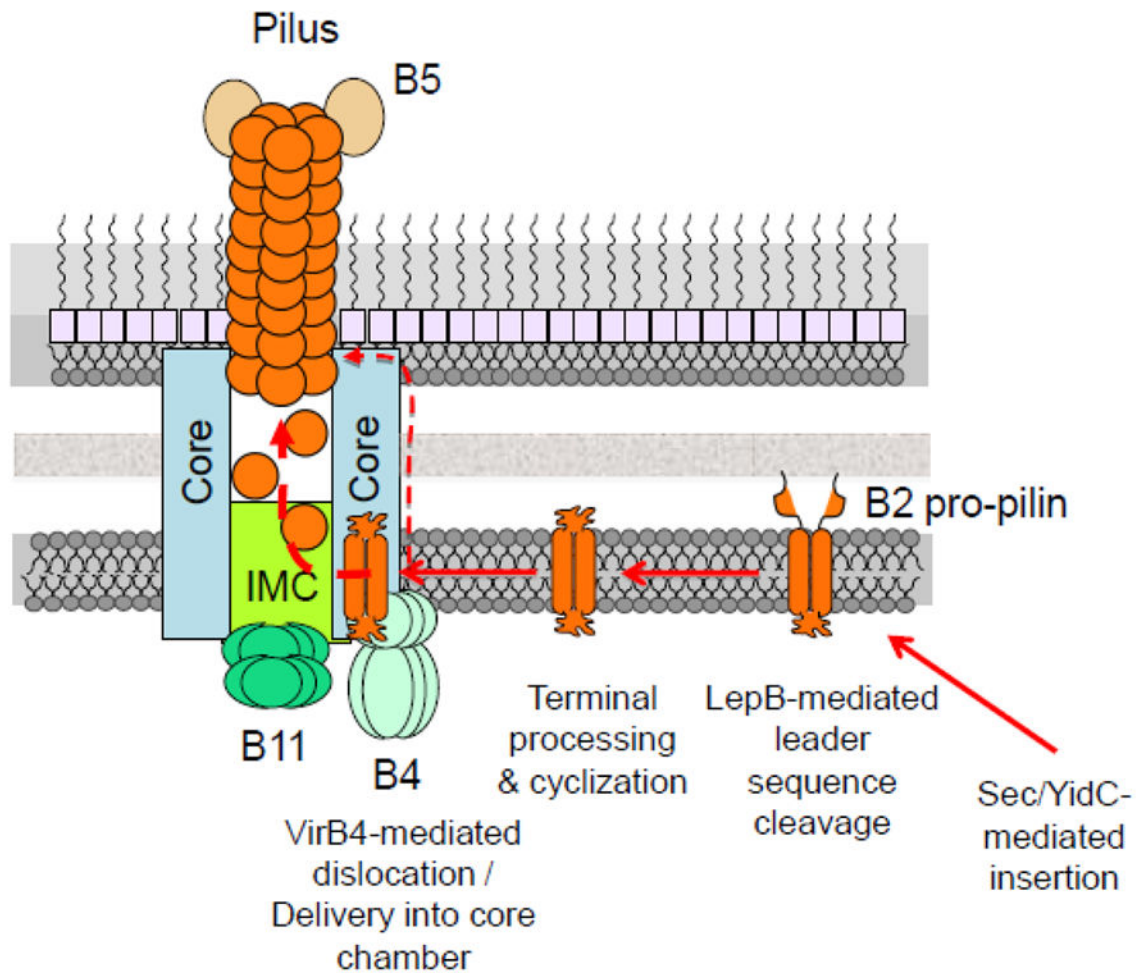
Type IV substrate translocation signals and accessory factors. Most substrates are translocated through the T4CP-dependent pathway. DNA substrates are processed by Dtr processing factors through assembly of the relaxosome at the origin of transfer sequence (*oriT*). Lower left: Relaxases responsible for nicking at *oriT* can carry positively-charged C-terminal or internal translocation signals (TS's) for docking with the T4CP receptor. A conserved sequence found in the TraI<sub>R1</sub> TSA motif and present in TSA and TSB motifs of MobA<sub>R1162</sub>, TraI<sub>R1</sub>, and TrwC<sub>R388</sub> is shown. Upper left: A model of the TSA motif from TraI<sub>R1</sub> generated from an X-ray structure [64]. The motif bears structural homologies to SF1B helicase domains. Mutations of residues shown in stick representation and color-coded disrupted TSA translocation function: red, significant impairment; pink, moderate impairment; yellow, no effect; blue, stimulation of TSA translocation function. Molecular graphics and analyses were performed with the UCSF Chimera package [158] using the PDB ID (4L0J) of the TraI<sub>R1</sub> TSA domain structure [64]. Lower right: DNA substrates and effector proteins can be recruited directly to the T4CP (Chaperone-independent) or via association with chaperones or spatial adaptors. Protein substrates can carry one or more of a combination of C-terminal, N-terminal or internal translocation signals; chaperones and spatial adaptors are in parentheses and the relevant species or plasmid is in parentheses (*A.t.*, *A. tumefaciens* VirB/VirD4; *L.p.*, *L. pneumophila* Dot/Icm; *B.h.*, *Bartonella henselae* VirB/VirD4; *H.p.*, *H. pylori* Cag). Upper right: In the T4CP-independent pathway, *B. pertussis* S1–S5 subunits are secreted across the inner membrane by the Sec system and assemble in the periplasm as the pertussis toxin (PT), which then docks by an unknown mechanism with the Ptl T4SS for extrusion across the outer membrane. Most T4SS substrates dock with the T4CP and are delivered in one step through the IMC/OMC channel.



**Fig. 4.**

X-ray and CryoEM structures of T4SS machine subunits and subassemblies. The schematic shows the T4SS translocation channel and conjugative pilus. Available crystal structures of homologs of individual VirB and VirD4 T4CP subunits are shown. A CryoEM structure of the pKM101-encoded core complex and an X-ray structure of the distal half (outer layer) of the core complex along with a magnified view of the pore-forming antennae projection (AP) are presented. Structures were reproduced with permission as follows: T4CP (TrwB<sub>R388</sub>), [86]; VirB11 (Cag $\beta$ <sub>*H.p.*</sub>), Yeo et al. [111]; VirB4 (TrwK<sub>R388</sub>), Pena et al. [105]; VirB5 (TraC<sub>pKM101</sub>), Yeo et al. [159]; VirB8 (VirB8<sub>*B.s.*</sub>), [121]; Core complex CryoEM (pKM101 TraN/TraO/TraF), Fronzes et al. [33]; Core complex X-ray (pKM101, O-layer) Chandran et al. [34].





**Fig. 5.**

Postulated biogenesis pathway for the *A. tumefaciens* *virB*-encoded pilus. VirB2 pilin is inserted into the membrane and processed in several steps to yield a pool of cyclized pilins. VirB4, aided by VirB11, catalyzes dislocation of mature pilins and feeds the pilin monomers into the site of pilus assembly within the core complex. Pilus polymerization initiates either on an inner membrane platform composed of VirB6 and VirB8 or an outer membrane platform composed of the cap structure of the core complex. An alternative pathway proposes that pilin monomers are shunted through the periplasm to the core's cap.