Published in final edited form as:

Mol Membr Biol. 2005 ; 22(0): 51–61.

Structural and dynamic properties of bacterial Type IV secretion systems (Review)

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Abstract

The type IV secretion systems (T4SS) are widely distributed among the Gram-negative and –positive bacteria. These systems mediate the transfer of DNA and protein substrates across the cell envelope to bacterial or eukaryotic cells generally through a process requiring direct cell-to-cell contact. Bacteria have evolved T4SS for survival during establishment of pathogenic or symbiotic relationships with eukaryotic hosts. The *Agrobacterium tumefaciens* VirB/D4 T4SS and related conjugation machines serve as models for detailed mechanistic studies aimed at elucidating the nature of translocation signals, machine assembly pathways and architectures, and the dynamics of substrate translocation. The *A. tumefaciens* VirB/D4 T4SS are polar-localized organelles composed of a secretion channel and an extracellular T pilus. These T4SS are assembled from 11 or more subunits, whose membrane topologies, intersubunit contacts and, in some cases, 3-dimensional structures are known. Recently, powerful *in vivo* assays have identified C-terminal translocation signals, defined for the first time the translocation route for a DNA substrate through a type IV secretion channel, and supplied evidence that ATP energy consumption contributes to a late stage of machine morphogenesis. Together, these recent findings describe the mechanics of type IV secretion in unprecedented detail.

Keywords

Conjugation; pathogenesis; traffic ATPases; macromolecular transport; DNA transfer

Introduction

The bacterial type IV secretion systems (T4SS) comprise a large family of translocation systems dedicated to the movement of DNA or protein substrates across the cell envelope [1,2]. The T4SS are broadly distributed among most species of Gram-negative and –positive bacteria [1,3]. In one classification scheme, the T4SS have been grouped according to sequence relatedness of machine components. Accordingly, systems assembled from proteins related to the archetypal VirB/D4 T4SS of *Agrobacterium tumefaciens* are classified as type IVA and those related to the Dot/Icm T4SS of *Legionella pneumophila* are classified as type IVB [4]. Other T4SS bearing little or no sequence relationships with the types IVA and IVB systems, e.g., those encoded by conjugative transposons and plasmids of Gram-positive bacteria [3], will be classified once their subunit compositions become better defined. The T4SS can also be grouped according to function. A large number of T4SS mediate the conjugative transfer of plasmids and other mobile DNA elements to bacterial

recipient cells. Others, designated as the ‘effector translocator’ systems, deliver protein or DNA substrates to eukaryotic target cells. The conjugation systems and most effector translocator systems deliver their substrates through direct contact with target cells. By contrast, a third subgroup termed ‘DNA release or uptake’ systems exchange DNA substrates with the extracellular milieu by contact-independent mechanisms [1]. To date, most structure–function studies have focused on the type IVA conjugation systems, although rapid progress is being made on type IVA and IVB effector translocator systems of medically important pathogens.

Various aspects of T4SS biology have been covered in recent excellent reviews [2,5–9]. Here, we will focus mainly on the type IVA T4SS, first summarizing novel systems identified in the past year whose functions reinforce the concept that these secretion machines have evolved for a wide range of biological activities. The remainder of the review will highlight recent progress toward answering longstanding questions of how and where at the cell surface the T4SS assemble, how T4SS recognize and recruit secretion substrates, and how type IV secretion substrates traverse the Gram-negative cell envelope.

Type IV family members

The *A. tumefaciens* VirB/D4 T4SS will serve as a framework for this discussion. The VirB/D4 T4SS is a *bona fide* bacterial conjugation apparatus adapted for the novel purpose of delivering an oncogenic T-DNA substrate as well as effector proteins to susceptible plant cells [10]. The ~12-kilobase *virB* operon encodes 11 proteins, VirB1 through VirB11, and the *virD* operon encodes 5 proteins including VirD4 [11]. The VirB and VirD4 proteins together elaborate the transenvelope secretion channel, whereas independently of VirD4 the VirB proteins elaborate an extracellular filament termed the T pilus [12]. The VirB proteins are termed the mating pair formation (Mpf) proteins, and VirD4 is termed the coupling protein (T4CP). The T-DNA is processed for transfer at the T-DNA border repeat sequences by the VirD2 relaxase and auxiliary factors, collectively termed the DNA transfer and replication (Dtr) proteins. The *A. tumefaciens* Dtr, T4CP, and Mpf subunits are related in sequence and function to subunits of conjugation systems, and the T-DNA borders to origin-of-transfer (*oriT*) sequences, of Gram-negative bacterial plasmids, e.g., F, RP4, R388, and pKM101 [6,13].

Many T4SS have been shown to deliver DNA and/or protein substrates to bacterial or eukaryotic target cells. In the past year, this list was expanded to include T4SS carried by *Mesorhizobium loti* [14], *Burkholderia cenocepacia* [15], *Xanthomonas axonopodis* pv. *citri* [16], and *Campylobacter jejuni* and *C. coli* [17]. The *M. loti* and *B. cenocepacia* T4SS contribute to the colonization of plant host cells. In *M. loti* strain R27, the 11 *virB* and *virD4* genes are carried on a symbiosis island. In addition to extensive similarities in regulation, operon structure, and subunit composition, the *M. loti* strain R27 and *A. tumefaciens* VirB/D4 T4SS export functionally redundant effector proteins to plant cells. For example, *M. loti* exports the effector protein Msi061 and *A. tumefaciens* exports VirF. Both effectors possess F-box motifs that, upon delivery into the plant cell, are postulated to mimic the activities of eukaryotic F-box receptors for attracting specific proteins to the SCF (Skp1-Cdc53-F-box protein) complexes for ubiquitination and proteolysis [14,18]. The *A. tumefaciens* VirB/D4 T4SS also was shown to translocate *M. loti* Msi061 to plant cells, suggesting that the *A. tumefaciens* and *M. loti* VirB/D4 T4SS recognize common translocation signals [14]. Interestingly, through adaptation of these closely related T4SS, *A. tumefaciens* induces a pathogenic response whereas *M. loti* establishes a symbiotic relationship with the eukaryotic host.

B. cenocepacia strain K56-2 carries two T4SS, the Ptw system encoded by plasmid pK56-2 and a second encoded by chromosome II [15]. The Ptw system is composed of homologs of VirB4, VirB6 through VirB11, and VirD4, as well as homologs of the F plasmid TraN and TraU subunits and a homolog of the R388 plasmid TrwC relaxase. The chromosomal T4SS is highly similar in genetic composition and arrangement to the *A. tumefaciens* VirB/D4 T4SS, with the exception that *virD4* is probably part of the same transcriptional unit as the 11 *virB* genes. Mutational studies provided evidence for the involvement of the Ptw system in eliciting the plant tissue watersoaking phenotype [15]. *Erwinia amylovora* and *Pantoea stewartii* subsp. *stewartii* also induce a plant watersoaking phenotype, but both of these species are thought to export the elicitors DspE and WtsE, respectively, to plant cells via type III secretion systems (T3SS) (see [15]). *B. cenocepacia*, *E. amylovora* and *P. stewartii* thus appear to have coopted either T3SS or T4SS to elicit a common pathogenic outcome on susceptible plant hosts.

A very recent study supplied compelling evidence that *Helicobacter pylori* activates the NF- κ B signaling pathway through delivery of peptidoglycan to gastric epithelial cells [19]. The host intracellular protein Nod1 is involved in epithelial cell sensing of peptidoglycan as well as recognition of *cagPAI*-positive *H. pylori*. Though not yet shown directly, these findings suggest that *H. pylori* Cag T4SS exports not only the previously-identified effector protein CagA, but also peptidoglycan [19]. If this turns out to be true, the type IV substrate repertoire now includes a nonprotein or non-DNA signaling molecule.

Type IV machine components: Cellular locations, interaction networks, and structures

At this time, there is no high-resolution structure for any T4SS analogous to that recently presented for a T3SS [20]. Nevertheless, fairly comprehensive architectural models of the T4SS can be generated through topological, structural, and interaction studies of machine components. This combination of approaches supplies evidence for at least three stable subassemblies of VirB/D4 components (Figure 1).

Energy subcomplex – VirD4, VirB4, VirB11

VirD4 T4CP, VirB11, and VirB4 are the three energetic components of the VirB/D4 T4SS. Each of these subunits possesses a characteristic nucleoside triphosphate binding site (Walker A) motif required for substrate translocation [7]. Homologs of each subunit bind ATP, but ATP hydrolysis activity has been demonstrated convincingly only for homologs of VirB11 (*H. pylori* HP0525, RP4 TrbB, R388 TrwD, R64 PilQ, *L. pneumophila* DotB) [21–23]. Members of the VirD4 T4CP family form ring structures discernible by electron microscopy. An X-ray structure solved for a soluble domain of TrwB_{R388} presents as a homohexameric ring with a central channel of 20 Å in diameter. The structure bears a striking resemblance to the F1-ATPase $\alpha\beta\beta_3$ heterohexamer [24]. The VirB11 homologs TrbB_{RP4}, TrwD_{R388}, and *H. pylori* HP0525_{Cag} also form homohexameric rings. The double-stacked ring of HP0525 seen by X-ray crystallography appears to be highly conserved, even among distantly-related ATPases associated with other transport or fimbrial biogenesis systems, e.g., *L. pneumophila* DotB of the Dot/Icm T4SS [22], *Aquifex aeolicus* PilT associated with type IV pilus retraction [25], *Vibrio cholerae* EpsE associated with type II secretion [26], and *Actinobacillus actinomyces-temcomitans* TadA associated with Flp pilus biogenesis [27]. Both EpsE and HP0525 are structurally similar to members of the AAA ATPase superfamily [9,26]. Little structural information is available for the VirB4 subunit family, with the exception that *A. tumefaciens* VirB4 self-interacts and its N terminus likely spans the inner membrane [28–30].

The three energetic subunits form pairwise interactions, as shown by two-hybrid interaction assays (Figure 1). For example, *A. tumefaciens* VirB4 interacts with VirB11 [29], *Rickettsia sibirica* VirB4 with VirD4 [31], and *Xanthomonas campestris* VirD4 with VirB11 [16]. Additionally, native and Walker A mutant forms of VirD4, VirB4, and VirB11 interact, as shown by co-immunoprecipitation, suggesting that ATP binding or hydrolysis does not drive at least the initial assembly of these ternary complexes [32]. Several studies also have demonstrated VirD4–VirB10 and VirB4–VirB10 interactions [29,31–36]. The physiological significance of the VirD4–VirB10 interaction is discussed below.

Core subcomplex – VirB6, VirB7, VirB8, VirB9, VirB10

Five VirB proteins are implicated as forming a ‘core’ transenvelope structure on the basis of phylogenetic relationships and cell localization and protein-protein interaction data [7]. Two recent advances in our understanding of this core complex include the development of a VirB6 topology model and evidence that VirB10 resembles the energy transducer protein TonB both in overall structure and function. *A. tumefaciens* VirB6 is highly hydrophobic and spans the membrane several times, as deduced by results of computer analyses and reporter protein fusion studies [37,38]. However, these studies left ambiguities in the topology that now have been resolved with Cys accessibilities studies of phenotypically-silent Cys substitution mutations [37]. Together, the data support a model for VirB6 as a polytopic membrane protein with a periplasmic N terminus, 5 TMS, and a cytoplasmic C terminus. A particularly notable feature of the topology model is a large central periplasmic loop, designated loop P2, which is now known to play an important role in substrate translocation (see below). VirB6 contacts with other channel subunits have not been detected by yeast two-hybrid screens, probably because of its hydrophobic character. However, VirB6 was shown to interact with the VirB7 lipoprotein and VirB9 by co-immunoprecipitation [39], and with other VirB ‘core’ subunits by gel filtration chromatography and blue native gel electrophoresis [40]. Additionally, VirB6 exerts stabilizing effects on other VirB subunits and it co-localizes with VirD4 and the T pilus at the cell poles of *A. tumefaciens* [12,39,41]. These findings together with results of DNA transfer assays described below strongly indicate that VirB6 assembles as a central component of the ‘core’ structure that likely corresponds to the VirB/D4 secretion channel.

The VirB7 lipoprotein forms a disulfide bridge with VirB9, and the heterodimer sorts to the OM where it exerts stabilizing effects on other machine subunits [7]. VirB8 and VirB10 are bitopic IM subunits. Recently, structures of periplasmic fragments of both subunits were resolved by X-ray crystallography (G. Waksman, pers. comm.). Over its length, VirB10 shares several structural features with TonB, including a small N-terminal cytoplasmic domain, a single TMS, a Pro-rich region, and a region of sequence conservation at the C-terminal end [36]. For TonB, the Pro-rich motif contributes to a rigid, extended structure in the periplasm that might permit simultaneous contacts with partner subunits at the inner and outer membranes [42,43]. Similarly, *A. tumefaciens* VirB10 interacts with the IM subunits VirB8, VirD4, and VirB4, and with the OM-associated VirB7–VirB9 heterodimer [32–34,36,44] (Figure 1). VirB10 also functionally resembles TonB by linking energy at the IM to the assembly or gating of the type IV secretion channel for substrate translocation (see below).

Recent studies also have identified protein-protein interactions among machine components of the F plasmid T4SS [45–47]. This T4SS is a chimeric system composed of several VirB/D4 subunits and other components of an unrelated ancestry [6]. Studies of the F-specific subunits have identified a protein interaction network, composed of TrbB, TrbI, TraW, TraU, TraF, and TraH, that likely spans the cell envelope [47] (Figure 1). This putative structure is of special interest because the F plasmid-encoded T pilus extends and retracts,

presumably to draw donor and recipient cells together for mating pair formation [6]. Mutations in the Trb/Tra components listed above disrupt pilus outgrowth and retraction, suggesting that this subcomplex regulates dynamic aspects of pilus formation [47]. It is likely that an F-specific protein subassembly serves such a function because other conjugation systems, e.g., the *A. tumefaciens* VirB/D4, RP4 Tra, and R388 Trw systems, appear to slough rather than retract their pili [7].

T pilus subcomplex – VirB2, VirB5, VirB7

VirB2 is the major subunit of the T pilus [48]. The VirB7 lipoprotein and VirB5 are minor pilus components, although it is not known how these subunits associate with the T pilus [49,50]. Recently, a crystal structure of a VirB5 homolog, TraC of pKM101, was presented [51]. This is a single domain protein with a mostly α -helical, elongated structure. Analyses of TraC mutants conferred altered conjugation frequencies as well as defects in infection by bacteriophages PRD1 and I κ e. These findings led to a proposal that the VirB5-like proteins bind along the pilus, including the pilus tip (the site of I κ e attachment) and base (the site of PRD1 attachment), and that these sites of contact might promote both pilus biogenesis and adherence to recipient cells [9,51].

Dynamics of T4SS machine assembly and function

The available structural data and protein interaction networks present a somewhat static view of the type IV systems (Figure 1). Recent studies, however, have begun to capitalize on development of novel *in vivo* assays to explore dynamic aspects of machine assembly and function.

Substrate recognition and recruitment

For example, studies examining the role of the T4CP in substrate recruitment exploited the finding that the VirD4 T4CP localizes to the *A. tumefaciens* cell poles [41]. Accordingly, to assay for T4CP receptor activity, the VirE2 effector protein was tagged with GFP, or both VirD4 and VirE2 were tagged with complementary half-GFP's. Cells producing these constructs displayed polar fluorescence that was dependent on VirD4 but independent of the VirB Mpf subunits, confirming the proposal that the VirD4 T4CP functions as a receptor for protein substrates [52]. Additionally, it was discovered that T4SS will translocate Cre recombinase [53] or adenylate cyclase when fused to the N termini of secretion substrates [54]. By monitoring intercellular translocation of reporter proteins, investigators are beginning to define the nature of type IV secretion signals carried by the exported protein substrates. In general, these translocation signals are located at the C termini of the substrates. Yet, the signals identified to date show little sequence conservation other than clusters of Arg residues, as shown for substrates of the *A. tumefaciens* VirB/D4 T4SS [53], or hydrophobic residues, as shown for substrates of the *L. pneumophila* Dot/Icm T4SS [54]. In *Bartonella henselae*, the VirB/D4 T4SS exports substrates (*Bartonella* effector proteins or Beps) through recognition of a bipartite motif composed of a positively-charged C terminus and one or more BID (Bep intracellular delivery) domains. These BID domains are often, but not exclusively, located at the C termini of the Beps [55].

Definition of the T-DNA translocation pathway by TrIP

Recently, another novel assay termed transfer DNA immunoprecipitation (TrIP) was developed to trace the path of a DNA substrate through the type IV secretion channel [56]. Adapted from the chromatin immunoprecipitation (ChIP) technique, TrIP employs a three-stage reaction involving: (i) formaldehyde treatment of intact cells to crosslink channel subunits to the T-DNA substrate as it exits the cell, (ii) detergent-solubilization and immunoprecipitation of individual T4SS subunits, and (iii) PCR amplification for detection

of the T-DNA in the immunoprecipitates. With this assay, it was shown that the T-DNA substrate forms close contacts with 6 of the 12 VirB/D4 components, VirD4 T4CP, VirB11 ATPase, polytopic VirB6, bitopic VirB8, VirB2 pilin, and VirB9 [56]. Further TrIP studies of T4SS mutants enabled formulation of a sequentially- and spatially-ordered translocation pathway for the T-DNA substrate. This pathway is described below and depicted in Figure 2.

Step I – Substrate recruitment to the T4SS—In the initial TrIP studies, VirD4 was shown to interact with the T-DNA independently of the VirB Mpf subunits, establishing that VirD4 is a receptor not only for protein substrates (see above) but also for the T-DNA [56]. Moreover, a VirD4 Walker A mutant was shown to retain both T-DNA as well as protein substrate receptor activity [32] (K. Atmakuri, P.J. Christie, unpublished findings). VirD4 thus appears to function as a general receptor for DNA and protein substrates through a mechanism(s) that operates independently of other Mpf components or ATP energy.

Step II – Transfer to the VirB11 hexameric ATPase—TrIP studies of various T4SS mutant strains established that VirD4 transfers the T-DNA substrate to the VirB11 ATPase [56]. Moreover, a VirD4 Walker A mutant protein successfully transferred the DNA substrate to VirB11 and, similarly, native VirD4 transferred the substrate to a VirB11 Walker A mutant. These findings argue against a requirement for ATP utilization by either of these energetic subunits for this transfer step [32]. Two further lines of study identified additional requirements for DNA transfer to VirB11. First, analyses of *virB* gene deletion mutants showed that VirB7 synthesis is essential for this transfer step [56]. As noted above, VirB7 contributes to assembly of a stable secretory apparatus through dimerization with VirB9. VirB7 might act indirectly through stabilization of the transenvelope structure to enable productive contacts between VirD4 and VirB11 [56], although it is also interesting to consider that the lipoprotein exerts its effects by interacting directly with the VirD4-VirB11 complex. Second, an *in vivo* reconstitution study showed that strains producing certain subsets of VirB/D4 components support DNA transfer from VirD4 to VirB11. Specifically, DNA transfer to VirB11 was observed in strains producing VirD4 and VirB11 plus VirB7 and either VirB8 and VirB9, or VirB10. Precisely how these ‘core’ components interface with the energetic components at the IM to stimulate substrate transfer from the VirD4 receptor to VirB11 is a fascinating topic for further study.

Step III – Transfer to the integral inner membrane proteins VirB6 and VirB8—The TrIP studies place VirB6 and VirB8 at an intermediate point in the postulated DNA translocation pathway, dispensable for DNA transfer to VirD4 and VirB11 but necessary for transfer to VirB2 and VirB9. Additionally, VirB6 and VirB8 must be co-synthesized for establishment of DNA substrate contacts with either of these subunits, suggesting that VirB6 and VirB8 coordinate their activities to mediate this transfer step [56]. To further define the contributions of VirB6 to substrate transfer, various VirB6 insertion and deletion mutations were assayed for effects on substrate translocation. Results indicated that: (i) periplasmic loop P2 contributes to formation of substrate contacts with VirB6, (ii) a domain adjacent to loop P2 contributes to substrate transfer from VirB6 to VirB8, and (iii) two terminal domains of VirB6 contribute to substrate transfer to VirB2 and VirB9 [37]. It can be speculated that loop P2 might form part of the secretion channel structure, whereas other domains of VirB6 form critical intersubunit contacts required for assembly of the distal portions of the channel. A combination of T4SS mutant analyses and *in vivo* reconstitution studies further showed that substrate transfer to VirB6 and VirB8 requires each of the three energetic components -VirD4, VirB4, and VirB11 – together with VirB6, VirB7, VirB8, and either VirB9 or VirB10. These ‘core’ subunits likely promote formation of critical contacts between the IM channel subunits, but again the molecular details of these interactions await

further study. Finally, Walker A mutations in any one of the energetic components abolished substrate transfer from VirB11 to VirB6 and VirB8, strongly indicating that VirD4, VirB4, and VirB11 mediate this step of substrate transfer through a coordination of ATP binding or hydrolysis activities [32,56].

Step IV – Transfer to the periplasmic and outer membrane-associated proteins VirB2 and VirB9

The TriP studies place VirB2 and VirB9 at the distal end of the proposed DNA translocation pathway. Additionally, as observed for VirB6 and VirB8, both VirB2 and VirB9 must be co-produced for establishment of DNA substrate contacts with either subunit, and thus VirB2 and VirB9 likely function together to mediate the latter step(s) of substrate transfer [56]. Only a portion of VirB2 pilin produced by a cell polymerizes as the T pilus, whereas the remaining cell-associated form – the fraction resistant to extraction from the cell by shearing – co-partitions with the IM, OM, and periplasmic proteins [57]. An interesting set of mutations in VirB11, VirB6, and VirB9 were shown to selectively block assembly of the T pilus without affecting substrate transfer [39,57] (S. Jakubowski, P.J. Christie, unpublished findings). These so-called ‘uncoupling’ mutations do not affect accumulation of cellular VirB2, strongly indicating that it is this cellular pilin pool and not the T pilus that contributes to substrate translocation to recipient cells. Finally, several T4SS subunits, including VirB3, VirB5, and VirB10 do not form detectable interactions with the T-DNA, suggesting that these subunits are probably not channel components. Nevertheless, the corresponding gene deletions abolish substrate translocation from VirB6 and VirB8 to VirB2 and VirB9, indicating that VirB3, VirB5, and VirB10 still are essential for substrate trafficking through the periplasm [56].

Energetics of DNA translocation: VirB10, a TonB-like ATP energy sensor subunit

The energetics of DNA translocation across bacterial membranes has been investigated in bacteriophage, transformation, and conjugation systems [58–61]. In each of these systems, DNA transfer is dependent in part on proton motive force, as demonstrated by an inhibition of transfer in the presence of protonophores such as carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). In early studies of conjugation, CCCP-treated *E. coli* donors carrying the F plasmid failed to form stable mating aggregates, suggesting that transmembrane potential ($\Delta\psi$) or a pH gradient energizes some aspect of machine assembly or function [58]. Additional electrochemical measurements showed that the addition of arsenate, an ATP synthase inhibitor, to RP4-carrying *E. coli* cells conferred defects (e.g., resistance to phage binding) suggestive of a block in assembly of the Mpf structure. Thus, evidence exists for contributions of both proton motive force and ATP energy to morphogenesis of the conjugation machines.

Very recently, it was reported that *A. tumefaciens* VirB10 undergoes a structural transition in response to ATP utilization by the VirD4 T4CP and the VirB11 ATPase [36]. Moreover, VirB10 was shown to interact with VirD4 independently of ATP energy, whereas stable complex formation between VirB10 and the OM-associated VirB7-VirB9 dimer requires ATP utilization by both VirD4 and VirB11. Accompanying the energy-dependent formation of this presumptive transenvelope VirD4-VirB10-VirB7-VirB9 structure, the T-DNA substrate translocates from the IM portion of the secretion channel composed of VirB6 and VirB8 to that in the periplasm composed of VirB2 and VirB9. VirB10 thus supplies a TonB-like function by converting energy at the IM into a mechanical force required for a latter stage of machine biogenesis. Reminiscent of TonB, VirB10 might transduce IM energy to the OM-associated VirB7-VirB9 complex to mediate formation or opening of a channel gate for passage of the DNA substrate to the cell surface (Figure 3) [36,62,63].

It is noteworthy that TonB couples IM proton motive force to OM transport [62,63], while VirB10 couples ATP energy to latter stages of DNA substrate translocation [36]. This is the first report of an ATP energy sensing mechanism by a TonB-like protein. However, studies of several T2SS as well as the bacteriophage f1 extrusion system also have implicated bitopic subunits as energy coupling subunits required for translocation (Figure 3). For example, the GspC subunits of T2SS interact with IM protein platforms composed in part of VirB11-like GspE ATP-binding subunits and the GspD channel-forming secretin complex at the OM [64,65]. In the *Aeromonas hydrophila* T2SS, complex formation between the ExeA ATPase and TonB-like ExeB promotes multimerization of the secretin ExeD, prompting the proposal that the ExeAB complex energizes morphogenesis and/or gating of the OM secretin [66,67]. Finally, protein pI of bacteriophage f1 appears to be a chimeric protein composed of an N-terminal ATP-binding subunit and a C-terminal TonB-like subunit. Both cellular ATP and an intact Walker A motif are required for phage extrusion, suggestive of pI-mediated energy transduction to gate the pIV secretin complex at the OM [68]. Taken together, these observations point to specific functional relationships between IM ATPases and cognate TonB-like subunits for assembly of evolutionarily diverse macromolecular trafficking systems.

Conclusion and perspectives

Bacteria have evolved T4SS to translocate a diverse array of macromolecular substrates across the cell surface. Recent studies have begun to describe the assembly pathways and architectures of these complex machines. Enabling *in vivo* assays also are supplying exciting new information about the dynamics of substrate recognition and recruitment, and the genetic and energetic requirements for defined steps of substrate translocation. Among the important remaining questions are the following. How are DNA and protein substrates configured for export and how are C-terminal (or other) translocation signals recognized? How do two ringshaped homohexameric ATP-binding subunits and a third ATP-binding subunit interface to drive substrate translocation across the IM and beyond? Are protein and DNA substrates delivered through the same translocation pathway? What is the structural and functional relationship between the secretion channel and extracellular pili or other surface structures? What determinants specify assembly of type IV secretion machines at the cell poles? The current pace of research with multi-faceted approaches, together with an influx of youthful energy, should secure answers to these fascinating questions within the coming few years.

Acknowledgments

The authors thank members of the Christie laboratory for helpful critiques of this manuscript. Work in this laboratory is funded by NIH Grant GM47846. We also thank Drs G. Waksman and C. Dehio for permission to cite unpublished findings, and the following investigators for permission to reproduce published images presented in Figure 3: M. Coll, S. Darst, H. Deisenhofer, W. Hol, B. Luisi, A. Pluckthun, T. Pugsley, and G. Waksman.

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Figure 1.

Protein interaction networks between type IV secretion subunits. The localization and topologies of the components of the archetypal *Agrobacterium tumefaciens* VirB/D4 T4SS are represented. Shaded areas correspond to subcomplexes – energy (yellow shading), core (cream), pilus (green), F plasmid-specific (tan) – described in the text. Arrows indicate interactions detected among the subunits by biochemical/structural approaches – (co-immunoprecipitation, co-purification, crystal structure; blue arrow), yeast two-hybrid or peptide linkage (red arrow) and lambda cI repressor fusion or bacterial two hybrid (green arrow). The letter(s) accompanying the arrow denotes the system where the binary interaction was detected (A, *A. tumefaciens*; B, *Bartonella henselae*; Hp, *Helicobacter pylori*; P, *Bordetella pertussis*; R, *Rickettsia sibirica*; Xa, *Xanthomonas axonopodis* pv. *citri*; W, N, F, X and H, IncW, IncN, IncF, IncX and IncH plasmid incompatibility group conjugation systems). Interactions detected by two-hybrid screens between components localizing in different compartments are not shown, e.g., VirB11-VirB1, VirB11-VirB10 C-terminal domain, VirB11-VirB8 periplasmic domain, VirB11-VirB9. The F-plasmid transfer system Tra/Trb components are probably involved in pilus extension/retraction (F, H, U, W represent TraF, TraH, TraU and TraW; B and I represent TrbB and Trb1). For studies reporting these interactions, see refs. [6,7,16,24,28,29,31–35,38–40,44,46,50,69].

Figure 2.

The T-strand translocation pathway, as identified by TriP. Immunoprecipitation of the VirD2-T-strand transfer intermediate with channel components (dark gray) from wild-type and mutant strains define a temporally- and spatially-ordered transfer pathway (Hatched arrow, blue arrow online). The remaining VirB subunits (light gray) participate in machine biogenesis and/or regulate substrate passage among the channel components [56]. This figure is reproduced in colour in *Molecular Membrane Biology* online.

Figure 3. Schematic representation of energetic components in macromolecular trafficking systems. Proton-motive force driven machines (upper panel): *E. coli* Tol-Pal system (left) involved in Group A colicins import and membrane stability, *E. coli* TonB system (right) required for group B colicins, B12 vitamin and iron-charged siderophores import. The energy is powered by two accessory proteins (TolQR and ExbBD) that probably act as a proton-conducting complex [70]. ATP-driven machines (lower panel): T4SS (left), T2SS (center) required for export of protein or nucleoprotein substrates across the envelope, and f1 filamentous phage extrusion system (right). The T4SS is exemplified by the *A. tumefaciens* VirB/D4 apparatus, whereas T2SS are exemplified by the Terminal Branch of the General Secretory Pathway (GSP) and the *Aeromonas aerophila* Exe machine. Subunits that bind or hydrolyze ATP are indicated with an arrow. The crystal or cryo-microscopy protein structures are reproduced from the following references [24,26,43,67,71–76] with permission from the authors, Elsevier Ed, MacMillan Publishers Ltd, Nature Publishing Group, and the American Society for Biochemistry and Molecular Biology. This figure is reproduced in colour in *Molecular Membrane Biology* online.