BIOLOGICAL AND STRUCTURAL DIVERSITY OF TYPE IV SECRETION SYSTEMS

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Abstract

The bacterial type IV secretion systems (T4SSs) are a functionally diverse superfamily of secretion systems found in many species of bacteria. Collectively, the T4SSs translocate DNA and monomeric and multimeric protein substrates to bacterial and eukaryotic cell types. T4SSs are composed of two large subfamilies, the conjugation machines and the effector translocators that transmit their cargoes through establishment of direct donor-target cell contacts, and a third small subfamily capable of importing or exporting substrates from or to the milieu. This chapter will summarize recent mechanistic and structural findings that are shedding new light on how T4SSs have evolved such functional diversity. Translocation signals are now known to be located C-terminally or embedded internally in structural folds; these signals in combination with substrate-associated adaptor proteins mediate the docking of specific substrate repertoires to cognate VirD4-like receptors. For the *Legionella pneumophila* Dot/Icm system, recent work has elucidated the structural basis for adaptor-dependent substrate loading onto the VirD4-like DotL receptor. Advances in definition of T4SS machine structures now allow for detailed comparisons of nanomachines closely related to the *Agrobacterium tumefaciens* VirB/VirD4 T4SS with those more distantly related, e.g., the Dot/Icm and *Helicobacter pylori* Cag T4SSs. Finally, it is increasingly evident that T4SSs have evolved a variety of mechanisms dependent on elaboration of conjugative pili, membrane tubes, or surface adhesins to establish productive contacts with target cells. T4SSs thus have evolved extreme functional diversity through a plethora of adaptations impacting substrate selection, machine architecture, and target cell binding.

INTRODUCTION

The bacterial type IV secretion systems (T4SSs) are a large, versatile family of macromolecular translocation systems functioning in Gram-negative (G−) and Gram-positive (G+) bacteria (1). These systems mediate the transfer of DNA or monomeric or multimeric protein substrates to a large range of prokaryotic and eukaryotic cell types (Fig. 1A). Conjugation systems, the earliest described subfamily of T4SSs (2), transfer mobile genetic elements (MGEs) between bacteria. They pose an enormous medical problem because MGEs often harbor cargoes of antibiotic resistance genes and fitness traits that endow pathogens with antibiotic resistance and other growth advantages under selective pressures (3-5). 'Effector translocators', a more recently described T4SS subfamily (6, 7),

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are deployed by pathogenic bacteria to deliver effector proteins to eukaryotic cells during the course of infection (8-11). The conjugation and effector translocator systems, as well as newly discovered interbacterial killing systems, transmit their cargos through direct donor-target cell contact (12-14). A few other T4SSs designated as 'uptake or release' systems acquire DNA substrates from the milieu or release DNA or protein substrates into the milieu (Fig. 1A) (1, 6).

The T4SSs are defined by the presence of a minimum set of conserved or 'signature' subunits (8). The *Agrobacterium tumefaciens* VirB/VirD4 T4SS, whose Vir subunit nomenclature is widely adopted in this field when referring to the conserved subunits of T4SSs, is assembled from VirB1 through VirB11 and VirD4 (Fig. 1B) (15). In G− species, T4SSs are composed of these Vir-like subunits, although many systems have appropriated other subunits or domains from unknown ancestries presumably for specialized functions (16-18). In G+ species, six VirB/D4-like subunits (VirB1, VirB3, VirB4, VirB6, VirB8, VirD4) are required to build 'minimized' systems spanning the single cytoplasmic membrane and cell wall (8, 19). Vir subunits can be grouped as: i) two or three conserved ATPases (VirB4, VirB11, VirD4) that coordinate the recruitment and processing of substrates, catalyze structural changes in the T4SS channel necessary for substrate passage, and in some cases regulate pilus biogenesis (12, 20-23); ii) integral inner membrane (IM) subunits (VirB3, VirB6, VirB8) that presumptively form an IM channel; iii) a transglycosylase (VirB1) that contributes to (G− species) or is required for (G+ species) assembly of the channel across the murein layer (8, 24, 25); and iv) outer membrane (OM)-associated subunits (VirB7, VirB9, VirB10) that form a structural scaffold for the portion of the channel spanning the periplasm and OM of G− species (26, 27). In this chapter, we will summarize results of recent mechanistic and high-resolution microscopy studies that are providing exciting new insights into the biogenesis and structural arrangement of T4SSs and how they have evolved such extreme biological diversity.

Substrate recognition: substrate signals and adaptors/chaperones

T4SSs recruit specific repertoires of substrates through recognition of translocation signals (TSs) and accessory factors bound to substrates. For conjugative DNA transfer, the recruitment and delivery of DNA substrates through cognate T4SSs can be summarized briefly as follows. First, an accessory factor binds the origin-of-transfer (*oriT*) sequence carried by an MGE. Accessory factors generally fall into the ribbon-helix-helix (RHH) family of DNA binding proteins, as exemplified by the TraM protein encoded by F plasmid and TrwA encoded by plasmid R388 (hereafter, the origin of the named T4SS or protein will appear in subscript, e.g., $TraM_F$. $TrwA_{R388}$) (28-31). The accessory factor, through a combination of DNA bending and direct protein-protein interactions, recruits a protein termed the relaxase to *oriT* to form the catalytically active relaxosome (32, 33). The relaxase cleaves the DNA strand destined for transfer DNA (T-strand), and as a consequence of nicking the relaxase remains covalently bound to the 5' end of the T-stand. The accessory factor and relaxase together promote docking of the relaxosome with a cognate VirD4 substrate receptor. In the F plasmid transfer system, the $TraM_F$ accessory factor binds a short motif at the C terminus of VirD4-like TraD, whereas the $TraI_F$ relaxase carries internal TSs that form only when TraI adopts its tertiary structure (34). Other relaxases may additionally

or alternatively carry C-terminal TSs, which are typically composed of clusters of positively charged or hydrophobic residues (35-40). These TSs mediate relaxase interactions with the VirD4 receptors, although the structural bases for these interactions are not yet defined. Other specialized accessory factors are members of the ParA superfamily of partitioning proteins; these proteins also physically couple the relaxosome with the VirD4 receptors through establishment of multiple protein - protein contacts (41-43). Once the relaxosome docks with the VirD4 substrate receptor, by mechanisms that are not yet known, the relaxase is unfolded (23) and the accessory factor(s) is released, and the relaxase pilots the covalently bound T-strand through the T4SS to recipient cells. In recipient cells, the relaxase catalyzes recircularization of the T-strand through a reversal of the strand-breaking reaction, followed by second-strand synthesis and replication of the transferred element.

Among the effector translocators, some systems translocate only one or a few effector proteins, whereas others deliver several hundred into eukaryotic target cells where they function in a myriad of ways to subvert host cell physiologies (Fig. 1A) (1, 6, 9). As with the conjugation systems, effector translocators recognize their substrate repertoires through a combination of intrinsic and C-terminal TSs carried by the effectors, and binding of adaptors or chaperones associated with the effectors. In addition to their role in physically coupling the effector with the VirD4 receptor, adaptors and chaperones block effector aggregation or prevent nonproductive protein interactions in the bacterium prior to effector translocation (44-49). Up until recently, effector translocators were thought to function exclusively to deliver protein effectors to eukaryotic cells where they disrupt host cell physiological processes that aid in infection. Recently, however, members of this subfamily were shown to translocate toxin components of toxin-antitoxin (TA) modules to kill other bacteria in the vicinity (Fig. 1A). *Xanthomonas spp.*, for example, deploy a VirB/VirD4-like T4SS to deliver toxins whose bacteriolytic activities can degrade peptidoglycan in target cells lacking the corresponding antitoxin (13). In *Bartonella spp.*, the VbhT toxin is similarly transmitted via a VirB-like T4SS to target bacteria (14). Interestingly, VbhT carries a C-terminal TS identical to that previously determined to be involved in T4SS trafficking of interkingdom effectors during the course of *Bartonella spp.* infections. These findings establish an evolutionary link between toxins transmitted interbacterially for niche establishment and effectors delivered to eukaryotic cells for pathogenic ends (14).

The VirD4 receptor

VirD4-like substrate receptors couple DNA or protein substrates to the T4SS, and consequently these subunits are also called type IV coupling proteins or T4CPs (Fig. 2A, B) (50, 51). T4CPs are phylogenetically related to the SpoIIIE and FtsK ATPases functioning in DNA transport across membranes during sporulation and cell division, respectively (52, 53). These ATPases are typically configured as homohexamers with an N-terminal transmembrane domain and a nucleotide binding/hydrolysis domain (NBD), giving rise to an overall F1-ATPase architecture (53, 54). T4CPs also carry a sequence variable all-alpha domain (AAD) situated at the base of the NBD, and many carry a second, variable C-terminal domain (CTD) (51, 54). The AAD functions in substrate binding and specificity (55, 56), whereas the CTDs can also confer substrate specificity (28), or spatiotemporal control over substrate selection and translocation through the T4SS (57, 58). Two structural

studies have shed important light on interactions between T4CP CTDs and cognate substrates. First, in the F plasmid transfer system, the accessory factor $TraM_F$ was shown to physically couple the F relaxosome to the TraD receptor through simultaneous binding of F's *oriT* sequence and a 13-residue sequence at the end of TraD's CTD (28, 59). Second, in the more complex *L. pneumophila* Dot/Icm system, which is capable of delivering over 300 effectors to eukaryotic target cells during infection, the VirD4-like DotL receptor has a long ~200 residue CTD that binds DotM, DotN, the IcmSW adaptor complex, and LvgA (Fig. 2B) (48, 49, 58, 60, 61). These bound factors collectively stabilize DotL and mediate recruitment of distinct subsets of effectors to the Dot/Icm T4SS. Modeling of the elongated CTD-adaptor subassembly onto DotL's NBD hexameric sphere gives rise to a bipartitate bell-shape structure that presumably sits at the base of the translocation channel to recruit and feed substrates into the channel (Fig. 2B) (58). Interestingly, however, to date neither the DotL-adaptor complex nor other VirD4 hexamers have been visualized in association with cognate T4SS machines (see below) (18). These and other biochemical findings (62-64) have supported a model that T4CPs associate only transiently with cognate channels as a function of substrate binding.

Structural advances: subunits and subassemblies

Structures now exist for intact or soluble domains of the conserved ATPases (VirD4, VirB4, VirB11) and several channel/pilus subunits (VirB5, VirB8, VirB9, VirB10), obtained by X-ray crystallography, NMR, or negative-stain electron microscopy (nsEM) (54, 65-72). Larger subassemblies termed outer membrane core complexes (OMCCs) have also been visualized by nsEM from systems phylogenetically related to the VirB/VirD4 T4SS (27, 73) as well as more distantly related systems, e.g., the *L. pneumophila* Dot/Icm and *H. pylori* Cag T4SSs (74, 75). The OMCCs of the VirB/VirD4-like systems are ~1.1 MDa complexes composed of 14 copies of the VirB7-, VirB9-, and VirB10-like subunits. These complexes are arranged as large barrels of ~185 Å in width and height (27). The outer-layer (O-layer) of the OMCC from the pKM101-encoded T4SS (Tra_{pKM101}) was solved by X-ray crystallography, revealing a network of intra and intersubunit contacts and a distal cap composed of 14 copies of a helix-loop-helix domain of VirB10 termed the 'antenna projection' (AP) (26). The cap is postulated to span the OM, and its central pore of ~32 Å to comprise the OM channel though which substrates pass to the cell exterior. Interestingly, chimeric T4SSs composed of the IM-spanning portion of the Tra_{pKM101} T4SS joined to heterologous OMCCs from other VirB/VirD4-like T4SSs are capable of translocating DNA substrates between bacteria, confirming that the observed structural conservation of OMCCs from these systems extends to the level of function (73). Very recently, a structure of the entire OMCC of a *Xanthomonas citri* T4SS was solved at 3.3 Å by cryoelectron microscopy (CryoEM); it shows in unprecedented atomic detail an extensive VirB7/VirB9/VirB10 interaction network and also identifies flexible linkers and weak contacts that are postulated to account for intrinsic flexibility of the OMCC necessary for signal-activated channel gating (20, 76, 77).

The VirB$_{3}$-$_{10}$ structure

A much larger substructure elaborated by the Trw_{R388} T4SS was solved by nsEM at a resolution of 20 Å (78). This structure is composed of homologs of the VirB3-VirB10 subunits and was designated the VirB$_{3-10}$ or T4SS$_{3-10}$ complex (Fig. 2C, E). The ~3.5 MDa

structure consists of the OMCC joined by a thin stalk to an even larger IM complex, designated the IMC, of 25.5 nm in diameter and 10.5 nm in thickness. The entire structure, with a length of ~34 nm, spans the entire cell envelope such that the OMCC's cap forms the OM pore and the upper portion of the IMC spans the IM. The IMC platform is composed of 12 copies each of VirB3, VirB5, and VirB8, and 24 copies of VirB6. This platform connects to two barrel-like structures of 10.5 nm in width and 13.5 nm in height that correspond to two side-by-side hexamers of VirB4 extending into the cytoplasm (Fig. 2C, E). The $VirB_{3-10}$ structure lacks the conjugative pilus elaborated by conjugation machines in G− species (see below), as well as the VirB2, VirB11, and VirD4 homologs required for substrate transfer. Interestingly, however, in a recent update a $VirB_{3-10}$ structure was solved that additionally has one or two dimers of VirD4 situated between the VirB4 hexameric barrels (79). These dimers might correspond to an assembly intermediate of the T4CP that engages with the channel in the absence of bound substrate. It is also intriguing to consider that an early X-ray structure of a soluble domain of the $TrwB_{R388}$ coupling protein (54), which has guided our thinking for many years regarding the hexameric structure of T4CPs, might not reflect the oligomeric and active states of the VirD4 ATPases assembled *in vivo* (79).

The overall $VirB_{3-10}$/VirD4 dimer structure lacks a detectable channel. However, results of a ChIP-based crosslinking assay termed <u>T</u>ransfer DNA <u>I</u>mmuno<u>P</u>recipitation (TrIP) using the model *A. tumefaciens* VirB/VirD4 system, allowed for provisional assignments of channel composition. As DNA substrates are translocated through the VirB/VirD4 channel, they can be formaldehyde-crosslinked sequentially with the VirD4 and VirB11 ATPases, then the VirB6 and VirB8 IMC subunits, and finally with the VirB9 and VirB2 pilin subunits in the periplasm (Fig. 2A) (20, 80-83). In subsequent studies, evidence also was presented for DNA substrate close contacts with VirB4-like subunits (71, 84). Thus, seven VirB/VirD4 subunits depicted in Fig. 2A are envisioned to comprise the translocation channel, while the remaining VirB components contribute indirectly to channel assembly or function.

The *L. pneumophila* Dot/Icm structure.—The *L. pneumophila* Dot/Icm T4SS is assembled from VirB-like subunits as well as approximately 20 additional subunits (Fig. 1B) (85, 86). Not surprisingly, therefore, the Dot/Icm structure recently visualized by *in situ* cryoelectron tomography (CryoET) is much larger than the $VirB_{3-10}$ substructure (Fig. 2D, F) (18, 87). The OMCC is 42 nm wide and 31 nm in height, and presents as a wheel-like structure with 13-fold symmetry as opposed to the 14-fold symmetries of the VirB/VirD4 OMCCs (18). The entire wheel is embedded in the inner leaflet of the OM, and a central pore of 6 nm projects across the outer leaflet of the OM. The wheel extends into the periplasm, where it is connected to a cylinder that extends to the IM, establishing contact with the IMC. Most strikingly, refinement of the IMC showed that it adopts a 6-fold symmetry and forms two concentric rings of 16 nm and 27.5 nm at the cytoplasmic entrance to the translocation channel (Fig. 2D) (18). In side-view, 6 inverted V structures extend into the cytoplasm, such that the inner arms of the V's form the inner ring and the outer arms form the outer ring. These V structures are composed of VirB4-like DotO, and thus the cytoplasmic complex consists of a hexamer of 6 DotO dimers (Fig. 2F). Furthermore, VirB11-like DotB was shown to dynamically associate at the base of the DotO inner ring by a mechanism dependent on ATP hydrolysis. The cytoplasmic complex is therefore composed

of a central DotO hexamer onto which the DotB hexamer binds, presumably when the machine is activated for substrate transfer. This symmetric IMC architecture differs strikingly from the asymmetric IMC of the VirB$_{3-10}$ structure marked by side-by-side VirB4 barrels (Fig. 2E, F). Gratifyingly, the Dot/Icm structure identifies for the first time a continuous T4SS channel extending from the cytoplasmic entrance (marked by the DotB lumen) to the cell surface (marked by the OMCC pore) (Fig. 2B, D, F) (18). It is also interesting to note that the Dot/Icm T4SS assembles at the cell poles and, moreover, polar delivery of effectors into the eukaryotic host cell evidently is required for successful *L. pneumophila* infection (88).

T4SS-associated mechanisms for target cell attachment

Conjugation systems of G– species elaborate conjugative pili that extend for as long as 20 μM from the donor cell (12). Flexible pili elaborated by F plasmids extend or retract to draw potential recipients into direct contact (89), whereas more brittle pili produced by other conjugative plasmids are either sloughed or broken from the cell where they accumulate and induce cellular aggregation (12, 90). To date, only one effector translocator system, the *H. pylori* Cag T4SS, also has been shown to elaborate pili in the presence of host epithelial cells (91-93). Interestingly, this Cag T4SS additionally elaborates large sheathed structures or 'membrane tubes' (94) that were recently visualized by *in situ* CryoET. Features of these tube structures led the authors to suggest they might arise by the extension of a pilus from an IM platform. As the pilus protrudes across the OM, the distorted membrane surrounds the pilus forming a sheath or tube that projects from the cell surface (95).

Surprisingly, the role of the pilus in substrate transfer is still not firmly established. On the one hand, there is some evidence in the F plasmid transfer system for DNA transfer between distant cells attached together by the F pilus, suggesting that the F pilus can function as a translocation channel (96). The structure of the F pilus was recently solved by CryoEM, and strikingly the lumen is lined with inner membrane phospholipid (PL). This discovery has important implications regarding the mechanism of F pilus assembly and retraction, but the presence of PL also imparts an overall weak negative charge to the inner lumen of possible importance for conveyance of the DNA substrate through the pilus (97). On the other hand, several observations argue against a role for the pilus as a conduit for substrates. First, in the *A. tumefaciens* VirB/VirD4 T4SS and related T4SSs, "uncoupling" mutations have been isolated that selectively block pilus production without impeding substrate transfer, strongly indicating that extended pili are not required for DNA transfer (73, 83, 98). Second, conjugation systems functioning in G+ species do not elaborate pili, yet can transfer DNA between cells at very high frequencies (8, 99, 100). Third, recently it was shown that the *E. coli* pKM101 conjugation system employs cell surface adhesins as an alternative to conjugative pili to mediate formation of donor-target cell contacts (101). This latter finding is of special interest given the paucity of evidence for pilus production by effector translocators other than the *H. pylori* Cag T4SS. It is enticing to propose that most members of the effector translocator subfamily have dispensed with energetically costly pilus production in favor of appropriating chromosomally-encoded surface adhesins to establish productive bacterial - eukaryotic cell membrane contacts.

CONCLUDING REMARKS

Mechanistic and structural studies are rapidly shaping a deeper understanding of how the T4SS superfamily evolved such functional diversity. Most notably, recent advances have been made in structural definition of T4SSs that are phylogenetically distantly related to the 'canonical' VirB/VirD4-like T4SSs, e.g., *L. pneumophila* Dot/Icm. Despite these advances, many fundamental questions remain: i) How do substrates dock with the T4SS and how are they processed for transfer? ii) What is the route of translocation and what are the signaling requirements for channel activation? iii) What mechanisms mediate productive donor-target cell contacts and what is the architecture of the mating junction? As basic studies continue to investigate these questions, we also note with excitement the emergence of translational studies aimed at designing T4SS machine inhibitors or repurposing T4SSs as therapeutic delivery systems (102-104). The integration of basic and translational approaches assures a bright and vibrant future for the fascinating T4SS nanomachines as well as the scientists devoted to their study.

ACKNOWLEDGEMENTS

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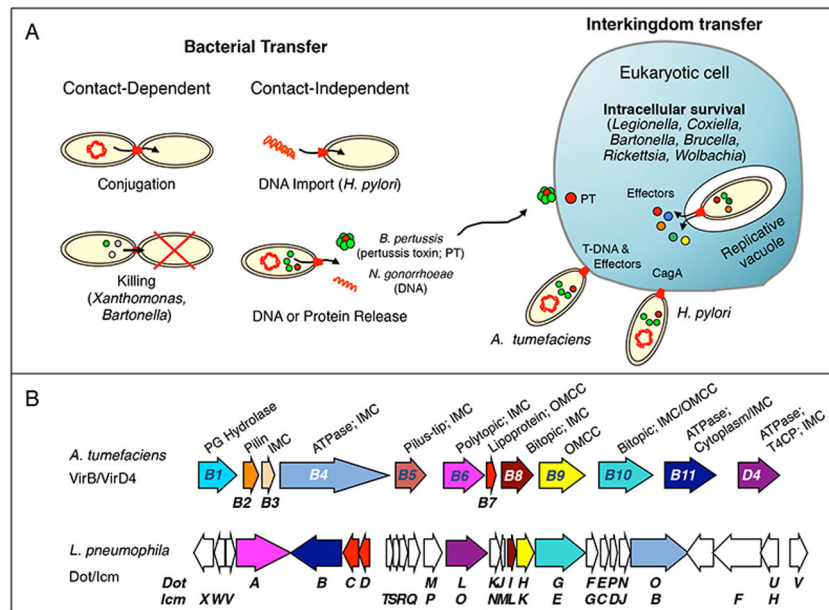
FIG. 1.

Functional and compositional diversity of the bacterial type IV secretion systems (T4SSs). **A)** Left: Contact-dependent conjugation systems and recently-described killing systems deliver DNA or protein substrates directly to bacterial target cells. Contact-independent systems mediate DNA import, DNA export, or export of the multimeric pertussis toxin. Right: Various pathogenic bacteria and symbionts have evolved T4SSs to deliver effector proteins or DNA–protein complexes into eukaryotic host cells to subvert host physiological processes. **B)** Gene arrangements and architectures of the *A. tumefaciens* VirB/VirD4 and *L. pneumophila* Dot/Icm secretion systems with color-coding of the genes encoding homologous subunits; unshaded genes are unique to the Dot/Icm system. The VirB/VirD4 subunit enzymatic functions and associations with inner membrane complex (IMC), outer membrane core complex (OMCC), or pilus are listed. PG Hydrolase, peptidoglycan hydrolase; T4CP, type IV coupling protein.
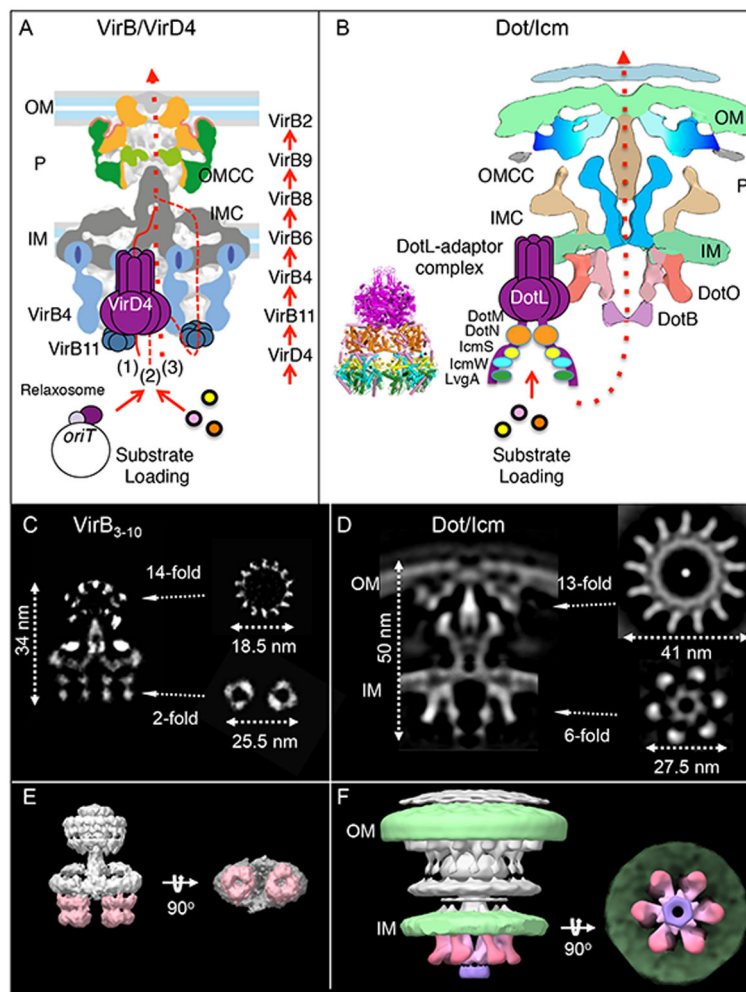
FIG. 2.

Architectures of the phylogenetically distant VirB/VirD4 and Dot/Icm T4SSs. **A)** A schematic of the VirB$_{3-10}$ structure elaborated by the Trw$_{R388}$ T4SS and solved by single-particle negative-stain electron microscopy (nsEM). A hexamer of the VirD4 receptor is fitted between the two hexameric barrels of the VirB4 ATPase. The VirD4 receptor recruits mobile genetic elements (MGEs) such as conjugative plasmids through recognition of components of the relaxosome (relaxase, accessory factors) assembled at the origin-of-transfer (*oriT*) sequence. VirD4 recruits protein substrates (colored dots) through direct or adaptor-mediated contacts. Substrates engage with the VirD4 receptor and are then delivered sequentially through a translocation channel composed of the VirB proteins listed at the right, as deduced from the TrIP assay (78). The route of transfer across the inner membrane is not known; substrates might be conveyed through the VirD4 hexamer (route 1, solid line), the VirB4 hexamer (2, small dashed line), or a channel composed of the VirB6 and VirB8 subunits (3, dotted line). Substrates then pass through the periplasm and across the outer membrane via an OMCC channel. **B)** A schematic of the *L. pneumophila* Dot/Icm T4SS solved by *in situ* cryoelectron tomography (CryoET) (16). The centrally stacked hexamers of the VirB4-like DotO and DotB form the cytoplasmic entrance to a channel that spans the entire cell envelope. The bell-shaped DotL-adaptor complex comprised of the hexameric

nucleotide binding domain (purple) and C-terminal domain bound with DotN (brown), IcmS (yellow), IcmW (aqua), and LvgA (green); reprinted with permission by Kwak *et al*. (58). The DotL-adaptor receptor complex was not part of the visualized Dot/Icm T4SS (16,), and is provisionally positioned adjacent to the Dot/Icm T4SS. Upon loading of substrates, the DotL-adaptor complex is postulated to present effectors to the DotB/DotO energy center for delivery through the central channel. **C-F)** Comparison of the R388-encoded $VirB_{3-10}$ substructure and the **L. pneumophila** Dot/Icm T4SS. **C)** A central section through the longitudinal plane of the $VirB_{3-10}$ single-particle reconstruction with cross-sections of the OMCC and IMC at the positions indicated. **D)** A central section through longitudinal plane of a global average structure of *L. pneumophila* Dot/Icm T4SS with cross-sections at the positions indicated. **E)** A 3D surface rendering of the $VirB_{3-10}$ substructure shown in side and bottom views. The side-by-side hexameric barrels of the VirB4 ATPase are colored pink. **F)** A 3D surface rendering of the Dot/Icm T4SS shown in side and bottom views. The bacterial membranes are in green and the DotO and DotB hexameric ATPases comprising the entrance to the translocation channel are in shaded pink and purple, respectively.