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Bacterial type IV secretion: conjugation systems adapted to deliver effector molecules to host cells

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Abstract

Several bacterial pathogens utilize conjugation machines to export effector molecules during infection. Such systems are members of the type IV or 'adapted conjugation' secretion family. The prototypical type IV system is the *Agrobacterium tumefaciens* T-DNA transfer machine, which delivers oncogenic nucleoprotein particles to plant cells. Other pathogens, including *Bordetella pertussis, Legionella pneumophila, Brucella* spp. and *Helicobacter pylori*, use type IV machines to export effector proteins to the extracellular milieu or the mammalian cell cytosol.

Gram-negative bacteria have adapted at least two cell-surface organelles for use in the delivery of macromolecules across kingdom boundaries by a cell-contact-dependent mechanism. The type III secretion systems are assembled from core components of the flagellar machine[1]. The type IV systems, the subject of this review, are built from core components of conjugation machines. This is a promiscuous secretion family both in terms of the translocated substrates – large nucleoprotein conjugation intermediates, an A/B toxin and monomeric proteins – and the phylogenetic diversity of cells targeted for substrate delivery – bacteria, fungi, plants and animals. In this review, we will summarize recent structure–function studies of the type IV systems, with an emphasis on the *Agrobacterium tumefaciens* T-DNA transfer machine.

Type IV family members

The type IV systems were initially defined on the basis of homologies between components of three different macromolecular complexes: the *A. tumefaciens* T-DNA transfer system required for exporting oncogenic T-DNA to susceptible plant cells; the conjugal transfer

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Note added in press

Recently, Chen *et al.*[51] identified VirE2 and VirD2 proteins in supernatant fractions independently of the *A. tumefaciens* VirB proteins. The authors speculate that VirE2 and VirD2 are translocated by a route that does not require the VirB proteins. Because only trace amounts (~1%) of the total cellular VirD2 and VirE2 proteins were detected in the supernatant, we suggest the alternative possibility that these proteins are associated with membrane vesicles or other cell surface structures that are released into the supernatant under laboratory growth conditions.

(Tra) system of the conjugative IncN plasmid pKM101; and the *Bordetella pertussis* pertussis toxin exporter, Ptl (Refs 2,3). As shown in Fig. 1, the list of type IV systems has recently been greatly expanded, with the identification of additional systems involved in DNA and protein translocation. Some of these systems are composed of a complete set of proteins homologous to the *Agrobacterium* VirB proteins; the corresponding genes are often colinearly arranged in the respective operons, highly suggestive of a common ancestral origin. Other transfer systems, such as the RP4 plasmid and the *Agrobacterium* pTi Tra systems, seem to be chimeras of VirB protein homologs and Tra proteins of an unrelated ancestry.

The *Legionella pneumophila* Dot/Icm system, an important macromolecular transporter associated with virulence, was originally proposed to be related to the *A. tumefaciens* T-DNA transfer system. Very interestingly, recent work has shown that the Dot/Icm system is in fact very closely related to the Tra region of the IncI ColIb-P9 plasmid of *Shigella flexneri* (Fig. 1). At least 16 *dot*/*icm* genes encode plasmid ColIb Tra homologs and gene organization is generally conserved in these two transfer systems[4]. The Dot/Icm system is unrelated to the VirB-based systems and yet is postulated to export novel effector proteins during the infection process[5]. Based on these observations, we propose the type IV classification be expanded to include functional secretion systems assembled from Tra proteins of any ancestral origin. We further propose the assignment of type IV subgroups, such that machines assembled from VirB homologs are type IVA and those from IncI Tra homologs are type IVB, allowing for future classification of other type IV systems assembled from other Tra systems.

A survey of the unfinished bacterial genomes database (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html) reveals additional candidate type IV systems among bacterial pathogens including *Bordetella bronchiseptica*, other *Brucella* spp., *Bartonella* spp. and *Actinobacillus actinomycetemcomitans*. Intriguingly, putative type IV systems are also present in a number of non-pathogenic bacteria, including *Caulobacter crescentus, Sinorhizobium meliloti, Rhizobium etli* and *Thiobacillus ferroxidans*, although their functions remain unknown. Finally, *L. pneumophila* contains a type IVA system (the Lvh system), which appears to play no role in virulence but is able to transfer a mobilizable IncQ plasmid[6].

Type IV systems as protein secretion machines

Currently, type IV systems are known to export three types of substrates: DNA conjugation intermediates, the multisubunit pertussis toxin (PT), and monomeric proteins including primase, RecA, the *A. tumefaciens* VirE2 and VirF proteins, and the *Helicobacter pylori* CagA protein (Table 1).

Many type IV systems transfer DNA, but it is important to note that the conjugation intermediate is not naked DNA but rather single-stranded (ss) DNA associated with one or more proteins. The co-transported protein(s) are involved in the processing of DNA at the origin of transfer (*oriT*) to form the conjugation intermediate. A transesterase, with contributions from one or more auxiliary proteins, initiates the processing reaction by

binding and generating a strand-specific nick at the plasmid *oriT* or the functionally equivalent *A. tumefaciens* T-DNA borders. Upon nicking, the transesterase remains covalently bound via a tyrosine to the 5′ end of the single-stranded (ss) DNA. Given that conjugal DNA transfer is a polar 5′–3′ reaction, the transesterase probably mediates the interaction between the ss DNA transfer intermediate and the transfer machine[7]. In the case of *A. tumefaciens*, the VirD2 transesterase is exported with T-DNA into the plant host cell, where nuclear localization sequences present on VirD2 mediate delivery of the T-DNA to the plant nucleus[3]. Thus, the type IV DNA-transfer systems are more aptly viewed as protein secretion machines, with the transesterase supplying the substrate recognition signal(s) and a 'piloting' function for directing its cargo, covalently bound ss DNA, through the translocation channel.

Recent work has also added to a body of early evidence that conjugation systems can transfer proteins independently of DNA to recipient cells. A clever genetic assay based on monitoring phage λ release from lysogenic recipients demonstrated transfer of RecA during the transfer of the RP4 plasmid to *Escherichia coli* recipients[8]. Similarly, there is strong genetic evidence that the *A. tumefaciens* T-DNA transfer machine exports VirE2, a ss-DNA-binding protein (SSB) and VirF, another virulence factor. Genetic work further suggests the protein substrates and conjugation intermediates compete for available T-DNA transfer machines[3,9].

What is the nature of the interaction between conjugation machines and their DNA and protein substrates? For plasmid mobilization, a coupling protein [e.g. TraG (RP4 and Ti plasmids), TrwB (R388), TraD (F) and VirD4 (*A. tumefaciens* T-DNA transfer system)] is thought to link the DNA processing and transfer reactions both spatially and temporally. Studies of chimeric transfer systems composed of a translocation apparatus and a heterologous coupling protein suggest that the coupling protein provides the basis for recognition and export of specific plasmid substrates[10–12]. For DNA-independent protein transfer, a chaperone-like protein is now known to be involved in mediating the transfer of the *A. tumefaciens* VirE2 SSB protein via the T-DNA transporter to plant cells. VirE1 is a small (7.5 kDa), acidic protein that interacts directly with an internal domain of VirE2 and is required for export of the SSB. There is also evidence that VirE1 stabilizes VirE2 *in vivo* and prevents VirE2 aggregation *in vitro*[13–15]. These characteristics, as well as the physical properties of VirE1, are reminiscent of the Syc chaperone or 'bodyguard' proteins involved in type III secretion[16]. Thus, by analogy to the type III systems, DNA-independent export of protein substrates via the type IV machines might generally require initial complex formation between the substrate and a cognate chaperone-like protein.

Effector proteins implicated in virulence of mammalian hosts

The protein substrates of type IV systems important for the virulence of bacterial pathogens of mammals are quite distinct from those associated with conjugation systems (Table 1). For example, the *B. pertussis* Ptl system secretes pertussis toxin (PT), which is a multisubunit A/B toxin composed of five subunits, S1–S5. The S1 subunit, or A domain, shares active-site ADP-ribosylating activity and structure with diphtheria toxin (DT), cholera toxin (CT) and other A/B toxins[17]. The B domain is a pentamer of the remaining S2–S5 subunits in a ratio

of 1:1:2:1, respectively. Interestingly, despite the overall similarity in subunit composition between the T-DNA transfer and Ptl secretion systems, the TDNA and PT export routes differ significantly. Whereas T-DNA and other conjugation intermediates are thought to be delivered across the cell envelope in a single step via a transenvelope mating channel, PT export is a two-step translocation reaction. PT subunits are first secreted across the cytoplasmic membrane via the general secretion pathway, then the PT holotoxin, assembled in the periplasm, is exported via the Ptl system across the outer membrane. Upon export, the B domain interacts with host cell glycoprotein receptors and mediates translocation of the A domain across the host cell membrane. In the host cell cytosol, PT ADP-ribosylates the α subunits of $G_{i,o}$ proteins, thus interfering with receptor-mediated activation of the G protein and associated signaling pathways[18].

Very recently, *H. pylori* was shown to export the 145-kDa CagA protein via a type IV secretion system encoded by the *cag* pathogenicity island[19,20]. *H. pylori* is the causative agent of peptic ulcer disease and a risk factor for the development of gastric adenocarcinoma. *H. pylori* attaches to human gastric epithelial cells and induces a number of changes in host cell physiology, including effacement of microvilli, cup/pedestal formation, cytoskeletal rearrangements, and interleukin (IL)-8 production[21]. Type I strains of *H. pylori* carrying the *cag* pathogenicity island were shown to induce signal transduction pathways, resulting in tyrosine phosphorylation of proteins adjacent to the site of bacterial adherence[22]. Two studies have now reported that CagA is one of these tyrosine-phosphorylated proteins. Intriguingly, a proportion of *H. pylori* cells found attached to the host cell surface were associated with a high concentration of CagA, with the appearance of a cylindrical structure associated with regions of active actin reorganization. These findings suggest that CagA cylinders might induce host cytoskeletal rearrangements[19,20].

Several type IV systems of mammalian pathogens have been identified on the basis of a requirement for virulence. However, the effector(s) exported by these systems remains unknown. The *L. pneumophila* Dot/Icm system was identified by screening for mutants defective in multiplication in host macrophages[23,24]. Loss of *dot/icm* function results in bacteria mistargeting to a late endocytic or lysosomal compartment. Presumably, the *dot/icm* system exports a virulence factor(s) that is required to prevent phagosome–lysosome fusion. As noted above, the *dot/icm* system is unrelated to the VirB systems. Yet, this system conjugally transfers plasmid RSF1010 to *L. pneumophila* recipients[23,24]. RSF1010 transfer is not thought to be relevant to *dot/icm* gene function during the infection process. Nevertheless, conjugation proficiency does provide a convenient assay for monitoring functionality of this, and possibly other, type IV systems associated with virulence.

Architecture of the T-DNA transfer machine

Conjugation machines of Gram-negative bacteria consist of two surface structures, the mating channel through which the DNA transfer intermediate and proteins are translocated and the conjugal pilus for contacting recipient cells[2,7]. Various conjugative pili have been visualized, but to date there is no ultrastructural information about the mating channel. Recent work on the *A. tumefaciens* T-DNA transfer system has focused on identifying interactions among the VirB protein subunits and defining steps in the transporter assembly

pathway. As depicted in Fig. 2, there are three functional groups of VirB proteins: (1) proteins localized exocellularly forming the T-pilus or other adhesive structures; (2) mating-channel components; and (3) cytoplasmic membrane ATPases. Although all of these proteins probably assemble as a supramolecular complex, as yet there is no direct evidence for a physical association between the conjugative pilus and the mating channel.

Exocellular proteins

In an accompanying review in this issue of *Trends in Microbiology* (pp. 361–369) E-M. Lai and C.I. Kado present a comprehensive update on the structure and function of the *A. tumefaciens* T-pilus. VirB2, the major subunit of the T-pilus, is processed from a 12.5-kDa proprotein to a 7-kDa mature peptide that partitions with the cytoplasmic membrane during cell fractionation. Interestingly, a recent study demonstrated that VirB2 and its homolog, TrbC of plasmid RP4, undergo novel cyclization reactions following processing such that the amino and carboxy termini are joined via an intramolecular covalent head-to-tail peptide bond[25]. Upon an unknown signal, VirB2 polymerizes to form the T-pilus, a thin (10 nm) filament found in abundance on cells grown at 18°C but rarely seen on cells grown above 28°C (Ref. 26; Lai and Kado, this issue). Recent work has shown that VirB5 co-purifies in low amounts with the T-pilus and thus is a probable minor pilus subunit[27]. Similar findings were reported for the VirB5 homolog, TraC of plasmid pKM101 (Ref. 28).

VirB1 has a motif found among lytic transglycosylases, and therefore might cause local lysis of the peptidoglycan during transporter assembly. VirB1*, a proteolytic product corresponding to the carboxy-terminal 73 residues of VirB1, localizes exocellularly and chemically crosslinks with VirB9. VirB1* is not detected in purified T-pilus preparations, and thus might mediate close contacts between donor and recipient cells[29,30].

Putative channel components

Several lines of evidence suggest that VirB6–VirB10 as well as one or more of the three ATPases (see below) are probable channel subunits[3]. VirB6, a highly hydrophobic protein, is thought to span the cytoplasmic membrane several times and presently is the best candidate for a channel-forming protein[3]. VirB7, an outer membrane lipoprotein[31], interacts with itself and with VirB9 via disulfide bonds between unique reactive cysteines present in each protein[32,33]. The VirB7–VirB9 heterodimer localizes at the outer membrane and plays a critical role in stabilizing other VirB proteins during assembly of the transfer machine[34]. Recent yeast two-hybrid studies supplied evidence for pairwise interactions between VirB8, VirB9 and VirB10 (Ref. 35). VirB9 is also required for formation of chemically crosslinked VirB10 oligomers probably corresponding to homotrimers. VirB10 has a short amino-terminal cytoplasmic domain and a large carboxy-terminal periplasmic domain and thus is proposed to link cytoplasmic and outer membrane VirB subcomplexes[36].

Cytoplasmic membrane ATPases

Two VirB proteins, VirB4 and VirB11, possess conserved Walker A nucleotide-binding motifs required for function, and purified forms of both proteins possess weak ATPase activities[3]. Recent work suggests both proteins assemble minimally as homodimers *in vivo*. Self-interaction is mediated by a domain in the amino-terminal third of VirB4 and by

domains located in each half of VirB11 (Refs 37–39). Further studies have now established that VirB11 and its homologs, TrbB of plasmid RP4, TrwD of plasmid R388, and RP0525 of the *H. pylori* Cag pathogenicity island, form higher-order homomultimers[39–41]. Examination of complexes by electron microscopy led to the intriguing discovery that TrbB, TrwD and RP0525 assemble as homohexameric rings in solution[40,41]. TrbB hexamer formation is dependent on an intact Walker A motif, suggesting that ATP-induced conformational changes in TrbB might contribute to transporter morphogenesis or substrate translocation[40].

As noted above, VirD4 is a member of a family of putative ATPases thought to couple DNA processing and transfer reactions and to supply the basis for plasmid substrate discrimination[42,12]. However, VirD4 homologs are also found among systems thought to function exclusively in protein export (see Fig. 1). For example, a VirD4 homolog is required for *H. pylori* CagA protein export[20]. Thus, these proteins are not limited to DNA translocation reactions and might instead play a more general role in substrate trafficking via type IV machines.

Perspectives and the future

The excitement surrounding the type IV secretion pathway builds with the identification and demonstration of functionality of each new member. Not only are these systems widespread in nature, they are also highly versatile as evidenced by their various uses by mammalian and plant pathogens. The *A. tumefaciens* T-DNA transporter seems to be the most promiscuous of these machines in its capacity to deliver DNA and proteins to an impressively wide array of cell types – numerous species of plants, bacteria, *Saccharomyces, Aspergillus, Fusarium, Trichoderma, Neurospora* and *Agaricus*[3,43]. The mammalian pathogens use their type IV systems for distinct purposes: *B. pertussis* to export PT to the extracellular milieu, *H. pylori* to deliver CagA to the mammalian cell cytosol and *L. pneumophila* and other intracellular pathogens probably to export an effector molecule(s) following uptake by macrophages. Comparative studies of these systems will certainly unveil common mechanistic principles as well as unique features appropriated for novel purposes.

Studies of the types II–IV secretion systems have revealed remarkable examples of convergent evolution. For example, in function, the type IV systems very closely resemble the type III systems, which utilize a flagellar export machine to inject effector molecules into host cells[1,16]. Both systems deliver substrates by a process requiring physical contact with target cells. Both systems require coupling or chaperone- like proteins for delivery of substrates to the respective transfer machines. Both systems are generally thought to export substrates in a one-step reaction via a transenvelope channel. Finally, both systems elaborate extracellular pili or filaments that contribute in some way to substrate delivery. However, at least one fundamental difference exists between these two systems – the type IV systems can export long ss DNA polymers to recipient cells, whereas there is currently no evidence for transmission of nucleic acids via type III machines.

The type IV systems also mechanistically resemble the type II systems (reviewed in Ref. 44). Most notably, the *B. pertussis* PT export pathway is an exception to the one-step

translocation route envisaged for type IV machines in that it involves a two-step translocation route much more reminiscent of the type II secretion systems. Indeed, cholera toxin (CT), an A/B toxin that is structurally highly related to PT (Ref. 17), is exported via a type II secretion pathway[45]. In addition, both the type IV and II systems permit transmission of nucleic acids across the cell envelope. A recent report showed that the same type II system (Eps) that mediates CT export also serves as the conduit for secretion of the filamentous phage CTXφ across the outer membrane of *Vibrio cholerae*[45]. Thus, although the type IV secretion systems are configured for exporting plasmid DNA in association with conjugation proteins, a type II system has now been shown to export a phage genome in association with phage proteins.

Many fundamental questions surrounding the type IV systems await further exploration. The Tra operons encoding the type IV conjugation systems are often induced by extracellular or growth-phase-dependent signals[2,3,7,46], as are the genes for the *B. pertussis* Ptl system[2,18]. What extracellular signals regulate expression of genes for type IV systems implicated in virulence of intracellular pathogens such as *L. pneumophila* or *Brucella* spp.? What is the molecular basis for dual recognition of protein and nucleoprotein substrates? What specific cell–cell interactions enable the delivery of substrates to such a wide array of target cell types? Answers to these questions will not only supply basic knowledge about this fascinating transporter family but might also pave the way for development of strategies aimed at incapacitating these systems to suppress virulence, or for manipulating bacterial pathogens as vectors for the targeted delivery of therapeutic DNA or proteins.

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Questions for future research

- What is the basis for substrate recognition? Are chaperone-like proteins of general importance for type IV protein transfer?
- What is the architecture of the channel and the nature of the contact with extracellular pili? Do all type IV systems elaborate extracellular pili or other appendages?
- What host signals are required for elaboration of type IV transfer machines? In addition to transcriptional regulation, what translational or post-translational regulatory processes are required for transporter biogenesis?
- Are type IV systems viable targets for antibiotic intervention or vectors for delivery of therapeutic molecules?
- Can the principle of interkingdom transfer of effector DNA or proteins via conjugation machines be generalized to the Gram-positive bacteria?

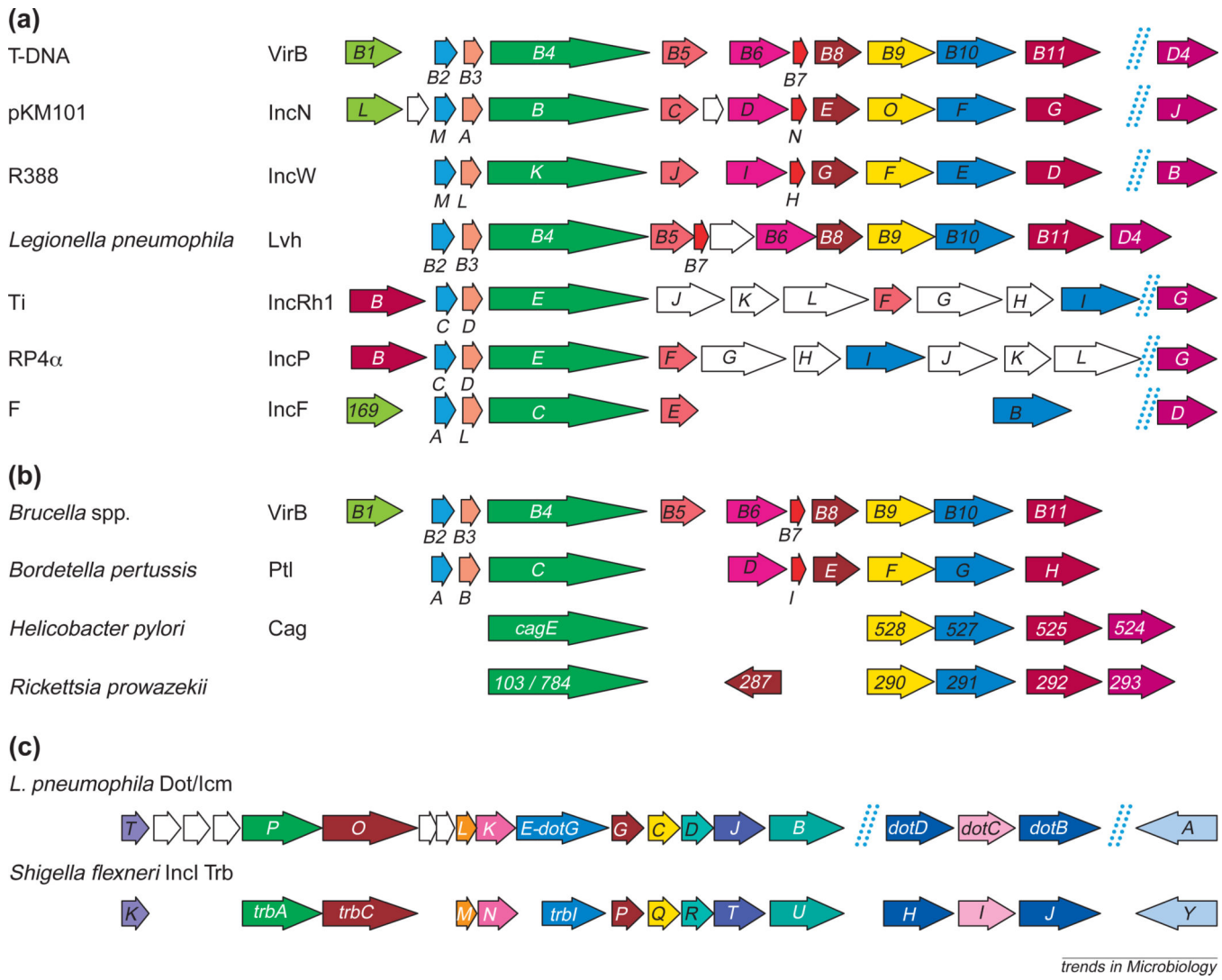

Fig. 1.

Type IV systems demonstrated or postulated to direct DNA and/or protein translocation. The *Agrobacterium tumefaciens* T-DNA transfer machine is composed of products of the *virB* operon and the *virD4* gene. Genes encoding VirB and VirD4 homologs are color-coded for the other type IV systems. Dashed lines indicate the genes are physically unlinked. **(a)** Representative conjugation systems. **(b)** Transfer systems thought to function as exporters of effector proteins during infection. **(c)** The *Legionella pneumophila* Dot/Icm system encodes *icmE*/*dotG* and *dotB*, weak homologs of VirB10 and VirB11, but is clearly ancestrally related to the transfer region of the *Shigella flexneri* ColIb IncI plasmid. Additional homologies not shown exist between *L. pneumophila* IcmW, IcmX, IcmV and DotK and the IncI plasmid TraV, TraW, TraX and TraL proteins, respectively. Sources: *A. tumefaciens virB* (see Ref. 3), pKM101 (Refs 2,47), R388 (Ref. 42), RP4 (Ref. 48), F (Ref. 7), *L. pneumophila lvh* (Ref. 6), *Brucella virB* (Ref. 49), *Bordetella pertussis ptl* (Refs 2,18), *Helicobacter pylori cag* (Ref. 21), *Rickettsia prowazekii* (Ref. 50), *L. pneumophila dot/icm* (Refs 23,24).

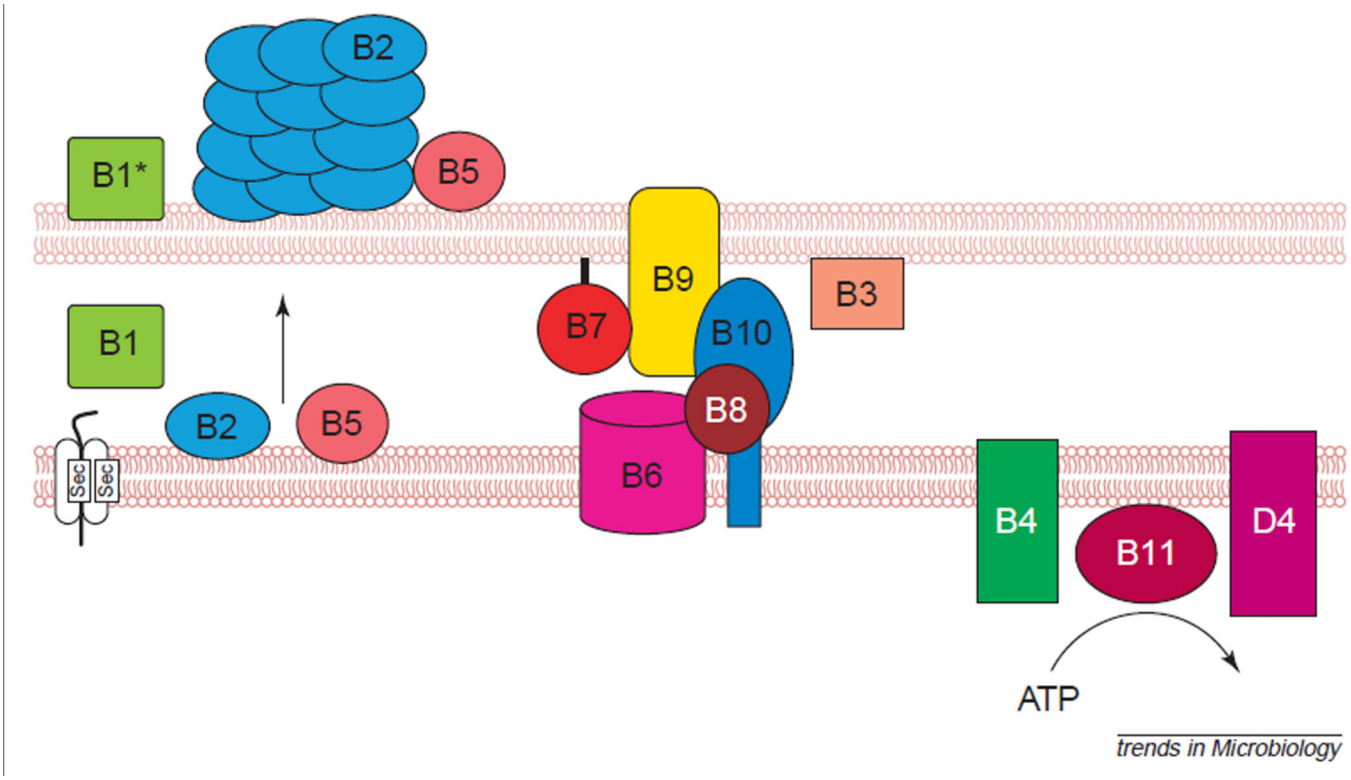

Fig. 2.

Locations of VirB components of the T-DNA transfer system. The VirB and VirD4 proteins are grouped according to probable functions: exocellular proteins mediating attachment (VirB1*, VirB2 pilin and VirB5), channel proteins (VirB3, VirB6, VirB7, VirB8, VirB9 and VirB10), and ATPases (VirB4, VirB11 and VirD4).

Table 1

Substrates of type IV systems[a]

System	Substrate(s)	Consequence(s) of transfer
Plasmid conjugation	Transfer intermediate (ss DNA) covalently bound at 59 end to transesterase; primase; SSB; RecA	Dissemination of virulence and antibiotic-resistance genes; transferred proteins contribute to establishment of plasmid in recipient
Agrobacterium tumefaciens T-DNA transfer	T-DNA (ss T-DNA/VirD2); VirE2; VirF	Oncogenesis (crown gall disease); T-DNA delivery to plant nucleus; broadened host range
Bordetella pertussis Ptl	Pertussis toxin	ADP-ribosylation of GTP-binding proteins
Helicobacter *pylori* Cag	145-kDa CagA protein	Tyrosine phosphorylation of CagA; formation of CagA cylinders; host cell cytoskeletal rearrangements
Legionella pneumophila Dot/Icm	Unknown effector	Allows survival and growth in macrophages; prevent phagosome/lysosome fusion?
	IncQ plasmid	Plasmid acquisition by *L. pneumophila* recipients
Lvh	IncQ plasmid	Plasmid acquisition by *L. pneumophila* recipients
Brucella, Bartonella, Rickettsia	Unknown	Aid in intracellular survival?

[a]Abbreviation: SSB, single-stranded-DNA-binding protein.