Minireview

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Bacterial Injection Machines*

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The delivery of virulence factors directly into host cells is a fascinating aspect of pathogenesis. For Gram-negative bacteria to translocate virulence factors into host cells, at least three membranes must be passed (two bacterial and a host plasma membrane). Bacterial injection machines deliver virulence factors to a specific cellular location where they intersect and influence host mechanisms. This minireview focuses on the Gram-negative bacterial translocation systems that mediate type III and type IV secretion. Remarkably, although these systems are complex multiprotein structures, there is significant similarity and analogy in function, and thus a conserved mechanistic theme in pathogenicity emerges.

Currently there are seven identified types of macromolecular secretion systems in Gram-negative bacterial pathogens (1, 2). This minireview focuses on the two systems that deliver macromolecules directly into eukaryotic cells: type III secretion system (T3SS)[1] and type IV secretion system (T4SS). The delivered macromolecules are referred to as effectors, as they affect and alter the host cellular process. Gram-negative bacterial effectors cross several biochemically distinct barriers, including the bacterial inner membrane, peptidoglycan layer, and outer membrane as well as the host plasma membrane, and even potentially intracellular host membranes. Plant pathogen effectors have the additional complexity of crossing the plant cell wall. The biochemistry of these delivery systems will be discussed, including what is known about how they are assembled and how they function.

Type III Secretion Systems

In the 1980s and 1990s researchers studying *Yersinia*, a genus that causes human diseases ranging from bubonic plague to gastrointestinal disease, found that the bacteria produced proteins that were thought to be associated with the outer membrane called Yops. Yops lacked classical signal sequences and were not secreted via a *sec*-dependent pathway and thus were assumed to be delivered by a new type of secretion system, which later became known as a T3SS, representing its order of discovery in secretion systems. In the last 10 years T3SS have been identified in more than 20 bacterial pathogens that infect plants and animals (Table I). Although there is a high degree of conservation among the components of the type III apparatus in different bacterial species, the pathogens often carry a distinct set of virulence factors with a variety of functions that can be translocated into either animal or plant cells. The overall theme of these T3SS is the direct delivery of proteins that alter and in effect "hijack" the infected host cell for the pathogen (reviewed in Refs. 3 and 4).

Type III Apparatus Components

Over 20 proteins are proposed to form a functional T3SS (Fig. 1*A*) (4, 5). YscN is thought to energize the secretion machinery, as it shares homology with the F_0F_1-ATPase and has an ATP-binding site. YscN from *Yersinia* and its homologue InvC from *Salmonella typhimurium* have been shown to have ATPase activity as mutations in the catalytic domain cause a loss of secretion (6, 7). YscN homologues are predicted to be located in the cytoplasm where they interact with membrane-bound components of the type III secretion apparatus, thereby energizing the system (4). It has been speculated that the ATPase polymerizes, by itself or with other components, to form the lower part of the T3SS, but this has not been shown. Lending support to this model, YscN has been shown to form a complex with three other cytoplasmic and/or inner membrane-associated Ysc proteins (8).

Many of the proteins involved in forming the T3SS have been localized or are predicted to be inner membrane proteins with varying numbers of transmembrane domains. For example the *Yersinia* YscV (LcrD) contains eight transmembrane domains and a large cytoplasmic C-terminal domain (9, 10). YscJ family members carry *sec*-dependent signal sequences and are lipoproteins (4, 11). The *Pseudomonas syringae* homologue HrcJ is associated with both inner and outer membranes (12), suggesting that it spans the periplasmic space.

Homologues of YscC (*e.g.* InvG, HrcC) are the only components of the type III apparatus that are clearly found in the outer membrane (12–15). YscC belongs to a family of proteins (secretins) that are involved in transporting large molecules across the outer membrane probably by forming a channel (4). YscC and its homologues form a ring-shaped oligomeric complex in the outer membrane with approximately a 20-nm diameter. Experimentally it has been shown that InvG has a cleavable signal sequence at residue 25, indicating that secretins are exported by the *sec*-dependent pathway (16). It has been demonstrated that small outer membrane lipoproteins are required to increase the efficiency for the correct localization and functioning of the YscC homologues (13, 14, 17–19). Recently we have shown that correct insertion and function of enteropathogenic *Escherichia coli*'s (EPEC) EscC secretin in the outer membrane requires cytoplasmic and inner membrane components of the type III apparatus, namely EscN and EscV (20).

Syringe and Needle-like Structure

Electron microscopic analysis has revealed the T3SS to be an organelle that consists of a base (or syringe) that spans the bacterial membranes and peptidoglycan layer composed of two pairs of rings that are joined by a central channel, and a hollow needle-like structure that protrudes outside the bacteria and in some cases has been observed to contact the host cells (reviewed in Refs. 21 and 22). There is a remarkable structural similarity to the bacterial flagellar basal bodies (see "Origin of Translocation Systems"). Mutants in the needle protein cannot secrete or translocate effectors, suggesting that either the needle keeps the pore open or that the needle extends into the "syringe." Whereas needle proteins exist in all of the animal pathogens, a homologous protein has not been found in the plant pathogens although the pilus protein HrpA seems to play a similar functional role (23).

Elegant immunogold electron microscopy experiments have been conducted with the plant pathogens *Erwinia* and *P. syringae* demonstrating very clearly that the effectors actually go through the needle/pilus conduit and can be visualized at the tip of the structure (24, 25). This indicates that the type III apparatus and needle are a hollow conduit for protein delivery.

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[1] The abbreviations used are: T3SS, type III secretion system(s); T4SS, type IV secretion system(s); Ysc, *Yersinia* type III secretion complex; EPEC, enteropathogenic *E. coli*.

TABLE I
Type III secretion systems are found in a wide variety of human, animal and plant pathogens

Bacterial pathogen	Disease
Animal pathogens	
Bordetella species	
B. pertussis	Whooping cough and other respiratory diseases
B. bronchiseptica	Respiratory diseases
B. parapertussis	Respiratory diseases
Burkholderiapseudomallei	Meliodosis (septicaemia, pneumonia, infections)
Chlamydia species	
C. trachomatis	Infectious blindness, sexually transmitted disease
C. pneumoniae	Upper respiratory tract infections, possibly artherosclerosis
C. psittaci	Primarily animal pathogen
Pathogenic *E. coli*	
Enteropathogenic *E. coli*	Diarrheal diseases
Enterohemorrhagic *E. coli*	Diarrheal diseases, hemolytic uremic syndrome
Rabbit EPEC	Rabbit pathogen
Dog EPEC	Dog pathogen
P. aeruginosa	Opportunistic pathogen: cystic fibrosis, burn victims
Salmonella serovars	
S. typhi	Typhoid fever in humans
S. typhimurium	Humans: gastroenteritis, bacteremia, enteric fever Mouse: typhoid fever
S. dublin	Cattle pathogen
S. pullorum	Poultry pathogen
S. arizonae	Reptile pathogen
S. enteritidis	Gastroenteritis in a broad host range
S. choleraesuis	Broad host range
Shigella species	
S. dysenteriae	Bacillary dysentery
S. flexneri	Diarrheal diseases
Yersinia species	
Y. pestis	Bubonic plague
Y. enterocolitica	Gastrointestinal syndromes
Y. pseudotuberculosis	Self-limiting gastroenteritis
Plant pathogens	
Erwinia species	
E. amylovora	Soft rot of plants
E. chrysanthemi	Fire blight of rosaceous plants
P. syringae	Bacterial speck disease
Ralstonia solanacearum	Bacterial wilt of solanaceous plants
Xanthomonas campestris	Bacterial spot disease of pepper and tomato
Fish pathogens	
Aeromonas salmonicida	Furunculosis
Endosymbiont	
Rhizobium species	Cultivar-specific nodulation of leguminous plants
Sodalis glossinidius	Intracellular endosymbiont of the tsetse fly

The Translocon: Pore in the Host Cell Membrane

All of the animal pathogen T3SS have one or more proteins that are thought to form a pore in the host cell membrane, called the "translocon" (reviewed in Ref. 26). It is important to note that although mutations in needle components result in no secretion or translocation of effectors, mutation of translocon components yields wild-type levels of secreted but not translocated effectors. Most of the translocon proteins contain one or two predicted transmembrane domains and are associated with host cell membranes. Furthermore, in EPEC the needle sheath protein EspA and the translocon EspB have been shown to interact by a number of binding assays (27), suggesting a continuous channel. Additionally, *Yersinia* and *P. aeruginosa* have another type of translocon protein that does not contain α-helical transmembrane domains but is required for pore formation. Translocon proteins have not been described in plant pathogens, but HrpF from *Xanthomonas* is a candidate as it is not needed for secretion but is required for translocation and forms pores in lipid bilayers (28).

Recognition of Type III Effectors: mRNA, Protein, and Chaperones

T3SS effectors do not have an obvious signal sequence. However, there is evidence for the existence of three different kinds of secretion signals: the 5′-region of the mRNA, the N terminus of the effector, and/or the ability of a chaperone to bind the effector before secretion (reviewed in Ref. 29). The minimal requirement for secretion of some effectors is the N-terminal 10–15 residues (30, 31), whereas the minimum needed for translocation is 50–75 N-terminal residues (30, 31). The so-called mRNA hypothesis is very controversial. Two groups have shown that certain effectors have a 5′-mRNA fold that directs translocation (32–35), but others have refuted these observations with equally convincing experiments showing that the N-terminal amino acids are the signal (36).

Many functions have been attributed to T3SS chaperones, but the exact role or roles of the entire family of chaperones remain to be determined (reviewed in Refs. 29 and 37). Although most chaperones have only one cognate effector, there are exceptions with differing numbers of effectors. Deletion of a chaperone usually results in less of the cognate effector in the cytoplasm of the bacteria and less secreted/translocated. For some effectors, chaperone binding prevents degradation, whereas for others it has been suggested that chaperone binding prevents premature association. Another model suggests that chaperones escort the effector to the type III apparatus and play a role in the hierarchy of translocation (38). Chaperones could maintain effectors in a secretion-competent state. The needle of the T3SS is likely too small to allow folded proteins to pass through, but recent data suggest that effectors are in a partially folded conformation (reviewed in Ref. 39).

Type IV Secretion Systems

T4SS are a recent discovery in pathogenic delivery systems (reviewed in Refs. 40 and 41). These systems deliver proteins and DNA (often complexed together), but they can also deliver only proteins. The prototype and most well studied T4SS is involved in the transfer of oncogenic DNA into plant cells by *Agrobacterium tumefaciens* (reviewed in Ref. 42). Although the function and perhaps the mechanism of the T4SS are similar to the T3SS, there is little to no conservation in the proteins that comprise the apparatus. The lack of conservation suggests the mechanism at a molecular and physical level may differ significantly between the two. Like the T3SS, the T4SS is considered "promiscuous" in terms of the variety of substrates and the diversity of target cells (43) (Table II). It should be noted that although T4SS have recently been identified in a number of bacterial species, their substrates/effectors are often unidentified.

Type IV Apparatus Components

The locus involved in T-DNA transfer in *A. tumefaciens* contains 11 VirB proteins, 2 of which are ATPases, as well as an additional ATPase, VirD4, which is involved in the export of DNA (Fig. 1*B*) (reviewed in Ref. 41). It is important to note that not all of the type IV-containing family of bacteria have homologues of all of these proteins, and so far a conserved core of 5 proteins exists (homologues of VirB4, -B7, -B9, -B10, and -B11) (44). The type IV apparatus components have been localized using standard biochemical fractionation techniques like sucrose gradients, alkaline phosphatase insertions, and protease susceptibility, with most of the studies being done on *A. tumefaciens*.

Several proteins are found in the inner membrane and some are integral membrane proteins like VirB4 and VirB6, whereas others are associated with the inner side of the inner membrane like VirB11 (reviewed in Ref. 40). VirB7–B10 fractionate to both the inner and outer membranes (45), and it has been suggested that these proteins act in concert to span both membranes. In fact, VirB7 is a lipoprotein that is important for structural integrity of many other components of the T4SS (46). VirB10 interacts with VirB9, and results suggest that the outer membrane VirB7-VirB9 complex interacts with the inner membrane proteins VirB8 and VirB10 to form the translocation channel (47).

Energy Requirement

The T4SS has at least two and possibly three ATPases/NTPases. The energy may be required for the translocation process, to open the channel, as a coupling factor, or for chaperone functions (reviewed in Refs. 40 and 41). Similar to what has been observed for the T3SS, studies have demonstrated that NTPase activity is crucial for export. VirB4 and VirB11 have Walker A nucleotide binding motifs and have ATPase activity (48). Interestingly, recent studies support a model whereby there can be bi-directional DNA transfer aided by the mul-

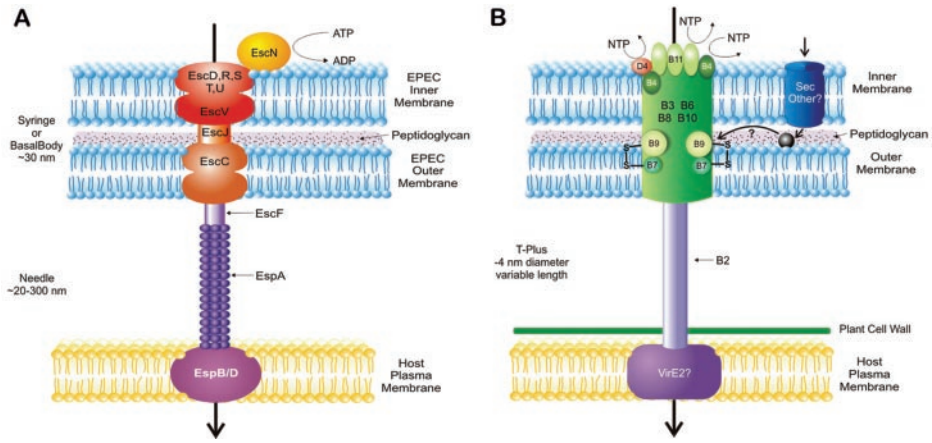

FIG. 1. **Models of type III and type IV secretion systems.** *A*, diagram of the T3SS highlighting apparatus components of EPEC and the direct nature of translocation to host cells. *B*, diagram of *A. tumefaciens* T4SS highlighting apparatus components and the possibility of having two (or more) mechanisms of export across the bacterial inner membrane.

TABLE II
Type IV secretion systems translocate macromolecules across a variety of kingdoms

System	Consequence of transfer
Bacteria → bacteria	
Plasmid conjugation (F, pKM101, R388, pAtC58, RP4 plasmid)	Dissemination of virulence and antibiotic resistance genes
Bacteria → plant	
A. tumefaciens T-DNA transfer	T-DNA delivery to plant; oncogenesis, broadened host range
Bacteria → animal	
Bordetella pertussis Ptl	Transfer of pertussis toxin (ADP-ribosylation of GTP-binding proteins); whooping cough
H. pylori CagA	Transfer of CagA; host cell cytoskeletal rearrangements; peptic ulcers
Legionella pneumophila Dot/Icm	Allows survival and replication in macrophages; Legionnaire's disease
Brucella species	Aid in intracellular survival? Effector?
Bartonella species	Aid in intracellular survival? Effector?
Rickettsia species	Aid in intracellular survival? Effector?
Actinobacillus species	Tight adherence to host? Flp pili effector?
Symbiont	
Wolbachia species	Cause reproductive abnormalities in host?

timeric structure of VirB4 but that it is the ATP-dependent activity that renders it an export-only system (49). VirB11 ATPase homologues from T4SS in Ti-plasmid, RP4, and *Helicobacter pylori* have been shown by electron microscopy to form a homohexameric ring structure with a 12-nm diameter and a 3-nm central channel (50). The crystal structure of the *H. pylori* ATPase HP0525 (homologous to VirB11) has been solved and suggests that this family of ATPases may function as chaperones similar to GroEL (51).

Type IV Secretion System Pilus and Translocon

In *A. tumefaciens* the T-pilus appears to be 4 nm in diameter and have a variable length (52), and it is made up of VirB2, which is a small 7.2-kDa protein (53, 54), a structure reminiscent of the T3SS needle/pilus. Recently, it has been suggested that VirB7-VirB9 complexes may link the T-pilus components (VirB2) to the core of the translocation machinery (55). VirE2 has been suggested to fulfill the translocon or host-cell pore-forming function of the T4SS because it forms large anion-selective, voltage-gated channels and allows the transport of single-stranded DNA (56).

Chaperone/Coupling Protein

Chaperones are involved in the export of both DNA and proteins by the T4SS. There is evidence for the chaperone VirE1 stabilizing VirE2 and inhibiting aggregation (57), suggesting a function in preventing premature association, as has been suggested for the T3SS chaperones. It has been suggested that coupling proteins like the VirD4 family act by specifically recognizing and exporting T4SS substrates (reviewed in Ref. 43).

Pertussis T4SS: Anomaly or the Norm?

Pertussis toxin transport seems to be an anomaly in the T4SS substrates because there are detectable periplasmic intermediates (reviewed in Ref. 40). Pertussis toxin subunits require the *sec*-dependent general secretion pathway for export to the periplasm and then the T4SS for secretion out of the bacterium. Additionally, the pertussis toxin system is unlike the other T4SS in that it functions independently of host contact because of the ability of the holotoxin to autotranslocate itself into the host cell rather than relying on T4SS delivery.

Although periplasmic intermediates of effectors have not been found in the T3SS, recent developments in the T4SS field suggest that the pertussis toxin two-step translocation requirements are not anomalous. Pantoja and colleagues (58) have demonstrated that T4SS substrates in *A. tumefaciens* that lack a signal peptide form a soluble complex in the periplasm with another protein, VirJ, and then interact with components of the T4SS. This suggests a two-step model for type IV secretion in which effectors are exported via different pathways into the periplasm and then translocated across the outer membrane and host cell membrane via the T4SS.

Origin of Translocation Systems

Many but not all of the proteins comprising the T3SS of pathogenic bacteria have homologues that are known to be involved in flagellar morphogenesis. Flagella are complex filamentous cell surface organelles that rotate, resulting in swimming motility. The flagellum includes a long hollow filament (20–25 nm in diameter), a curved hook structure, and a membrane-embedded basal body. At least 50 gene products are involved in flagellum biogenesis (for a detailed review see Ref. 59).

Comparatively, T3SS needle complexes are not rotary structures like flagella, although these respective secretion systems are both involved in secreting numerous different substrates, which eventually polymerize to form a cell surface structure. In fact, isolated T3SS needle complexes (from many bacterial species) resemble flagella basal bodies when viewed by electron microscopy (16, 60, 61). Proteins that form these basal bodies are some of the most highly conserved components in all T3SS and are homologous to specific flagellar proteins. One example is YscJ (and its homologues in all T3SS), which are lipoproteins that share sequence similarity with FliF from the flagellar secretion system. This family of proteins is involved in forming an oligomeric membrane-embedded ring structure.

An interesting question is how have these distinctive secretion systems evolved with similar components. It is possible that in the evolution of a bacterial species other proteins somehow became targeted to the flagellar apparatus and were secreted. This hypothesis is difficult to test, but there is some evidence to suggest that this is the case. In *Yersinia enterocolitica* it has been shown that a small specific number of virulence substrates are secreted through the flagellar pathway (62). A complex functional secretion system for a cell surface organelle has been in place prior to the appearance of lower eukaryotes, because bacteria have likely been motile cells for millions of years.

Bacterial pathogens have evolved strategies to infect the diverse eukaryotic cell types still using the basic T3SS machinery. The extracellular components of T3SS that directly contact the host cell (*i.e.* the needle and translocon components) are not as highly con-

served among bacteria when compared with the basal body components (21, 63). Moreover, the structural needle proteins do not appear to share sequence homology with flagellin proteins. Thus, it is believed that in the flagellar secretion systems and T3SS of bacteria, the basal body components make up an ancient secretory apparatus, and the extracellular components have been forged by selective pressures brought about by sensing the immediate environment (motility) or the host-pathogen interaction.

Bacterial Conjugation and Type IV Secretion

Interestingly, some of the components involved in the secretion of virulence substrates via the T4SS are similar to those required for the transfer of plasmid DNA by bacterial conjugation. All 12 components of the plasmid R388 are homologous to components of the *A. tumefaciens* T4SS, and 8 components of the plasmid RP4 conjugation system share homology to the T4SS (44). These include a family of secretion NTPases and coupling proteins such as TraG and TrwB (similar to VirD4).

It is believed that bacterial conjugation is a T4SS-like process. In fact, it has been demonstrated that the T4SS-like DNA transfer pathway can mediate the DNA-independent translocation of a protein (Sog primase) between *E. coli* cells (64). Similarly, Vergunst *et al.* (65) demonstrated that the agrobacterial system translocates free proteins as well as nucleoprotein complexes. Moreover, these studies strongly indicate that a system for the transfer of one kind of substrate (*e.g.* DNA) could be employed by a different type of substrate (*e.g.* effector protein). This paradigm where an existing translocation system is utilized for a virulence purpose is also hypothesized to have occurred for the flagellar secretion pathway and T3SS (62). It has also been proposed that T4SS were initially for conjugation with other prokaryotes and later evolved to facilitate virulence relationships with eukaryotes (66).

Conclusions and Outlook

Both type III and type IV translocation machines are in many ways ingenious solutions to the dilemma of how to get bacterial proteins across the Gram-negative envelope as well as into potential target cells. The T3SS field is advancing rapidly, and it is hoped that soon it will be understood how this system is assembled and functions to deliver virulent proteins directly into host cells. The most recent developments in the T4SS suggesting a two-step transfer with a periplasmic intermediate suggest that more novel findings are to come. The suggestion of a defined hierarchy of secretion/translocation is more apparent in the T3SS and likely also occurs in T4SS. Although the evolution of both these systems is not completely understood, the leap from flagellar assembly to type III secretion and bacterial conjugation to type IV secretion may not be such a big jump in the genetically plastic world of prokaryotes.

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