Bacterial Type III Secretion Systems: Specialized Nanomachines for Protein Delivery into Target Cells

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Abstract

One of the most exciting developments in the field of bacterial pathogenesis in recent years is the discovery that many pathogens utilize complex nanomachines to deliver bacterially encoded effector proteins into target eukaryotic cells. These effector proteins modulate a variety of cellular functions for the pathogen's benefit. One of these protein-delivery machines is the type III secretion system (T3SS). T3SSs are widespread in nature and are encoded not only by bacteria pathogenic to vertebrates or plants but also by bacteria that are symbiotic to plants or insects. A central component of T3SSs is the needle complex, a supramolecular structure that mediates the passage of the secreted proteins across the bacterial envelope. Working in conjunction with several cytoplasmic components, the needle complex engages specific substrates in sequential order, moves them across the bacterial envelope, and ultimately delivers them into eukaryotic cells. The central role of T3SSs in pathogenesis makes them great targets for novel antimicrobial strategies.

Contents

INTRODUCTION

Many bacteria have evolved specialized machines to deliver effector proteins into target eukaryotic cells with the capacity to modulate a variety of cellular activities (7, 59, 94). Type III protein secretion systems (T3SSs) are arguably the best characterized of these protein injection machines (20, 32, 62). In the pregenomic era, these systems were most often identified in the context of searches for genes involved in intimate host-pathogen interactions (60, 68, 112). However, in the postgenomic era, it is now clear that T3SSs are widespread in nature, playing important roles not only in pathogenic but also in symbiotic interactions in the context of vertebrate, plant, or insect hosts (21, 34, 129). Although the secretion machine is highly conserved across bacterial species, the effector proteins that it delivers are specific for each pathogen or symbiont that encodes them (59). In this article, we describe the main components of these fascinating nanomachines and discuss what is known about their function. We do not attempt to comprehensively review the literature. Rather, we describe what in our view are the most important aspects of the structure and function of these machines. This article focuses on T3SSs involved in protein delivery into eukaryotic cells, which we view as substantially different in many important aspects from related systems involved in motility (53). In addition, we do not cover the diverse activities mediated by the effector proteins delivered by these machines. Finally, we recognize that the nomenclature of the genes involved in T3SSs in different bacteria can be confusing and can hamper comparisons between different systems. Therefore to facilitate the reading of this article we provide a table (**Table 1**) listing the nomenclature of homologs in the most-studied systems as well as a previously introduced unifying nomenclature (70). When necessary, we provide gene or protein names, with emphasis on homologs from T3SSs of *Salmonella*, *Shigella*, and *Yersinia* spp., which are arguably the most studied.

EVOLUTIONARY ORIGINS OF THE TYPE III SECRETION SYSTEM

Recent phylogenetic analyses support the notion that T3SSs are an evolutionary exaptation of the flagellar apparatus (114) and that this process may have proceeded in two steps (1). The first step, the products of which can still be detected in *Myxococcales* spp. (80), led to a structure that is competent for protein secretion although it is no longer able to carry out motility functions. The second step, which may have occurred more than once, involved the recruitment of secretins,

Table 1 Nomenclature of type III secretion components in different bacteria[a]

Yersinia spp.	*Shigella* spp.	*Salmonella enterica*		*Pseudomonas syringae*	Sct common nomenclature[b]	Flagellar apparatus	Proposed function
		SPI-1	SPI-2				
YscC	MxiD	InvG	SsaC	HrcC	SctC	–	Needle complex outer rings
YscD	MxiG	PrgH	SsaD	HrpQ	SctD	–	Needle complex inner rings
YscJ	MxiJ	PrgK	SsaJ	HrcJ	SctJ	–	Needle complex inner rings
YscR	Spa24	SpaP	SsaR	HrcR	SctR	FliP	Export apparatus (protein channel?)
YscS	Spa9	SpaQ	SsaS	HrcS	SctS	FliQ	Export apparatus (protein channel?)
YscT	Spa29	SpaR	SsaT	HrcT	SctT	FliR	Export apparatus (protein channel?)
YscU	Spa40	SpaS	SsaU	HrcU	SctU	FlhB	Export apparatus; substrate switching
YscV	MxiA	InvA	SsaV	HrcV	SctV	FlhA	Export apparatus (proton channel?)
YscK	MxiK	OrgA	–	HrpD	SctK	–	Cytoplasmic sorting platform
YscQ	Spa33	SpaO	SsaQ	HrcQA + B	SctQ	FliM + FliN	Cytoplasmic sorting platform
YscL	MxiN	OrgB	SsaK	HrpE	SctL	FliH	Links ATPase to sorting platform (?)
YscN	Spa47	InvC	SsaN	HrcN	SctN	FliI	ATPase
YscO	Spa13	InvI	SsaO	HrpO	SctO	–	Cytoplasmic component; function unknown
YscF	MxiH	PrgI	SsaG	HrpA	SctF	–	Needle filament component
YscI	MxiI	PrgJ	SsaI	HrpB	SctI	–	Inner rod component
YscP	Spa32	InvJ	SsaP	HrpP	SctP	FliK	Inner rod assembly; substrate switching
LcrV	IpaD	SipD	–	–	–	–	Tip complex; translocase deployment
YopB	IpaB	SipB	SseC	HrpK	–	–	Effector translocase
YopD	IpaC	SipC	SseD	–	–	–	Effector translocase
YscW	MxiM	InvH	–	–	–	–	Pilotin (assembly of outer rings)
YopN	MxiC	InvE	SsaL	HrpJ	SctW	–	Controls translocase secretion

[a]Abbreviations: –, homolog not present; SPI, *Salmonella* pathogenicity island.
[b]Previously proposed common nomenclature (70).

a family of outer membrane proteins that are involved in phage release or protein secretion. Composed of more than 20 proteins, T3SSs are among the most complex protein secretion systems known. Such complexity may have emerged from the need to modulate complex cellular processes requiring the delivery of several bacterial proteins to the same eukaryotic cell. The delivery of multiple proteins to the same cell in a coordinated fashion demands adaptations much more complex than those that have evolved, for example, to deliver a single exotoxin to a target cell.

THE NEEDLE COMPLEX AND ASSOCIATED ELEMENTS

The core component of the T3SS is the needle complex, a 3.5-MD multiring structure that spans the bacterial envelope (82) (**Figure 1**). Originally identified in *Salmonella* Typhimurium (82), this structure has been subsequently visualized in many other bacteria (17, 38, 128) with a highly conserved architecture. In recent years, high-resolution cryo–electron microscopy (cryo-EM) combined with the molecular docking of the atomic structures of some of its components has provided a remarkable high-resolution view of this structure (58, 95, 98, 99, 126, 127, 132) (**Figure 1**). In addition to the structure embedded in the bacterial envelope, several cytoplasmic components associate with the needle complex in a dynamic fashion to mediate specific steps of the secretion process. Finally, passage of effector proteins through the target eukaryotic cell membrane requires the assembly of a protein channel (the translocon), composed of proteins deployed by the T3SS itself.

General Architecture of the Needle Complex

The needle complex is composed of a base substructure embedded in the bacterial envelope and a needle-shaped extension that protrudes from the bacterial surface (24, 81, 95, 98, 99, 118, 148). The base itself is approximately 25 nm wide and 30 nm long and is composed of two rings associated with the inner membrane, inner rings 1 (IR1) and 2 (IR2), which connect to two outer membrane rings, outer rings 1 (OR1) and 2 (OR2), through a neck (**Figure 1**). The needle itself is approximately 50 nm in length and is linked to the base through the inner rod, which docks into a socket-like structure within the base. The entire structure is traversed by a channel ~20 Å in diameter that serves as a conduit for proteins traveling this pathway (118). In addition to the core needle complex, there are other substructures often lost during its biochemical isolation. At the tip of the needle there is the tip complex, presumably involved in the sensing of the target cell and in the deployment of the protein translocases (see below) (52, 110). A group of inner membrane proteins (the export apparatus) located at the center of the inner rings presumably serves as a channel that mediates the passage of type III secreted proteins through the inner membrane (139). Finally, several cytoplasmic proteins are linked to the cytoplasmic side of the needle complex, presumably forming a defined structure, as recently suggested by electron tomographic visualization of the needle complex in situ (2, 76, 84).

Structural Organization of the Needle Complex

The base. Despite its architectural complexity, the core needle complex is composed of a relatively small number of proteins (82). The lower rings (IR1 and IR2) are composed of two proteins [PrgK/YscJ/MxiJ and PrgH/YscD/MxiG in *Salmonella*, *Yersinia*, and *Shigella* spp., respectively (**Table 1**)], whereas the outer rings (OR1 and OR2) and neck are composed of just one protein (InvG/YscC/MxiD), a member of the secretin family of outer membrane proteins (**Figure 1**). In

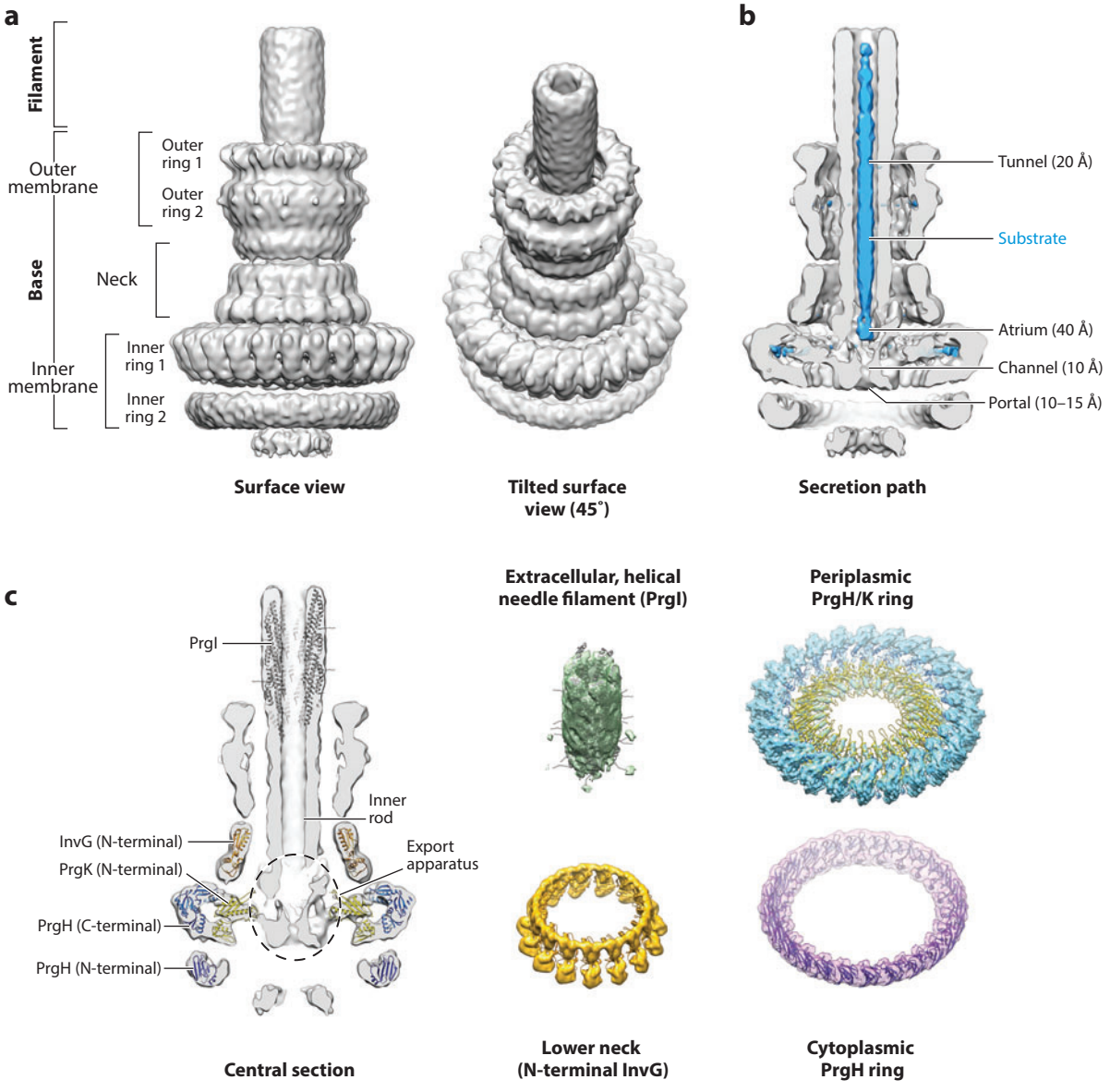


Figure 1

Needle complex structure from *Salmonella* Typhimurium. (*a*) Surface views of the 3-D reconstruction of the cryo–electron microscopy (cryo-EM) map of the *S.* Typhimurium needle complex. The different substructures are noted. (*b*) Surface view of a half-sectioned needle complex containing a trapped substrate within the central tunnel (118). Relevant structural details and dimensions are noted. (*c*) Docking of the atomic structures of the different needle complex components onto the 3-D cryo-EM map.

Salmonella spp., the needle complex exhibits a threefold symmetry in which 15 subunits of the outer ring and neck connect to 24 subunits of the inner membrane ring (127). This observation implies that there is a local symmetry mismatch between the neck and inner ring subunits. A different stoichiometry has been proposed for needle complexes from *Shigella* spp. (69), although it is unclear whether this represents real differences in the organization of needle complexes from

different bacteria. PrgH and PrgK are assembled into two concentric rings 27 and 18 nm in diameter, respectively (126, 132) (**Figure 1**). PrgK is composed of a larger N-terminal lipid-anchored periplasmic domain separated from a short (and in some homologs absent) cytoplasmic domain by a transmembrane segment. PrgH shares a similar architecture of two domains separated by a single transmembrane segment but with an inverted topology. However, its cytoplasmic domain is much larger than PrgK's and forms the IR2 ring of the needle complex. The secretin that forms the outer rings of the needle complex possesses a long periplasmic domain that makes the neck of the base substructure and directly contacts the inner rings (126).

The atomic structures of the soluble domains of the base components revealed that these three proteins share a small domain with an $\alpha\beta\beta\alpha\beta$ configuration (132). The fact that all these three proteins organize in a ringlike fashion has led to the proposal that this domain may be responsible for ring formation. This hypothesis was suggested by the observation that one of these protein homologs, EscJ (homolog of PrgK), crystalized as a superhelical structure that, when collapsed in its axis, results in a ring of dimensions compatible with those predicted for the smaller ring within IR1 (126, 132). However, this domain is present in proteins that do not form rings, and, more important, this domain has been shown to be dispensable for ring formation (2). Therefore the relationship between the presence of this domain and ring formation is still unclear.

The needle. The needle substructure is assembled from multiple (~100) copies of a single ~80-residue subunit, PrgI/YscF/MxiH, arranged in a helical fashion (31, 63, 83, 95) (**Figure 1**). In its native form, the length of the needle ranges from 30 to 70 nm, and its width ranges from 10 to 13 nm. The crystal structures of needle protomers from different T3SSs show a conserved organization consisting of an α-helical hairpin made of two α-helices of roughly the same size linked by a short segment most often containing two prolines separated by two amino acids (the so-called PXXP motif) (40, 117, 152). The structure of the in vitro assembled PrgI needle filament from *Salmonella* spp. obtained by solid-state nuclear magnetic resonance (NMR) spectroscopy and Rossetta modeling has provided a high-resolution view of this substructure. The NMR structure shows an ~80-Å-wide filament with an ~25-Å-diameter lumen with a right-handed helical organization consisting of ~5.7 subunits per turn and a helical pitch of ~24 Å (95). The assembled subunits show a short (5 amino acids) N-terminal extended domain followed by an α-helix, the PXXP loop (pointing toward the tip), and a C-terminal α-helix. The subunits are stabilized by multiple intersubunit and intrasubunit contacts resulting in a rather rigid structure. Of note is the presence of a small kink (residues Val-20 through Asn-22 in PrgI) that interrupts the N-terminal α-helix that is not observed in the crystal structure of the soluble protomer. It is tempting to hypothesize that this structure may be involved in signal transduction upon activation of the secretion machine (see below). The solid-state NMR structure shows that the N-terminal domain of PrgI faces the exterior of the needle filament whereas the C-terminus faces the lumen. Residues that line the lumen of the channel are highly conserved and mostly polar, and analysis of their electrostatic potential reveals alternating positive- and negative-charge regions. Although the significance of this observation remains to be determined, it is intriguing to hypothesize that such alternating charge distribution may be important for substrate progression within the channel. Although a different organization was proposed for the needle structure of *Shigella* (57), a recent solid-state NMR study has confirmed that the *Shigella* and *Salmonella* needles exhibit the same architecture (43).

The inner rod. The inner rod is also built from a single ~90-amino-acid subunit, PrgJ/YscI/MxiI in *Salmonella/Yersinia/Shigella* spp., respectively (98). The atomic structure solved by NMR of the PrgJ monomer from *Salmonella* is available, but in its soluble form this protein is largely

unfolded (153). However, PrgJ is predicted to have a similar structure to the needle subunit PrgI, and in silico modeling has shown that these two proteins share a similar α-helical hairpin shape flanked by flexible regions. In fact, the two monomeric structures align very well around critical domains required for filament assembly (91). The modeled structure of the inner rod (based on the needle filament) suggests that, because of significant divergence at its amino terminus, the inner rod may not be able to elongate beyond two turns of the helix (~11 subunits). However, this structure has not been visualized at high resolution, and its actual length is currently unknown.

Inner membrane export apparatus. All T3SSs contain five highly conserved inner membrane proteins that are essential for their function (InvA, SpaP, SpaQ, SpaR, and SpaS in *Salmonella* spp.) (6, 57, 61, 65, 66, 116). Recent cryo-EM studies have correlated the presence of a defined density in the lumen of the inner rings of the needle complex with the presence of the inner membrane export apparatus (139). These results indicate that at least a subset of these inner membrane proteins are located within the needle complex, presumably serving as a protein channel to facilitate the export of target proteins through the inner membrane. Although these proteins are usually considered as a group, it is likely that they perform specialized functions. For example, one of these membrane proteins (InvA in *Salmonella* spp.) has a large cytoplasmic domain that, as shown by structural studies, can form a circular nonamer (2). Indeed, the presence of this protein has been correlated with the presence of a toroidal density immediately below the cytoplasmic IR2 of the needle complex. Although the functional significance of this observation is unknown, it is tempting to hypothesize that this ring structure may aid the preparation of substrates before their translocation through the inner membrane channel. Another member of this group of membrane proteins (SpaS in *Salmonella* spp.) has a unique C-terminal domain that functions as a protease for its own autocatalytic processing (49, 56, 88, 105). This processing event has been linked to altered secretion; therefore, this protein has been postulated to play a role in the establishment of the secretion hierarchy (see below).

Cytosolic components. There are several cytosolic proteins that are essential for secretion and are conserved across all T3SSs (29, 30, 51, 57, 109, 145) (**Figure 2**). Although interactions among some of these components have been detected (73, 87, 131), the organization of these proteins within the bacterial cytoplasm remains poorly understood. In the flagellar apparatus, some of these components form a defined structure known as the C ring, which is involved in switching the direction of flagellar rotation (48, 78). However, there is no definitive evidence of the existence of a stable C ring equivalent in T3SSs, and lack of a C-ring-like structure has been recently observed in tomographic reconstructions of T3SSs from different bacteria in situ (76, 84, 118). In fact, homologs of the flagellar C ring component (FliG) that anchors this structure to the flagellar body are absent from T3SSs. Nevertheless, it is likely that the homologs of C ring components form a complex that may dynamically associate with the needle complex. In fact, evidence for such a complex or platform has been obtained (87) (see below). Although some densities seen in tomograms of needle complexes in situ have been assigned to these components (2, 76, 84), there is still no direct demonstration of how these complexes are organized in the three-dimensional space. Another highly conserved cytosolic component is an ATPase with structural similarity to F0F1 ATPases (51, 145) that is thought to be involved in substrate recognition and unfolding (4) (see below). Low-resolution tomograms of T3SS needle complexes and associated structures in situ have correlated the presence of a density beneath the needle complex with the ATPase (2, 76, 84). Although it is certainly expected that the ATPase associates with the needle complex at least at some point during its functional cycle, the data available are not sufficient to establish its precise subcellular location. Indeed, at least in the flagellar system evidence is accumulating

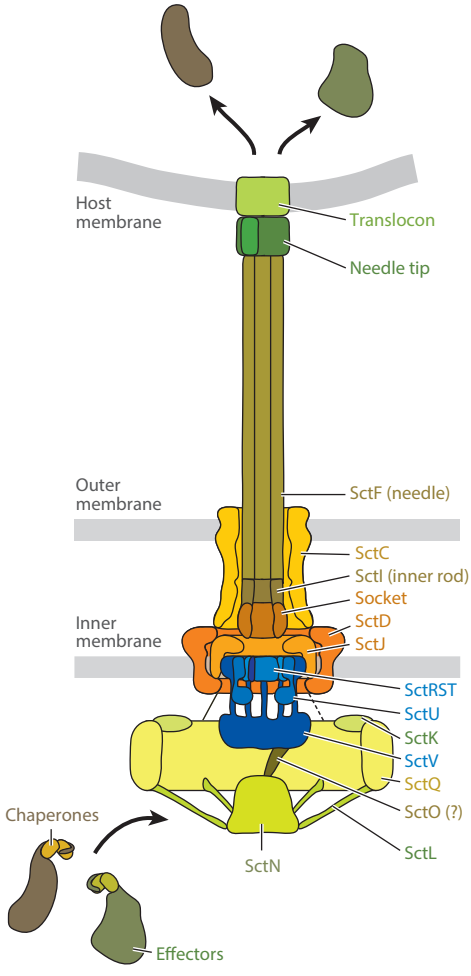


Figure 2

Diagram of the needle complex and associated structures. A previously suggested common nomenclature (70) was used to indicate the potential localization of the different components and facilitate comparison across different systems.

that the location of some of the cytoplasmic components is dynamic (42, 92), and therefore it is possible that the location of the ATPase may change during its functional cycle. An attractive model is that a linker protein (OrgB in *Salmonella* spp.) recruits the ATPase to the sorting platform (SpaO/OrgA in *Salmonella* spp.), which may bring it in close proximity to the needle complex and export apparatus. In support of this model, interactions among the relevant proteins have been demonstrated (46, 73, 87, 131). An alternative hypothesis implicates another cytoplasmic component (InvI in *Salmonella* spp.), which would work in a manner analogous to the stalk that links F0F1 ATPases to the plasma membrane. This proposal is largely (but not solely) based on the observation that the crystal structure of FliJ (the homolog in flagella) exhibits structural similarity with the stalk protein (72). However, the structural feature that led to this proposal is in essence a coiled-coil domain, a feature not distinctive enough to extend functional analogy to these proteins. Indeed, the coiled-coil domain of FliJ shows greater similarity to coiled-coil

domains of functionally unrelated proteins. Therefore more studies are needed to ascertain how much the analogy to ATP synthases can be extended to the T3SS components.

The needle tip structure and needle extension. The needle filament is either capped at its tip by a single protein (52, 110) or extended by yet another filament that is longer than the needle itself (79). Based on their structure similarities the tip proteins can be classified in two related groups: the SipD/IpaD (from *Salmonella* and *Shigella* spp.) and the LcrV/PcrV (from *Yersinia* and *Pseudomonas* spp.) families (**Figure 3**). The SipD/IpaD family displays a distinct domain organization: an N-terminal α-helical hairpin, a central coiled-coil domain, and a C-terminal region composed of mixed structural elements (54, 74). The LcrV/PcrV family has the conserved central coiled-coil domain but lacks the N-terminal α-helical hairpin and has an extended globular domain at its amino terminus that is absent from the SipD/IpaD family (44). Specific functions have been proposed for some of these defined structural elements. For example, it is thought that the highly conserved central coiled coil links the tip proteins to the end of the needle filaments, presumably through interactions that in many ways may resemble those that link the needle protomers in the needle filament (96, 119, 151). The α-helical hairpin has been proposed to function as a self-chaperone preventing the self-oligomerization of IpaD/SipD within the bacterial cytoplasm (74). In the LcrV/PcrV family, which lacks this domain, the chaperone function is thought to be carried out by a cytoplasmic protein (LcrG/PcrG) (41, 100) that is absent in bacteria encoding members of the IpaD/SipD family. Under low-resolution EM the LcrV needle tip shows a "head, neck and base" configuration (19). In contrast, the IpaD tip complex visualized by low-resolution cryo-EM exhibits a fivefold symmetric scepter-like structure with a diameter of 78 Å at its widest point (52). Docking of the crystal structure of the IpaD monomer was possible only if a large conformation change of the C-terminal domain was introduced into the model, suggesting that the tip protein may undergo significant conformational changes upon assembly into the tip complex.

A substantial variation in the structure at the tip of the needle filament occurs in T3SSs from some pathogenic strains of *Escherichia coli*. In these T3SSs the needle extends into a long filament that presumably serves as a link between the bacteria and its target cell (79). In *E. coli* the filament is made of a single protein, EspA, which is structurally similar to the flagellar protein FliC and assembles into a helical structure similar to the needle filament (149). However, in contrast to the rigid needle, the helical filament extension appears to be flexible because despite having a fixed twist of 5.6 subunits per turn, its axial rise varies substantially, from 3.6 Å to 5.6 Å (141). The functional significance of this observation is currently unknown.

The translocon. The last step in the T3SS-mediated delivery of effector proteins into eukaryotic cells is their passage through the target host cell membrane (see below). This process is mediated by the protein translocases (SipB/SipC, IpaB/IpaC, and YopB/YopD in *Salmonella*, *Shigella*, and *Yersinia* spp., respectively) (28, 121). After secretion by the T3SS, the translocases insert into the target host cell membrane, where they presumably form a protein channel (16, 111). Although the protein translocases are not well conserved at the primary amino acid sequence level, they are all α-helical proteins with transmembrane helices (71, 108, 123). The crystal structures of the amino terminus of IpaB and SipB revealed the presence of a trimeric coiled-coil domain formed by three antiparallel α-helices, an organization reminiscent of other membrane-active proteins such as colicins or viral envelope glycoproteins (13). The mechanism by which the translocases insert in the membrane is not understood, although it is likely that it is orchestrated by the tip complex. Indeed, in the absence of the tip protein, the translocases cannot insert into the host target membrane, although they can be efficiently secreted (28, 67, 97, 138).

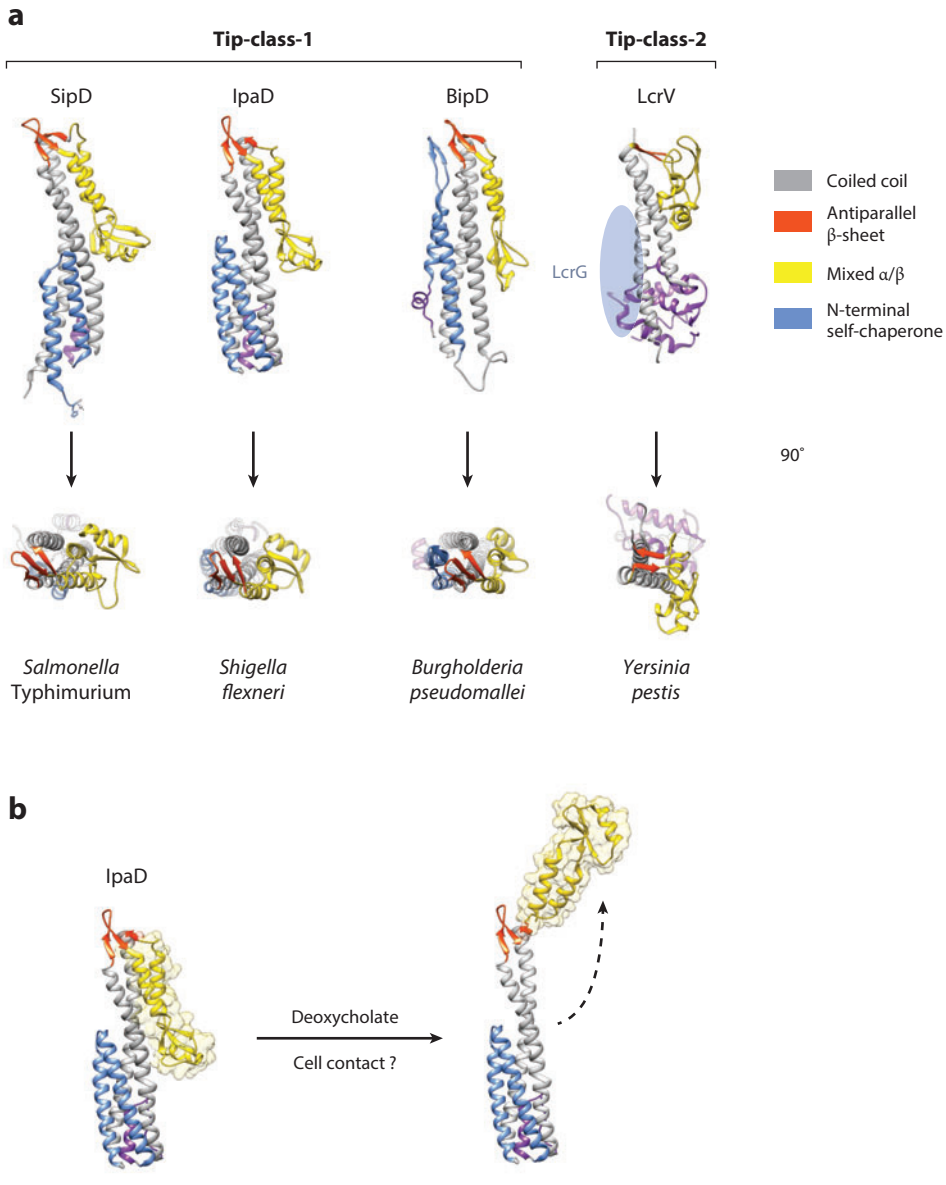


Figure 3

The tip complex of type III secretion systems. (*a*) Crystal structures of tip proteins from different bacteria. Relevant structural features are noted. (*b*) Conformational changes in the IpaD tip protein induced by the binding of deoxycholate, which is thought to mimic the activation event that occurs upon contact with target cells.

NEEDLE COMPLEX ASSEMBLY

Assembly of the needle complex and associated structures occurs in a step-wise fashion (46, 47, 135, 139) (**Figure 4**). The *sec* machinery mediates the export or membrane insertion of all the base and inner membrane export apparatus components prior to their assembly into the final base substructure. Assembly starts at the inner membrane with the formation of a complex of a subset

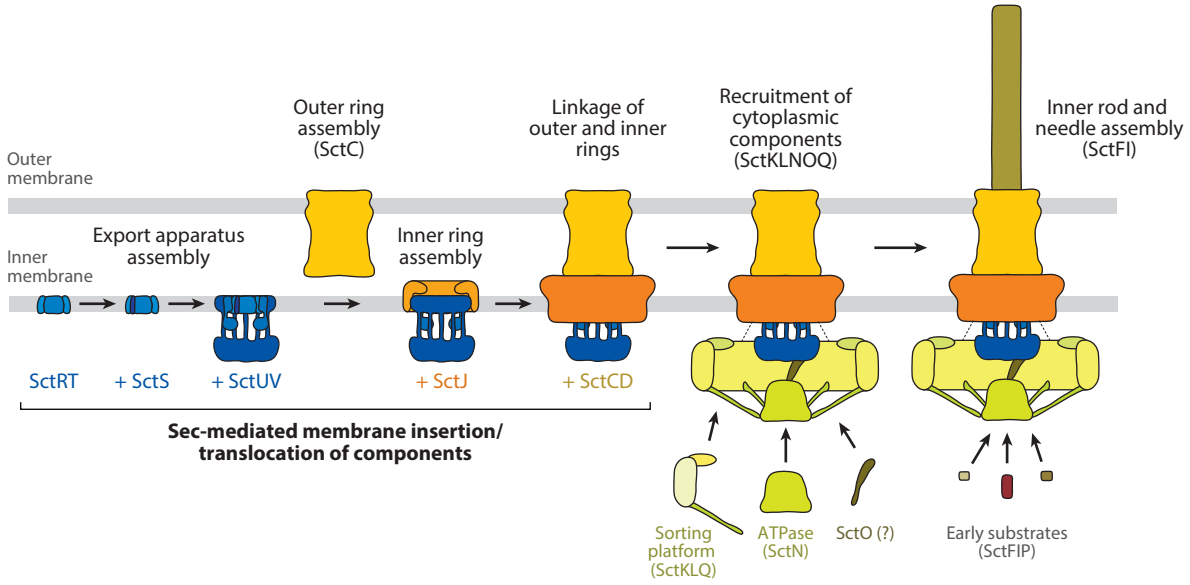


Figure 4

Model for the assembly of the type III secretion needle complex and associated structures. To facilitate comparison of the assembly pathway in different type III secretion systems, a previously suggested nomenclature of the different components was used (70) (see **Table 1**).

of the export apparatus components (SpaP and SpaR in *Salmonella* spp.) (139), followed by the incorporation of additional export apparatus components, which finally template the assembly of the lower rings, first the inner ring of IR1 (made of PrgK in *Salmonella* spp.) and then the outer rings of IR1 and IR2 made of PrgH (see **Figure 4**). Several observations support this model. Although assembly of the needle complex can occur in the absence of the inner membrane export apparatus, the efficiency of assembly is drastically reduced. Furthermore, at least four of the five inner membrane apparatus components (SpaP, SpaQ, SpaR, and SpaS in *Salmonella* spp.) cannot be incorporated into previously assembled bases, indicating that functional needle complexes cannot be assembled without the prior deployment of the export apparatus (139). The outer rings and neck are formed by a single protein of the secretin family (InvG in *Salmonella* spp.), a process that in most (but not all) systems requires the assistance of an accessory lipoprotein of the pilotin family (InvH in *Salmonella* spp.). The outer and inner rings of the base substructure can assemble independently, although their stability in the absence of one another is compromised (46). Therefore, it is likely that these two structures are assembled independently but are rapidly linked to one another to form a stable structure.

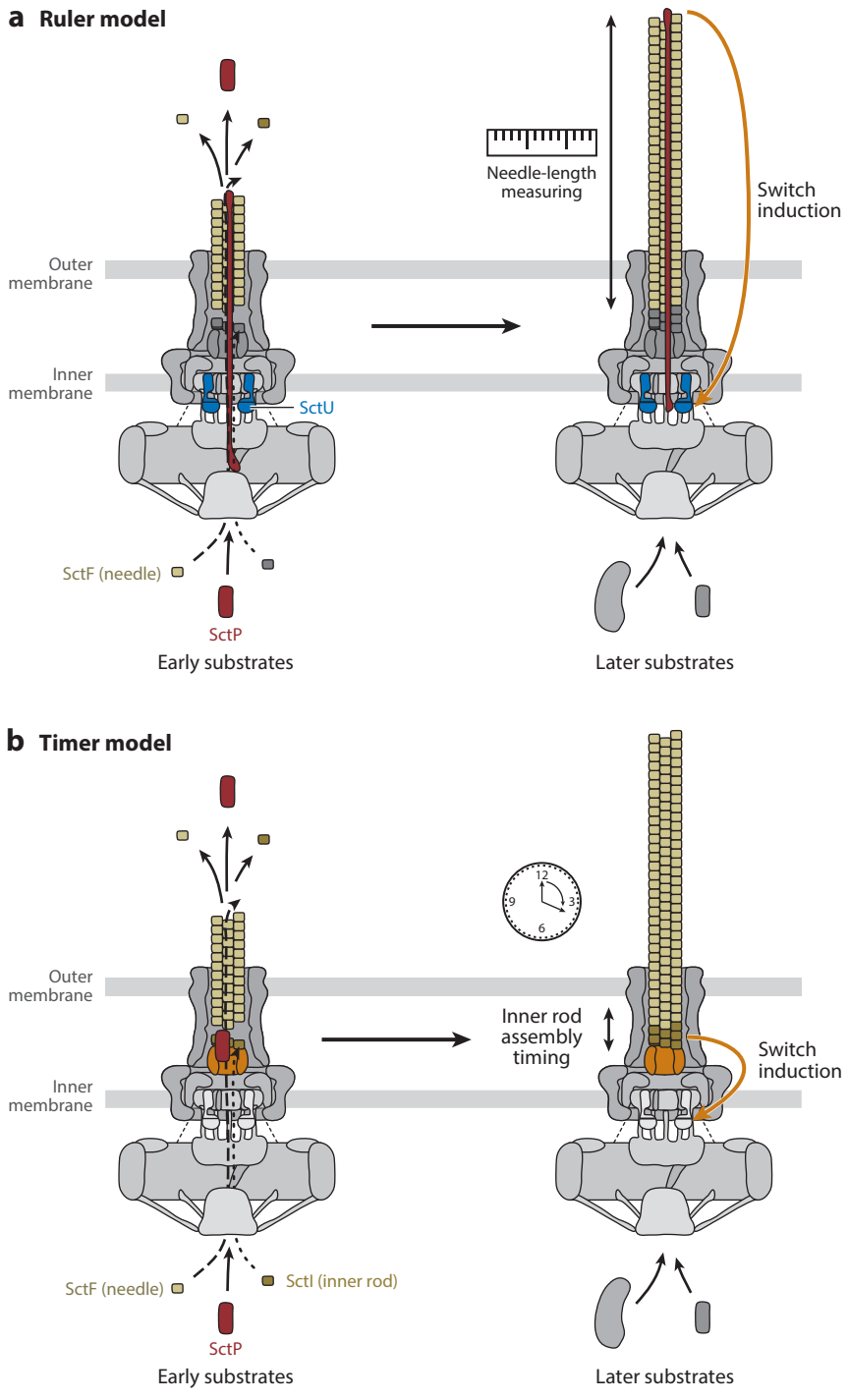
Once the base substructure is assembled, several cytoplasmic proteins must be recruited so that the base can become competent for type III secretion. Although this process is poorly understood, the available evidence suggests that some of these cytoplasmic factors may be preassembled into a complex prior to their recruitment to the base. For example, it is likely that the components of the sorting platform (OrgA and SpaO) exist in the cytoplasm in a preassembled state (87). Whether the ATPase is part of that preassembled complex is unclear, but it is known that the recruitment of the ATPase to the needle complex requires the components of the sorting platform (2, 46). How the sorting platform is recruited to the needle complex and even what specific interactions bring this cytoplasmic complex to that site are not known. Although it has been proposed that the

cytoplasmic domain of the InvA family of proteins is involved in the recruitment of cytoplasmic components (107), recent electron tomography studies on flagellar T3SSs suggest otherwise (2). Most likely, components of the needle complex itself serve as an anchor for this recruitment given that in its absence there is no recruitment of cytoplasmic components to the membrane.

Once the cytoplasmic components are recruited, the base substructure can function as a limited-specificity type III protein secretion machine that can only recognize the inner rod and needle protomers as well as an accessory protein required for proper needle complex assembly (InvJ/YscP in *Salmonella* and *Yersinia* spp.). As with flagella, assembly of the needle substructure occurs by sequential addition of subunits at the growing tip (37, 117). Unlike flagella, however, assembly of the T3SS needle does not require a capping protein that facilitates addition of the subunits at the tip. Assembly of the inner rod is less well understood but, unlike assembly of the needle substructure, it requires the function of an accessory protein (InvJ in *Salmonella* spp.), which like the flagellar capping proteins does not form part of the final structure and is discarded in the culture supernatant (91, 98). In the absence of InvJ, assembly of the inner rod does not take place, although needles of improper length (see below) can efficiently assemble. At some point during the assembly process the type III secretion machine switches substrate specificity so that it can no longer recognize the early substrates (needle and inner rod protomers) and becomes competent for the secretion of middle (i.e., the tip protein and translocases) and late substrates (i.e., effector proteins). The mechanisms by which the secretion machine is reprogrammed are incompletely understood, although the accessory protein InvJ/YscP is required for this process. In its absence, the needle complex assembles abnormally long needles because it is unable to switch substrates (83). The function of this accessory protein is incompletely understood and the subject of some controversy. For example, YscP has been proposed to function as a molecular ruler, measuring the length of the needle and triggering substrate switching once an appropriate length of the needle is achieved (75) (**Figure 5**). How this protein would measure the length is not understood, but it has been proposed that the fully extended form of YscP located within the lumen of the secretion channel performs the measuring by interacting with proteins at the tip and at the base of the needle complex (YscU in *Yersinia* spp., see below), triggering substrate switching. Support for this model comes from the observation that alterations in the length of YscP result in needles of different lengths (75). A significant caveat for these experiments is that measurements were not made with isolated needle complexes but on shed needles, which can be subject to artifacts. In addition, artificial elongation of YscP could result in partial loss of function that could lead to longer needles (see below), which can further complicate the interpretation of these results. Finally, this model is not compatible with the fact that needle length in wild-type needle complexes varies substantially, following a rather broad length distribution (98). This distribution would not be expected if a molecular ruler mechanism were involved in length determination and, instead, it suggests a stochastic process. An alternative model has been proposed based on the observation that InvJ is required for the assembly of the

Figure 5

Proposed models for the mechanism of substrate switching and needle-length control in the assembly pathway of the needle complex. (*a*) In the molecular ruler model, a regulatory protein (depicted in *red*) is engaged by the secretion machine while remaining tethered to the cytoplasmic side and the tip of the needle complex by its termini. Once fully extended, the regulatory protein triggers conformational changes on the cytoplasmic side of the secretion machine that lead to substrate switching. (*b*) In the timer model, the regulatory protein (depicted in *red*) facilitates the assembly of the inner rod and its firm anchoring to the base, which results in a conformational change on the cytoplasmic side of the needle complex leading to substrate switching. In this model, the termination of assembly of the inner rod determines substrate switching. See the text for a more detailed discussion.


a Ruler model
Needle-length measuring
Switch induction
Outer membrane
Inner membrane
SctU
SctF (needle)
SctP
Early substrates
Later substrates
b Timer model
Inner rod assembly timing
Switch induction
Outer membrane
Inner membrane
SctF (needle)
SctI (inner rod)
SctP
Early substrates
Later substrates

inner rod, a process that leads to the firm anchoring of the needle filament to the base (91, 98) (**Figure 5**). Anchoring of the needle results in substantial conformational changes on the cytoplasmic face of the needle complex (98, 99), which is hypothesized to, directly or indirectly, trigger substrate switching. Therefore in this model the termination of the assembly of the inner rod is the critical event that determines substrate switching. In this case the role of InvJ in needle determination is indirect, through its role in inner rod assembly. It has been proposed that SpaS/YscU, a component of the export apparatus, may also be involved in the mechanisms of substrate switching (18, 49, 120, 147). As discussed above, the SpaS/YscU protein family possesses a long cytoplasmic domain with autocatalytic protease activity (56). Mutations in the catalytic site result in a strain that is competent for secretion of effectors but unable to secrete the protein translocases (49). It has been proposed that the autoproteolytic cleavage of SpaS, which is hypothesized to be triggered by its putative interaction with InvJ/YscP, may determine substrate switching. Although there is no question that the catalytic mutant of SpaS exhibits altered secretion, needle length control is unaffected in this mutant, which is inconsistent with its proposed role in needle length determination.

SUBSTRATE RECOGNITION BY THE TYPE III PROTEIN SECRETION MACHINE

Proteins destined to travel the type III secretion pathway are targeted to the secretion machine by a set of secretion signals that ensure specificity (11). One of the secretion signals encompasses the first 20–25 amino acids (104, 130). This signal is highly variable in sequence and can often tolerate significant changes without affecting function (10, 122), an observation that led to the proposition that the 5′ mRNA of some type III secreted proteins was responsible for their targeting (8, 9). However, it is now believed that the amino acid sequence acts as the targeting element and that the tolerance for mutagenesis stems from the fact that the targeting signal is indeed unstructured. Furthermore, bioinformatic analyses have identified different common features in this amino acid sequence (101). These features include enrichment in serine and threonine and depletion of charged and hydrophobic residues such as leucine. A second signal, at least in some type III secreted proteins, serves as a binding site for specific chaperones and spans from residues ~25–100 (26, 130, 142, 143). Unlike other chaperones, such as the GroEL and DnaK/Hsp70 protein family, T3SS-associated chaperones lack nucleotide-binding or hydrolysis activities. T3SS chaperones exhibit limited primary amino acid sequence similarity to one another, although they share structural similarity as well as some physical properties, such as small size and an acidic pI (134). Based on their tertiary structure and specificity of binding, these chaperones have been classified in two general groups referred to as class I and class II (33, 115, 136). An additional distinction is usually made among class I chaperones, between those that bind a single protein (unicargo) and those that bind several (multicargo). T3SS chaperones usually form homodimers along a helical interface and exhibit extended hydrophobic patches and a large hydrophobic groove that accommodates the amino-terminal region of their cognate effectors (14, 133). A notable feature of this interaction is that the chaperone-bound effector domain is completely nonglobular, although it retains significant amounts of secondary structure. Because substrates are transported in at least a partially unfolded state (118), it is believed that this configuration aids the secretion process. Although the primary sequence of chaperone-binding domains varies greatly, some common motifs have been identified. For example, a β-strand motif is present at the amino-terminal region of many effectors, and a conserved chaperone-binding domain consensus sequence $[(LMIF)_1XXX(IV)_5XX(IV)_8XN_{10}]$ that overlaps with the β-motif has also been identified (35, 93). Multicargo chaperones exhibit a similar interacting interface with their cognate effectors although usually burying less surface (93). In the absence of chaperones, particularly those with a single cargo, the cognate effector proteins

are degraded within the bacterial cytoplasm (102, 143). This is often not the case for effectors chaperoned by multicargo chaperones (50, 89). In this case it is possible that the stability of these effectors in the absence of cognate chaperone may be necessary to facilitate complex assembly given that they are usually encoded away from their cognate chaperones. This is most often not the case for single-cargo chaperones, which not only are encoded in the immediate vicinity of their cognate cargo but also, in some instances, have specific translation regulatory mechanisms in place to coordinate their synthesis with that of their effector (22). It is now well accepted that the main function of these chaperones is to target their cognate effectors to the secretion pathway. Absence of these chaperones results in lack of secretion of their corresponding effectors. Furthermore, removal of the chaperone-binding domain also prevents secretion through their cognate type III secretion pathway (124, 146), although mistargeting to the flagellar secretion pathway (by the amino-terminal secretion sequence) can occur (90). Class II chaperones usually interact with protein translocases and, in some T3SSs, with protomers of the needle or of the extended appendages such as EspA (36, 102). The structural feature that characterizes this type of chaperones is the presence of tetratricopeptide repeats (23). Although it is clear that these chaperones stabilize or prevent detrimental interactions of their cognate target proteins, their role in secretion is still unclear.

The mechanisms by which substrates of the type III secretion systems are ultimately recognized are poorly understood. The chaperone-effector complexes are most likely recognized and targeted to the secretion machinery by a group of cytoplasmic proteins that are associated with the needle complex such as the sorting platform (see above). It has also been shown that the ATPase interacts with chaperone/effector complexes (64) and that it is able to dissociate them (4), a necessary step before secretion given that the chaperones remain in the cytoplasm after secretion of the cognate effector. Furthermore, chaperones are essential for the recruitment of the secreted proteins to the sorting platform (87). It is therefore possible that the chaperones are involved in the establishment of the secretion hierarchy, perhaps by exhibiting different affinity to the relevant sorting platform components. Subsequent to their recognition by the secretion machine, proteins must be at least partially unfolded prior to or simultaneously with their delivery to the secretion channel, which is too narrow to accommodate folded proteins. The ATPase is capable of unfolding effector proteins in vitro, so it is possible that it plays an equivalent role in vivo (4).

ENERGIZING THE TYPE III SECRETION SYSTEM

Although there are no reliable measurements of the speed at which proteins are moved through individual type III secretion machines, the available estimates suggest that the process must be fast (125). Furthermore, the type III secretion machine is able to deliver proteins that have been engaged in the bacterial cytoplasm directly into the host target cell. Undoubtedly these activities must demand a significant amount of energy. There are at least two possible sources of energy for this system. One source is likely derived from the hydrolysis of ATP by the conserved T3SS-associated ATPase, because it is known that its catalytic activity is essential for secretion (51, 145). How ATP hydrolysis could be coupled to the secretion process is incompletely understood. However, given that at least in vitro these ATPases can unfold the effector proteins (3), it is possible that the energy stored in the unfolded proteins contributes to the progression of substrates through the secretion channel. Several pieces of evidence indicate that the proton motive force (PMF) is also required for type III secretion (144). However, how the PMF is potentially coupled to the secretion process is unknown. It has been proposed that a conserved component of the inner membrane export apparatus in the flagellar system can function as a proton-protein antiporter that uses the two components of proton motive force, $\Delta\psi$ and ΔpH, for protein export (106). However, definitive demonstration of this hypothesis awaits further investigation. Recently a

radically different mechanism has been proposed to explain the movement of flagellar subunits within the flagellar channel (55). This model proposes a pulling mechanism derived from the crystallization of subunits at the growing flagellar tip that would harness the entropic energy of the unfolded subunits. In this model, subunits would be linked in a head-to-tail configuration within the flagellar channel so that the crystallization of subunits at the tip would pull all the subunits in the channel. Although this model could potentially account for the movement of the subunits that are destined to form the needle substructure of the needle complex, it is unclear how this model could explain the movement of effector proteins. Effector proteins do not crystalize at the tip, and their diversity makes the proposed head-to-tail arrangement within the secretion channel hard to accommodate. Furthermore, this model is at odds with previous thermodynamic calculations, which led to the conclusion that diffusion could account for the movement of flagellar subunits within the flagellar channel (137, 150). Therefore, more experiments will be required to explore the universality and validity of this new proposal.

SENSING AND FIRING: TYPE III SECRETION MACHINES IN ACTION

Type III secretion machines require an activating signal before they can be competent for protein secretion and delivery. Although the activation process is poorly understood, there is compelling evidence that activation occurs upon contact with target cells (103, 154). Such a mechanism presumably ensures that effector proteins are delivered directly to target cells and not to the extracellular space, where they would be functionally irrelevant. How cell contact activates the secretion machine is unknown, but most likely the tip complex is involved in the sensing process (15). In fact, some compounds that bind the tip complex, such as bile salts or Congo red, can stimulate type III secretion (12, 113), presumably by introducing specific conformational changes in the tip protein that probably resemble those induced by cell contact (25, 45, 140) (**Figure 3**). The signal presumably sensed by the tip complex must be transduced to the cytoplasmic side of the secretion machine, a process most likely mediated by conformational changes in the needle and inner rod substructures of the needle complex (15). In support of this hypothesis, several mutations in the needle and inner rod proteins have been identified, which result in derepressed and/or otherwise altered patterns of protein secretion (27, 39, 77, 91, 138). Activation of the T3SS ultimately leads to the deployment of the translocases on the target cell membrane, which in turn will mediate the passage of effectors through the target cell plasma membrane. In this model, the secretion machine must engage the translocases prior to the effectors, a mechanism that most likely involves a cytoplasmic sorting platform. Consistent with this model, prior to activation of the secretion machine, only the translocases are found on the sorting platform, and it is only in the absence of the translocases that effector proteins can be detected at this location (87). Deployment of the translocases leads to intimate attachment of the bacteria to the target host cell, which presumably aids the translocation process (86). An alternative two-step model has been proposed in which effector proteins are first delivered to the bacterial surface and a second step, akin to the mechanisms of AB toxins, in which the effectors are moved through the plasma membrane by the protein translocases (5). This model was proposed based on the observations that, under certain conditions, effectors are seen on the surface of *Yersinia* spp. and effector proteins artificially deposited on the bacterial surface can be translocated into eukaryotic cells. However, in other bacteria no effectors are seen on the bacterial surface prior to target cell contact (85, 103, 154). Furthermore, it is possible that the observed translocation of exogenously applied effector proteins (5) is the result of the artificial capture of translocation intermediates on the bacterial surface. More experiments will be required to support this model, which is inconsistent with a substantial amount of available data.

OUTSTANDING QUESTIONS

Although a great deal is known about the structure of the type III secretion machine, there are still protein densities observed in the high-resolution cryo-EM map that are unaccounted for. Some of these densities are likely to represent transmembrane domains of export apparatus components, whose crystal structures are not yet available. Short of solving the atomic structure of the entire needle complex, the solution of the atomic structures of these membrane proteins is clearly one of the major challenges for the future. The actual mechanism of secretion is still poorly understood, and there are many outstanding questions that are likely to guide and inspire future research. How is the host cell sensed and how is the signal transduced to the secretion machinery, in particular to its cytoplasmic components? If the needle and inner rod are indeed the signal transducers, how do they accomplish this function? Are they as rigid as their structures suggest, or do they transduce signals by yet-undetected conformational changes? How does the sorting platform select its substrates and deliver them to the secretion channel? Are there differences in the recognition mechanisms of translocases and effectors? How do the substrates move through the secretion channel, and what is the source of energy that drives the secretion process? How do the effectors traverse the target cell plasma membrane? The last 10 years have seen remarkable progress in the understanding of the structure and organization of the type III secretion machine. However this knowledge has been largely focused on snapshots of the machine, with limited studies focusing on the dynamic aspects of the secretion process. With the availability of powerful live-imaging tools, it is expected that during the next few years we will be able to catch the type III secretion machine in action. Finally, the central role of the T3SS in the pathogenesis of several bacterial pathogens of great importance to public health has stimulated efforts to develop novel therapeutic strategies targeted to this machine. It is expected that during the next 10 years these efforts may begin to translate into effective therapeutics.

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