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The Structure and Function of Type III Secretion Systems

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ARTICLE SUMMARY

Type III secretion systems (T3SS) afford gram-negative bacteria a most intimate means of altering the biology of their eukaryotic hosts — the direct delivery of effector proteins from the bacterial cytoplasm to that of the eukaryote. This incredible biophysical feat is accomplished by nanosyringe “injectisomes,” which form a conduit across the three plasma membranes, peptidoglycan layer and extracellular space that form a barrier to the direct delivery of proteins from bacterium to host. The focus of this chapter is T3SS function at the structural level; we will summarize the core findings that have shaped our understanding of the structure and function of these systems and highlight recent developments in the field. In turn, we describe the T3SS secretory apparatus, consider its engagement with secretion substrates, and discuss the post-translational regulation of secretory function. Lastly, we close with a discussion of the future prospects for the interrogation of structure-function relationships in the T3SS.

INTRODUCTION

Type III secretion systems (T3SS) afford gram-negative bacteria a most intimate means of altering the biology of their eukaryotic hosts — the direct delivery of effector proteins from the bacterial cytoplasm to that of the eukaryote (1, 2). T3SS utilize a conserved set of homologous gene products to assemble the nanosyringe “injectisomes” capable of traversing the three plasma membranes, peptidoglycan layer and extracellular space that form a barrier to the direct delivery of proteins from bacterium to host. While the injectisome is architecturally similar across disparate gram-negatives, its applications are a study in diversity: T3SS are employed by both symbionts and pathogens; they target animals, plants, and protists; and they are used to manipulate a wide array of cellular activities and pathways.

T3SS have attracted intense scientific interest since the seminal work documenting their discovery was published over two decades ago (3-5). Given their role in the virulence of several human and plant pathogens (e.g. *Salmonella enterica*, *Shigella flexneri*, *Yersinia* spp., pathogenic *Escherichia coli*, *Vibrio* spp., *Pseudomonas* spp., *Chlamydia* spp.), T3SS are attractive targets for the discovery or design of novel anti-infective agents and vaccine approaches. Conversely, as T3SS accomplish the biophysical feat of protein transduction across multiple membranes, their re-engineering for *in vivo* delivery of therapeutic proteins or *in vitro* production of protein reagents provides an exciting prospect for future biomedical

application. In either case, the manipulation of T3SS for human benefit will require highly refined mechanistic models of T3SS function. Drawing on research from multiple disciplines and employing complementary techniques, such models are beginning to emerge. In particular, the application of structural biochemical approaches to the T3SS has provided numerous insights into the assembly and function of this system.

The focus of this chapter will be on T3SS function at the structural level; we will summarize the core findings that have shaped our understanding of the structure and function of these systems and highlight recent developments in the field. In turn, we will describe the T3SS secretory apparatus, consider its engagement with secretion substrates, and discuss the post-translational regulation of secretory function. Lastly, we close with a discussion of the future prospects for the interrogation of structure-function relationships in the T3SS.

ARCHITECTURE OF A NANOSYRINGE

The genomic islands and virulence plasmids that support T3SS encode proteins of four broad classes: the components of the secretory system itself, the effector substrates, their chaperones, and transcriptional regulators. Working in concert, these components form a complete secretory system that de-chaperones and secretes substrates in a defined hierarchy and delivers them to the host cytoplasm. The repertoire of effector proteins secreted by a given T3SS is species-specific, as is the transcriptional network regulating T3SS expression (6). A discussion of these elements is beyond the scope of this review and has been expertly reviewed elsewhere (7-16). In contrast to the diverse, species-specific catalog of effector proteins and transcriptional regulators, the nanosyringe-like secretory machinery is well conserved across species, and advances in our mechanistic understanding of one species' injectisome are often applicable to others.

The core secretion machinery of the T3SS is comprised of a homologous set of approximately two-dozen gene products. Because of the high degree of homology of some components of the system, a universal nomenclature was previously suggested to facilitate cross-species comparisons (17), and recently others in the field have endorsed this naming system (18, 19). Similarly, we will employ this nomenclature (Table 1) wherever possible. Additionally, a subset of these proteins have conserved homologues in the flagellar apparatus (Table 1), which uses its own T3SS machine to assemble the flagellar filament (20). Given that these T3SS subtypes appear to have diverged from a common ancestor some hundreds of millions of years ago (21), their conservation is noteworthy. While the focus of this review is the injectisome T3SS subtype, we will draw on the flagellar literature where it offers insights into injectisome function.

The core conserved proteins of the T3SS form a double-membrane-spanning syringe-like structure (22, 23), including its extracellular needle-like appendage, and the associated cytoplasmic and membrane-integral secretion machinery (Figure 1). It is these components that are collectively responsible for the delivery of effector proteins into the cytosol of the eukaryotic host cell (24, 25). While our mechanistic model of this cytoplasm-to-cytoplasm secretion system continues to evolve, (ultra-)structural and biochemical characterization of its core components has yielded significant insights.

The basal body

The bacterial double membrane and peptidoglycan layer are spanned by a stack of protein annuli known as the basal body (Figure 1). It is comprised of an outer membrane-anchored layer (SctC) and an inner membrane-anchored layer (SctD and SctJ) that interface at a “neck” (26, 27). In electron microscopic (EM) reconstructions of the injectisome, SctC forms two distinct outer rings (OR1 and OR2), SctD and SctJ together form the distal inner ring (IR1), and the cytoplasmic amino-terminus of SctD forms the innermost ring (IR2) (26). The highest resolution cryo-EM models of the *Salmonella* basal body reveal an overall three-fold rotational symmetry, with a resultant symmetry mismatch between the inner and outer layers: each basal body contains 24 SctD molecules, 24 SctJ molecules, and 15 SctC molecules (27). While the 24-fold symmetry of the inner membrane rings appears conserved across T3SS (28, 29), the stoichiometry of the SctC outer membrane ring may vary between species (12-15 molecules per basal body), such that some systems have an overall 12-fold rotational symmetry (29, 30).

SctC is homologous to the Type II secretion system secretins (5, 31), and like other secretin family members requires a pilotin lipoprotein for its optimal localization and assembly (32-34). The membrane-embedded, β -rich region at the SctC carboxy-terminus can be isolated and has been visualized by EM (30), but it has yet to be characterized at moderate or high-resolution. The periplasmic amino-terminus of SctC contains a modular domain architecture (35) that interacts with the inner membrane ring (36, 37).

SctD and SctJ form the inner membrane rings (26). Each is anchored to the membrane by a single transmembrane helix, and SctJ is additionally lipidated near its amino-terminus (38). Like the amino-terminal periplasmic region of SctC, the periplasmic domains of SctD and SctJ are comprised of a modular multidomain architecture (35). Despite differences in connectivity and little sequence homology, the mixed α/β domains of SctC, SctD, and SctJ show a similar three-dimensional structure: two α -helices pack against the same face of a three strand β -sheet (35, 39). Superhelical crystal packing of the *E. coli* SctJ periplasmic region provided initial insights into the mechanism of inner membrane ring assembly (38), and the modular arrangement of these domains seems to promote oligomerization (40); however, none of these domains have been shown to clearly form annuli in solution, suggesting that additional constraints (e.g. protein-protein interactions or lipid membrane planarity) are critical for ring formation. Similarly, despite their 1:1 stoichiometry in the basal body, the periplasmic domains of SctD and SctJ have not been crystallized in complex.

Might there be a specific functional advantage for the modular domain architecture common to the SctCDJ periplasmic regions? Recent *in situ* electron tomography of the *Yersinia* and *Shigella* injectisomes shows that the basal body has the ability to stretch in response to osmotic expansion of the periplasmic space (29). This resilience could be of potential importance for the maintenance of intact T3SS injectisomes under physiologic stresses and membrane deformations (29). Molecular dynamic simulations suggest that relative motions of the SctD periplasmic domains could account in part for this flexibility (29), but this hypothesis requires empiric support.

The amino-terminal cytoplasmic domain of SctD forms the innermost ring of the T3SS basal body. High-resolution structural analyses have determined this domain to have a forkhead-associated fold that interacts with cytoplasmic components of the T3SS (41-44). Forkhead-associated domains are β -sandwiches that typically serve as phosphothreonine binding scaffolds, suggesting a means of signal-dependent recruitment of the cytoplasmic secretory apparatus to the basal body. However, the potential phosphopeptide binding residues are not conserved among T3SS, and the precise nature of the interactions between SctD and the cytoplasmic apparatus remains to be determined.

Within the central lumen of the basal body annuli, SctI is believed to form a cylindrical “inner rod” (26) structure that may support the extracellular needle filament. Computational methods have suggested a predominantly α -helical structure for SctI similar to that of the needle filament protomer (below); however, structural analyses of *Salmonella* and *Shigella* SctI in solution showed little secondary or tertiary structure (45). It remains to be determined whether SctI can adopt a stable fold within the confines of the basal body. The functional significance of the inner rod in the regulation of needle length and secretion substrate switching will be discussed below.

Direct structural characterization of the T3SS basal body epitomizes the challenges associated with the interrogation of high-molecular weight macromolecular machines: the assembly spans two membranes and a layer of peptidoglycan, and *in situ* electron tomographic analyses suggest that the basal body is capable of substantial conformational dynamism (29). As direct, high-resolution structural characterization of the assembled T3SS basal body has not yet been possible, a multidisciplinary approach integrating cryo-EM maps, x-ray crystallographic domain structures, biochemical analyses and computer modeling has yielded a high-probability static model for injectisome architecture (46) (Figure 2). Such “hybrid” models will allow the testing of molecular-level hypotheses about ring assembly until the structure of the T3SS basal body has been determined at high-resolution *in toto*.

The inner membrane machinery

Five highly conserved inner membrane proteins (SctRSTUV) are necessary for the function of the pathogenic T3SS; however their individual functions are unclear. It is worth noting these proteins show a high degree of sequence homology to components of the evolutionarily related flagellar T3SS (Table 1), and may represent a functional core, serving critical chemical roles in initiating or powering protein secretion.

Among the SctRSTUV cohort, SctU and SctV possess cytoplasmic domains in addition to their transmembrane helices, and these domains have been best characterized to date. The cytoplasmic region of SctV contains a modular array of small domains (47-49) and crystallographic data suggest SctV may nonamerize (49). Intriguingly, such an oligomer is well suited to fit in a torus of SctV-associated density observed 5-10nm beneath the basal body in EM reconstructions of the T3SS (29, 49). Lea and colleagues (49) have forwarded the hypothesis that this SctV homo-oligomer may serve as a “cage” to facilitate the complete unfolding of folded or partially unfolded secretion substrates, but this possibility has not yet been experimentally validated. The cytoplasmic region of SctU is considerably smaller than

that of SctV and contains an autoprotease (50); its potential role in the regulation of secretion will be discussed below.

SctRSTUV are important in the organized, stepwise assembly of the T3SS basal body. Galán and colleagues (51) employed a combination of genetic and structural approaches to show that SctRSTUV help to organize the SctDJ inner membrane rings. Subsequently, the SctC ring and cytoplasmic machinery (below) are recruited, and the inner rod and needle polymers assembled (19). While SctRSTUV are individually not strictly necessary for the formation of the SctCDJ basal body, the efficiency of basal body assembly is significantly ameliorated in their absence (51).

The needle filament

A needle-like filament tens of nanometers in length protrudes from the extracellular face of the T3SS basal body (22, 52). The needle is formed by a helical assembly of the protein SctF, with an outer diameter of 8nm and an inner pore diameter of 2.5nm (53). The apparent similarity of the T3SS basal body and needle filament to a macroscopic syringe makes it tempting to speculate that the T3SS directly injects its substrates into the host cell cytoplasm, with the needle filament serving as a conduit for the passage of partially unfolded effector proteins. Until recently, this hypothesis lacked direct empirical support, and alternative “non-injectisome” models for T3SS effector delivery had been proposed (54). Analyzing substrate-trapped injectisomes by cryo-EM, Marlovits and colleagues (55) and Kolbe and colleagues (56) demonstrated the presence of additional density in the lumen of the T3SS needle filament, consistent with the passage of partially unfolded substrate molecules through the needle.

High-resolution structures of monomeric SctF mutants (57) or chaperone-bound SctF (58, 59), have allowed the characterization of the needle protomer fold. SctF is a hairpin of alpha helices with an intervening conserved PXXP motif. The oligomeric nature of the needle filament had posed a practical barrier to high-resolution structure determination for the intact assembly. Recent hybrid approaches combining cryo-EM with solid-state nuclear magnetic resonance spectroscopy (NMR) and computational modeling have since afforded such a model for both the *Salmonella* (53) and *Shigella* (60) needle filaments. In these models, the SctF amino-terminus is oriented towards the convex needle exterior, the carboxy-terminus towards the lumen, and the apex loop connecting the two alpha helices points away from the bacterium. It should be noted that this arrangement is in contrast to prior lower resolution models (61), which oriented the SctF amino-terminus towards the needle lumen. This correction is significant: the orientation of SctF protomers in the solid state NMR models is such that the lumen walls are formed by highly conserved residues, consistent with the passage of secretion substrates through the lumen (53, 60).

Assembly of needle filaments of a given length is necessary for the proper infectivity of T3SS-bearing pathogens, possibly matching the dimensions of host-pathogen adhesion complexes (62). How, though, is the length of the needle filament controlled? SctP regulates the length of the needle filament in several species (63-65). In *Yersinia spp.*, the number of residues in SctP correlates with needle filament length, leading to the hypothesis that SctP functions as a “molecular ruler” (65). That is, SctP might attach at one end to the basal body

or cytoplasmic apparatus and at the other end to the growing needle filament, and once SctP was stretched beyond a given length, it would signal to the secretion apparatus to change substrates.

In contrast to the molecular ruler model, work in *Salmonella* suggest that SctP regulates needle length through control of inner rod assembly (52). *Salmonella* lacking SctP show decreased density in the inner rod-supporting socket region, lack a polymerized inner rod, and generate long needles (52, 66). Accordingly, Galán and colleagues hypothesized that SctI and SctF are secreted simultaneously and completion of inner rod assembly terminates needle growth in a SctP-dependent fashion. Consistent with this “timer” model of length control (19), overexpression of SctF or SctI leads to longer or shorter needles, respectively (52). Moreover, alanine-scanning mutagenesis analyses of the inner rod protein SctI in *Salmonella* revealed numerous point mutations that increased needle length without compromising secretory function, perhaps by slowing the rate of inner rod polymerization (66). Intriguingly, the elongate needles generated by most of these mutants remained attached to the basal body (66), in contrast to those produced by *sctP* deletion mutants, which are easily sheered off (52). This observation is consistent with the hypothesis that the polymerized inner rod joins with the needle filament, anchoring it to the basal body (66).

The needle tip and translocon pore

At the tip of the T3SS needle filament is a pentameric cap formed by the hydrophilic translocator protein (67, 68). The needle tip is believed to interact directly with the host cell surface to facilitate the insertion of a multimeric pore (69, 70), thus completing the cytoplasm-to-cytoplasm protein conduit. The structure and function of the needle tip is of particular biomedical interest, as the hydrophilic translocator protein is a protective antigen in anti-*Yersinia* vaccine formulations (71) and is targeted by a recently developed passive immunization strategy for the treatment of *Pseudomonas aeruginosa* infections (72).

X-ray crystallographic analysis of the monomeric tip protein from several species reveals some conserved architectural features (73, 74): the tips of all species show an elongate coiled-coil region and a central mixed α/β subdomain. The overall structure of the tip protein shows some interspecies variation though, with *Salmonella/Shigella* SipD/IpaD possessing an amino-terminal autochaperoning subdomain (74) lacked by the *Yersinia/Pseudomonas* LcrV/PcrV tip proteins (19, 70). High-resolution models of the pentameric needle tip are not available, but low-resolution negative stain EM models have offered some insight into its gross architecture. While both appear pentameric, the *Shigella* tip complex is narrow and elongated relative to that of the *Yersinia* tip (67, 68), and fitting the *Shigella* IpaD monomeric crystal structure into the EM map required a significant rearrangement of the mixed α/β domain (68).

Attempts to model the tip protein-needle filament interaction at high-resolution using NMR or x-ray crystallographic data have so far proven challenging, with incompatibilities arising between the proposed models and other data sets (75-77). The crystal structure of a *Salmonella* SctF-SipD fusion protein identified a potential binding mode for the tip with the needle (75); however, modeling the fusion structure onto the solid state NMR model of the needle filament (53) resulted in steric clashes, suggesting that artifactual constraints imposed

by the protein fusion strategy biased the architecture of the complex (77). Regardless, a synthesis of the available data shows that the needle filament interacts with the tip protein at least in part through its elongate coiled-coil, a motif observed in all T3SS tip proteins described to date.

EM and biochemical analyses have shown that the *Shigella* tip complex is actually a heteropentamer containing four copies of the hydrophilic translocator IpaD and one copy of the hydrophobic translocator IpaB (76). A refined tip model incorporating this insight is similar to previous models, in that the amino- and carboxy-termini of IpaD are oriented towards the needle filament, with portions of the coiled-coil region contacting the needle. However, the refined model presents an IpaD orientation consistent with antibody binding data and the heteropentameric architecture explains the transition from a helical needle filament to a nearly flat-topped tip complex (76).

In contrast to the annular, pentameric tip complexes observed for other T3SS, enteropathogenic *E. coli* possess a long filamentous needle accessory comprised of EspA, the SipD/IpaD/LcrV/PcrV homologue (78). EspA forms a helical filament similar to the SctF needle, with an internal diameter of approximately 2.5nm (79), suggesting that it functions to extend the T3SS transport conduit. Filling a functional niche similar to needle filament length control in other T3SS, *E. coli* EspA polymers may adapt the injectisome to reach the target cell membrane beneath the intestinal glycocalyx (70).

The translocon permeating the host cell membrane is formed by the hydrophobic translocators SipB and SipC in *Salmonella* and their homologues (Table 1). Experiments in red blood cell membranes have shown that the needle tip is crucial for the insertion of the hydrophobic translocators into the host membrane and/or the organization of inserted translocators into functional pores (69, 80). While numerous experimental approaches have been employed to characterize the pore diameter and structure of the translocon (70), direct structural interrogation of native translocons is lacking. Intriguingly, the amino-termini of *Salmonella* SipB and *Shigella* IpaB contain extended coiled-coils reminiscent of the colicin family of bacteriocins (81), which are known to function in the delivery of protein toxins across bacterial membranes. However, the precise mechanisms of host cell recognition, membrane insertion, pore formation, and protein translocation remain unclear for the T3SS translocon.

SUBSTRATE RECRUITMENT AND SECRETION

T3SS secrete only a small fraction of the proteins present in the bacterial cytosol (82), and do so in a defined hierarchy. How does the T3SS select its substrates, how is their secretion hierarchy maintained, and what is the mechanism of secretion? A combination of genetic, biochemical, and structural data provide insight into the role of cytoplasmic injectisome-associated proteins in these processes.

Secretion chaperones

The amino-terminal ~100 amino acids of T3SS substrates possess two secretion signals: an unstructured extreme amino-terminus followed by a chaperone-binding region. While the

extreme amino-termini of T3SS substrates are highly variable, computational approaches have identified commonalities in the chemical composition of this secretion signal (83-85): the first ~15 amino acids in an effector sequence are enriched in serine, threonine, isoleucine, and proline. Indeed, an effector with a synthetic, amphipathic poly-serine/isoleucine secretion signal was secreted by *Yersinia* even in the absence of the second secretion signal (its secretion chaperone) (86). However, other studies have shown that chaperone-substrate interactions are necessary for targeting substrates specifically to the injectisome T3SS, as the extreme amino-terminal secretion signal sequence can facilitate injectisome substrate export through the flagellar apparatus in the absence of a chaperone-binding domain. This finding suggests that the extreme amino-terminal secretion signal is evolutionarily “ancient” and shared by both types of T3SS (87).

Downstream of the amino-terminal secretion signal, each secretion substrate is recognized by a specific chaperone protein that maintains the bound region of the substrate in a partially unfolded state (88). It is hypothesized that this nonglobular conformation primes the substrate for secretion through the narrow aperture of the T3SS conduit (89). Chaperones can be classified by their structure and substrate type (90), as follows: Class IA chaperones are mixed α/β homodimers that bind to one effector; Class IB are structurally similar to IA but bind multiple effectors; Class II are alpha helical tetratricopeptide repeat (TPR) proteins that bind to translocon proteins; and Class III are heterodimeric TPR proteins that bind SctF needle filament protomers. It should be noted that while the premature polymerization of SipD/IpaD tip proteins is prevented by their autochaperoning domain (74), tip proteins in *Yersinia* and *Pseudomonas* utilize a unique chaperone (LcrG/PcrG) to stabilize their tip protein monomers (91).

Like the extreme amino-terminal secretion signal, the chaperone-binding sequences of secretion substrates are variable. However, Class I chaperone recognition of a conserved β -strand(s) is a common feature of a diverse array of T3SS effectors (92), and it is conserved from animal to plant pathogens (93) (Figure 3). Indeed, the sparse sequence conservation associated with the “ β -motif” (92) can be used to recognize previously unknown T3SS effector proteins (94). In contrast, Class II and III TPR chaperones can recognize substrate sequences in either extended unstructured or α -helical conformations; the commonality here is that the TPR concavity is used to bind the substrate (90) (Figure 3).

The aforementioned substrate secretion signals are not only necessary for protein secretion through the T3SS, they are sufficient. Fusion of the secretion signal and chaperone-binding domain from endogenous T3SS substrates to heterologously expressed proteins results in their secretion through the T3SS (95), provided they can be properly unfolded for transit through the needle (55, 56). While this observation is noteworthy for its mechanistic insight into the targeting of virulence factors for secretion, it has allowed the benevolent re-engineering of the system to deliver protective antigens in vaccine design (96) and the large scale production of challenging protein reagents, like spider silk (97).

The ATPase

Both the injectisome and flagellar T3SS include an ATPase with notable sequence homology to the β subunit of the F_0F_1 ATPase (98). For the injectisome, this ATPase is

SctN; for the flagellar apparatus, FliI (Table 1). High-resolution structural analysis of the *E. coli* SctN catalytic domain showed similarities to V- and F-type ATPases and confirmed that SctN hexamerization would be required for efficient ATP hydrolysis (99). Both FliI (100, 101) and SctN (98, 102) form such oligomers, but neither has been characterized structurally as a catalytically active hexamer.

Interaction of chaperone-substrate complexes with the T3SS ATPase SctN causes the dechaperoning and unfolding of the substrate in an ATP hydrolysis-dependent fashion (103). Given that disruption of tertiary structure is necessary to fit protein substrates in the 2.5nm conduit of the needle filament (55, 56), it is not surprising that loss of function mutations in SctN cause the near complete abrogation of T3SS function (98, 103). Similarly, genomic deletion of *fliI* severely compromises flagellar filament assembly (104-107). Consistent with the role of SctN in preparing substrates for export, SctN/FliI-dependent density is observed directly beneath the T3SS basal body by EM (108, 109). The partial structural similarity of SctO/FliJ with the γ -subunit of F-type ATPases and the ability of FliJ to stimulate FliI hexamerization (101) has led to the hypothesis that this coiled-coil containing protein (110) might connect oligomeric SctN to the SctV export gate, thus linking the sub-basal body toruses of electron density (49). However, the mere presence of a coiled-coil is insufficient evidence to ascribe a γ -like function to SctO (19), and its precise role remains to be determined.

Given the ATPase activity of SctN/FliI, it is tempting to speculate that ATP hydrolysis provides the free energy for protein secretion; however, chemical and genetic analyses show that SctN/FliI is not the sole energizer of the T3SS. Experiments with the protonophore CCCP have shown that the inner membrane proton motive force is necessary for secretion by both injectisome (111) and flagellar (104, 105) T3SS. Additionally, the flagellar assembly defect of ATPase deletion mutants can be at least partially corrected by mutations that alter the export apparatus, increase substrate levels, or increase the magnitude of the proton motive force (104-106). A similar result was recently reported for SPI-1 T3SS in *Salmonella* (106). These results suggest that under sufficiently permissive conditions, the actual transit of substrates into and/or through the conduit is powered by the proton motive force. However, one must interpret these results with some caution, as ionophores can significantly perturb cellular physiology (112) and SctN-independent injectisome secretion of a substrate requiring dechaperoning has not yet been demonstrated (106). A reasonable synthesis of the available data might surmise that both ATP hydrolysis and the proton motive force are important for energizing T3SS: the SctN/FliI ATPase functions to dechaperone and begin unfolding the secretion substrates with optimal efficiency under (non-ideal) physiologic conditions, while the proton motive force is responsible for the apical transit of the nonglobular substrate (106).

Regardless of the quantitative contributions of either energy source, the mechanics of secretion remain poorly understood. There are no available high-resolution structural models of the interaction between chaperone-substrate complexes and the SctN ATPase. One computational model suggests a mode of ATPase-chaperone interaction based on structural similarities between Class I chaperones and the F-type ATPase γ -subunit (113). While this model and the accompanying biochemical data are consistent with the observation that

relatively carboxy-terminal residues of SctN interact with chaperones (113), its structural accuracy lacks empiric support. Recent SAXS data suggest an alternative model for the interaction of substrates, chaperones, and the ATPase. Complexes of the *Salmonella* effector-chaperone pair SopB-SigE are able to hexamerize in a concentration dependent manner with dimensions comparable to the hexameric models of the ATPase (114). While it is too early to say whether other chaperone-effector complexes can oligomerize (115), whether these oligomers can interact with SctN, or whether such interactions — even if possible — are physiologically relevant, these results raise the possibility of alternate ATPase-cargo stoichiometries.

Recent solution NMR analyses suggest an interesting role for chaperone structure in the targeting of substrates to the T3SS. In solution, the *E. coli* chaperone CesAB is a partially folded molten globule (116) that does not interact with the hexameric SctN (117). However, upon binding to its substrate — the EspA tip filament protein — CesAB becomes fully structured and is able to bind SctN (117). The binding site for SctN was mapped onto the CesAB-EspA heterodimer, where it covered regions of CesAB unstructured in the absence of substrate (117). Consistent with the hypothesis that substrate-induced folding of the chaperone allows for targeting to the ATPase, a mutation-stabilized, structured CesAB homodimer was able to bind SctN in the absence of substrate (117). While it remains to be determined whether similar disorder-to-order transitions effect SctN binding for other chaperone classes, these results are consistent with the role of chaperone-substrate interactions in targeting substrates for secretion. Additionally, the observation that substrate-chaperone complexes are recognized by hexameric SctN, but not its amino-terminally truncated monomeric form, suggests that ATPase hexamerization is critical for both hydrolytic catalysis and substrate recognition (117).

The sorting platform

Located at the peripheral cytoplasmic face of the flagellar basal body is a ring of robust density in EM reconstructions (109). Known as the “C-ring,” this annulus is composed of the flagellar proteins FliM, FliN, and FliG, and it plays a role in flagellar motor function (torque generation) and rotational switching (20). FliM and FliN have an injectisome homologue with some conserved domains (SctQ), but torque generation by the injectisome is controversial (118) and a robust C-ring is absent in tomographic reconstructions of the injectisome basal body (29, 109). However, immuno-EM analysis of purified *Shigella* injectisomes shows localization of SctQ to the cytoplasmic face (119), suggesting that it plays some role in protein secretion or the regulation of the secretory process. Indeed, recent cryo-electron tomographic analyses of the *Shigella* injectisome have identified six SctQ-dependent “pods” of density proximal to the cytoplasmic face of the basal body, forming a structure distinct from that of the flagellar C-ring (120).

Seminal biochemical and genetics work by Galán and colleagues revealed that SctQ forms a critical “sorting platform” for the T3SS (121). Affinity purification of SctQ from secretion-competent *Salmonella* produces high molecular weight complexes containing the SctN ATPase, regulatory proteins, chaperones and secretion substrates (121). Most notably, the sorting platform plays a role in the hierarchical secretion of substrates, queuing substrates in

their appropriate order. For example, in *Salmonella* with assembled injectisomes, the sorting platform is predominantly occupied by translocon proteins, but genomic deletion of the translocators allows the next tier of substrates (effector proteins) to access the sorting platform (121).

In addition to SctQ, formation of the sorting platform requires the proteins SctK and SctL (121). While the role of SctK is at present unclear, biochemical analyses of the flagellar apparatus shed light on the potential function of SctL. SctL is a homologue of the flagellar protein FliH (Table 1). The SctL/FliH family is predicted to have a conserved domain architecture: an amino-terminal disordered region is followed by a coiled-coil and then a mixed α/β domain (122). The carboxy-terminus of FliH has interacts with the amino-terminal oligomerization domain of FliI (122), inhibiting its ATPase activity (123). While sequence similarities between the FliH carboxy-terminal domain and the F-type ATPase δ -subunit suggest a role for FliH as a “stator” (124), it is not obvious that FliH interacts with oligomeric FliI, and the structural details of this interaction are not yet known. The amino-terminus of FliH interacts with FliN (125), and this FliH-FliN interaction is important (126) — if not absolutely necessary (127) — for the recruitment of FliI to the export apparatus. Given that the homologous injectisome complex (SctQ-SctL-SctN) forms a portion of the sorting platform and that chaperone-substrate complexes interact with the ATPase, these data suggest that one function of the SctQ sorting platform could be to localize chaperone-effector-ATPase complexes to the injectisome export apparatus. Indeed, Minamino, Namba and colleagues have hypothesized that the FliI ATPase exists in two forms: an ATP-hydrolyzing hexamer and a dynamic substrate-carrying monomer bound to FliH and the C-ring (128). Similarly, SctQ-injectisome interactions are dynamic in *Yersinia*, as injectisome-associated SctQ exchanges with a cytoplasmic pool with a half-time of approximately one minute (129).

Structural models of SctQ are a work in progress, and have focused to date on the carboxy-terminus of the molecule. In *Pseudomonas syringae*, SctQ is spread over two open reading frames (*hrcQA* and *hrcQB*), much like FliM and FliN in the flagellar system. The structure of the carboxy-terminal domain of HrcQB is quite similar to that of the carboxy-terminal domain of FliN (130, 131); both domains are homodimers of the “surface presentation of antigens” (SpoA) fold. The folded core of each protomer is an antiparallel β -sheet, and a loop from each protomer containing a β -strand and α -helix wraps around the β -sheet core of the other protomer. In *Yersinia*, SctQ is the product of a single open reading frame (as in most injectisomes), but the carboxy-terminal SpoA domain is duplicitously translated from an internal translation start site (132). The homodimer produced by this translation product is able to interact with full-length SctQ and, at least in the *Yersinia* system, is necessary for secretion *in vivo*. In both the flagellar (133, 134) and injectisome (135) systems, this SpoA domain tetramerizes as a dimer of dimers, but appears to do so in different orientations in each system. Cross-linking analyses suggest that the FliN SpoA tetramers form a “doughnut” at the base of the C-ring (133), but high-resolution support for this arrangement is lacking.

Despite the progress that has been made, numerous structural questions remain unanswered for the SctQ sorting platform. The function of the SpoA domain is unclear, SctQ-

SctQ(SpoA) interactions have yet to be structurally characterized, and the structural basis for the interaction of SctL with SctQ has not yet been determined. Moreover, while the amino-terminal domains of FliM have well characterized functions in the regulation of flagellar rotation switching (136, 137), these motor functions are likely flagella-specific and involve interactions with partners not conserved from the flagellar apparatus to the injectisome (e.g. FliG, CheY). Thus, the function of the SctQ amino-terminus is also unclear. Lastly, how and when SctQ or its soluble interaction partners interface with the basal body or export apparatus remains to be determined.

Substrate switching

T3SS substrates are secreted in a defined order that is necessary for the proper assembly and function of the system (138, 139): secretion of the needle filament (SctF) and inner rod (SctI) is followed by secretion of the needle tip protein and translocon pore proteins, which is followed by the secretion of effector proteins. Thus, it seems that there are several sequential substrate “switching” events that must occur for the hierarchical secretion of substrates to be maintained (139).

The first such switching event halts the extension of the growing needle filament upon completion of the inner rod and allows for secretion of the needle tip protein and translocators. As discussed above, the length of the needle filament is controlled by the assembly of the inner rod in a SctP-dependent fashion (52, 66). Full deletion of *sctP* locks the T3SS into a mode of exclusive SctF filament secretion; that is, deletion of *sctP* results in not only elongate needles, but a lack of translocon and effector secretion (63, 65, 140, 141). Indeed, deletion of *sctP* results in the absence of translocon components from the SctQ sorting platform (121). However, small deletions in the amino-terminal regions of SctP alter needle length without compromising translocon secretion, suggesting that some portion of SctP performs a crucial switching function (141). Deletions within the conserved mixed α/β region at the carboxy-terminus of the protein compromise translocon secretion (in addition to disrupting needle length regulation), and this presumptive domain has been termed the “type III secretion substrate specificity switch” (T3S4) domain (141).

The three-dimensional structure of an injectisome T3S4 domain has not yet been determined, but the flagellar FliK T3S4 domain has been solved by NMR (142). The carboxy-terminal domain of FliK possesses two α -helices folded against a four-strand β -sheet (142), and the predicted structural conservation of these secondary structural elements in SctP suggests that this model may be generalizable to the injectisome. While it is still unclear at the molecular level how SctP functions to promote specificity switching, its interaction partners suggest some viable hypotheses. For example, the SctP T3S4 domain interacts with the SctO protein (143), suggesting that it may be able to transmit regulatory information to the SctN ATPase or the SctV export gate. Moreover, the T3S4 domain interacts with the cytoplasmic autoprotease domain of SctU (144). Like SctP, SctU regulates the secretion of the inner rod protein (145). The interaction between these two proteins is intriguing given that SctU interacts with components of the SctQ sorting platform and the SctV export apparatus (146), again suggesting mechanisms for the relay of switching information throughout the secretory apparatus.

The second major switching event distinguishes between translocon components and effector proteins (121). Deletion of translocon components allows for the localization of effector proteins to the sorting platform, consistent with a model where a gradient of substrate and/or chaperone affinities for the sorting platform controls the hierarchy of secretion (121). The identification of several classes of secretion apparatus mutants that can secrete effectors but not translocon proteins offers some insights into the establishment of secretion hierarchy. Deletion of *sctW* in *Salmonella* results in the specific loss of translocon component secretion (138). SctW binds the translocon proteins and their chaperone in *Salmonella* (SicA) (138), and it is necessary for translocator binding to the SctQ sorting platform (121). These observations are consistent with SctW enhancing the affinity of translocon-containing complexes for the sorting platform. However, recent genetics data suggest the mechanism of hierarchy control for SctW may be more complex. A subset of the SctI alanine mutants identified by Lefebvre and Galán (66) have normal needle lengths but phenocopy *sctW* deletion, and an interaction between SctW and SctI was recently reported in *Shigella* (147). Together, these data raise the possibility of SctW binding not only the sorting platform but also portions of the basal body.

Further clouding the role of SctW in T3SS is the observation of species-specific effects of *sctW* mutation. In *Yersinia* and *Shigella*, SctW is secreted and *sctW* deletion does not specifically impair translocon protein secretion (148, 149). Moreover, the *Yersinia* SctW protein pair YopN/TyeA is part of a complex calcium response apparatus in the bacterial cytosol (150) that involves several *Yersinia*-specific proteins (151, 152). While the structures of the *Shigella* and *Yersinia* SctW homologues have been determined (153, 154), a fuller understanding of SctW function (and its species-specific nuances) will require structural characterization in complex with other injectisome components.

In addition to its role in the first switching event, SctU is also involved in the second switch. The cytoplasmic domain of the SctU family autocatalyzes cleavage between the asparagine and proline residues of its conserved NPTH cleavage site (139). Alanine mutations on either side of the cleavage site cause aberrant specificity switching: translocon proteins are no longer secreted but effector secretion remains intact (50, 148). An amphipathic linker connects the SctU transmembrane region to the cytoplasmic autoprotease, and this linker undergoes a disorder-to-order transition in the presence of anionic lipids (155). Introducing charge-altering mutations in the linker impaired T3SS function, suggesting that the ordering of the SctU linker against the bacterial inner membrane is crucial, perhaps favorably orienting the autoprotease domain for interactions with other members of the export apparatus (155). As mentioned above, SctU interacts with multiple members of the sorting platform, but the bases for these interactions — and the mechanisms by which they would effect specificity switching — are unclear.

Control of secretion

The T3SS can assemble a basal body, needle and tip, and then pause in a “primed” state until the relevant stimulus arrives and secretion resumes. This strategy prevents the wanton waste of translocon and effector proteins. Interrogating this additional level of complexity is important to our full understanding of the pathobiology of T3SS, and may suggest routes to

anti-virulence compounds that prevent the activation of otherwise structurally competent injectisomes.

Bile salts play a regulatory role in the T3SS used by several enteric pathogens. The interaction of bile salts with the *Shigella* tip complex promotes IpaB recruitment to the tip, forming the heteropentameric tip complex described above (76, 156). In contrast, bile salts suppress *Salmonella* SPI-1 T3SS function (157). These observations provide an intriguing correlation between host gastrointestinal physiology and pathogen virulence that ties environmental factors to the species-specific adaptation of the T3SS. Despite reports describing the interaction of bile salts with monomeric *Shigella* IpaD (158) and *Salmonella* SipD (75, 159), the structural basis for bile salt interaction with the intact tip complex has yet to be determined in either species, and so the mechanism of its regulatory activity remains unclear.

Contact with host cells stimulates the activation of T3SS in several species (160-162). In *Salmonella*, contact with target cells stimulates the secretion of the translocon proteins SipB and SipC (163), and in *Shigella*, interaction of the IpaD-IpaB tip with liposomes resembling host cell membranes induces IpaC secretion (164). It is tempting to speculate that contact of the needle tip with the host cell sends a mechanical signal to the basal body and/or export apparatus that reinitiates secretion (6, 19, 165). As the connecting factor between the host cell surface and the basal body, the needle filament itself is a promising candidate for force transduction. Specific needle filament protein mutations can trap the *Shigella* T3SS in a constitutively active secretion mode, and one might hypothesize that these mutations stabilize needle filaments in a post-contact activated conformation (166). However, the filaments formed by these mutants do not exhibit the gross conformational changes one might expect if the needle filament architecture were transducing this signal (166). Alternatively, local changes in the tip environment may permit the secretion of substrates trapped within the needle by a closed tip, restarting secretion without requiring signal transduction to the bacterial cytoplasm or basal body (165).

Work from the *Salmonella* SPI-2 T3SS suggests a tantalizing third (and non-mutually exclusive) possibility, that the needle is not only a conduit for protein secretion, but a passageway for the diffusion of chemical signals (167). *Salmonella* makes use of two T3SS: broadly, the SPI-1 T3SS promotes cell invasion and subsequently the SPI-2 T3SS facilitates the formation of the *Salmonella* Containing Vacuole (SCV), an intracellular environment for *Salmonella* survival and replication (168). Holden and colleagues noted that priming of the SPI-2 T3SS requires exposure of the bacteria to low pH (as would be experienced in the endosomal compartment), but that triggering of effector secretion required a return to neutral pH (167). It is noteworthy that the SPI-2 SctW protein was required for this transition (167), consistent with the apparent role of SctW in translocon-to-effector specificity switching in other systems (above). However, it is most intriguing that this switch required intact translocon components, suggesting that the neutral pH signal may be transduced from the host cell cytosol, through the translocon and needle, to the basal body and/or export apparatus (167).

In light of these findings from *Salmonella* SPI-2, one wonders whether the needle conduit could serve as a channel for other small molecule signals from the host cytosol: perhaps the needle of *Yersinia* spp. transmits the decreased calcium ion concentration of the eukaryotic cytosol, resulting in T3SS reactivation. Intriguingly, Plano and colleagues have shown that point mutations in the *Yersinia* needle filament protein result in calcium-independent, constitutive T3SS (169); however, the mechanism of this altered calcium response is unclear. One might hypothesize that these mutant needles are unable to associate with tip or translocator proteins, preventing access to — and transmission of — the low calcium environment of the host cytosol. Consistent with this hypothesis, a subset of the constitutively secreting mutants were deficient in delivery of T3SS substrates to the host (169). Alternatively, extracellular calcium might regulate T3SS by altering the conformation of the needle filament protein (akin to the aforementioned force transduction hypothesis), and these mutants might simply be unable to assume some putative calcium-dependent conformations.

FUTURE PROSPECTS

In the past 20 years, our models of T3SS structure and function have evolved substantially, from the first visualization of the injectisome architecture, to the high-resolution structural interrogation of many of its individual components. Combining these insights with a plethora of genetic and biochemical data, the molecular mechanics of this astounding secretory nanomachine are coming into focus. However, numerous questions remain — the answers to which are critical to our understanding of bacterial virulence, the design of new therapeutics, and the imaginative re-engineering of the system.

Despite the improvements in cryo-EM models of the injectisome, the precise architecture of the membrane embedded components of the T3SS is still unclear, as is the structural basis for their interactions with the soluble components of the system. The native structures of the filament-bound needle tip and the translocon in the host membrane must be determined to understand how the extracellular environment regulates secretion, how proteins penetrate to the host cytosol, and how to rationally design secretion-blocking vaccines. Although the constituents of the cytoplasmic sorting platform have been identified, the structural bases for their interactions are unknown: how the sorting platform assembles, how substrate-chaperone complexes engage the system, and how the numerous regulatory elements interact to govern a secretory hierarchy all remain to be determined.

Answering these questions is likely to require a hybrid approach, characterizing local interactions and large assemblies alike, and employing a range of structural and molecular techniques. However, it is clear that high-resolution models of intact macromolecular assemblies (e.g. basal body, needle tip, translocon pore) would greatly advance the field. Much like the role that atomic models of the ribosome have played in the interrogation of its multiple functional states, one can imagine the watershed of insight that would come from successful visualization of the injectisome or sorting platform in each of their several forms: needle-assembling, translocator-secreting, and effector-secreting. Ideally, these mechanistic insights will allow the uncoupling of some pathogenic gram-negative bacteria from virulence and the re-engineering of the nanosyringe for the benefit of biotechnology.

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Figure 1. Gross architecture of the T3SS

Cryo-EM reconstruction of the *Salmonella typhimurium* injectisome basal body at subnanometer resolution reveals its overall architecture. (A) Surface representation of the highest resolution cryo-EM map (EMD 1875, contour level 0.0233) published by Marlovits and colleagues (27). Dashed lines indicate the positions of bacterial membranes *in vivo*. Abbreviations used: OR, outer ring; IR, inner ring; OM, outer membrane; IM, inner membrane. (B) An axial section through the map in (A). (C) Transverse sections through the map in (A) at the level of the neck (top) and IR1 (bottom).

Figure 2. Hybrid models of basal body structure

(A) Computational modeling of the neck (SctC, PDB 3J1V), IR1 (SctD, PDB 3J1X), and IR2 (SctD, PDB 3J1W) annuli of the *Salmonella typhimurium* basal body. No high-resolution structural information is available for the basal body above the neck. (B) In this model, complementary electrostatic surfaces support ring building, as shown for the SctD periplasmic domains. Note the modular domain architecture (enumerated 1, 2, 3) for SctD_{periplasmic}.

Figure 3. Chaperone-substrate interactions

Structural distinctions between effector-chaperone and translocator-chaperone complexes.

(A) The structure of the *Salmonella* effector SipA chaperone-binding domain (CBD, red and yellow) in complex with the Class IB chaperone InvB (dark grey, light grey). PDB 2FM8 (47). The structurally conserved β -motif is highlighted in yellow. (B) The SipA β -motif is bound by a hydrophobic (grey) patch on the InvB surface (blue/grey). (C) Superposition of the CBDs from effectors from multiple species shows a common binding mode marked by the structurally conserved β -motif. The prototypical Class I chaperone SicP is shown in place of the various chaperones. PDB codes: YopN, 1XKP (153); YopE, 1L2W (170); YscM2, 1TTW (171); SptP-SicP, 1JYO (88); SipA, 2FM8 (47); HopA1, 4G6T (93). (D) The *Yersinia* translocator YopD CBD (red) lacks secondary structure and is bound by the concave cleft of the Class II chaperone SycD (grey). PDB 4AM9 (172).

Table 1**A unified nomenclature for the homologous core components of the T3SS**

Based on the nomenclature proposed by Hueck (17), with modifications and additions from others (16, 18, 19, 90).

Notes	Universal Nomenclature	<i>Salmonella SPI-1</i>	<i>Shigella</i>	<i>EPEC</i>	<i>Yersinia spp.</i>	<i>Pseudomonas aeruginosa</i>	<i>Flagellar Apparatus</i>
Basal Body	SetC	InvG	MxiD	EscC	YscC	PscC	
	SetD	PrgH	MxiG	EscD	YscD	PscD	
	SetJ	PrgK	MxiJ	EscJ	YscJ	PscJ	
Pilotin		InvH	MxiM		YscW	ExsB	
Inner Rod	SetI	PrgJ	MxiI	EscI	YscI	PscI	
Needle Filament	SetF	PrgI	MxiH	EscF	YscF	PscF	
Needle Length Regulator	SetP	InvJ	Spa32	EscP (Orf16)	YscP	PscP	FliK
Inner Membrane Machinery	SetV	InvA	MxiA	EscV	YscV (LcrD)	PcrD	FliA
	SetR	SpaP	Spa24	EscR	YscR	PscR	FliP
	SetS	SpaQ	Spa9	EscS	YscS	PscS	FliQ
	SetT	SpaR	Spa29	EscT	YscT	PscT	FliR
	SetU	SpaS	Spa40	EscU	YscU	PscU	FliB
Needle Tip and Translocon		SipB	IpaB	EspD	YopB	PopB	
		SipC	IpaC	EspB	YopD	PopD	
		SipD	IpaD	EspA	LcrV	PcrV	
ATPase	SetN	InvC	Spa47	EscN	YscN	PscN	FliI
Coiled Coil Linker	SetO	InvI	Spa13	EscO (Orf15, EscA)	YscO	PscO	FliJ
Sorting Platform	SetQ	SpaO	Spa33	SepQ	YscQ	PscQ	FliM/FliN
	SetK	OrgA	MxiK		YscK		
	SetL	OrgB	MxiN	EscL	YscL	PscL	FliH
Needle Length Regulator	SetP	InvJ	Spa32	EscP (Orf16)	YscP	PscP	FliK

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Notes	Universal Nomenclature	<i>Salmonella</i> <i>SPI-1</i>	<i>Shigella</i>	<i>EPEC</i>	<i>Yersinia</i> spp.	<i>Pseudomonas aeruginosa</i>	<i>Flagellar Apparatus</i>
Export Regulator	ScdW	InvE	MxiC	SepL/SepD	YopN/TyeA	PopN	