Received: 4 May 2023 | Revised: 5 June 2023 | Accepted: 7 June 2023

DOI: 10.1002/bies.202300078

BioEssays

REVIEW ESSAY

Prospects & Overviews

The sorting platform in the type III secretion pathway: From assembly to function

Jose Eduardo Soto | **María Lara-Tejero**

Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut, USA

Correspondence
Jose Eduardo Soto and María Lara-Tejero, Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT06536, USA.
Email: jose.soto@yale.edu; maria.lara-tejero@yale.edu

Abstract

The type III secretion system (T3SS) is a specialized nanomachine that enables bacteria to secrete proteins in a specific order and directly deliver a specific set of them, collectively known as effectors, into eukaryotic organisms. The core structure of the T3SS is a syringe-like apparatus composed of multiple building blocks, including both membrane-associated and soluble proteins. The cytosolic components organize together in a chamber-like structure known as the sorting platform (SP), responsible for recruiting, sorting, and initiating the substrates destined to engage this secretion pathway. In this article, we provide an overview of recent findings on the SP's structure and function, with a particular focus on its assembly pathway. Furthermore, we discuss the molecular mechanisms behind the recruitment and hierarchical sorting of substrates by this cytosolic complex. Overall, the T3SS is a highly specialized and complex system that requires precise coordination to function properly. A deeper understanding of how the SP orchestrates T3S could enhance our comprehension of this complex nanomachine, which is central to the host-pathogen interface, and could aid in the development of novel strategies to fight bacterial infections.

KEYWORDS
bacterial pathogenesis, nanomachine assembly, protein secretion, sorting platform, type III secretion system

INTRODUCTION

To gain access to valuable resources and privileged replicative niches, many bacteria establish close ecological interactions with eukaryotic organisms. These relationships range from beneficial symbiotic associations to deadly infections. One of the most common ways in which bacteria interact with eukaryotic cells and exert control over them, is through the acquisition of specialized multiprotein devices specifically devoted to transporting proteins across membranes.[1] Among these, the virulence-associated type III secretion system (T3SS) present in many gram-negative bacteria, enables the polarized transport of effector proteins across three distinct membranes (two bacterial and one host) leading to their direct delivery into host eukaryotic cells (Figure 1A,B).[2,3] The cocktail of injected effectors modulates important cellular functions such as cellular immunity, rearrangement of the cytoskeleton, and cellular signaling, among many, for the benefit of the bacterium.[4] The ecological outcome of the interaction between the bacteria and their host, whether it is pathogenic, commensal, or even symbiotic, is ultimately determined by the interplay between the collective biochemical activities of the translocated effector arsenal and the responses of the host.

Although the specific repertoire of injected effectors is diverse and tailored to the unique lifestyle of each species, the T3SS machinery itself is highly conserved across diverse organisms. Moreover, the evolutive origin of the virulence T3SS can be traced back to an exaptation event of the bacterial flagella,[5] which is the most widespread motility device in bacteria. During this exaptation process, several components

ABBREVIATIONS: cryo-EM, cryo-electron microscopy; cryo-ET, cryo-electron tomography; NC, needle-complex; SP, sorting platform; SPI, *Salmonella* pathogenicity island.; T3SS, type III secretion system.

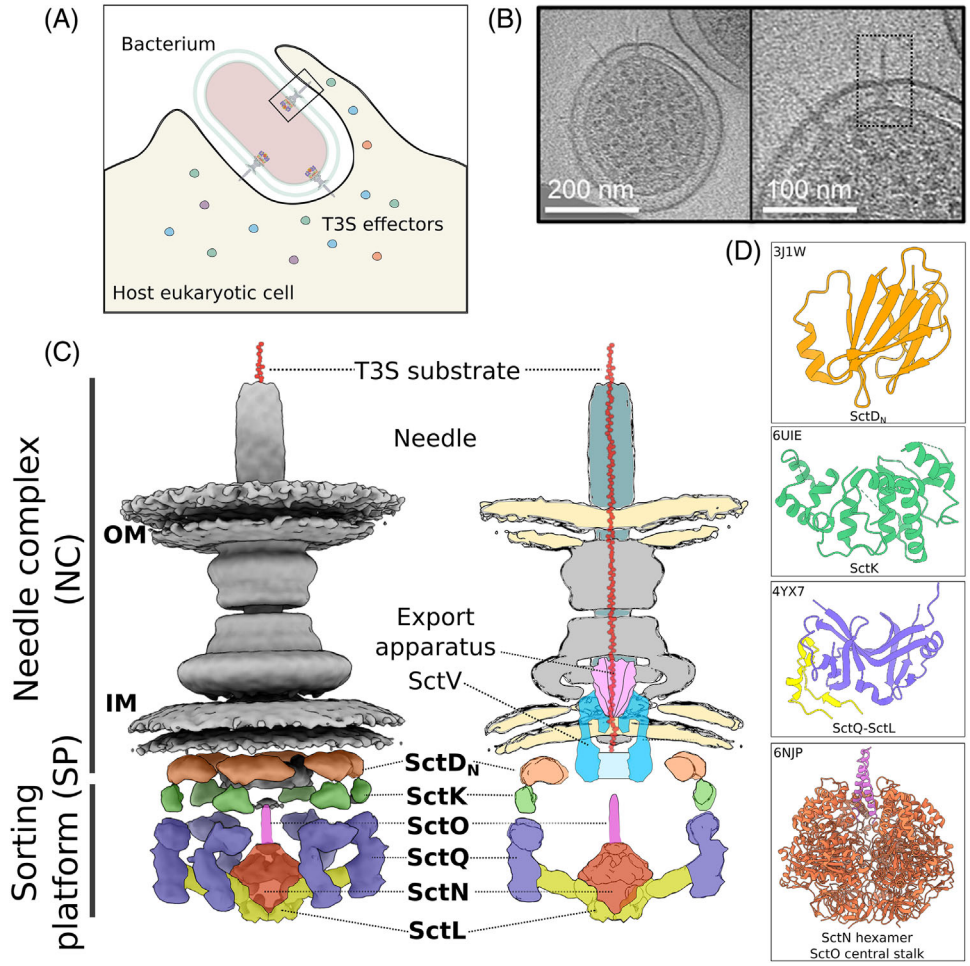

FIGURE 1 The overall architecture of the T3SS sorting platform. (A) Schematic diagram of a bacterium translocating effectors proteins into a eukaryotic host cell through the type III secretion system. (B) Tomographic slice of a *Salmonella* Typhimurium minicell carrying T3SS (left panel) along with the corresponding zoom-in view of the injectisomes (right panel). Reproduced from reference.[28] (C) Front view (left) and sagittal section (right) of the cryo-ET in situ structure of the *Salmonella* Typhimurium T3SS (EMDB-8544). IM, inner membrane; OM, outer membrane. (D) Ribbon representation of the solved atomic structures of key building blocks of the sorting platform. From top to bottom: SctD$_N$ (PDB 3J1W), SctK (PDB 6UIE), SctQ-SctL (PDB 4YX7), and SctN-SctO (PDB 6NJP).

of the flagella were repurposed or co-opted, while others were lost, and new components were acquired to convert a motility organelle into a dedicated protein translocation device. As a result, both homologous machines share numerous structural components.[6]

Box 1. A common language for T3SS research

Numerous pathogenic bacteria, including *Salmonella* spp., *Shigella flexneri*, *Yersinia* spp., *Escherichia* spp., *Chlamydia* spp., and *Pseudomonas* spp., employ a T3SS to deliver effector proteins into host cells and manipulate cellular processes to facilitate infection. Given the prevalence of T3SS as a common theme in bacterial pathogenesis, a vibrant body of research has been conducted on numerous bacterial species, resulting in the adoption of species-specific nomenclature. To facilitate insights extrapolation across different species carrying T3SS, we adopted here the proposed universal sct (secretion and cellular translocation) nomenclature[7,8] which is based on homology among T3SS components (Table 1).

Frequently referred to as *injectisome* due to its physical resemblance to a syringe, the T3SS is a massive molecular machine broadly comprised of two main subassemblies: a membrane-embedded needle-complex (NC) and a large cytosolic engine called the sorting platform (SP) (Figure 1C). The effective operation of the secretion system requires the proper assembly and coordination between these two structural modules. The NC acts as a syringe-shaped chassis, made up of a multi-ring base mounted in the bacterial envelope. The NC base anchors an extracellular needle-like structure that protrudes from the surface[9] with a length ranging from 20–150 nm depending on the bacterial species,[3] and that spans the distance between the bacterium and the eukaryotic host. The entire NC is traversed by a continuous hollow channel that provides a physical conduit for the passage of the unfolded T3S substrates.[10] Moreover, the multi-ring base lodges the export apparatus, a pseudo-helical structure that acts as a gate

TABLE 1 Nomenclature of sorting platform proteins in various T3SS-carrying bacterial species and their flagellar homologs.

Unified Sct name	Description	*Salmonella* SPI-1	*Salmonella* SPI-2	*Yersinia*	*Shigella*	*E. coli* (EPEC/EHEC)	*Rhizobium*	Flagelllum
SctD	Docking site for the SP	PrgH	SsaD	YscD	MxiG	EscD	Y4yQ	?[a]
SctK	Symmetry adapter	OrgA	SsaX (STM1410)	YscK	MxiK	EscK	NolU	?FliG[b]
SctQ	Pods protein	SpaO	SsaQ	YscQ	Spa33	EscQ	RhcQ	FliM/N
SctL	Cradle protein	OrgB	SsaK	YscL	MxiN	EscL	NolV	FliH
SctN	ATPase	InvC	SsaN	YscN	Spa47	EscN	RhcN	FliI
SctO	Central stalk	InvI	SsaO	YscO	Spa13	EscO	RhcZ	FliJ

[a]Sequence analysis and structural comparisons have found a limited degree of similarity between the flagellar FliF and SctD proteins.[67]
[b]Despite the absence of significant sequence similarity between SctK proteins and the flagellar FliG, they share a common function of anchoring their respective cytosolic complexes to the membrane-bound machinery. Moreover, the genes encoding these two proteins are generally located in the same genomic context between *sctJ* (*fliF*) and *sctL* (*fliH*).[68]

for the entry of the substrates into the secretion channel at the inner membrane.[11,12] T3S substrates carry an N-terminal secretion signal that allows recognition and transport through the export gate, with many having dedicated chaperones to maintain a secretion-competent state and escort them to the secretion apparatus.[13] Although the translocated effectors display a wide variety of enzymatic functions inside the host cell, they all share this common secretion mechanism.

Before entering the export gate, T3S substrates must first be recruited from the cytoplasm in a sequential manner by the secretion machine. To enter the secretion pathway, substrates need to dissociate from their cognate chaperone. Stripping of the chaperone accompanied by the unfolding of the associated substrate, allow for the travel of the unfolded substrate through the narrow needle channel.[14] These essential functions are carried out by the SP, a complex cytosolic engine that orchestrates the specific and ordered export of substrates.[15] In this review essay, we discuss the latest advances in SP structure and revise information about its assembly pathway. In addition, we discuss how this essential element of the injectisome may carry out its sorting function.

STRUCTURE OF THE SORTING PLATFORM

The post-assembly stability of the entire NC makes it suitable for isolation and single particle cryo-electron microscopy (cryo-EM) analysis. Therefore, significant progress has been made in unraveling its structural intricacies over the last two decades, culminating in atomic-level resolution structures of the entire NC.[16–19] However, our understanding of the architecture of the SP has been limited as this substructure is lost during the NC preparation, most likely due to its weak association with the NC base. This structural "labileness" makes challenging the direct application of single-particle cryo-EM analysis to study the SP.

Earlier studies focused on determining the location of the SctQ protein which, based on the similarity to the flagellar C-ring, was postulated to be the major component of the cytosolic complex. Using immunoelectron microscopy, SctQ was found to be located close to the bacterial cytoplasmic membrane just beneath the NC.[20] Live fluorescence microscopy further supported the perimembranous localization of SctQ in close association with the NC.[21] Biochemical analysis using LC-MS/MS revealed that the cytosolic proteins SctK, SctL, and SctN form a high-molecular-weight complex with SctQ[15] and ex vivo experiments established a serial SctK-SctQ-SctL-SctN-SctO chain of interactions[14,22–25] that closely resembles the FliG-FliM/N-FliH-FliI-FliJ complex in the flagellar C-ring.[26] Based on homology with the flagellar structure and in the absence of detailed structural information, the T3SS cytosolic complex was postulated to be arranged as a closed continuous ring.

The cryo-EM revolution, specifically the advances in cryo-electron tomography (cryo-ET), circumvented the need for harsh purification steps allowing for the direct visualization of the entire T3SS in its native environment. Applying genetically engineered bacterial minicells (Figure 1B), which are much thinner and thus yield higher resolution data, to a high throughput cryo-ET pipeline revealed the ultrastructure of the intact injectisomes of *Shigella flexneri*[27] and *Salmonella* Typhimurium[28] including the cytosolic components. These studies showed that the SP is organized as a chamber-like enclosure situated below the export gate (Figure 1C). In the *S.* Typhimurium pathogenicity island-1 (SPI-1) encoded T3SS, this structure measures 23 nm in height and 36 nm in width and consists of six discrete pods stemming from the NC that are arranged radially around a central nave. This central nave rests on a six-spoke-like structure or "cradle," which is in turn tethered to the injectisome by the six-fold symmetry scaffold provided by the pods (Figure 1C). The addition of traceable extra densities on the cytosolic components enabled the elucidation of the relative orientation and approximate position of each building block in the final assembled structure.[28] The pods are composed of SctK and SctQ proteins, with SctK mapping to the most membrane-proximal region and SctQ accounting for the bulk density of the rest of the pods. The spokes of the cradle are made up of SctL, while the central nave density is attributed to the hexameric ATPase SctN. The central stalk SctO penetrates the pore of the SctN hexamer physically linking the

ATPase activity to the multiring entrance of the export gate formed by an SctV nonamer. The overall chamber-like shape of the SP provides thus a structural scaffold to put in register the ATPase with the export gate offering a unique and privileged compartment where the sorting and initiation of substrates can occur.

Recently, the *Yersinia* injectisome was visualized in situ, showing noticeable morphological differences in its SP compared to those of *Shigella* and *Salmonella*.[29] While the cytosolic components of the *Yersinia* T3SS also form a chamber-like structure, the cytosolic platform appears as a more closed and continuous ring-like structure instead of the well-defined six discrete pods in the other bacteria. Taking into account that the *Yersinia* injectisome belongs to a distinct phylogenetic clade compared to *Shigella* and *Salmonella*,[5] this structural variation could indicate that there exists species-specific adaptation in the cytosolic ensembles of distinct bacteria with T3SS. Alternatively, the observed structural differences may simply reflect the snapshot of fully occupied SPs in action (see below *Orchestrating the secretion from the inside: the cytosolic complex as an SP*). Additionally, cryo-ET visualization of *Chlamydia trachomatis* injectisomes has shown that while the six-fold symmetry in the SP is conserved within this distant clade, it becomes stabilized upon contact with the host cell.[30] Future efforts to visualize the SP of phylogenetically distant injectisomes in different activation states will be essential to investigate the extent of their structural diversity.

In addition to the in situ visualization of the injectisome, which has yielded valuable insights into the overall architecture of the cytosolic components, high-resolution structures of individual proteins and partial complexes have shed light on the atomic details that cryo-ET alone is not able to capture. Specifically, the structures of the cytosolic domain of SctD and SctK individual monomer proteins[31–33] have contributed to our understanding of the structural organization of these pieces of the pods. Also, both the partial structure of the SctQ-SctL complex[34] and the partial structure of the central stalk in complex with the hexameric ATPase SctN[35] and with the soluble domain of SctV[36] were determined, providing important insights into the interactions that make up the SP. Despite these advances, the molecular details and the precise oligomeric composition of the pods and cradle remain largely uncertain. In an effort to fill in the gaps on how the SP building blocks are pieced together, we combined the predictive power of AlphaFold2[37,38] with an in vivo cross-linking strategy to provide an in-depth map of the interaction interfaces occurring in the SP.[39] The in vivo photocrosslinking strategy is based on the genetically encoded UV-photoreactive amino acid *p*-benzoyl-L-phenylalanine (*p*Bpa), which enables the tracking of protein-protein interactions in living cells at high spatial resolution.[40] This unnatural amino acid has a reactive radius of ~3.1 Å essentially allowing to define interactions at the residue level.[41] Furthermore, the in vivo crosslinking strategy offers distinct advantages over in vitro approaches, such as pull-downs and two-hybrid assays, previously utilized for studying the SP composition. By investigating the protein-protein interaction network of the SP under physiological conditions and at native levels of expression, we not only ensured the physiological relevance of the observed interactions but also enabled the detection of transient interactions within the dynamic cellular environment. Such strategy allowed us to gain a deeper understanding of how the SP subunits interact with each other, shedding new light on the structural intricacies of this functionally important cytosolic module (see below).

BUILDING BLOCKS OF THE SORTING PLATFORM

The inner membrane ring SctD

The SctD protein is a single-pass membrane protein with an N-terminal cytoplasmic domain and a prominent and rigid C-terminal periplasmic domain that plays a crucial role in the formation of the NC. Together with the lipoprotein SctJ, SctD forms a 24-mer inner membrane ring that associates with the outer membrane secretin SctC to establish the membrane base of the NC (Figure 1C). Although it is not formally considered part of the so-called soluble components, SctD plays a fundamental role in the assembly of the SP, as its N-terminal cytosolic domain ($SctD_N$) acts as the link between the NC and the SP. $SctD_N$ folds into a forkhead-associated (FHA) domain that provides the physical interface to properly connect the SP to the NC (Figure 1C,D). FHA domains are small protein-protein binding modules (~100 amino acids), structurally consisting of a β-sandwich domain formed by two β-sheets. The inter-strand loops connecting the β-strands display a high diversity in sequence and typically provide specificity toward phosphopeptides.[42] However, in most SctD proteins, the canonical FHA residues responsible for phospho-recognition are poorly conserved, suggesting that the binding mechanism of SctD is independent of phospho-recognition.[33] Further evidence for the distinct binding mode of SctD comes from the absence of phenotype when the SctD amino acids equivalent to the proposed canonical phospho-binding residues are mutated.[32,33] Additionally, we have shown that the non-canonical binding mode of SctD to SctK involves not only the interstrand loops but also one of the lateral faces of the SctD FHA domain.[39] These findings support a docking mechanism for SctD distinct from the classical FHA domains.

The symmetry adapter SctK

The soluble protein SctK is positioned in the most proximal region of the SP pods in close proximity to the base of the NC (Figure 1C). SctK direct binding to the FHA domain of SctD allows the tethering of the entire SP to the NC.[28] Moreover, SctK plays a pivotal role in adapting the 24-mer symmetry of the otherwise continuous $SctD_N$ ring to the overall six-fold symmetry of the SP[28] (see *Biogenesis of the SP*). Despite its importance for T3S, SctK is arguably the least studied component of the SP, partly due to its reluctance to be isolated in vitro. However, in a recent study, Muthuramalingam et al. were able to purify and solve the first atomic structure for any member of this protein family.[31] This work revealed that SctK is mostly composed of α-helices that fold into a unique globular kidney-shaped structure that does not resemble any other known protein folds (Figure 1D). Notably, a single SctK monomer

fits well into the top part of the cryo-ET densities that correspond to the pods, indicating that it can fulfill its role as a structural linker as a monomer. Such organization is consistent with the estimated in situ stoichiometry of six copies of SctK per injectisome.[43,44] Furthermore, in vivo photo-crosslinking experiments showed that a single SctK monomer simultaneously interacts with two $SctD_N$ on one side and one SctQ on the other, through interfaces that are located on opposite sides of the SctK protein,[39] directly linking the NC to the SP.

Noticeably, both SctK and $SctD_N$ exhibit the highest sequence variation among the T3S SP's building blocks and, unlike SctQ, SctL, SctN, and SctO, these two proteins do not have clear homologs counterparts in the flagellar system.[6] This evolutionary divergence is intriguing, as it may account for the gross structural difference observed between the discrete arrangement of the SP pods in the injectisome and the annular-like C-ring in the flagellar system. One could hypothesize that the distinctive pod-like structure of the injectisome is due to the particular arrangement of these two T3SS-specific proteins. Undoubtedly, these structural differences reflect the varying functionalities of the bacterial machines in question. While the flagellum is specifically adapted for motility and requires rotation, the virulence T3SS is dedicated to deliver effectors into the eukaryotic host and does not require rotational motion.

SctQ, the major component of the SP

Located just beneath the SctK-associated density, the SctQ protein is the core structural component of the cage-like structure as it builds up the central region of the pods (Figure 1C). In most of the archetypical T3SS studied so far, the *sctQ* gene typically encodes for two tandemly translated products: a full-length protein ($SctQ^L$) and a shorter isoform ($SctQ^S$) that comprises the final third of the full-length protein and that is the result of an internal translation start site.[34,45–47] $SctQ^L$ and $SctQ^S$ are thought to be homologs to the flagellar FliM and FliN proteins, respectively, which in turn are the core building blocks that make up the flagellar C-ring. Structural and functional studies have mostly focused on the C-terminal half of $SctQ^L$, showing that this domain possesses two surface presentation of antigens domains (named SPOA1 and SPOA2) critical for T3S. The SPOA1 and SPOA2 domains engage in intramolecular heterotypic interactions to provide a structural scaffold for the interaction of SctQ with SctL (Figure 1D). On the other hand, $SpaO^S$, which encompasses only the SPOA2 domain, forms a homodimer that associates with $SctQ^L$ resulting in the formation of $SctQ^L$-$2SpaO^S$ heterotrimeric complexes, which in turn can form stable complexes in vitro with SctL and SctN.[48] The essential role of $SctQ^S$ for T3SS function in *Yersinia*,[46] *Shigella*,[49] and *Xanthomonas*[50] suggests that this isoform is an integral part of the injectisome. However, in the case of *Salmonella* SPI-1[47] and SPI-2[45] encoded injectisomes, the absence of $SctQ^S$ results in a rather mild T3SS phenotype, suggesting a chaperone-like role promoting $SctQ^L$ stability rather than a structural role. While these discrepancies could be attributed to species-specific adaptations, it is conceivable that the observed differences could be the result of different experimental approaches. For example, in the case of *Yersinia*, T3SS function is assayed by the low calcium response. Switching *Yersinia* to calcium free medium and 37°C, results in a massive activation of the T3SS resulting in growth arrest. This experimental approach, likely quite different from a physiological activation, bears little resemblance to the experimental approaches in *Salmonella* and could explain the phenotypic differences observed. Therefore, more sensitive, and quantitative experimental approaches that more closely resemble the in vivo situation, are needed to determine whether $sctQ^S$ mutants in *Yersinia* and *Shigella* result in a complete loss-of-function phenotype or if there is some residual activity that contributes to T3SS functionality, similar to what has been observed in both SPI-1 and SPI-2 *Salmonella* injectisomes. Further studies are warranted to determine the precise role of $SctQ^S$ in T3SS function.

Although much less is known about the N-terminal region of $SctQ^L$, mutagenesis studies have identified critical amino acids in the first third of this protein.[47] In addition, our recent AlphaFold2-guided crosslinking studies revealed that the N-terminal region of $SctQ^L$ contains the binding domain for SctK,[39] which is in agreement with the topology observed in the cryo-ET data indicating that the N-terminal region of $SctQ^L$ faces the SctK density.[28]

Besides its structural role in making up the bulk densities of the pods, it has been shown in *Yersinia* that $SctQ^L$ exhibits a continuous dynamic exchange between the injectisome-bound state and a freely diffusing cytosolic pool.[51] Although this dynamic behavior is posited to be correlated with T3SS activity, it is not clear whether the cytosolic SctQ pool has a specific function or what that function might be. Fluorescence microscopy and single-molecule super-resolution measurements have provided an estimated stoichiometry of ~24 subunits of $SctQ^L$ bound to each injectisome.[43,51] However, based on the volume density attributed to each pod in the cryo-ET map, it seems unlikely that each pod can accommodate four $SctQ^L$ subunits. Furthermore, although the precise composition of the SP remains unknown, in vivo photo-crosslinking experiments have shown that at least one $SctQ^L$ subunit is simultaneously bound to both SctK and SctL through its amino- and carboxyl-terminal domains, respectively.[39] It is thus tempting to speculate that the pods visualized as cryo-ET densities, may represent a non-dynamic subpopulation of $SctQ^L$ stably committed to making the body of the pods, while the extra estimated subunits may account for a more dynamic subset of StcQ subunits freely exchanging between the cyotosol and the pods without becoming structural elements in the SP.

The ATPase complex

Emanating from the bottom of each pod, a spoke-like homodimer of the SctL protein projects inward creating a wheel-like scaffolding structure known as the "cradle" (Figure 1C). SctL features an elongated shape with a disordered N-terminal region that engages directly with the SPOA1-SPOA2 domains of $SctQ^L$,[34] and a globular domain in the C-terminal region that mediates its association with the ATPase SctN. Although the molecular details of the SctL-SctN interaction are not yet known, the crystal structure of the related FliH-FliI complex in

the flagellar system reveals that two FliH (SctL) monomers, each in a unique different conformation, bind simultaneously to a single FliI (SctN), thereby serving as a linker where the ATPase rests.[52] The ATPase SctN in turn, forms a homohexameric ensemble atop the cradle, aligned about ~10 nm beneath the multiring cytoplasmic conduit created by SctV (Figure 1C). SctN contains 3 sub-domains: an N-terminal oligomerization domain, a central catalytic domain featuring a Rossman fold, and a C-terminal domain that lines the central pore of the structure. This latter domain is oriented toward the export gate and is known to interact with substrate/chaperone complexes.[14] SctN binds to and dissociates the substrate/chaperone complexes prior to secretion, resulting in the subsequent unfolding of the secreted substrate to facilitate its passage through the narrow export channel.[14] A single SctO protein penetrates into the central cavity of the SctN hexamer crowning the ATPase complex (Figure 1C,D).[35] SctO adopts an extended antiparallel coiled-coil structure that spans the distance between SctN and SctV acting as a central stalk, coupling the energetics of ATP hydrolysis to the PMF-driven translocation of substrates through the export gate.

BIOGENESIS OF THE SORTING PLATFORM

The assembly of the SP is a highly regulated process that relies on the precise coordination between multiple membrane-associated and cytosolic building blocks. To form this large multi-subunit megacomplex with a mass exceeding 1 MDa in size, at least five cytosolic proteins (SctK/Q/L/N/O), each with unique stoichiometry, must work in concert. One of the first attempts to understand the structural requirements for SP assembly was provided in *Yersinia*. By combining fluorescent-labeled SctQ with an array of mutations in other injectisome genes, that study determined that SctQ recruitment to the assembled apparatus depends on the integrity of both the membrane-ring proteins and all the other soluble structural components, except for the central stalk SctO.[21] A follow-up study reported that the cytosolic platform proteins within the *Yersinia* injectisome form a highly interdependent network, as the proper localization of SctK and SctL also relied on the presence of the other soluble components.[44] Later studies using super-resolution imaging and in situ cryo-ET have provided a more detailed insight into the assembly process of the SP in the *S.* Typhimurium SPI-1 encoded T3SS. These studies showed that, in *Salmonella*, the presence of its direct binding partners SctK and SctL is sufficient for SctQ to form the pods of the SP.[28,43] On the other hand, the absence of the central components, SctN and SctO, has a negligible impact on the formation of the SP cage, indicating that these peripheral components have a minor role in scaffolding the cage and may be incorporated at later stages.[28,43] Interestingly, similar observations have also been made in the flagellar C-ring, where the absence of the ATPase results in the C-ring structure being present, albeit in a more flexible state.[53]

The in situ cryo-ET structure also unveiled the structural rearrangements that reconcile the disparity between the 24-fold symmetry of the NC and the overall 6-fold symmetry of the SP. In the absence of the SP, the cytosolic domain of SctD ($SctD_N$) exhibits a 24-mer solid ring configuration similar to what is observed in isolated NCs. However, when the SP plugs into the cytosolic portion of SctD, the $SctD_N$-ring structure reorganizes into six circularly disposed discrete patches, with each patch aligned to accommodate one of the SP pods.[28] The possible mechanistic basis for this rearrangement has been recently provided by in vivo photo-crosslinking coupled to structural modeling studies. A single SctK molecule was shown to bind two adjacent $SctD_N$ subunits, each arranged in an opposite orientation. This binding event elicits then a conformational change in the two neighboring non-bound $SctD_N$ subunits, bringing them closer together to form the 4-mer patches. This "domino effect" serves as the pivotal step in the rearrangement process, causing the 24-mer perfect ring to rearrange into the six discrete patches observed in the cryo-ET structure.[39] This mechanism solves the symmetry mismatch between these two structures that need to interact. In *Shigella*, it has been suggested that such remodeling does not take place.[54] Nevertheless, this hypothesis becomes challenging to reconcile with the clear requirement for such rearrangement to happen in order to align the distinct symmetries exhibited by the inner ring of the NC and the SP.

Although the aforementioned approaches allowed the examination of the structural requirements for assembling the injectisome-bound components, these studies were limited in their ability to provide information regarding the assembly state of specific pairwise subcomplexes. Employing an in vivo photo-crosslinking strategy, we were able to identify residues implicated in the $SctD_N$-SctK, SctK-SctQ, SctQ-SctL, and SctL-SctN pairwise interfaces.[39] Subsequently, these paired interactions were utilized as reporters for SP assembly, facilitating the systematic assessment of the SP structural integrity. The detection of a crosslink indicates that a specific protein-protein assembly step has occurred. Therefore, by assessing the crosslinking pattern in relevant mutant backgrounds, we were able to dissect the SP assembly pathway (Figure 2).[39] Applying this strategy, we discovered that the association between the cytosolic interface of $SctD_N$ and the symmetry adapter protein SctK occurs regardless of the presence of other cytosolic components. Similarly, we found that the binding of SctQ to SctL does not require any other SP component. These findings suggest that the formation of the SctD-SctK and SctQ-SctL intermediate subcomplexes represents the earliest steps in SP formation and may initiate the entire assembly process. Consistent with this notion, we observed that SctQ requires prior binding to SctL before being recruited to SctK. This observation explains the previously puzzling findings that the association of SctQ to the NC is disrupted, resulting in SctQ becoming predominantly cytosolic when SctL is absent[21,28,43] or unable to interact with SctL.[34] While the SctQ-SctK interaction has been reported in vitro,[22,54] our in vivo findings suggest that SctL binding triggers a conformational change at the N-terminus of SctQ that facilitates its engagement with SctK. Thus, preformed SctQ-SctL subcomplexes are recruited to the NC-bound SctK platform, facilitating the formation of the pods (Figure 2). The assembly of the pods then results in the formation of the cradle structure, which caps the SP on its cytoplasmic side and offers a docking site for the incorporation of the SctN ATPAse subunits. Our studies also indicate that the formation of

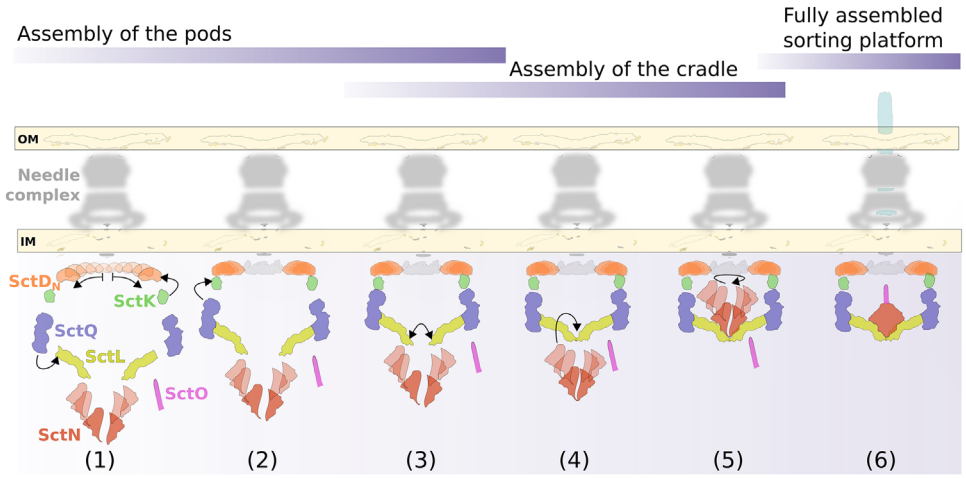

FIGURE 2 Progressive assembly of the T3S sorting platform. (1 and 2) The assembly process begins with two independent events: SctK binding to SctD$_N$ triggering the reorganization of SctD$_N$ into discrete patches; and SctL engagement to SctQ, forming a stable cytosolic complex competent for binding to SctD-bound SctK. (3) The assembly of the pods facilitates then the capping of the SctL cradle. (4 and 5) Recruitment of SctN subunits to the fully assembled cradle promotes its oligomerization and the subsequent recruitment of SctO, (6) leading to the formation of a functional type III secretion sorting platform. IM, inner membrane; OM, outer membrane.

the SctL cradle precedes the recruitment and hexamerization of SctN subunits, suggesting that the previously proposed SctL-SctN cytosolic subcomplexes may not occur in vivo.[55] Finally, at an unknown point and by an as-yet unexplored mechanism, the central stalk SctO is recruited, resulting in the assembly of a fully functional T3SS.

ORCHESTRATING THE SECRETION FROM THE INSIDE: THE CYTOSOLIC COMPLEX AS A SORTING PLATFORM

One of the most exciting unresolved questions in T3SS biology is how the T3S substrates find their way to the secretion channel. T3S effectors are delivered directly from the bacterial cytosol into the mammalian cell, bypassing the extracellular medium. Therefore, T3S is necessarily a hierarchical process, with T3S client proteins that can be broadly classified into early, intermediate, and late substrates based on the temporal engagement of the secretion pathway (Figure 3A). The first substrates of the incipient T3SS are the proteins SctI and SctF that will make the inner rod and needle, respectively, of the complete injectisome along with some regulatory proteins needed to assemble the filament substructure. Once the inner rod and needle substructures are completed, the tip protein SctA must then be secreted prior to any other substrate to form the tip complex. The tip complex, consisting of five SctA molecules,[56] senses the presence of a suitable target cell and signals the bacterial cytosol to initiate the secretion process. Upon contact with the cell, the substrates known as the translocases (SctE and SctB) are secreted leading to the formation of a eukaryotic membrane bound structure known as the translocon.[57] This structure remains anchored to the T3S needle, bridging the bacterial and eukaryotic cytosol. With the translocon in place, the direct translocation of effector proteins can now proceed.

The position of the cytosolic engine in direct contact with the cytosolic side of the NC suggests that it may play a role in the sorting and sequential loading of substrates destined to be secreted and effectors proteins destined to be translocated into the host cell cytosol. Insights derived from biochemical and genetic studies conducted in *S.* Typhimurium have shed light on the active role of the cytosolic components in orchestrating this process.[15] Affinity purification of SctQ from *Salmonella* strains poised for secretion, followed by LC-MS/MS and BN-PAGE studies, allowed to identify that, in addition to the structural components SctK/L/N, SctQ is occupied mostly by translocases (intermediate substrates), while effectors (late substrates) are largely absent. Notably, emulating the activated state of the T3SS by genetically deleting the translocases led to increased levels of effectors associated with SctQ. These observations indicate that SctQ, in conjunction with its associated cytosolic components, serves as an SP where distinct substrates categories are sequentially queued for their orderly secretion.

Proteins destined to the secretion pathway are associated in the bacterial cytosol to dedicated chaperones required for the secretion and in some cases the stability of the cognate proteins.[13] Experiments performed with the *S.* Typhimurium SPI-1 T3SS suggested that the chaperones are an essential element on the recruitment of substrates to the SP and that the secretion hierarchy may be the result of different affinities of distinct chaperone/substrate complexes for the SP.[15] However, the precise mechanism by which the SP performs its function remains obscure and needs to be studied in more detail. The SP ATPase, SctN, organizes as a hexamer on the cradle space formed by the SP protein SctL, and it is in register with the SctV multiring that paves the way towards the export gate.[11] Previously, it was shown that the SctN induces chaperone release and unfolding of the cognate secreted protein.[14] The position of the SctN hexamer in register with the secretion conduit facilitates the entrance of the unfolded effector

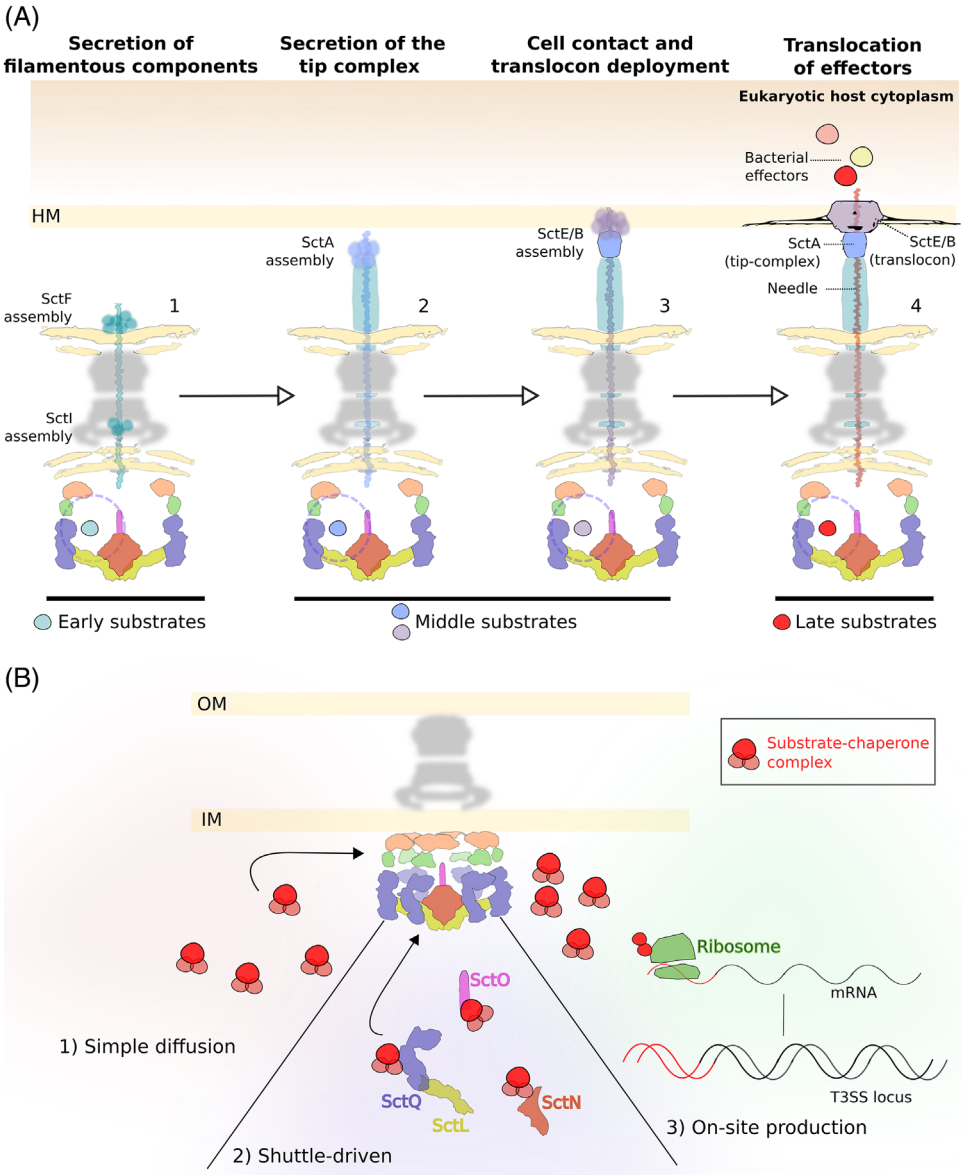

FIGURE 3 Mechanisms of substrate recruitment by the sorting platform. (A) Sequential loading of T3S substrates onto the sorting platform. (1) Initially, the cytosolic sorting platform engages the early substrates SctI and SctF, which form the inner rod and extracellular needle, respectively. (2) Once the needle is completed, the tip protein SctA is loaded onto the sorting platform and initiated into the T3 secretion pathway to form the pentameric tip complex. (3) Tip-host cell contact triggers then the secretion of the SctB and SctE translocases leading to the assembly and deployment of the translocation pore into the target host membrane (HM). (4) Once this direct conduit bridge has been established, the sorting platform becomes competent for loading effector proteins, and the T3SS is ready for deliver bacterial effectors into the host cell. (B) Proposed scenarios for targeting T3 substrates to the sorting platform. (1) T3 substrates escorted by cognate chaperones freely diffuse in the cytosol until they encounter an available sorting platform. (2) Substrate/chaperone complexes are recruited by the pool of free cytosolic components that act as dynamic carriers, facilitating their handover to the injectisome-bound cytosolic platform. (3) T3 substrates are produced on-site, in close proximity to the T3S machinery, leading to a localized region of high substrate concentration. This model demands the colocalization of the translation machinery with the membrane-bound injectisome, similar to what was observed in the T3SS2 of *V. parahaemolyticus*.[59] IM, inner membrane; OM, outer membrane.

into the secretion channel. A fascinating question that arises then is how the chaperone/effector complexes are brought to the ATPase for unfolding and secretion? Complexes of some SP proteins can be detected freely diffusing in the bacterial cytosol,[58] it is possible that these complexes usher chaperone/effector complexes to the SP for secretion. Consistent with this hypothesis, it has been proposed that the dynamic interchange between the injectisome-bound SctQ and the cytosolic pool could potentially reflect a shuttle-like mechanism, wherein SctQ facilitates the handover of cytosolic substrates to the injectisome.[51] However, it is challenging to reconcile the observed pace of exchange of the cytosolic components with the reported rate of substrate secretion.[51] Moreover, it remains unclear why the chaperone/substrate pair could not efficiently diffuse by itself toward the SP (Figure 3B). Nevertheless, the shuttle-mediated recruitment of substrates is an attractive model that invites further exploration in a broader range of T3SS-carrying bacteria.

There is recent evidence that suggests that in the case of *Vibrio parahaemolyticus*, the expression, translation, and assembly of its T3SS2 co-occurs next to the membrane in a process known as transertion, upon activation by the presence of bile salts.[59] Thus, effectors can potentially be produced on-site, bypassing the need for recruitment to the injectisome (Figure 3B). This is possible in the case of *V. parahaemolyticus* because the transcriptional regulator responsible for the expression of its T3SS2 is membrane bound. Although in bacteria lacking membrane-embedded T3SS's regulators the substrates seem to be distributed within the cytoplasm,[43] it remains an attractive hypothesis worthy of study since it would clearly simplify the model for recruitment and selection of substrates and effectors.

CONCLUSIONS AND OUTLOOK

Since its discovery 30 years ago, significant progress has been made in elucidating the structure and function of the T3SS. The SP is an essential and conserved feature of the T3SS, making it an appealing target for drug development. However, due to its intricate composition and difficult isolation, our understanding of the SP has lagged that of the rest of the T3SS machinery. The advent of cryo-ET provided a unique window into the architecture of the SP, enabling us to visualize its overall structure.[27,28] However, the current in situ data only offers low-resolution maps, limiting our ability to resolve the atomic spatial arrangement of the cytosolic complex. Thereby, to fully understand the mechanisms behind T3SS, it is of utmost importance to obtain the structural details of the SP at atomic or near-atomic scale. Moreover, recent advances in accurate protein structure prediction[37] have significantly accelerated structure-function studies of molecular machines, including the T3SS. Integrative approaches that combine complex modeling and in vivo protein-protein interaction data have begun to shed light on how the multiple subunits that make up the SP are pieced together and the chronological events that culminate in the assembly of this intricate machinery.[39] As we continue to witness the advances in AI-guided protein design, we anticipate that defining the SP and other interfaces within the T3SS will have important translational applications. These contact points represent ideal targets for the rational design of inhibitors capable of specifically blocking the assembly of virulence-related secretion systems.

Substrates that are destined to travel through the injectisome pathway interact directly, or through cognate chaperones, with several cytosolic components of the T3SS, including SctQ,[20,60] SctN,[14,61] SctO,[62–64] and the cytosolic domain of SctV[65,66] (Figure 3B). However, the exact route of trajectory that substrates follow from their synthesis to their entry into the export gate remains unclear. Future studies employing live-cell imaging among other in vivo strategies will be needed to reveal the itinerary of substrate trafficking and the precise role of the SP in orchestrating this process.

All in all, despite its sophisticated and dynamic nature, by integrating multidisciplinary approaches, we are beginning to unlock the secrets hidden within the chamber of the SP.

ACKNOWLEDGEMENTS

Jose Eduardo Soto was supported in part by the Pew Latin American Fellows Program in the Biomedical Sciences. This work was supported by grant AI126158 to María Lara-Tejero.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

María Lara-Tejero https://orcid.org/0000-0002-1339-0859

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How to cite this article: Soto, J. E., & Lara-Tejero, M. (2023). The sorting platform in the type III secretion pathway: From assembly to function. *BioEssays*, *45*, e2300078. https://doi.org/10.1002/bies.202300078