Trends in Biochemical Sciences

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Review

Type III Secretion: Building and Operating a Remarkable Nanomachine

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The Type III secretion system (T3SS) is a protein export pathway that is widespread in Gram-negative bacteria and delivers effector proteins directly into eukaryotic cells. At its core lie the injectisome (a sophisticated transmembrane secretion apparatus) and a complex network of specialized chaperones that target secretory proteins to the antechamber of the injectisome. The assembly of the system, and the subsequent secretion of proteins through it, undergo fine-tuned, hierarchical regulation. Here, we present the current understanding of the injectisome assembly process, secretion hierarchy, and the role of chaperones. We discuss these events in light of available structural and biochemical dissection and propose future directions essential to revealing mechanistic insight into this fascinating nanomachine.

Trends

The type III secretion system (T3SS) is essential for bacterial pathogenesis, symbiosis, and motility.

At its core, the T3SS contains a complex injectisome, built of >20 proteins that need to be properly assembled into a functional unit.

Injectisome biogenesis and T3S undergo sophisticated, multistepped regulation.

Elucidating the molecular mechanism of T3S stands to benefit from *in vitro* assays to mechanistically dissect the process.

From Essential to Specialized Bacterial Protein Secretion Systems

Bacterial ribosomes synthesize up to ~8000 different proteins [1]. Almost half of these become embedded in membranes and are secreted to the cell wall or to the external milieu [1–4]. Many bacterial processes, such as DNA replication, motility, transport, antibiotic resistance, scavenging of chemicals, and pathogenesis, depend on protein secretion. Many specialized protein export systems have evolved to tackle these processes [4]. Some of these systems allow proteins to be fully released extracellularly ('secretion'), and sometimes even inside host cells. The T3SS is widespread in many Gram-negative bacteria, including symbionts, such as *Rhizobium*, and pathogens that are responsible for a range of severe diseases, such as plague (*Yersinia pestis*), typhoid fever (*Salmonella typhi*), gastroenteritis (*Shigella flexneri*), and infantile bacterial diarrhea (enteropathogenic *Escherichia coli*; EPEC) [5].

T3S is essential for the pathogenic potential of Gram-negative bacteria by delivering essential 'effectors', such as toxins and enzymes, into the eukaryotic cytoplasm. Remarkably, some of these effectors travel across even more membranes inside the host, such as into mitochondria [6]. Some bacteria have more than one T3SS and synthesize up to hundreds of copies of the 30–40 T3SS proteins [5] (Table 1). Synthesis and export of these molecules need to be coordinated so that the injectisome can be properly assembled and become functional; synthesis of the proteins that build it and/or use it follows a strict hierarchy [7]. Thus, protein secretion through the T3SS is a highly regulated multistep process, making it one of the most complex bacterial protein secretion systems known.

Form and Parts of the Injectisome

The T3SS contains at its core the injectisome, a nanosyringe–like structure that transfers effectors into the host cell cytoplasm (Figure 1A). Overall, injectisomes share high structural

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Table 1. Unified Nomenclature, Localization, and Function of the T3S Injectisome and Flagellum Components[a]

		Hosts											Flagellar Apparatus
		Humans and Animals								Plants			
Common Name (Sct)	Function	EPEC/EHEC	*Pseudomonas aeruginosa*	*Yersinia*	*Shigella flexneri*	*Salmonella* sp. SPI-1	*Salmonella enterica* SPI-2	*Chlamydia pneumoniae*	*Burkholderia pseudomallei*[b]	*Pseudomonassyringae*	*Ralstonia solanacearum*	*Xanthomonas* spp.	
Extracellular Components													
SctF	Needle	EscF	PscF	YscF	MxiH	PrgI	SsaG	CdsF	BsaL	HrpA	HrpY	HrpE	/
$SctA_{t/f}$[c]	Tip/filament	$EspA_f$	$PcrV_t$	$LcrV_t$	$IpaD_t$	$SipD_t$	$SseB_t$	CT584 or $LcrV_t$	$BipD_t$	/[e]	/	/	$FliC_f$
SctB[d]	Translocator	EspB	PopD	YopD	IpaC	SipC	SseD	CopD1/2	BipC	/	XopA	XopA	/
SctE[d]	Translocator	EspD	PopB	YopB	IpaB	SipB	SseC	CopB1/2	BipB	HrpK	PopF1/ PopF2	HrpF	/
Basal Body													
SctC	OM ring	EscC	PscC	YscC	MxiD	InvG	SsaC	CdsC	BsaO	HrcC	HrcC	HrcC	FlgI; FlgH
/	Pilotin	/	ExsB	YscW	MxiM	InvH		/		/		/	/
SctI	Inner rod	EscI	PscI	YscI	MxiI	PrgJ	SsaI	/	BsaK	HrpB	HrpJ	HrpB2	FlgB; FlgC; FlgF; FlgG
SctD	IM ring	EscD	PscD	YscD	MxiG	PrgH	SsaD	CdsD	BsaM	HrpQ	HrpW	HrcD	FliG
SctJ	IM ring	EscJ	PscJ	YscJ	MxiJ	PrgK	SsaJ	CdsJ	BsaJ	HrcJ	HrcJ	HrcJ	FliF
SctR	Export apparatus	EscR	PscR	YscR	Spa24 (SpaP)	InvL/SpaP	SsaR	CdsR	BsaW	HrcR	HrcR	HrcR	FliP
SctS	Export apparatus	EscS	PscS	YscS	Spa9 (SpaQ)	SpaQ	SsaS	CdsS	BsaX	HrcS	HrcS	HrcS	FliQ
SctT	Export apparatus	EscT	PscT	YscT	Spa29 (SpaR)	InvN/SpaR	SsaT	CdsT	BsaY	HrcT	HrcT	HrcT	FliR
SctU	Export apparatus	EscU	PscU	YscU	Spa40 (SpaS)	SpaS	SsaU	CdsU	BsaZ	HrcU	HrcU	HrcU	FlhB
SctV	Export apparatus	EscV	PcrD	YscV	MxiA	InvA	SsaV	CdsV	BsaQ	HrcV	HrcV	HrcV	FlhA

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Table 1. (continued)

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Common Name (Sct)	Function	EPEC/EHEC	*Pseudomonas aeruginosa*	*Yersinia*	*Shigella flexneri*	*Salmonella* sp. SPI-1	*Salmonella enterica* SPI-2	*Chlamydia pneumoniae*	*Burkholderia pseudomallei*[b]	*Pseudomonassyringae*	*Ralstonia solanacearum*	*Xanthomonas* spp.	
Cytoplasmic Components													
SctQ	Cytoplasmic ring	SepQ	PscQ	YscQ	Spa33	SpaO	SsaQ	CdsQ	BsaV	$HrcQ_A$/$HrcQ_B$	HrcQ	$HrcQ_A$; $HrcQ_B$	FliM; FliN
SctL	Stator	EscL	PscL	YscL	MxiN	OrgB	SsaK	CdsL	OrgB	HrpE	HrpF	HrcL	FliH
SctN	ATPase	EscN	PscN	YscN	Spa47	InvC	SsaN	CdsN	BsaS	HrcN	HrcN	HrcN	FliI
SctO	Stalk	EscO	PscO	YscO	Spa13	InvI	SsaO	CdsO	HrpD or BsaT	HrpO	HrpD	HrpB7	FliJ
SctP	Molecular ruler	EscP	PscP	YscP	Spa32	InvJ	SsaP	CdsP	BsaU	HrpP	HpaP	HpaC	FliK
SctW	Gate-keeper	SepL	PopN	YopN/TyeA	MxiC	InvE	SsaL	CopN	BsaP	HrpJ	HpaA	HpaA	/
/	Regulatory compnent	SepD	/	/	/	/	SpiC	/	/	/	/	/	/
SctK	ATPase co-factor	/	PscK	YscK	MxiK	OrgA	/	/	OrgA	HrpD	/	/	/
Chaperones													
/	For early substrates	EscE; EscG	PscE; PscG	YscE; YscG				CdsE; CdsG					
/	For middle substrates	CesAB; CesD; CesD2	PcrG; PcrH	LcrG; SycD; SycB	IpgC; IpgC	SicA	SseA; SsaE	LcrH					FliS; FliT
/	For late substrates	CesT; CesF	SpcU; SpcS	SycE; SycT; SycH; SycN; YscB; YsaK	IpgE; Spa1; IpgA	SicP; SigE; InvB; SigE	SrcA; SscB	SycE	BPSS151	ShcA; ShcM; ShcF; ShcV; ShcO1; ShcS1; ShcS2	HpaB	HpaB	

[a]Only some T3S systems, better-characterized biochemically, from a few bacteria are included in this table. This table including active links and schematic diagrams of the injectisome can be accessed at the Subcellular Topology of E. coli Polypeptides Database (STEP db, http://stepdb.eu).
[b]*B. pseudomallei* can also infect plants.
[c]SctA proteins are 16–60% identical and highly similar between different pathogenic T3Ss and share 15–30% homology with the flagellin component FliC. We propose to rename them collectively 'SctA'. Despite homologies, they form morphologically different structures. $SctA_f$ stands for filament proteins, and the $SctA_t$ for tip protein.
[d]SctB and SctE, proposed here as unified names for the translocators were not part of the original proposal [9].
[e]'/' indicates that there is no homologous protein known.

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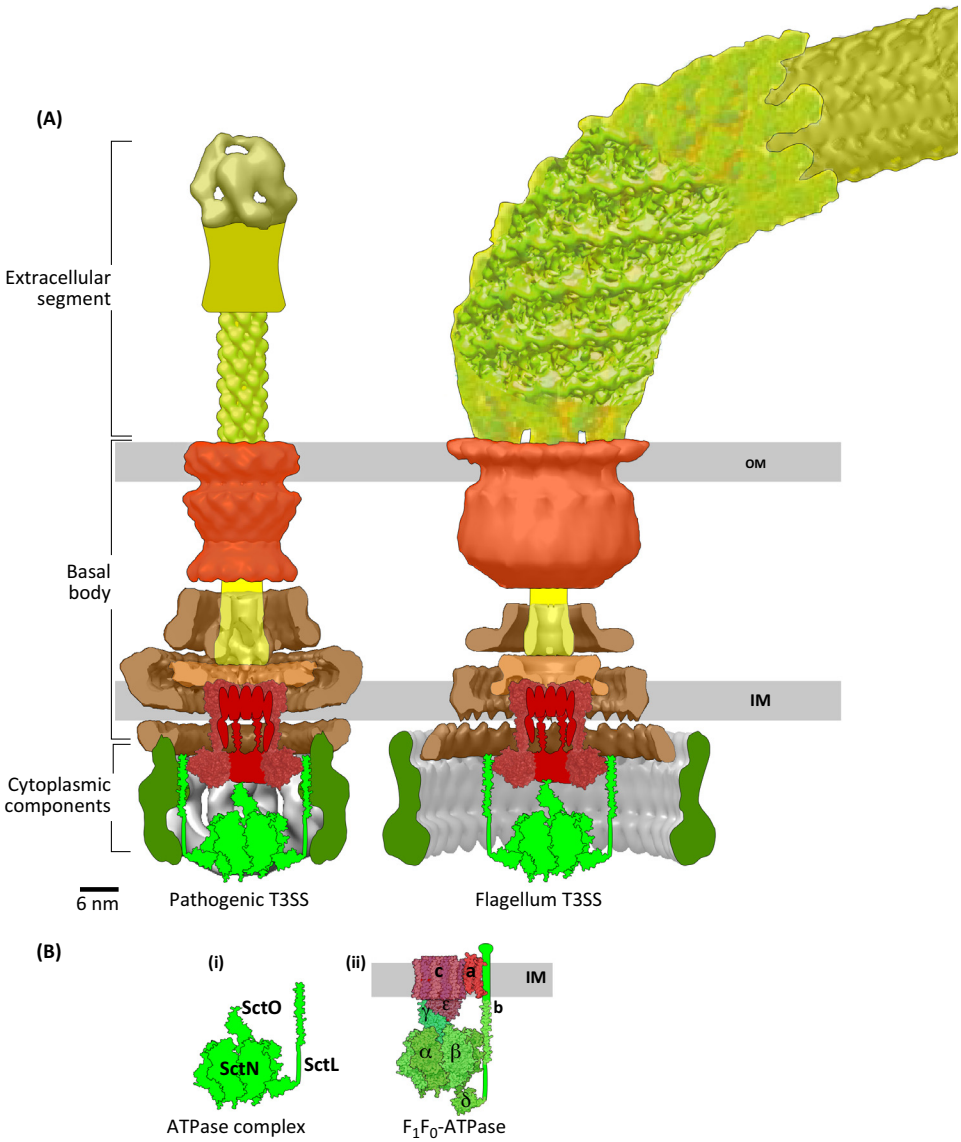

Figure 1. Schematic Comparison of the Type III Secretion (T3S) Injectisome with a Flagellum and the F_1F_0 ATPase. (A) Protein complexes were designed using electron microscopy (EM) structures as a matrix. The pathogenic injectisome is generated using: EMD accession numbers 1875, 5720, 5721, and 2669, for the basal body; the model from [12] for the major export component; EMD accession number 2805 for the tip complex; and EMD accession number 1416 for the needle. The flagellum is generated using: EMD accession number 5310 for the OM ring; EMD accession number 1887 for the basal body and C-ring; the model from [117] and EMD accession number 1647 for the hook; and EMD accession number 5007 for the filament [59,117–119]. Homologous components are presented with the same color in the structures. The T3Ss comprise the extracellular segment, the basal body, and the cytoplasmic components. (B) Schematic comparison between the ATPase complex of T3SS (i) with the F_1F_0 ATPase synthase (ii) demonstrates the high structural homology between them (F_1F_0 ATPase synthase model obtained from www.rcsb.org/pdb/101/motm.do?momID=72).

and genetic homology, and, hence, common ancestry [8], with the flagellar apparati (specialized devices used for cell motility; Figure 1A, right) and with parts of the F_1F_0 ATP synthases (Figure 1B, ii). Due to the conservation, data derived from either pathogenic or flagellar T3SSs from different bacteria are commonly combined to understand a unified mechanism of the secretion process. To unify the different protein and gene names and reduce confusion

(for a selection, see Table 1), common nomenclature rules have been proposed [9], including the Secretion and cellular translocation (Sct) moniker for the highly conserved genes. The Sct nomenclature has been exclusively adopted here and the inclusion of some new Sct proteins has been proposed (see below).

Analyses derived from X-ray crystallography, nuclear magnetic resonance (NMR), and cryo-electron microscopy (EM) visualization of several T3SS components [10–13] have provided important structural insights into injectisome formation [8,14,15]. The injectisome spans two bacterial membranes and one eukaryotic membrane. It has three distinct parts (Figure 1A): the extracellular segment; the basal body, which crosses both membranes of the Gram-negative envelope; and the peripheral inner membrane cytoplasmic components [5]. The extracellular segment that bridges the bacterial outer membrane to the host plasma membrane comprises the needle that carries on one end either a tip or a filament and the translocator pore. The basal body is built of stacked toroids: an outer membrane ring (OM ring) extends to the periplasm and associates with the inner membrane one (IM ring). The basal body and the extracellular segment form a conduit through which effectors are transferred. The cytoplasmic components are the ATPase complex and the cytoplasmic ring (C-ring), which are essential for protein secretion, protein sorting, and the unfolding of secretory proteins (Figure 1A). Injectisome assembly and function are complicated processes that are under sophisticated regulation.

How to Build an Injectisome: Sec- Versus T3SS-Dependent Phases

Assembly of the injectisome is precisely orchestrated in three discernible phases (Figure 2). In Phase I, the injectisome components enter the membrane and form the outer shell of the basal body. This process makes use of the Sec machinery, the essential system that the cell uses for 95% of its exported and membrane proteins (Figure 2, phase I–III) [1,2]. After the basal body frame is in place, the T3SS-dependent phase occurs, during which the injectisome is completed with an internal connector of the basal body toroids, elongation of the needle, and the formation of the translocator pore (Figure 2, phase IV and V) [16,17].

Sec-Dependent Formation of the Basal Body Shell

The basal body comprises membrane-embedded ring-like structures that connect the inner and outer membrane by forming a continuous tubular conduit [14,15]. The OM ring comprises the 12mer SctC, a member of the secretin family [16]. SctC has its N-terminal domain protruding deeply into the periplasm, forming a 16 nm-long funnel with a 7 nm-wide channel (Figure 3) [18,19]. In some cases, the small lipoprotein pilotin is essential for SctC oligomerization and stabilization (Table 1) [18,20].

The membrane and supramembrane (MS) ring comprises an inner and outer toroid. The inner toroid is formed by a 24mer of the periplasm-facing lipoprotein SctJ; the outer toroid is formed by a 24mer of the single transmembrane domain (TM) protein SctD, which 'jackets' both the SctJ ring and the export apparatus (Figure 3). The MS ring is stabilized through SctJ–SctD electrostatic interactions [15,21]. The SctJ pore is negatively charged and might serve as an adaptor for the inner rod protein SctI to anchor [10,22]. Upon MS ring assembly, SctD bridges the OM and the IM through its periplasmic domain (Figure 3, D_C) by interacting with the periplasmic domain of SctC and stabilizing the whole basal body structure [15,22–25]. The N-terminal domain of SctD is flexible and resides in the cytoplasm (Figure 3, D_N), where it is thought to act as a dock for the C-ring [26,27].

Five polytopic IM proteins (SctR, SctS, SctT, and SctU, minor components; SctV, major component) form the 'export apparatus'(Figure 3) that is surrounded by the MS ring [7,11]. SctR, SctS, and ScT mainly comprise TMs with short linkers, whereas SctV and SctU also contain large, cytoplasmic C-terminal domains [11,12,28]. SctV assembles into a ring-like nonamer, with a 5-nm

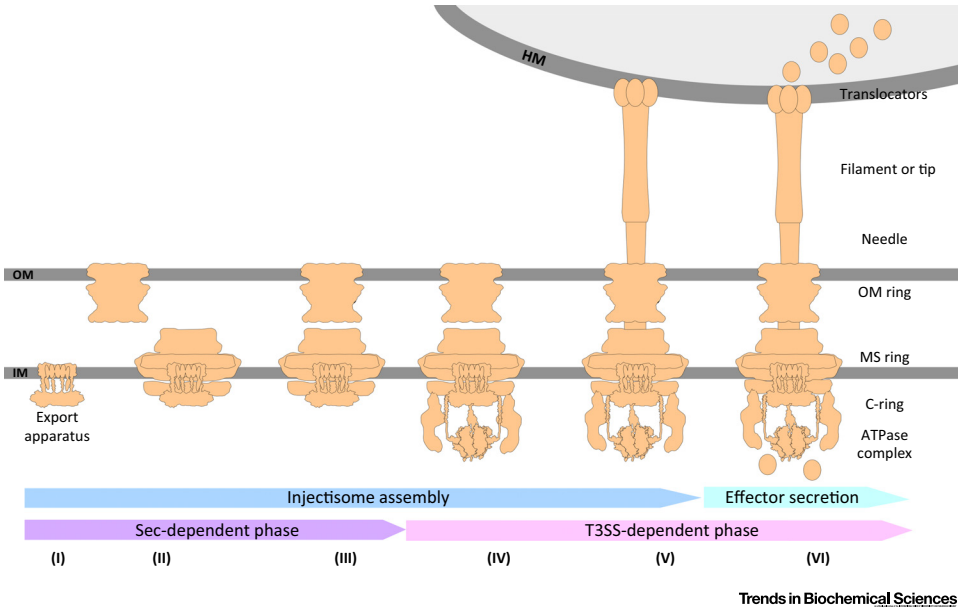

Figure 2. Schematic Representation of Injectisome Assembly. Schematic representation of injectisome assembly subdivided into Sec- and Type III secretion (T3S)-dependent phases. First, the export apparatus needs to be localized to the inner membrane (Sec dependent, phase I). Afterwards, the membrane and supramembrane (MS) ring is formed by enveloping the export apparatus. Meanwhile, the outer membrane (OM) ring has been localized in the outer membrane (Sec dependent, phase II). The two ring-like structures become bridged via intermolecular stabilizing interactions (Sec dependent, phase III). During the T3S phase, the ATPase complex is transferred to the membrane and interacts with the C-ring and the export apparatus (T3SS dependent, phase IV). The anchoring of the ATPase and C-ring to the membrane initiates the secretion of the early substrates (T3SS dependent, phase V). Once the needle is attached to the host membrane via the translocator assembly that also forms the pore, the late substrates (i.e., various effectors) are secreted directly inside the eukaryotic cytoplasm (T3SS dependent, phase VI).

pore, via intersubunit salt bridges [12]. Its C domain lies 6 nm from the membrane surface, and is connected to the rest of SctV by a slender stem [12] (Figure 3). Once embedded in the IM, the minor export apparatus is surrounded by the SctV ring [12,29,30].

The hierarchy of the OM ring, MS ring, and export apparatus assembly is somewhat controversial. Once synthesized, their proteins are localized to the inner membrane or translocated to the outer membrane. It has been proposed that the OM and MS rings are stably formed and attached to the membrane regardless of the existence of the export apparatus, and *vice versa* [14,22] (Figure 2, phase I). Given the need for IM integration of the 'export apparatus' proteins, presumably via signal recognition particle targeting and to the SecYEG channel [17], the MS ring likely envelops a pre-assembled export apparatus.

Subsequently, either the MS ring is the first to be assembled in the IM, followed by periplasmic interlocking with SctC, or the SctC ring is formed in the OM and then the MS ring components SctD and SctJ are assembled progressively and attached to the SctC ring, using it as a building scaffold (Figure 2, phase II,III) [7]. The two rings snap together during holomachinery assembly, possibly bridged by electrostatic interactions between their periplasmic domains [7,31].

T3SS-Dependent Phase of the Injectisome

Needle-Filament Formation and Length Regulation

Once the basal body is assembled, it is used for further secretion of the injectisome components, SctF and SctI (early secretory substrates; hereafter 'substrates'). SctF self-oligomerizes to form

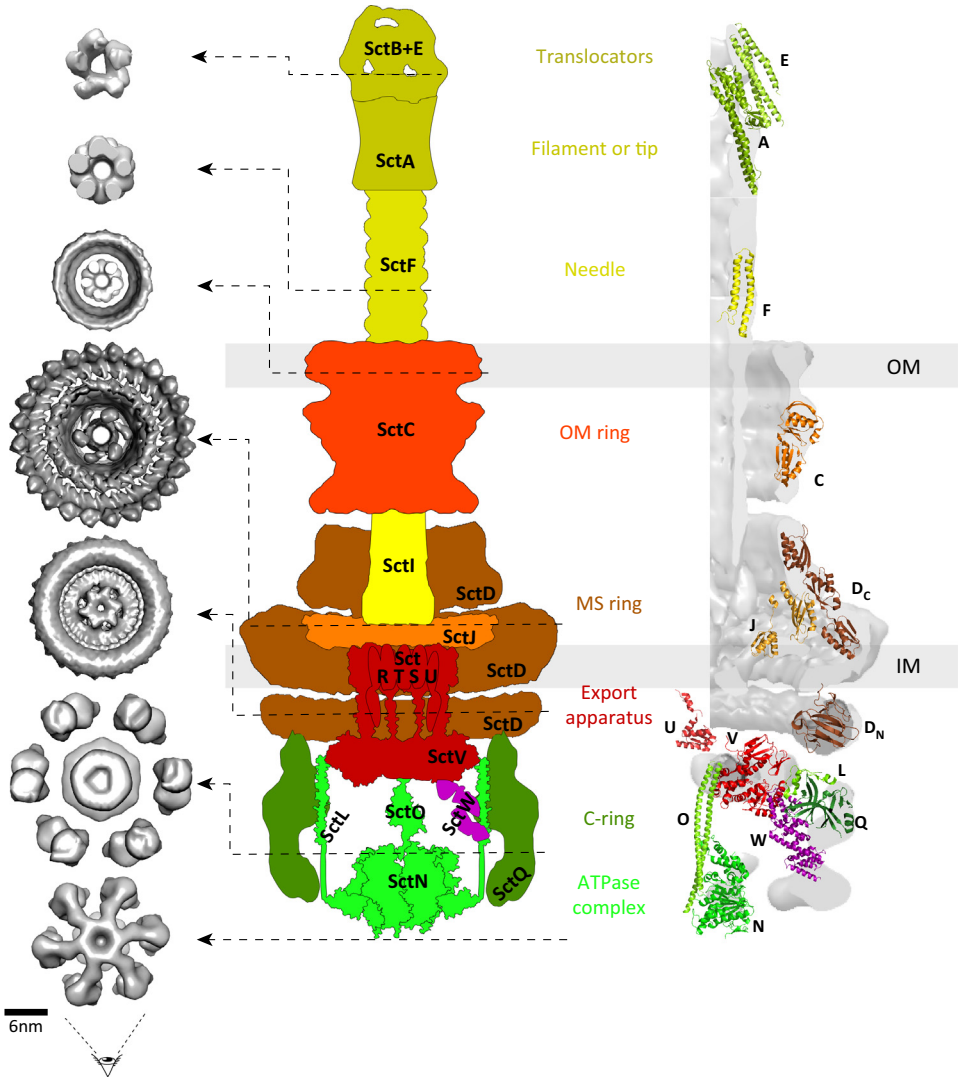

Figure 3. Schematic Representation of Low- and High-Resolution Structures of Injectisome Subunits. Cartoon representation of the pathogenic Type III secretion system (T3SS) using the common Sct nomenclature system (middle) [9]. The T3S injectisome comprises the translocators, tip complex and/or filament (olive green), the needle (dark yellow), the inner rod protein (yellow), the outer membrane (OM) ring (orange), the inner membrane (IM) ring (brown), the export apparatus (red), the cytoplasmic ring (dark green), and the ATPase complex (light green). Cross-sections of the injectisome are shown from the bottom to the top (left). A longitudinal cross-section of the right half of the injectisome is shown (right, gray). Electron microscopy (EM) sections of the pathogenic T3SS structure were generated using Chimera software. Available crystal structures of individual protein subunits or domains were manually placed inside the EM envelope for visualization and oriented as they are proposed to interact within the injectisome, using PyMOL Viewer. Where oligomerization is known to occur, only a single protomer is shown. EMD accession numbers: 1875, 2669, 1416, and 2805. Protein Data Bank (PBD) accession numbers: SctE, 3TUL; SctA, 4D3E; SctF, 2X9C; SctC periplasmic domain, 3GR5; SctD (D_C) periplasmic domain, 4ALZ; SctD cytoplasmic domain (D_N), 4A0E; SctJ, 1YJ7; SctU cytoplasmic domain, 3BZL; SctV cytoplasmic domain, 4A5P; SctQ cytoplasmic domain and SctL N-terminal domain, 4YXA; SctO, 4MH6; SctN, 2OBL; SctW, 2VJ5.

the extracellular needle (Figure 3). This needle is anchored to the basal body by the inner rod protein SctI (Figure 3) and extends to the external milieu for lengths of 80 nm–2 μm [32–34]. SctI is localized inside the SctJ channel (Figure 3) [35–37] and appears to form a cylindrical conduit upon oligomerization [38], acting as a foundation for the growing needle [37,39]. At the end of the

needle, either the tip protein or a filament is attached (Figure 3). The tip protein usually forms a pentamer on the top of the needle [40], whereas the filament is a sheath-like structure that can be elongated up to 25 nm [41,42]. Tip or filament proteins share sequence similarity and, hence, common ancestry between different pathogenic T3SS and flagella [40,43,44]. Although filament and tip proteins are structurally distinct, they appear to function similarly in T3S. Therefore, we propose the common name SctA, separated in two classes; $SctA_f$ for filament and $SctA_t$ for tip proteins (Table 1).

The needle or filament length varies between different bacteria and is dictated by the molecular ruler SctP. SctP is partially unfolded and 'measures' the length of the developing needle, resulting in a needle length that is apparently proportional to the length of the extended SctP polypeptide in each organism [42,45]. One model posits that SctP is sporadically secreted from the injectisome and somehow measures the needle from the outside (Figure 4A, left) [42]. A second, more prominent, model posits that SctP is anchored C terminally on the SctU protein of the export apparatus [39,46–48] and its N terminus follows the developing needle anchored to SctA [49,50] (Figure 4A, middle). According to a third model, dissected in the flagellum, the increased concentration of the needle analog, flagellin protein, at the export apparatus promotes its secretion by preventing the interaction between the SctP and the SctU protein of the export apparatus [51,52] (Figure 4A, right).

Formation of the Translocator Pore

Once the needle is formed and the filament or tip is attached to it, the translocators (we propose here to call them SctB and SctE) are secreted and anchored on SctA. SctB and SctE (middle substrates) are mainly hydrophilic and are inserted in the host plasma membrane, whereupon they oligomerize to form the translocator pore [53,54] (Figure 3). The translocator pore is a heteropentamer (four SctE: one SctB) with an internal pore 10-nm wide through which effectors (late substrates) are injected into the host cytoplasm [53,55–57] (Figure 2, phase VI).

C-ring and ATPase Complex Assembly and Localization

The C-ring comprises SctQ, a peripheral membrane-associated protein essential for T3S. SctQ forms a circular 30 nm-wide arrangement comprising six separated pods. The C-ring is docked on the MS ring on a peripheral IM pedestal formed by the N-terminal cytoplasmic domain of SctD, probably upon phosphorylation of SctQ (Figure 3) [26,27]. By contrast, the C-ring of the flagellar T3SS has a continuous cylindrical, and significantly wider (45 nm diameter), wall [58–60].

The C-ring acts as a receptacle for the 'ATPase complex,' which comprises an ATPase held in place by two accessory components: the stator and the stalk [31]. The SctN ATPase (Figures 1B and 3), homologous to the F_1F_0-ATPases (Figure 1B) [11,61], is a chemomechanical energy converter. SctN forms homohexameric rings with an internal diameter of 2.5–3 nm [61–66]. F_1F_0-ATPases comprise a membrane-embedded F_0 domain and a cytoplasm-exposed F_1 domain. One end of the γ central stalk of F_1 attaches to the rotating c oligomer of F_0, while the other inserts in the F1 ATPase hexamer composed of three α/β subunit pairs. An external stator (b_2 and δ subunits) is a second F_0–F_1 connector [67–69] that prevents the ATPase from rotating in vane when the central stalk rotates inside it (Figure 1B). Similarly, SctN localizes stably to the membrane via the elongated α-helical SctL stator and the SctO stalk, both attached to the cytoplasmic domain of SctV [65,70,71]. SctL has an additional role; it acts as a chaperone-activity regulator of the ATPase in the cytoplasm, prevents SctN oligomerization, and escorts it to the membrane [65,72]. EM studies suggest that, once at the membrane, six SctL stator units interact with the SctQ pods [8,12,60,73], linking them to the ATPase, while also docking to SctV for membrane anchoring via their N-termini (Figure 3) [71,74,75]. SctO stabilizes the SctN hexamer and stimulates its ATPase activity [65] and, by analogy to the γ stalk, might insert partly inside the ATPase pore (Figure 1B) [62,65].

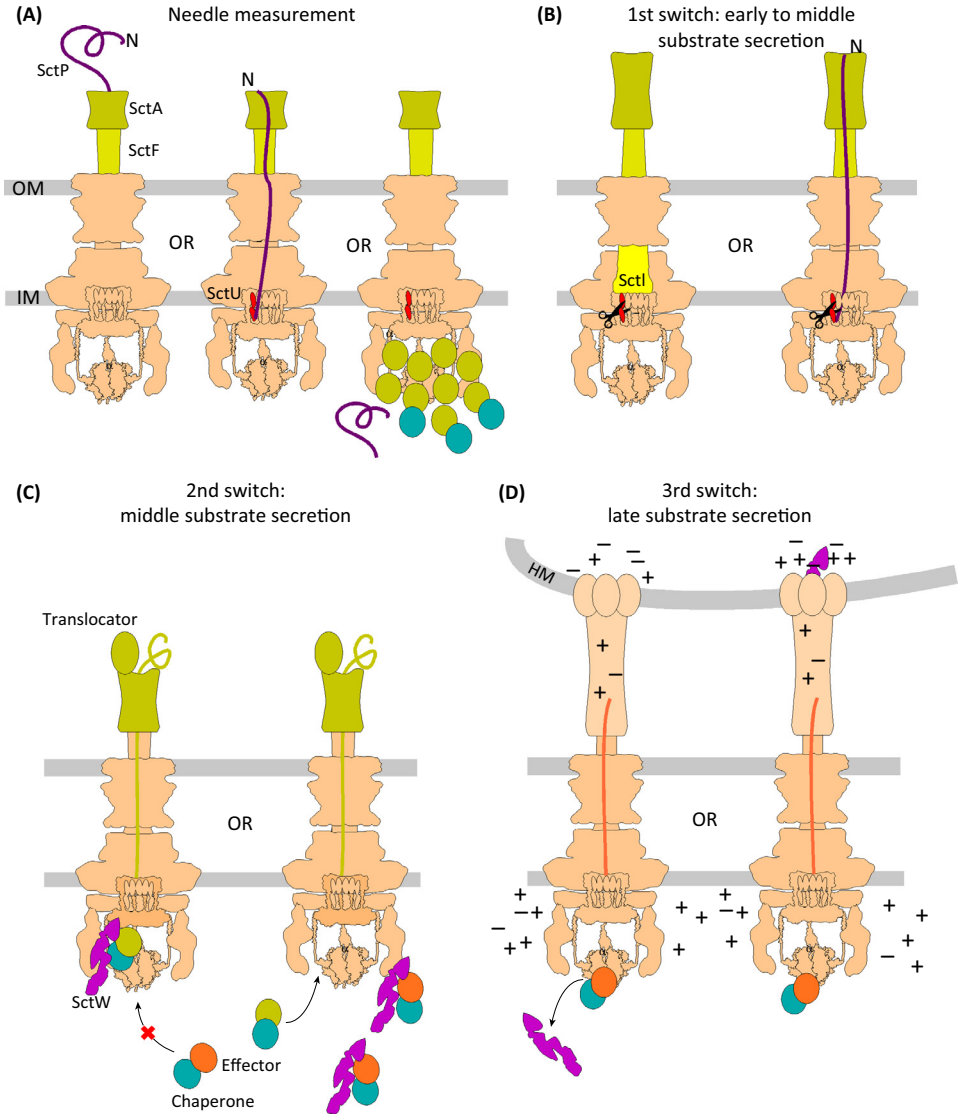

Figure 4. Proposed Models for Secretion Regulation or Switch. (A) Needle length-measuring models. Left: SctP (magenta) is secreted and attached to the developing tip of the needle (olive green). Middle: SctP is C-terminally anchored on the SctU cytoplasmic domain (red), and follows needle growth with its N terminus. Right: Increased concentration of needle protein subunits (olive green) in complex with its chaperone (blue) at the cytoplasmic domain of the export apparatus prevents the interaction between the SctP and SctU (cup-model). (B) SctU (red) cleavage is promoted by (left) conformational changes due to interactions between SctI (yellow) and SctF (olive green) or (right) due to the complete extension of SctP (magenta). (C) Left: SctW (magenta) shown bound to the membrane, either at the C-ring or the export apparatus. SctW may promote translocator protein secretion by interacting with chaperone–secretory protein complexes (blue and olive green, respectively). Right: SctW may prevent effector secretion by interacting with chaperone–effector complexes and blocking their membrane targeting (blue and orange). (D) Influx of ions ('+' and '–') from the host changes the local potential at the membrane, resulting in either the secretion of SctW (magenta) (left) or its disengagement from the membrane and cytoplasmic release (right). These changes may lower the binding affinities of the export apparatus for middle substrates and increase the affinities for late substrate effectors. Abbreviations: IM, inner membrane; OM, outer membrane.

Although the T3SS can be divided into distinct structural parts, its assembly and protein secretion are consecutive, multi-stepped, and complex processes. Once the Sec-dependent phase of assembly is completed, the T3SS-dependant phase is highly regulated in all steps by various factors, including protein components of the system and/or environmental cues.

Regulation of Secretion through the T3SS

Almost all of the T3SS-related genes are clustered into operons found in specific loci on the bacterial chromosome or plasmids. Their transcription is coordinated and upregulated under secretion-permissive conditions, through specialized sigma factors and transcriptional regulators [76,77], resulting in the simultaneous presence of the majority of the system components in the cytoplasm [76]. Consequently, the T3SS needs mechanisms to prevent unspecific and untimely protein secretion and maintain the strict early-to-late substrate hierarchy of the process (see Outstanding Questions).

Recognition Targeting

Several T3SS secretory proteins contain noncleavable signal sequences at their N termini (Figure 5A) [78], but these sequences share little sequence or biophysical features and do not countersign the proteins for secretion hierarchy or membrane targeting [79]. The export apparatus components SctU and SctV, the C-ring, the ATPase complex, and the gatekeeper SctW are responsible for mediating different steps in the secretion pathway, such as the order of

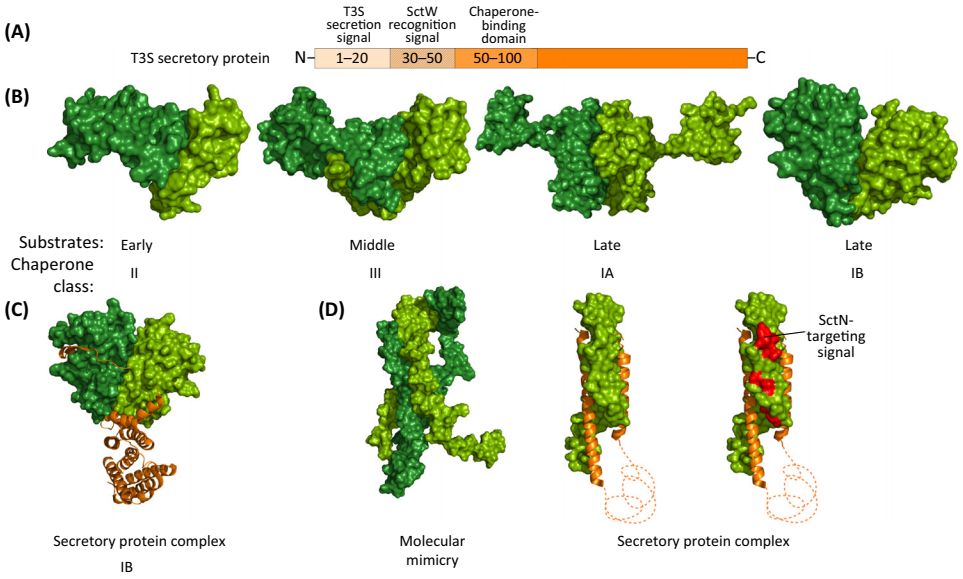

Figure 5. Specialized Chaperones of Type III Secretion (T3S), from Structure to Function. (A) General scheme of T3S-related recognition signals on secretory protein sequences. Within the N-terminal region lies a T3S secretion signal, usually 20 amino acids long, which is poorly conserved among secretory proteins ([78,120]). Downstream of the signal sequence lies the chaperone-binding domain, usually from position ~50 to 100. This stretch is specifically recognized by the specialized chaperones and its length varies between different secretory proteins. Middle substrates (translocators) also have a SctW recognition signal that is located usually between the signal sequence and the chaperone-binding domain (cross-hatched area). This signal somehow ensures their secretion before that of the effectors. (B) Representative structures of different dimeric chaperones (green and light green for each protomer) of T3SS are shown following the protein secretion hierarchy (early to late substrates). Chaperone classes are indicated. Protein Data Bank (PBD) numbers from left to right: IpgC dimer (*Shigella* sp.), 3GYZ; YscE–YscG dimer (*Yersinia* sp.), 2P58; CesT dimer (enteropathogenic *Escherichia coli*; EPEC), 1K3E; Spa15 dimer (*Shigella* sp.), 2XGA. (C) Representative structure of a chaperone–secretory protein complex (green and orange, respectively). According to the model, a dimeric chaperone can bind one secretory protein. PDB accession number 2FM8 [InvB–SipA complex (*Salmonella* sp.)] [121]. (D) Chaperones can be autoinhibited by self-dimerization. Here, CesAB of EPEC is autoregulated using molecular mimicry of the secretory protein bound state. The CesAB–CesAB dimer (green and dark green represent each protomer) on the left as determined using in solution nuclear magnetic resonance. Crystal structure of CesAB in complex with secretory protein EspA. CesAB chaperone is shown in green and secretory protein EspA in orange. Unresolved structure in EspA is shown with orange-dashed line (middle and right). Similar overall structures were obtained in both cases. PDB accession numbers: 2LHK (CesAB–CesAB) and 1XOU (CesAB–EspA). Upon binding to the secretory protein, a SctN targeting signal (red) is exposed on the surface of the chaperone due to conformational changes at the surface (right).

secretion, membrane targeting, and export. However, little is known about the precise mechanism that is followed to segregate the secretory proteins in each step during secretion [12,15,29] (see Outstanding Questions).

Secretion Switches

Protein secretion through the injectisome occurs in consecutive steps and different switching mechanisms ensure the secretion hierarchy. SctU has been proposed as the first regulatory switch. Once SctF is secreted and the needle is in place, the injectisome is ready to secrete all the other proteins. To signal this, the cytoplasmic domain of SctU undergoes autoproteolysis, thus promoting conformational changes thought to alter the electrostatic surface of the export apparatus [80,81].

Two models attempt to explain this mechanism. According to the first, the inner rod SctI (Figure 4B, left) interacts with SctU at the periplasmic phase of export apparatus, leading to its translocation into the basal body and association with the secretin SctC [82]. Once there, SctI somehow regulates the needle length and promotes SctU autoproteolysis (Figure 4B, left) [37,39,48,82,83]. The second model (Figure 4B, right) proposes that, once the needle or filament structure reaches the correct length, autoproteolysis of SctU, on which the fully extended SctP is anchored, occurs [50,52,84]. Either way, cleaved SctU is thought to reduce the affinity of the apparatus for the early substrates, resulting in the switch to middle substrate secretion [11,80,85].

The switch from middle to late substrate secretion is attributed to the gatekeeper SctW. In the absence of SctW, translocators (middle substrates) are not secreted, whereas effectors (late substrates) are oversecreted [79,86]. Hence, SctW is needed for translocator secretion through an unknown mechanism. One proposal for this is that SctW is localized at the export apparatus and/or the C-ring [79,87]. There, possibly selectively, it interacts with the translocators, sorts them, and promotes their preferential secretion (Figure 4C, left) [79,87–89]. Otherwise, SctW, possibly while in the cytoplasm, 'captures' directly chaperone–effector complexes, thereby preventing their access to the translocation pore (Figure 4C, right), [42,88,90]. Translocators have SctW-recognized sequences downstream of their export signals [79] (Figure 5A). This lends strong support for an active role of SctW in translocator recognition and selection.

Once the translocator pore in the host plasma membrane is formed, ion flow to the bacterial cytoplasm is thought to alter the local potential [91]. This alteration leads to either dissociation of SctW from the membrane (Figure 4D, left) [92,93] or its secretion from the injectisome (Figure 4D, right) [94]. Detachment of SctW from the membrane drives the switch to late substrate secretion [91,95,96].

Protein Targeting to the Membrane

Apart from the switching regulators, the major component of the export apparatus SctV acts as a regulatory or targeting receptor, by interacting with chaperone-secretory protein complexes via its cytoplasmic nonameric ring [12,14,95,97]. It has been proposed that conformational changes that occur upon SctU cleavage, SctW dissociation, and/or the local potential changes at the membrane can regulate secretion steps by varying the binding affinities of the cytoplasmic domain of SctV for each chaperone–secretory protein complex. This mechanism was also proposed for the flagellar T3SS [14,31,98,99].

Additionally, C-ring formation is believed to somehow serve in the sorting of exported proteins and their transfer to the ATPase and/or to SctV [88]. Interactions observed between SctQ and SctL may be important for regulating the hierarchy of secretion (hence, also referred to as 'sorting platform') [27,60,73,88]. However, the assembly and association of the C-ring–ATPase complex are highly dynamic and they easily detach from the membranes [27]. As a result, they are not

constitutively present at the injectisome entry point [8,27]. This makes it less likely that they provide an essential contribution to sorting. It might be that C-ring formation is essential for downstream events, such as stabilization and activation of the ATPase complex at the membrane.

Nevertheless, the *in vivo* analyses, EM studies, co-purification, and protein–protein interaction studies to date cannot fully address the multi-level, complex mechanism mediating secretion regulation. For this, *in vitro* assays with purified components and a reconstituted injectisome would be required.

Chaperone Holdases are Essential for Protein Secretion

Secretory proteins of the T3SS depend on cytoplasmic chaperones for their efficient secretion [100,101]. Chaperones form stable complexes with their secretory proteins, preventing the latter folding or aggregating; thus, they display holdase activity. They are small, monomeric or dimeric, and share little sequence or structural similarities (Figure 5B) [102–106]. Their genes are almost always located next to those of the secretory proteins [107]. Depending on the substrates that they recognize, they fall into three classes: those that interact with early (Class II), middle (Class III) [100,103,108], and late secretory substrates (Class I). Class I chaperones can specifically recognize either one or several homologous effectors (Class IA), or bind to different unrelated effectors (Class IB) (Table 1) (Figure 5B,C) [100,105].

Class III chaperones usually contain three tandem tetratricopeptide repeats (TPRs), forming two antiparallel α-helices and imperfect amino-acid repeats, which are often involved in protein–protein contacts [66,102,104,108,109]. Some T3SS chaperones, such as CesAB of EPEC, prevent unspecific interactions by a self-association mechanism that mimics the state the chaperone acquires when bound to the secretory protein [104] (Figure 5D).

T3SS chaperones can associate, either alone [105] or in complex with their cognate secretory substrates [66], with components of the injectisome. In the latter case, the targeting signals are conformational; that is, they are present in the chaperone structure but are only exposed upon allosteric changes brought about by secretory protein binding (Figure 5D) [66]. Therefore, it has been proposed that chaperones may facilitate membrane targeting of secretory proteins and/or control the hierarchy of secretion, by increasing the local concentrations of secretion substrates at the base of the injectisome or by competing for the same membrane receptor component [64–66,97,99,105]. However, the molecular mechanism behind the targeting process remains unclear and the multiplicity of possible interactions complicates dissection *in vivo* (see Outstanding Questions).

The ATPase Complex and Energy Requirements for Protein Secretion

The ATPase SctN is believed to catalyze disassembly of secretory protein–chaperone complexes and/or secretory protein unfolding necessary for transport through the export apparatus [66,110]. Not all secretory proteins are expected to acquire folded structures before export. SctN appears to interact with some secretory proteins and chaperones alone, or with their complexes [64,66,111]. Moreover, it was demonstrated that ATP hydrolysis is essential for secretory protein unfolding, which is necessary for transport [110]. It is expected that proteins must be in non-native states to cross the 2–3-nm pore of the inner channel of the export apparatus, but the molecular basis of the mechanism remains unknown [66,105,110,111].

Although ATP hydrolysis is important, it is not the only energy source for T3S [112]. In the absence of the ATPase or its stalk proteins, secretion is driven primarily by the proton motive force (PMF or $\Delta\mu H^+$), which is the electrochemical potential difference of protons across a membrane that is generated during electron transport in the bacterial plasma membrane

[95,112–114]. Therefore, it was proposed that the PMF drives protein transport across both bacterial membranes [74,112,113].

Concluding Remarks

T3SS is essential for the pathogenesis of many Gram-negative bacteria. Tight, sophisticated regulation is needed to coordinate more than 40 proteins that include injectisome subunits, host contact proteins, and injected effectors. Remarkable progress has been made towards understanding the structure and molecular mechanism of the injectisome. The view revealed is that of a complicated nanomachine that starts its assembly using the ubiquitous Sec system and completes its construction using itself as an export apparatus. As a finishing touch, the last components to be secreted provide a means of attachment of the injectisome to the host plasma membrane and a specialized translocator channel. Once the conduit that connects the two organisms is complete, secreted effector proteins cross through this channel into the eukaryotic host cytoplasm. Despite this progress, we are still missing detailed mechanistic understanding of the precise order of events, the mechanism of secretion, the pathway that secretory proteins take from their docking to transmembrane crossing, and the energetics of that pathway. Elucidating the molecular mechanisms that underlie T3S is essential and may allow us to exploit T3S for specialized protein delivery [115,116]. To address these issues, it is essential to reconstitute the T3SS pathway *in vitro* and obtain high-resolution structures of increasingly larger subassemblies of the device.

Outstanding Questions

Given that T3SS proteins share no distinct signals on their sequence, how are these proteins recognized? How is their secretion hierarchy regulated?

T3S chaperones maintain the translocation competence of secretory proteins and may mediate protein targeting to the export apparatus. How do chaperones mediate targeting?

Assembly and membrane attachment of the cytoplasmic ring of the injectisome are dynamic. Is the C-ring essential for protein targeting to the membrane? If not, which protein(s) act as membrane receptor(s) for the secretory proteins?

Does the ATPase have roles beyond dissociating chaperone–secretory protein complexes? Does PMF drive protein translocation through the injectisome, as it does in flagella?

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