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Architecture, function, and substrates of the type II secretion system

Konstantin V. Korotkov^{1,*} and Maria Sandkvist^{2,*}

¹Department of Molecular and Cellular Biochemistry, University of Kentucky, Lexington, Kentucky, United States

²Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan, United States

Abstract

The type II secretion system (T2SS) delivers toxins and a range of hydrolytic enzymes including proteases, lipases and carbohydrate-active enzymes to the cell surface or extracellular space of Gram-negative bacteria. Its contribution to survival of both extracellular and intracellular pathogens as well as environmental species of Proteobacteria is evident. This dynamic, multi-component machinery spans the entire cell envelope and consists of a cytoplasmic ATPase, several inner membrane proteins, a periplasmic pseudopilus and a secretin pore embedded in the outer membrane. Despite the trans-envelope configuration of the T2S nanomachine, proteins to be secreted engage with the system first once they enter the periplasmic compartment via the Sec or TAT export systems. Thus, the T2SS is specifically dedicated to their outer membrane translocation. The many sequence and structural similarities between the T2SS and type IV pili suggest a common origin and argue for a pilus-mediated mechanism of secretion. This minireview describes the structures, functions and interactions of the individual T2SS components and the general architecture of the assembled T2SS machinery and briefly summarizes the transport and function of a growing list of T2SS exoproteins. Recent advances in cryo electron microscopy, which have led to an increased understanding of the structure-function relationship of the secretin channel and the pseudopilus are emphasized.

INTRODUCTION

The type II secretion system (T2SS) is one of several extracellular secretion systems in gram-negative bacteria. While highly prevalent in γ - and β -proteobacteria, the T2SS is also recognized to lesser extent in members of the δ and α classes (1, 2). It is known for its prolific protease secretion activity. In addition, the T2SS mediates extracellular delivery of a variety of toxins, lipases and enzymes that break down complex carbohydrates thus conferring a survival advantage to pathogenic as well as environmental species (2–4). The T2SS is not restricted to extracellular pathogens such as *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Vibrio cholerae*; it is also present and contributes to growth of intracellular pathogens including *Legionella*

*For correspondence: kkorotkov@uky.edu (Konstantin V. Korotkov);, mariasan@umich.edu (Maria Sandkvist).

pneumophila, which replicates in both aquatic amoebae, alveolar macrophages and epithelial cells (5–7). The obligate intracellular pathogen *Chlamydia trachomatis* does also depend on T2SS components for extracellular secretion; however, its T2SS is atypical as some components are missing or are too different from homologs in other species to be identified using BLAST algorithms (8, 9).

With 12–15 different components distributed in the cytoplasm, cytoplasmic membrane (CM), and outer membrane (OM), the large multiprotein T2SS spans the entire Gram-negative cell envelope (Fig. 1A). While many of the T2SS constituents are structurally and functionally related to those of type IV pili systems (10), some of the components are unique to the T2SS and are therefore likely to have a specific role in the secretion process. Energy through the hydrolysis of ATP is provided by GspE, a cytoplasmic hexameric ATPase that interacts with the cytoplasmic domains of GspL and GspF, two CM components (Fig. 2) (11–21). GspL, in turn, forms a tight complex with GspM, a structural homolog (22–27). The CM complex also consists of GspC (Fig. 2), which reaches into the periplasmic space making contact with the secretin that forms the OM conduit consisting of fifteen copies of GspD (Fig. 3A) (28–38). A gene for GspN, a fifth CM component, is present in T2SS operons of a subset of Gram-negative species; however, its removal has often no discernable effect on secretion and its function remains unknown (39, 40). Interestingly, *Xanthomonas campestris* lacks GspC. Instead, it expresses GspN, which may substitute for GspC (41). In addition, the function of some T2SSs is supported by the CM proteins GspA and GspB, which contribute to GspD assembly and transport to the OM possibly by increasing the pore size of the peptidoglycan or anchoring it to this structural meshwork (42–44). Finally, GspG forms a periplasmic pseudopilus that extends from the CM and is likely capped by the minor pseudopilins GspH, GspI, GspJ and GspK, components that initiate the formation of the pseudopilus (Fig. 2) (45–50). Prior to assembly of the pseudopilus, which involves extracting the pseudopilins from the CM and polymerizing them into short helical pilus-like fibers, they are N-terminally cleaved and methylated by the prepilin peptidase GspO (PilD) (51–53).

Proteins to be secreted by the T2SS are initially produced as precursors with N-terminal signal peptides that are removed following translocation across the CM by the Sec or TAT pathways (54–57). While tethered to the CM or following release to the periplasmic compartment they then undergo folding and, in some cases, oligomerization into larger complexes (58–65). Figure 1B shows examples of T2SS substrates with known structures. Many T2SS substrates, particularly proteases, are also produced with a removable propeptide, in addition to the signal peptide, that functions as an intramolecular chaperone and/or inhibitor (64, 66–68). Other T2SS substrates require dedicated, often CM tethered, chaperones that assist in the folding and/or engagement with the T2SS prior to OM translocation (see the crystal structure of the *Burkholderia glumae* lipase in complex with a soluble form of its chaperone in Figure 1B) (69–74). Here, we discuss the latest findings relating to the T2SS substrates, the structure and assembly of the secretin, and the mechanism of secretion focusing on the role of the pseudopilus.

TRANSPORT AND FUNCTION OF T2SS SUBSTRATES

The secretion of exoproteins by the T2SS is considered a two-step process, where the two steps — transport across the CM and OM — can be genetically and physically separated (61, 62). All T2SS substrates have to be exposed to the periplasmic compartment to be recognized by the T2SS. Some enter the T2SS as soluble periplasmic intermediates while others are extracted directly from the CM. Examples of the latter include the prolipoproteins pullulanase and SslE produced by *Klebsiella pneumoniae* and Enteropathogenic *Escherichia coli* (EPEC), respectively, which are expressed with signal peptides that contain a lipobox with a conserved cysteine (75–77). The cysteine is acylated and the signal peptide is removed. The lipidated cysteine remains with the mature protein and is further modified by an N-acyl transferase prior to engagement with the T2SS (78). In contrast to many other, soluble T2SS substrates, which are released from the cells following OM transfer, these lipoproteins remain primarily associated with the bacterial cell surface (76, 79). Presumably they are retained with the OM through their lipidated N-termini because a pullulanase variant produced with a typical Sec signal peptide is solubly released following OM translocation (62). Another example of surface retention includes the cell association of heat-labile enterotoxin (LT) produced by enterotoxigenic *Escherichia coli* (ETEC), which binds via the B subunit oligomer to LPS in a Kdo core-dependent manner (80, 81). Although the B subunits of LT and cholera toxin are nearly identical, cholera toxin does not remain associated with the *V. cholerae* surface because its Kdo core is phosphorylated, thus preventing the binding. A third form of surface retention is typified by the pectin lyase PnlH, which is anchored in the *Dickeya dadantii* OM by a non-cleavable TAT signal peptide (82). *V. cholerae* provides yet another means by which T2SS substrates associate with the cell surface. This involves the production of proteins with a C-terminally located tripartite motif, GlyGly-CTERM, which consists of residues rich in glycines and serines followed by a stretch of hydrophobic amino acids and positively charged residues (83, 84). A recent study has shown that the GlyGly-CTERM domain of one of these proteins, VesB, is cleaved off in the CM by an intramembrane protease, rhombosortase, and the newly generated C-terminus is capped with a glycerol-phosphoethanolamine unit that may be acylated (84). This is followed by OM translocation and surface localization of VesB. Expression of VesB without its GlyGly-CTERM domain results in the release of VesB to the extracellular compartment, signifying the importance of the GlyGly-CTERM extension and C-terminal modification for VesB surface retention. While the above methods exemplify ways to retain T2SS substrates on the bacterial surface, these proteins can also be found in various amounts in culture supernatants. This is due to release of OM vesicles, the formation of micelles or removal of the proteins from the cell surface by extracellular proteases (79, 84).

The contribution of the T2SS to environmental growth and virulence of human, animal and plant pathogens is apparent when one considers the secreted proteins and their activities (Table 1). Devastating diseases such as cholera and childhood diarrhea caused by ETEC are mediated by cholera toxin and LT and result in severe dehydration and even death when not treated (85, 86). By inducing watery diarrhea, the enterotoxins aid in the spread and transmission of these diseases. Another means by which T2SS substrates benefit bacteria is through nutrient acquisition in the eukaryotic host and environment. The release and

generation of nutrients is the result of the action of pore-forming toxins such as aerolysin which causes osmotic host cell lysis and a range of enzymes including proteases, lipases, DNases, and carbohydrate-degrading enzymes that digest host components and tissue (40, 63, 87–92). For example, plant cell-wall-degrading enzymes such as cellulases, pectinases and pectate lyases, promote growth of phytopathogenic bacteria resulting in crop losses (55, 87, 93). *L. pneumophila* aminopeptidase LapA is another recently identified example of a T2SS protein that contributes to nutrient acquisition by generating amino acids and thus supporting intracellular growth of *L. pneumophila* in amoebae (91). Degradative enzymes also aid pathogens in gaining access to new niches. Enzymes that target mucins, which cover and protect cells of the digestive tract, generate direct access to the epithelial cell surface for colonization of enteric pathogens and facilitate delivery of toxins (94–96). Recent work has also recognized the contribution of many proteases and other effectors in immune evasion. The *B. cenocepacia* ZmpA and ZmpB are examples of proteases that cleave antimicrobial peptides, and the *L. pneumophila* T2SS reduces the output of cytokines and chemokines during infection, in part due to secretion and activity of the metalloprotease ProA (97–99). Another secreted metalloprotease, StcE, protects EHEC from host defense mechanisms including complement-mediated killing by cleaving C1 esterase inhibitor and neutrophil-associated proteins, while the *A. baumannii* metalloprotease CpaA interferes with blood coagulation by inactivating factor XII, which may result in the escape from clearance by intravascular clots and dissemination of *A. baumannii* (73, 100–103). In the environment, bacterial life frequently occurs as matrix encased biofilm. The major component is often polysaccharides, but the matrix also contains specific proteins secreted by the T2SS that contribute to the formation, architecture and stability of the biofilm (76, 104–109).

SECRETIN STRUCTURE AND ASSEMBLY

The secretin of the T2SS forms the OM channel for the passage of secreted proteins. Topologically, it contains an N-terminal N0 domain, homologous repeat N1–N3 domains and a C-terminal secretin core domain (Fig. 3A). The majority of T2SS secretins have an additional C-terminal S-domain required for interaction with pilotins, which delivers them to the OM (see below and Fig. 3B). Early electron microscopy (EM) studies revealed the general architecture of secretins as a multimeric channel with an open periplasmic chamber, a closed central gate and a top chamber (31, 110–113). The crystal structures of the N0-N1-N2 domains of ETEC GspD (30) allowed modeling of the N0-N1-N2-N3 domains in the cryo-EM structure of *V. cholerae* GspD (31). However, only the recent progress in cryo-EM methodology enabled to solve the full-length secretin structures of GspD from *E. coli* K12 and *V. cholerae* (34), *P. aeruginosa* (35), and EPEC (36), and of the GspD–pilotin complex from ETEC (37) (Fig. 3A). The general structural features are well conserved between secretins of the *Vibrio*-type, the *Klebsiella*-type (114) and the more sequence divergent *Pseudomonas*-type (Fig. 3A) (35). The secretins form cylindrical structures ~150 Å in diameter and ~195 Å in length that display 15-fold symmetry, in contrast with previously reported 12-fold symmetry, a discrepancy likely caused by the limited resolution of the earlier studies. The major difference between the secretins is the presence of an extracellular gate (cap) of unknown function in the *Vibrio*-type secretins (Fig. 3A) (34, 36).

In the secretin monomer, the domains are arranged in line that is tilted at $\sim 30^\circ$ (Fig. 3A). The N0 domain is not modeled in the available reconstructions as it was disordered, although weak smeared density was observed in some 2D class averages (34). Together, the secretin core domains form a double barrel structure that include inner and outer barrels. Each secretin monomer contributes 4 shorter beta-sheets to the inner barrel and 4 longer beta-sheets to the outer barrel. The β -hairpin extensions from the inner barrel form the periplasmic gate in the secretin channel. A mechanism of periplasmic gate opening during secretion has been suggested based on a partially open structure of a G453A mutant of *V. cholerae* GspD, which showed an upward rotation of the β -hairpins (34). This is consistent with a recent structure of a homologous type III secretion system secretin in the open form, which revealed that the β -hairpins of the periplasmic gate are in upward position (115). The aromatic and aliphatic residues on the top of the outer barrel contribute to the hydrophobic belt that creates the transmembrane region. The S domain provides overall stability to the secretin oligomer by interacting with an adjacent protomer. The N3 domains form a ring below the secretin core domains with which they make extensive contacts. The interactions between N1-N2 and N2-N3 domain rings, on the other hand, are limited to the linkers and several specific contacts, which led to lower resolution of 3D reconstructions in this region.

The majority of T2SSs contain a *Klebsiella*-type pilotin, a fatty-acylated protein which has an α -helical structure that provides a hydrophobic groove for interactions with the second α helix from the S domain of the secretin (Fig. 3B) (116). In contrast, *Vibrio*-type secretins utilize an alternative, structurally unrelated pilotin, although this pilotin fulfills the same function (114, 117). The structure of the *Vibrio*-type pilotin in complex with the secretin adopts an open conformation compared to the apo-pilotin to accommodate the α -helix from the S domain (Fig. 3B) (37). While pilotins direct secretins to the OM via the lipoprotein sorting system (118), the mechanism by which the secretin protomers acquire the assembled state and insert into the OM independently of the BAM complex is less clear (119, 120). However, a recent study that subjected the N3 domain of the *K. oxytoca* GspD to mutational analysis underscores the importance of the N3 domain in the early steps of secretin assembly (121). The results suggest that the N3 domain provides stability to the pre-pore, a prerequisite for OM insertion and pore formation of the secretin.

PSEUDOPILUS AND ASSEMBLY PLATFORM

Topologically, all pseudopilins consist of an N-terminal hydrophobic helix that extends into variable C-terminal globular domains. The major pseudopilin GspG contains a conserved Ca^{2+} -binding site (Fig. 2) (50, 122). Disruption of Ca^{2+} -binding by either amino acid substitutions or Ca^{2+} ion removal with chelating agents affects the stability of pseudopilin monomer, pseudopilus assembly and substrate secretion (50, 122). Interestingly, the minor pseudopilin GspK also contains a Ca^{2+} -binding site (Fig. 2); however, the functional significance of this feature has yet to be determined (48). The minor pseudopilins GspK–GspI–GspJ form a quasi-helical trimeric complex that is thought to be located at the pseudopilus tip (Fig. 2) (48). It has been demonstrated that this complex serves as a priming site for the pseudopilus assembly (123). Minor pseudopilin GspH possibly acts as an adapter between the GspK–GspI–GspJ tip and the poly-GspG fiber (47). The most recent model of the *K. oxytoca* GspG pseudopilus based on a cryo-EM reconstruction revealed a right-

handed helical fiber with a 10 Å rise (50). The N-terminal hydrophobic helices of the GspG subunits arrange within the core of the pseudopilus, with the C-terminal domains and the Ca²⁺-binding sites located at the surface (Fig. 2). Interestingly, the N-terminal hydrophobic helix is connected to the C-terminal domain by an extended linker, a feature distinct from the type IV pilus.

While a number of GspE homologs from the type IV pili systems have been structurally characterized in hexameric form (124–126), the structure of hexameric GspE has been elusive. Employing a ‘scaffold’ Hcp1 fusion strategy allowed to visualize GspE in two hexameric conformations: a symmetrical C6 form and an extended C2 form (Fig. 2) (20). These conformations may reflect structural transitions in GspE during ATP hydrolysis and transfer of mechanical energy to support the pseudopilus assembly. The details of this process are not completely understood although it is believed to involve GspF, GspL and GpsM, which have all been shown to interact with the pseudopilus (13, 18, 127, 128). While the structures of the extramembrane domains of GspC, GspF, GspL and GpsM have been solved (Fig. 2), the structure of the assembly platform formed by these components has not yet been reported.

IMPLICATIONS FOR MECHANISM

Despite the progress in understanding the structure-function relationship of the various components of the T2SS, the mechanism by which this important secretion system transports both soluble and lipidated proteins across the OM remains poorly understood. As folding of the T2SS substrates is a prerequisite for engagement with the T2SS, a secretion signal is thought to be formed in the folded structures; however, the structures of T2SS substrates greatly vary and a common, general secretion signal has yet to be identified. The protein-protein interaction domain PDZ of GspC has been suggested to recognize and recruit the T2SS substrates once they arrive in the periplasmic compartment; yet, interactions between the T2SS substrates and GspD, GspH–GspI–GspJ–GspK that forms the tip of the pseudopilus and the CM proteins GspL and GpsM have also been demonstrated (129–134). These interactions are, for the most part, consistent with two prevailing models for driving proteins through the secretin pore; the piston machinery and the Archimedes’ screw (135). In the piston model the pseudopilus tip supposedly pushes the substrates through the secretin in a linear fashion, although this mechanism cannot fully account for the required retraction of the pseudopilus and recharging of substrate as the T2SS lacks a retraction ATPase (48, 129, 132, 135, 136). In the Archimedes’ screw model, the rotary motion via interactions with the poly-GspG shaft of the pseudopilus threads the T2SS substrates out through the secretin pore; however, this model requires a continuous degradation and replenishment of GspG (137, 138). Dedicated removal of GspG-bound calcium and subsequent destabilization of GspG may result in degradation (50), but given the rigid structure of the double-barrel secretin domain, the dimensions of the pseudopilus and the mounting evidence for the rotation of the pseudopilus itself, perhaps a composite model should be considered, in which the rotary motion of the pseudopilus drives the secretion of the substrates that are pushed out by the pseudopilus tip.

CONCLUSION

In conclusion, while structural information is now available for most of the T2SS components and many T2SS substrates and the general architecture of the T2SS is understood, there are still multiple unanswered questions about the precise stoichiometry of this secretion complex, the detailed mechanism of pseudopilus assembly and possibly disassembly, and the molecular basis for substrate recognition. Before long; however, answers to these quandaries are expected to be revealed as the field is progressing rapidly due to technology advances.

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Figure 1. Overview of the general architecture of the T2SS and its substrates.

A. A schematic diagram of topology and location of the conserved core components of the T2SS. The accessory components GspN, GspA and GspB are not shown. **B.** A selection of the T2SS substrates of variable functions. Protein toxins: *V. cholerae* AB₅ cholera toxin (160); *P. aeruginosa* exotoxin A (161). Hydrolytic enzymes: *V. cholerae* VesB (68); *B. glumae* lipase in complex with chaperone (shown in purple) (71); *K. oxytoca* pullulanase (77); *D. dadantii* pectate lyase C (162); EHEC metalloprotease StcE (163); *L. pneumophila*

aminopeptidase LapA (91). Scaffolding protein: *V. cholerae* biofilm matrix protein RbmA (164, 165).

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Figure 2. Structures of the T2SS assembly platform and pseudopilus components.

ATPase: hexameric *V. cholerae* GspE with C6 and C2 symmetries (20). A close-up view shows the Zn²⁺-binding site, which is required for the function of GspE (14, 166). Inner-membrane components: cytoplasmic domain of *V. cholerae* GspF (19); cytoplasmic domain of GspL in complex with N1 domain of *V. cholerae* GspE (16); periplasmic domain of *V. parahaemolyticus* GspL (26); periplasmic domain of *V. cholerae* GspM (25); the homology region (HR) domain of ETEC GspC (32); and the PDZ domain of *V. cholerae* GspC (29). The structure of periplasmic domain of *P. aeruginosa* GspL (XcpY) has been recently

published (167). Pseudopilus components: *K. oxytoca* GspG pseudopilus model based on the cryo-EM reconstruction (50), a close-up view shows the Ca^{2+} -binding site of *K. oxytoca* GspG; *V. cholerae* minor pseudopilin GspH (47); the trimeric complex of ETEC GspK–GspI–GspJ (48), a close-up view shows a double Ca^{2+} -binding site of GspK. The structure of a homologous XcpX–XcpV–XcpW complex from *P. aeruginosa* has been recently reported (168).

Figure 3. Structures of the T2SS secretins and pilotins.

A. The side and top views of ETEC GspD-AspS complex (37); EPEC GspD (36); *E. coli* K12 GspD (34); and *P. aeruginosa* GspD (35). A single secretin protomer is highlighted with N1, N2 and N3 domains in shades of blue, secretin domain in green and S domain in magenta. Several AspS protomers (brown) were omitted to clearly show the location of the S domain. The cap sub-domain in the *Vibrio*-type secretins is highlighted in orange. The N0 domains (purple) were not resolved in the available cryo-EM reconstructions due to flexibility. Instead, its approximate location is indicated (169). Note that the N1-N2 domains of EPEC GspD (36) and the N1 domain of *P. aeruginosa* GspD (35) have been placed as rigid fit models. **B.** Structures of pilotins in complex with the secretin S domains (magenta). Structures of *Vibrio*-type ETEC AspS (37) and *Klebsiella*-type *D. dadantii* GspS (116) are shown.

Table 1.

Examples of T2SS substrates

Protein	Type	Activity	Reference Examples
Toxins	Enterotoxin (cholera toxin; <i>E. coli</i> heat-labile enterotoxin)	ADP-ribosylation of Gsa. subunit leading to increased adenylate cyclase activity and raising cAMP levels, which activates protein kinase A followed by phosphorylation of the CFTR channel. This leads to efflux of chloride ions and water release into the intestinal lumen; Secretory diarrhea	(85, 86)
	Exotoxin A	ADP-ribosylation of elongation factor 2; Inhibition of protein synthesis in host cells	(139)
	Pore forming toxin (aerolysin; cytolysin)	Host cell membrane depolarization and lysis	(63, 140–142)
Proteases	Metalloprotease; Serine protease; Cysteine protease, Aminopeptidase	Cleavage of proteins or peptides; Breakdown of host extracellular matrix; Tissue damage; Detachment from host cells; Nutrient acquisition; Evasion of host defense system; Translocation to new niche.	(73, 90, 91, 96, 97, 99–101, 103, 139, 143–146)
Lipid-modifying enzymes	Lipase, phospholipase, Glycerophospholipid cholesterol acyltransferase	Breakdown of lipids to fatty acids and glycerol; Nutrient acquisition; translocation to new niche. Breakdown of phospholipids and destabilization of host cell membranes.	(40, 70, 73, 88, 91, 139, 147, 148)
Carbohydrate-active enzymes	Chitinase, Amylase, Cellulase, Pullulanase, Xylanase, Pectinase, Pectin methylesterase, Pectate lyase, Levansucrase	Breakdown of polysaccharides such as chitin, cellulose, pectin, amylose, pullulan, xylan; targeting of O-GlcNAcylated proteins in the host; Nutrient acquisition; Depolymerization of plant cell wall; Wilting; Soft rot	(55, 87, 93, 147, 149, 150)
Phosphatases	Alkaline phosphatase; Acid phosphatase	Dephosphorylation; Phosphate acquisition; Phosphate solubilization	(151–153)
Nucleic acid targeting enzymes	DNase, RNase	Hydrolysis of DNA and RNA; generation of nutrients that include carbon, nitrogen and phosphate that support bacterial growth; Evasion of neutrophil extracellular traps	(92, 154, 155)
Metal reductase	C-type cytochromes	Reduction of insoluble metal oxides; Electron transport; Anaerobic respiration	(156, 157)
Others	Chitin-binding protein; collagen-like protein; Biofilm associated proteins	Adherence; Biofilm formation	(105, 106, 158, 159)