The structure and mechanism of the bacterial type II secretion system

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Funding information
Wellcome Trust, Grant/Award Number: 215553/Z/19/Z; Wellcome Trust, Grant/Award Number: 215553 and 19

Abstract
The type II secretion system (T2SS) is a multi-protein complex used by many bacteria to move substrates across their cell membrane. Substrates released into the environment serve as local and long-range effectors that promote nutrient acquisition, biofilm formation, and pathogenicity. In both animals and plants, the T2SS is increasingly recognized as a key driver of virulence. The T2SS spans the bacterial cell envelope and extrudes substrates through an outer membrane secretin channel using a pseudopilus. An inner membrane assembly platform and a cytoplasmic motor controls pseudopilus assembly. This microreview focuses on the structure and mechanism of the T2SS. Advances in cryo-electron microscopy are enabling increasingly elaborate sub-complexes to be resolved. However, key questions remain regarding the mechanism of pseudopilus extension and retraction, and how this is coupled with the choreography of the substrate moving through the secretion system. The T2SS is part of an ancient type IV filament superfamily that may have been present within the last universal common ancestor (LUCA). Overall, mechanistic principles that underlie T2SS function have implication for other closely related systems such as the type IV and tight adherence pilus systems.

Keywords
assembly platform, cryo-EM, secretin, structure, type II secretion system, type IV pilus system

1 | INTRODUCTION

The T2SS is a macromolecular complex that spans the cell envelope of many Gram-negative bacteria. It is part of a much larger superfamily of type IV filament containing systems all of which share homologous components and conserved mechanistic principles. These include the type 4a and 4b pilus (Craig et al., 2019), tight adherence (Tad) pilus (Tomich et al., 2007), mannose-sensitive hemagglutinin pilus (Marsh and Taylor, 1999), competence pilus (Piepenbrink, 2019) and archaeal T4 pilus and flagellum (Makarova et al., 2016). These systems are ancient and fundamental to both bacterial and archaeal kingdoms with phylogeny analyses suggesting a lineage split from within the last universal common ancestor (LUCA) (Denise et al., 2019). The T2SS is particularly concentrated in the Alpha-, Beta-, Gamma- and Delta-proteobacteria, as well as the Bacteroidetes and Deferribacteres (Denise et al., 2019). It is a key virulence factor in many human pathogens including Acinetobacter baumannii (Elhosseiny and Attia, 2018), Klebsiella
These are “ESKAPE” pathogens known for their ability to evolve anti-microbial resistance and are the leading cause of nosocomial infections (Santajit and Indrawattana, 2016). Many diverse effectors and toxins depend on the T2SS for secretion (Cianciotto, 2009; Shutinoski et al., 2010; Sikora et al., 2011). Such substrates are involved in adhesion, biofilm formation, nutrient acquisition, colonization, and invasion (Cianciotto and White, 2017).

Overall, the T2SS is of fundamental importance as it represents a key mechanism by which bacteria induce local and long-range modifications to their external environment with significant ramifications for human disease and drug development (Waack et al., 2017). This microreview summarizes recent advances in T2SS structure and mechanism. It provides a snapshot of how the T2SS spans the cell envelope and examines how pseudopilus assembly may drive substrate secretion.
1.1 Type 2 secretion system components

The canonical T2SS operon contains ~13 genes often arranged in a single operon, named gspC to gspO (Nivaskumar and Francetic, 2014; Pugsley, 1993). The T2SS may be subdivided into three subcomplexes (1) an outer membrane complex (OMC), which includes the secretin channel formed by GspD and the pilotin GspS; (2) an inner membrane complex called the assembly platform (AP), which includes GspC, GspF, GspL, and GspM (GspCFLM) plus the cytoplasmic ATPase GspE; (3) the pseudopilus, which constitutes the major pseudopilin GspG, and the minor pseudopilins GspH, GspJ, and GspK (GspGHJK). GspO, the prepilin peptidase is embedded in the inner membrane and processes the pseudopilins GspGHJK into a mature form ready for pseudopilus assembly (Dupuy and Pugsley, 1994; Francetić et al., 1998). In addition, a membrane protein called GspN is present in some T2SS and forms part of the AP within the inner membrane. Some bacteria additionally encode two inner membrane peptidoglycan-interacting proteins GspA and GspB, which are involved in locating GspD to the outer membrane (Howard et al., 2019; Strozen et al., 2011).

1.2 Outer membrane complex ultrastructure and assembly

GspD forms a multi-subunit secretin channel with C15 symmetry that spans the outer membrane and protrudes into the periplasm (Chernyatina and Low, 2019; Hay et al., 2018; Hay et al., 2017; Howard et al., 2019; Yan et al., 2017; Yin et al., 2018). The secretin constitutes four N-terminal periplasmic domains called N0, N1, N2, and N3, a secretin core, and a C-terminal S-domain. Two types of secretin, the Klebsiella-type (Figure 1a,c) and the Vibrio-type (Figure 1b,d) are distinguished. Whereas the secretin core is highly ordered, flexibility increases toward the N-terminus with most cryo-electron microscopy (EM) secretin structures exhibiting partially disordered N2 or N1 domains and entirely absent densities for the N0 domain (Hay et al., 2018; Hay et al., 2017; Howard et al., 2019; Yan et al., 2017; Yin et al., 2018) (Figure 1b-d). While the fold of the N0 domain was known from N0-N2 domain crystal structures (Korotkov et al., 2011; Korotkov et al., 2009b; Van der Meeren et al., 2013), these structures did not fit the cryo-EM maps (Yan et al., 2017). Therefore, the position of N0 relative to the rest of the secretin was unknown. The purification of a ~2.4 MDa T2SS complex from K. pneumoniae comprising GspD with its pilotin GspS/PulS, plus GspE, GspL, GspM, GspN, and GspC (GspCDELMNS) yielded a complete secretin structure with ordered N0 domains (Chernyatina and Low, 2019) (Figure 1a). The secretin N0-N3 domains form pentadecameric stacked rings. N1-N3 domains are aligned with a ~36° angular offset to the channel axis. These ~8 kDa domains are connected to each other and the secretin core by short flexible linkers. At the level of the N3 domain, the internal lumen of the secretin is constricted with a disordered loop, which may function as a selectivity filter for exported substrates. The N0 domain consists of two helices flanked on each side by β-sheets that pack laterally to form the secretin base (Figure 1a,e). Recently, the structure of the PilQ secretin from the Vibrio cholerae type IV competence pilus was determined by cryo-EM (Weaver et al., 2020). Although PilQ does not share N1-N3 domains with the T2SS, it does have an N0 domain. Interestingly, PilQ N0 domains pack similarly to T2SS N0 domains at the secretin base (Figure 1e). This packing arrangement may, therefore, be a conserved feature in other type IV filament system secretins, although it is not conserved in the T2SS where the secretin N0 domain helices pack laterally (Hu et al., 2018). In the K. pneumoniae T2SS, the N0 and N1 domains are connected by a substantial 26 amino acid linker called Loop 7, which forms a wedge that packs between neighboring N1 domains and stabilizes them. Wedge formation, stability of the K. pneumoniae secretin N-domains was likely promoted by additional components within the GspCDELMNS complex. The homology region domain of GspC (GspCHR) was bound to GspD N0 domains (Figure 1a) as observed in the enterotoxigenic Escherichia coli (ETEC) GspD N0-N1 domain and GspCHR co-crystal structure (Korotkov et al., 2011). Additionally, in the K. pneumoniae secretin cryo-EM map, an unidentified plug density was observed occluding the secretin lumen at the level of the N0-N1 domains (Chernyatina and Low, 2019). Given the position of the plug, it likely contributed to the stabilization of the secretin periplasmic domains. A similarly positioned plug density was observed in the cryo-electron tomography (cryo-ET) reconstruction of the Legionella pneumophila T2SS (Ghosal et al., 2019). Intriguingly, in the type 4 pilus system (T4PS), the N-terminal von Willebrand factor A domain of PilY1 constitutes a secretin plug while its C-terminus locates with the minor pilins at the pilus tip to form a priming complex (Treuner-Lange et al., 2020).

The secretin core spans the outer membrane and constitutes a barrel with an inner and outer layer of β-sheets (Yan et al., 2017). A central gate separates the external milieu from the inner lumen (Figure 1f) and must open to allow the substrate to pass through the channel. The gating in the T2SS might work similarly as for the type III secretion system (T3SS), where the central gate has been shown to hinge open (Hu et al., 2018; Yan et al., 2017) (Figure 1f). Vibrio-type secretins feature an additional cap gate at the top of the secretin (Figure 1b, d). The C-terminal S-domain forms a triple helix or helix-strand-helix motif located on the outside of the secretin core interacting with the pilotin (Figure 1a,b).

Pilotins are secretin-specific lipoprotein chaperones supporting the oligomerization, assembly, and correct insertion of GspD subunits into the outer membrane (Dunstan et al., 2013; Nickerson et al., 2011). They may be co-located with other T2SS genes in a single operon as for yghG in ETEC (Strozen et al., 2012) or separated and positioned elsewhere in the chromosome (Chernyatina and Low, 2019; Koo et al., 2012). Pilotins are sorted to the outer membrane via the Lol machinery after the cleavage of their signal sequences (Okuda and Tokuda, 2011). A conserved cysteine at the pilotin N-terminus undergoes lipidation, which serves as an anchor within the inner leaflet of the outer membrane (Zückert, 2014). There are two types of pilotins identified in the T2SS, the Klebsiella-type, and the Vibrio-type. The Klebsiella-type pilotins have a fold comprised
of four α-helices (Gu et al., 2012; Korotkov and Hol, 2013; Rehman et al., 2013; Tosi et al., 2011) (Figure 1a). In contrast, the Vibrio-type pilotins constitute five stranded β-sheets flanked by four α-helices (Dunstan et al., 2013; Yin et al., 2018) (Figure 1b). Each pilotin has a groove into which the C-terminal helix of the S-domain inserts. The lack of a pilotin can lead to the mislocalization of the secretin to the inner membrane or its degradation (Hardie et al., 1996a; Hardie et al., 1996b; Viarre et al., 2009). T3SS and T4PS pilotins (Golovanov et al., 2006; Lario et al., 2005) are not conserved with those of the T2SS so that overall, pilotins are secretion system specific and vary in both sequence and structure. Interestingly, some T2SS secretins assemble in the outer membrane without the requirement of a pilotin, for example, *Aeromonas hydrophila* (Howard et al., 2019; Strozen et al., 2011). In such cases, the accessory components GspA and GspB facilitate secretin assembly and membrane insertion through the binding and modulation of the peptidoglycan layer.
1.3 | The inner membrane components of the T2SS

The inner membrane module of the T2SS is composed of three components, a group of integral membrane proteins called the AP, a pseudopilus, and a cytoplasmic ATPase (Figure 2). The AP is composed of the monotopic membrane proteins GspM, the bitopic membrane proteins GspC (Bleves et al., 1999) and GspL, and the polytopic membrane protein GspF. In some T2SS, an additional possible monotopic membrane protein called GspN associates with GspL and GspM (Lee et al., 2001; Lee et al., 2000). Currently, the role of GspN is unclear given mutants in both A. baumannii and Klebsiella oxytoca do not affect substrate secretion (Possot et al., 2000; Waack et al., 2017).

GspC has multiple domains (Figure 2a) including a short N-terminal cytoplasmic stalk, a transmembrane helix followed by a long linker, the periplasmic GspCperiplasm, and a C-terminal PDZ domain (GspCperiplasm) (Gérard-Vincent et al., 2002; Korotkov et al., 2011; Korotkov et al., 2006). The transmembrane helix dimersises within the inner membrane (Login and Shevchik, 2006). Then through the binding of GspCperiplasm to the N0 domains at the secretin base (Figure 1a), GspC likely forms a periplasmic cage-like structure that interconnects the AP with the OM (Chernyatina and Low, 2019). While the crystal structure of GspCperiplasm revealed two stacks of three anti-parallel β-sheets (Korotkov et al., 2011), the GspCperiplasm crystal structure contained a typical bacteria-specific PDZ fold (Liao et al., 2000) comprising six β-sheets flanked by two helices (Korotkov et al., 2006). GspCperiplasm is not conserved and is sometimes either absent or replaced with a coiled-coil domain. Either of these domains is required for the recognition and secretion of particular substrates (Bleves et al., 1999; Bouley et al., 2001). For example, GspCperiplasm mutants were unable to secrete pectate lyases although they were still competent to secrete other substrates such as PemA or Cel5 at either normal or reduced levels (Bouley et al., 2001).

GspL and GspM contain a single membrane helix connected to a periplasmic domain (GspLperiplasm and GspMperiplasm). The crystal structures of Vibrio paraheamolyticus GspLperiplasm and GspMperiplasm both revealed a ferredoxin-like fold (Abendroth et al., 2009a; Abendroth et al., 2004b) (Figure 2a). Intriguingly, the equivalent fold was also observed in the T4PS proteins PilN and PilO (Karuppiah et al., 2013; Sampaleanu et al., 2009), which suggested a common evolutionary ancestry and possible functional equivalence (Ayers et al., 2009). GspLperiplasm and GspMperiplasm formed dimers in the crystals although different interfaces were used for oligomerization. These interfaces may be present in vivo as cross-linking studies in Dickeya dadantii indicated GspLperiplasm and GspMperiplasm homodimer and heterodimer formation (Lallemand et al., 2013). GspL also includes a globular N-terminal cytoplasmic domain (GspLcytoplasm) (Figure 2a). The crystal structure of Vibrio cholerae GspLcytoplasm revealed three β-sheet-rich subdomains I, II, and III (Abendroth et al., 2004a). The subdomains I and III have an RNase H-like fold with five β-sheets flanked by α-helices and interestingly share structural homology with the 1A and 2A subdomains of the actin superfamily (Van den Abendroth et al., 2004a; Ent et al., 2001). GspLcytoplasm is unable to bind ATP though as it lacks an actin-like 2B subdomain. While GspLcytoplasm is a close structural ortholog of T4PS PiliM, they differ as PiliM includes an actin-like 2B subdomain that binds ATP but cannot hydrolyze it (Karuppiah and Derrick, 2011).

GspF is a polytopic membrane protein predicted to span the inner membrane three times and to contain two N-terminal cytoplasmic domains connected by a periplasmic loop. A short C-terminal tail is also accessible from the periplasm (Abendroth et al., 2009b; Arts et al., 2007; Thomas et al., 1997). This membrane topology was substantiated by alkaline phosphatase fusion assays (Arts et al., 2007). The crystal structure of the V. cholerae GspF (EpsF) first cytoplasmic domain showed a novel helical fold that dimerized and coordinated calcium through a conserved interface (Abendroth et al., 2009b) (Figure 2a). An equivalent fold was also predicted for the second cytoplasmic domain due to substantial shared sequence similarity (Peabody et al., 2003). A recent study in P. aeruginosa indicated that purified GspF (Xcps) adopts a dimeric structure (van Putte et al., 2018). Ultimately, the cellular oligomerization state of GspF within the T2SS is uncertain as the T4PS ortholog Picl has been isolated both as a dimer and tetramer in different systems (Bischof et al., 2016; Collins et al., 2007; Karuppiah et al., 2010).

GspE is a cytoplasmic ATPase that acts as a motor to power pseudopilus assembly and substrate extrusion (Camberg et al., 2007; Camberg and Sandkvist, 2005; Patrick et al., 2011; Sandkvist et al., 1995). It is closely related to the T4PS ATPases PilB and PilT (Mancl et al., 2016; Planet et al., 2001; Satyshur et al., 2007) and is an essential component of the T2SS (Sandkvist et al., 1995). Sequence and structural analysis of GspE revealed three domains, the N1E, N2E, and C-terminal CTE connected by linkers (Lu et al., 2014; Lu et al., 2013; Robien et al., 2003) (Figure 2c). Superposition of the GspE structures from Vibrio vulnificus and V. cholerae highlighted the flexibility of the linker between the N2E and CTE domains (Lu et al., 2014; Lu et al., 2013) (Figure 2c). The CTE domain may be further segmented into three subdomains called C1E, C2E, and CME. The C1E subdomain contains the nucleotide-binding Walker A and B motifs as well as additional aspartate and histidine motifs. Importantly, a unique tetracycline motif in the CME coordinates zinc, which is also conserved in T4PS assembly ATPases (Robien et al., 2003). Deletion of the tetracycline motif or substitution of any of the cysteine residues abrogated secretion in V. cholerae (Rule et al., 2016). The GspE structure in the absence of the N1E domain was determined in two states including a crystallographic hexamer with C6 symmetry (Figure 2d) and a non-crystallographic hexamer with C2 symmetry (Figure 2e). However, crystallization required the assisting hexameric fusion protein Hcp1. ATPase activity was upregulated upon hexamer formation which, along with biochemical studies (Camberg et al., 2007; Shiue et al., 2006), supported the concept that a hexamer was the functional unit within an assembled T2SS (Lu et al., 2013). Ultimately though, the functional oligomeric state of GspE in vivo has not yet been conclusively shown. Large scale conformational changes were observed between the C6 and C2 symmetry states that were reminiscent of other related ATPases in both the T4PS and archaeal motors (Collins et al., 2018; McCallum et al., 2019; McCallum et al., 2017; Reindl et al., 2013; Satyshur et al., 2007). Overall, the dynamic nature of GspE and its inherent flexibility is suspected to be vital in coupling nucleotide-induced...
conformational change in the mechanical loading of pseudopilins into the pseudopilus.

The pseudopilus functions to extrude substrates through the T2SS secretin channel. The tip of the pseudopilus is formed by a GspUK trimeric complex that may bind one or two subunits of the minor pseudopilin GspH (Korotkov and Hol, 2008) (Figure 2b). The rest of the pseudopilus constitutes a helical filament formed by the major pseudopilin GspG (Campos et al., 2010; Köhler et al., 2004; López-Castilla et al., 2017). All the pseudopilins have a characteristic fold comprising an N-terminal α-helix, a short variable region, and a four-stranded C-terminal antiparallel β-sheet (López-Castilla et al., 2017; Raghunathan et al., 2014; Yanez et al., 2008). GspGHI has a conserved glutamic acid at amino acid position 5 (Glu5) within the mature sequence. In the case of GspG and GspH, Glu5 mediates both recruitment of these pseudopilins to the AP via direct contact with GspM, and stabilisation of neighbouring pseudopilins within the pseudopilus (Nivaskumar et al., 2014; Nivaskumar et al., 2016). GspG contains a conserved calcium-binding site, which is essential for subunit folding and stability (Korotkov et al., 2009a; López-Castilla et al., 2017). GspK also binds calcium ions and has a unique additional helical domain that likely caps the pseudopilus tip (Korotkov and Hol, 2008).

1.4 Architecture of a type II secretion system

The T2SS is a multi-component complex that spans the cellular inner and outer membranes and periplasm. Other than the GspD secretin core which is a rigid and ordered structure, a high level of flexibility and dynamism underpins most T2SS components. This flexibility and the transient nature of the protein-protein interactions appear crucial for function. Recently, cryo-ET provided the first glimpse of a T2SS assembled in situ (Ghosal et al., 2019). Combined with cryo-ET on the T4PS (Chang et al., 2017; Chang et al., 2016; Gold et al., 2015; Treuner-Lange et al., 2020), we now have a topology map for the position of most components for both the T2SS and T4PS. In these systems, the secretin was observed in the outer membrane with its N-terminal domain protruding into the periplasm. Between the secretin base and the inner membrane, densities consistent with a ring-like structure were observed and assigned to the periplasmic domains of GspL and GspM in the T2SS, or PipN and PipO in the T4PS. A central stem density was positioned within the GspLM ring, which likely relates to the pseudopilus tip proteins GspHJK or their equivalent in the T4PS (Chang et al., 2016; Treuner-Lange et al., 2020). Densities for the cytoplasmic domains of GspL or T4PS PipM were nestled on the inner membrane surface and were consistent with another ring-like structure. In the T2SS, the putative GspE hexamer was suspended ~5 nm below the inner membrane (Ghosal et al., 2019), presumably tethered in place by the long linker between the GspE N1E and N2E domains (Figure 2c). The N1E domain then connects GspE to GspLcyto in a 1:1 stoichiometric complex (Abendroth et al., 2005; Lu et al., 2014). This contrasts with the T4PS ATPases PipB and PiIT, which are positioned flush with the membrane (Chang et al., 2016). Based on this data, it was possible to generate a model showing T2SS architecture (Figure 3) using known structures (Figures 1 and 2).

Within the model, the stoichiometry for GspD and the associated pilotin was 1:1 with 15 copies of each per OMC (Chernyatina and Low, 2019; Yin et al., 2018). The K. pneumoniae GspCDELMSN complex yielded relative stoichiometries for the GspCELMN AP components (Chernyatina and Low, 2019). GspE:L:M:N was in 1:1:1:1 ratio based on protein gel densitometry measurements (Luo et al., 2006), which translated into a copy number of six for each protein given GspE likely functions as a hexamer in an assembled T2SS (Chernyatina and Low, 2019; Ly et al., 2013). Protein gel densitometry also indicated that there were at least two GspC subunits for each GspELMN subunits, which translated to a copy number of at least twelve GspC subunits per T2SS. As each GspC reaches across the periplasm from the inner membrane and binds to a single GspD N0 domain at the secretin base, this suggested a simple mechanism for overcoming the symmetry mismatch between the overall sixfold symmetry of the AP and the 15-fold symmetry of the OMC (Chernyatina and Low, 2019). It is possible that up to fifteen GspC subunits may be incorporated within each T2SS as each GspD N0 domain at the secretin base has the capacity to bind a single GspC_{15} (Chernyatina and Low, 2019; Korotkov et al., 2011; Wang et al., 2012). Alternative mechanisms for overcoming symmetry mismatch have been suggested, which may not be mutually exclusive, including the formation of pseudo C6 symmetry within the secretin periplasmic domains (Douzi et al., 2017; Hay et al., 2017). Overall, GspC appears to represent the core mechanism for connecting the inner membrane to the OMC, which highlights the flexible and dynamic nature of the T2SS.

An outstanding question is how GspL, GspM, GspC, and GspN when present, are arranged within the inner membrane when the T2SS is assembled. Pull-down and yeast-two hybrid experiments in D. dadantii have shown direct interactions between the transmembrane regions of GspL, GspM and GspC, as well as the interaction between GspL and GspM periplasmic domains potentially through their ferredoxin-like domains (Lallemand et al., 2013). Studies in other systems such as V. cholerae (Johnson et al., 2007; Sandkvist et al., 1999; Sandkvist et al., 2000), Erwinia chrysanthemi (Py et al., 2001) and P. aeruginosa (Robert et al., 2005) also supported the interaction between GspL and GspM. In addition, GspN was shown to associate with GspL and GspM in both Xanthomonas campestris (Tsai et al., 2002) and in K. pneumoniae (Chernyatina and Low, 2019). A picture, therefore, emerges where GspC, GspL, GspM, and GspN form a complex within the inner membrane. In the K. pneumoniae GspCDELMSN complex, a modular and dynamic hexameric hub architecture was suggested for AP components where complexes of GspCLMN were flexibly arranged around the central GspE hexamer (Chernyatina and Low, 2019). Both GspL and GspM formed homodimers and heterodimers in vitro (Abendroth et al., 2009a; Douet et al., 2004; Lallemand et al., 2013; Py et al., 1999; Sandkvist et al., 1999) and it is possible that this arrangement is reproduced within an assembled T2SS. GspF is positioned at the center of the AP where it makes contact with GspL and GspE as shown in P. aeruginosa and E. chrysanthemi (Arts et al., 2007; Py et al., 2001). Bacterial two-hybrid analyses have also connected GspF with GspM as well as components of the pseudopilus including GspG and GspH (Nivaskumar et al., 2016). The pseudopilus is, therefore, located...
centrally associated with GspF where it is well positioned to extend across the periplasm into the secretin lumen.

1.5 | Substrate recruitment and secretion mechanism

T2SS substrates are initially transported across the inner membrane by the Sec or Tat systems (Pugsley et al., 1991; Voulhoux et al., 2001). From the periplasm, the substrate is then targeted for secretion through poorly understood and diverse mechanisms that appear specific to each substrate with no universal signal sequence. T2SS substrates include toxins, lipases, metalloproteases, and digestive enzymes (Korotkov and Sandkvist, 2019). Recently, the pathogen Acinetobacter baumannii was shown to secrete multiple T2SS substrates including the lipases LipA and LipH along with the protease CpaA (Tilley et al., 2014; Urusova et al., 2019) (Figure 2a). These substrates are essential for A. baumannii virulence. Interestingly, both LipA and CpaA were paired with specific periplasmic chaperones called LipB (Frenken et al., 1993) and CpaB (Harding et al., 2016; Kinsella et al., 2017), which were anchored in the inner membrane and required for T2SS recruitment and secretion. Bioinformatics suggests that multiple T2SS effectors are actually paired in dimeric operons with putative membrane-bound chaperones, as identified in V. cholerae, P. aeruginosa, and Burkholderia pseudomallei (Harding et al., 2016).

Many studies have identified GspC as being critical for substrate recognition and recruitment into the T2SS (Bleves et al., 1999; Bouley et al., 2001; Douzi et al., 2011; Gérard-Vincent et al., 2002; Pineau et al., 2014) (Figure 4, steps 1 and 2). In some cases, direct interactions occur between the substrate and both GspC<sub>HR</sub> and GspC<sub>PDZ</sub> suggesting multiple contacts (Douzi et al., 2011; Pineau et al., 2014). In P. aeruginosa, the effector CbpD has recently been shown to be recruited first to GspM (XcpZ) and then to GspL (XcpY)
Substrate binding may stimulate GspL and GspM heterodimerization and transmembrane rotation so that allosteric conformational changes are induced on the cytoplasmic side. Such signal transduction may prime GspLcyto and other components of the AP for pseudopilus assembly and active secretion (Lallemand et al., 2013; Michel-Souzy et al., 2018). Not all substrates may follow the same physical and temporal protein-protein pathway between GspC, GspL, and GspM during secretion. Ultimately substrates bind to the GspHIJK pseudopilus tip and make contact with the secretin N-domains (Douzi et al., 2011; Pineau et al., 2014; Reichow et al., 2011; Reichow et al., 2010; Shevchik, 1997) (Figure 4, step 3). This priming complex may be similar to the T4PS in its retracted state with PilY1 topologically analogous to T2SS substrate given it simultaneously contacts both minor pilins and plugs the secretin base (Treuner-Lange et al., 2020).

One of the outstanding questions in the T2SS field relates to how pseudopilus assembly occurs within the AP and how this assembly is then coupled with substrate extrusion through the secretin channel. The inherent dynamism of the AP has hindered structural studies which might otherwise have provided a molecular snapshot of how the different components engage each other and undergo conformational changes. For example, current structures of GspE depended on the fusion protein Hcp1 to oligomerize and to capture a hexameric state (Lu et al., 2013). In contrast, how the T4PS ATPase motors extend and retract pili is better understood (Craig et al., 2019) and may provide insight into how the T2SS pseudopilus assembly works given evolutionary relatedness and the likelihood of shared mechanistic principles. In the T4PS a rotary mechanism has been suggested for pilus extension where ATP hydrolysis in the motor PilB is coupled with a clockwise sequence of conformational changes in the hexameric ring (Mancl et al., 2016; McCallum et al., 2019; McCallum et al., 2017; Solanki et al., 2018). These conformational changes may be directly transmitted to PilC (equivalent to GspF in the T2SS) so that it sequentially rotates and shifts 8–10 Å.

**FIGURE 4** T2SS substrate secretion mechanism. 1, When the T2SS is inactive, the pseudopilus tip comprising GspHIJK resides in the inner membrane possibly associated with GspF. 2, Substrate is recruited into the secretion system through interactions with GspC and GspM, and GspL and makes contact with the pseudopilus tip. 3, Substrate is loaded and the T2SS is primed for secretion. Given the dimensions of the pseudopilus tip and periplasm, loaded substrate is positioned within the secretin lumen contacting GspD N0 and N1 domains. The GspE hexamer has engaged GspF ready to initiate pseudopilus assembly. 4, GspE ATP nucleotide cycling drives pseudopilus assembly possibly through a GspF rotary mechanism. In such a model, the pseudopilus itself is not expected to rotate during assembly. GspG subunits are processed by the prepilin peptidase GspO (not shown), diffuse into the T2SS via GspM, and are spooled into the growing pseudopilus. 5, Substrate is extruded through the secretin and is secreted. It is estimated that ~24 copies of GspG are required for the pseudopilus tip to contact the secretin central gate, which may be a sufficient length for the substrate to be released. Pseudopilus assembly stalls GspE possibly through a GspK steric hindrance mechanism triggering retraction. 6, GspG diffusion from the base of the pseudopilus represents a passive mechanism for pseudopilus disassembly. GspE is disengaged from GspF. The T2SS returns to an inactive resting state.
orthogonally from the membrane toward the periplasm. The effect is to spool incoming pilins, or pseudopilins in the case of the T2SS, from the inner membrane reservoir and to extrude them by this distance out of the membrane into the growing pilus or pseudopilus (Figure 4, step 4). Notably, this 8–10 Å translation correlates well with the 8–10 Å rise observed between pilin or pseudopilin subunits assembled in both the T4PS pilus (Kolappan et al., 2016; Li et al., 2012; Wang et al., 2017) and T2SS pseudopilus (López-Castilla et al., 2017; Nivaskumar et al., 2014) (Figure 2b). In this model, the rotary motion would be specific to PilC or GspF and does not include T4PS pilus or T2SS pseudopilus rotation. An alternative model where the pilin is extruded from the membrane by compression has also been proposed for the T4PS and may also be relevant for the T2SS (Craig et al., 2019). Based on the dimensions of the L. pneumophila T2SS (Gholasal et al., 2019), it is estimated that a pseudopilus incorporating ~24 GspG subunits is required for the GspHIJK pseudopilus tip to reach the secretin central gate, which may be sufficient for the substrate to then be extruded in a piston-like mechanism (Shevchik, 1997) (Figure 4, step 5). How the pseudopilus retracts or disassembles in the T2SS is another fundamental question that is not currently well understood. In the T4PS, retraction is actively driven through an ATPase such as PilT (Merz et al., 2000), which replaces PilB and likely processively reverses the assembly mechanism to unspool pilins from the pilus and to return them to the inner membrane reservoir. In the T2SS there is no known dedicated retraction ATPase. To account for this, one option is that the pseudopilus actually continually assemblies at the base, while concurrently disassembling at the tip, thereby functioning similar to an Archimedes’ screw (Nivaskumar and Francetic, 2014). The pseudopilus requires bound calcium ions for stability and in this type of model, pseudopilus disassembly may be coupled with a reduced calcium concentration in the external environment in comparison to the periplasm (López-Castilla et al., 2017). Alternatively, periplasmic proteolysis may simply control pseudopilus length. While the Archimedes’ screw model is viable, the mechanistic principles upon which the T4PS functions, where the pilus cycles through extension and retraction cycles, supports a T2SS pseudopilus piston-like model. In this case and in the absence of a dedicated retraction ATPase, it is suggested that pseudopilus retraction occurs passively through spontaneous disassembly (Craig et al., 2019; Ng et al., 2016) (Figure 4, step 6). Stalling of the GspE motor may be induced by steric hindrance of GspK at the pseudopilus tip contacting the central gate. Indeed, GspK (XcpX) has been shown to control pseudopilus length in P. aeruginosa (Durand et al., 2005). Inactivation of the GspE motor would switch the equilibrium from pseudopilus assembly toward a disassembled resting state where GspG subunits diffuse out of the pseudopilus back into the inner membrane reservoir. The retraction of the GspHIJK pseudopilus tip to the inner membrane would mark one full secretion cycle where the secretion system is now competent to recruit further substrate (Figure 4, step 1). The limited length of a fully extended pseudopilus facilitates passive disassembly. Studies on various T4P systems support such a passive disassembly model. For example, retraction of pili many microns in length have been described in three T4P systems carrying pilI mutations (Clausen et al., 2009; Ellison et al., 2018; Zöllner et al., 2019). Additionally, the pili of the V. cholerae toxin-coregulated pilus system, which is one of the simplest of T4P systems, are retractile even in the absence of a dedicated ATPase (Ng et al., 2016). While the Tad pilus system has no dedicated retraction ATPase, it may have compensated by evolving two PilC/GspF paralogs called TadB and TadC, whose role may be to deliver bi-directional pilus dynamics from a single motor (Ellison et al., 2019; Tomich et al., 2007).

2 | CONCLUSION

Since the discovery of the T2SS over two decades ago, significant progress has been made in understanding its structure and mechanism. A combination of X-ray crystallography and more recently cryo-EM means that most of the T2SS components have had their structure at least partially determined. Structural biology is important for dissecting the T2SS mechanism as it provides an essential means to understand the chemistry of individual proteins and protein complexes, while also providing a powerful framework from which to interpret and base biochemical and cell biology analyses. Arguably some of the most critical structures for showing how the T2SS functions remain outstanding. For example, the assembly platform represents the stator for the GspE motor and provides the scaffold to house GspF and the pseudopilus. Many of the assembly platform components have had their cytoplasmic and periplasmic components structurally determined but crucially in the absence of their membrane domains. It is known that a web of interactions occurs between GspL, GspM, GspF, and GspE, many of which occur within the membrane. The next frontier will be to resolve full-length complexes of these proteins. These should show snapshots of how the motor engages GspF and transitions power to pseudopilus assembly. Such complexes should also show how conformational change in the cytoplasmic side are allosterically coupled with movements on the periplasmic side, and vice versa, with implication for substrate recruitment and pseudopilus assembly control. Such structures, combined with higher resolution in situ cryo-ET, will ultimately yield a detailed molecular model and greatly enhanced the mechanistic understanding of the T2SS.

ACKNOWLEDGMENTS

This work was funded by a Wellcome Trust Senior Research Fellowship to HL (215553/Z/19/Z). The authors state that they have no conflict of interest.

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**How to cite this article:** Naskar S, Hohl M, Tassinari M, Low HH. The structure and mechanism of the bacterial type II secretion system. *Mol Microbiol*. 2021;115:412–424. [https://doi.org/10.1111/mmi.14664](https://doi.org/10.1111/mmi.14664)