The role of intrinsic disorder and dynamics in the assembly and function of the type II secretion system

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ABSTRACT

Many Gram-negative commensal and pathogenic bacteria use a type II secretion system (T2SS) to transport proteins out of the cell. These exported proteins or substrates play a major role in toxin delivery, maintaining biofilms, replication in the host and subversion of host immune responses to infection. We review the current structural and functional work on this system and argue that intrinsically disordered regions and protein dynamics are central for assembly, exo-protein recognition, and secretion competence of the T2SS. The central role of intrinsic disorder-order transitions in these processes may be a particular feature of type II secretion.

1. Introduction

Gram–negative bacteria have evolved sophisticated multi-protein assemblies that can transport molecules across their outer membrane for manifold purposes including: offense, defense, nutrient acquisition, competition and communication. Some of these external assemblies, such as: pili, fimbriae, polysaccharides and flagella enable adhesion to host and abiotic surfaces, facilitate motility and allow invasion of the host [1-3]. Other bacterial systems can recognize specific cargo and transport this cargo into the extracellular milieu or directly into other cells to release nutrients and subvert the machinery of the other cells. Over ten outer-membrane protein assembly and secretion systems have been identified across Gram-negative bacteria which include the flagellum, the β-barrel assembly machinery (BAM) complex, the Wza translocon that assures secretion of capsular polysaccharides, the lipopolysaccharide assembly transport system (Lpt), the chaperone-usher and type IV pilus assembly systems, and the type I through to type IX secretion systems [3-9]. Although some of these machines are involved in essential cellular processes (e.g. BAM), others facilitate specific functions related to colonization of niche environments and persistence and can be acquired through horizontal gene transfer [10]. As such these outer-membrane machines and their cargos are often key virulence determinants and directly involved in pathogenesis.

The relatively large number of secretion systems that occur across Gram-negative bacteria reflects the importance of communicating with the environment. Gram-negative bacteria contain two membranes, inner or cytoplasmic and outer, that delimit together an intervening periplasmic space with peptidoglycan mesh. Therefore, transport out of the cell must either proceed directly from the cytosol through a single step involving a tunnel through the periplasm, or using a two-step process with a periplasmic intermediate. For example, effectors released through type III secretion systems (T3SS) must unfold during a one-step translocation process and then fold into their native conformation post ‘injection’ inside the host cell [11]. On the other hand, substrates of the type II secretion system (T2SS) are fully folded before being recruited, they are often oligomeric, contain disulfide bonds and can harbor complex cofactors [12-14]. Consequently, these proteins first enter the periplasm via either the

Sec or TAT translocon [15, 16] before their release from the cell in their final native state by the type II secretion system.

2. The type II secretion system

The T2SS was initially considered as the main terminal branch of the general secretory pathway (Gsp), where unstructured substrates first enter the periplasm via the Sec translocon [15], fold, and are then secreted by the T2SS. However, it has since been discovered that some T2SS substrates can also be exported into the periplasm by the TAT (twin-arginine translocation) pathway, which translocates proteins in their native folded state [15, 16]. Nonetheless, the term Gsp is often still used to refer to the T2SS components, from GspA to GspO and GspS.

T2SSs have been identified in both commensal and pathogenic bacteria belonging to α, β, γ and δ-proteobacteria, and in some strains, several T2SSs are present [17]. For instance, *Legionella pneumophila* and *Vibrio cholerae* each carry a single T2SS [18] while *Dickeya dadantii* and several *Escherichia coli* pathotypes carry two [19, 20]. Often, the same T2SS secretes multiple proteins, e.g. the *D. dadantii*, *V. cholerae* and *L. pneumophila* T2SSs each secrete 20 to 30 substrates with functions that include biofilm formation; adhesion to, invasion of and replication in their hosts; and subversion of host immune responses to infection [21-23]. In contrast, the T2SS of the human pathogen *Klebsiella oxytoca* secretes just one known substrate [24].

In this review, we will concentrate our analyses on the *V. cholerae* and *V. parahaemolyticus* Eps, *E. coli* Gsp, *D. dadantii* Out, *P. aeruginosa* Xcp, *K. oxytoca* Pul, *Aeromonas hydrophila* Exe, *Xanthomonas campestris* Xps and the *L. pneumophila* Lsp systems; since significant structural and functional data is available for these bacteria.

2.1 Genetic organization

There are 12 core components of the T2SS that are essential for biogenesis and secretion (**Fig. 1A**). These are the outer membrane secretin (GspD), the inner-membrane platform (GspC, GspF, GspL and GspM), the cytosolic ATPase (GspE), the pseudo-pilus composed of (GspG, GspH, GspI, GspJ and GspK) and the prepilin

peptidase, GspO, which is responsible for processing of pseudo-pilin subunits. In addition, *Vibrio*- and *Dickeya*-like systems also express pilotins, AspS and GspS, small lipoproteins each with a unique structure, which enhance the kinetics of secretin targeting and assembly in the outer-membrane [20, 25-29]. In *P. aeruginosa* Hxc and *X. campestris* Xps T2SSs, the secretin contains its own lipid anchor and they can pilot themselves [30]. A crystal structure is also available for *P. aeruginosa* protein PA3611, which shares structural homology with the *V. cholerae* pilotin, AspS, although its function as a pilotin has still to be confirmed experimentally [31]. However, no pilotin has yet been identified for the *L. pneumophila* Lsp system.

2.2 Species variations

Some T2SSs also express auxiliary genes within core components (**Fig. 1A**). In *Aeromonas* and *Vibrio*, GspA and GspB span the inner membrane once and form together a large multimeric complex that is thought to modify or organise the peptidoglycan to allow assembly of the GspD secretin [32-35]. GspA is an ATPase; its periplasmic domain interacts with peptidoglycan and forms a complex with GspB. The GspAB complex is essential for type II secretion in *Aeromonas* while its presence is not apparently obligatory in *Vibrio* [34]. However, in cross-complementation experiments, GspAB from *V. cholerae* restored secretin assembly and secretion in an *A. hydrophila gspA* mutant suggesting that GspAB performs the same role in *Vibrio* and *Aeromonas*. Supporting this idea that GspA and GspB act together, in *Vibrio vulnificus* they are naturally fused into a single polypeptide, the periplasmic portion of which possess a canonical peptidoglycan-binding domain [36]. However, in *D. dadantii, K. oxytoca* and some other bacteria, only GspB is present. OutB of *D. dadantii* interacts with the cognate secretin OutD but its precise function remains unclear [37]. These ancillary proteins might therefore stabilize or aid the assembly of the outer membrane secretin, tether the complex to the peptidoglycan or help the complex form across the peptidoglycan mesh [33-35]. Interestingly, although no additional genes are present in the *L. pneumophila* T2SS, its outer membrane secretin LspD is predicted to contain a supplementary peptidoglycan binding domain at its extreme N-terminus [38].

2.3 Architecture of the T2SS

Over the past 20 years X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy and cryo-electron microscopy (EM) have delivered atomic structures for almost all components of the T2SS, the exemplar structures spread over several bacterial systems. However, the structure of an intact T2SS complex has yet to be determined. The T2SS, T3SS, type IV pilus system (T4PS), DNA uptake systems and the filamentous phage-assembly system all feature an outer-membrane oligomeric protein called a 'secretin' [39]. In *V. cholerae* the secretin, EpsD, features in both type II secretion but also the extrusion of filamentous bacteriophage [40]. Furthermore, the T2SS is ancestrally related to the T4PS and many of the T2SS components have sequence and structural homologs in the T4PS [41].

Analysis of *V. cholerae* GspD and *K. oxytoca* PulD cryo-EM maps at low resolution reveals a dodecameric arrangement of subunits [42-45], which is consistent with cryo-EM and cryo-electron tomography (ET) studies with the ancestrally related *Neisseria meningitidis* and *Myxococcus xanthus* T4PS, respectively [46, 47]. However, the 7 Å cryo-EM structure of the T4PS PilQ secretin is a 14-mer [48]. Furthermore, near atomic resolution structures have recently been published for the *E. coli* K12 and *V. cholerae* GspD proteins, which display predominantly 15-fold symmetry [49] and this is also observed in the *Salmonella* T3SS secretin [50]. Differences in recombinant expression strategies and sample preparations could lead to these discrepancies in oligomeric state; for example, replacement of lipid bilayers with detergent micelles may introduce artefacts in how secretins oligomerize. It is also possible that oligomeric variability is an innate property of these systems, for instance, 28% of the *E. coli* K12 GspD oligomers have 16-fold symmetry [49]. This remains a puzzle and the native stoichiometry of the T2SS is still to be unambiguously established.

The atomic model of the *M. xanthus* type IVa pilus system, based on data collected *in situ*, has revealed that the inner and outer membrane platforms both display the same 12-fold symmetry [47]. However, it is not yet clear whether this symmetry match is also important in the functioning of the T2SS; we currently know too little about the mechanics of secretion to understand if a symmetry match or a symmetry mismatch would be optimal. Using all available structural data, we have generated a model of the *V. cholerae* T2SS in its closed state where we have orientated structures or

homology models using the *M. xanthus* T4PS model, based on cryotomography, as a template (**Fig. 1B**). This results in dodecameric symmetry within the inner and outer-membrane platforms. The ATPase is assumed to have 6-fold symmetry for the purpose of generating this cartoon [51] and only core components that are present in all types of T2SS are shown (e.g. the GspC C-terminal PDZ domain that modulates the specificity of some secretion systems is not shown).

2.4 Current model of type-II dependent secretion

In contrast to many other secretion systems that transport unfolded proteins, the T2SS recruits and transports fully folded proteins. Consequently, it has been suggested that the secretion signal is conformational in nature; it could be a patchwork of structural signals embedded on the substrate surface yet conserved across various substrates [52-54]. Studies on different T2SSs have pointed to several, distant and often large regions of various substrates, which are essential for secretion and able to promote secretion of heterologous cargos [55-62]. Recent structure-guided mutagenesis, cross-linking and functional studies in *D. dadantii* shed a new light on the nature of the type II secretion signal and showed that a short 9-residue intrinsically disordered loop of pectate lyase PelI acts as a specific secretion signal that interacts directly with GspC and GspD and controls substrate recruitment by the T2SS [60]. Furthermore, a concerted bioinformatics approach has suggested an occurrence of equivalent secretion motifs in other T2SS substrates [60]. A recent structure/functional study on the PulA substrate of *K. oxytoca* has also revealed that several structurally dynamic regions of this large multi-domain protein are important for its secretion [61]. Therefore, it seems likely that at least in some systems a few surface exposed, intrinsically disordered and highly dynamic regions of secreted substrates act as composite secretion signals *via* their transient folding on appropriate T2SS components.

Recruitment of substrates in the periplasm would necessitate their interaction with one or several T2SS components. GspC, GspD and pseudopilins have been shown to interact with the secreted substrates but it is generally assumed that the initial recognition is primarily performed by GspC [60, 63-65], which results in their transport into the interior of the T2SS (**Fig. 1C**). Although as the cargo are generally

very large (**Fig. 1B**) there are presumably substantial conformational changes to allow them to enter the departure vestibule [43].

Interactions have been observed between substrates and GspD periplasmic domains in *D. dadantii* Out, *V. cholerae* Eps, and *P. aeruginosa* Xcp, although it is unknown whether these contacts represent periplasmic recruitment (in conjunction with GspC), positioning within the T2SS vestibule, gating of the secretin pore, or a combination of these steps [43, 62, 63]. Once inside the secretion apparatus, substrates are thought to sit on the tip of the pseudopilus [63] and with the recruitment of GspE and the hydrolysis of ATP the pseudopilus grows and forces the substrate out of the cell [66].

3. Intrinsic disorder and dynamics within the T2SS

The T2SS transports soluble substrates into the extracellular space but it can also mediate the attachment of some substrates to the cell surface [67]. In this review, we will focus on the export of 'soluble' cargo, although it is likely that all type II dependent translocation events, including those that result in surface attachment, share a common secretion mechanism. In particular, here we will emphasize our current knowledge of how the interplay between intrinsic disorder and dynamics within the core T2SS components allows for the secretion of cargo and to what extent this is also observed in other secretion systems.

3.1 Biogenesis and role of the GspD outer membrane pore

GspD is formed of three regions: an N-terminal periplasmic N-domain region, the secretin domain and a short C-terminal S-domain (**Fig. 2A,B**). In the pentadecameric GspD structures, these regions assume an almost linear arrangement but are tilted at an approximately 30° angle with respect to the channel axis (**Fig. 2B**), which generates a highly stable assembly. The secretin domain forms a pore in the outer-membrane and allows for gated secretion of proteins into the extracellular space, whilst the S-domain provides stability to the mature structure by embracing the adjacent subunits [49, 50]. Four N-domains termed N^0 to N^3 (numbered from the N-terminus) extend from the inner leaflet of the outer membrane into the periplasm (**Fig. 2B**). They act to funnel selected protein substrates into the membrane pore. In other secretin containing systems (T3SS, T4PS, filamentous phage) the arrangement and

number of these domains varies, consistent with different functioning of these systems [39]. In the T2SS, the N-domains communicate with the inner-membrane platform, penetrate the peptidoglycan and also interact with substrates during their secretion [42, 60, 62-64, 68, 69].

3.1.1 Dynamic partnering between N-domains

Several structures of GspD have been published, ranging from individual domains to almost complete GspD chains. To aid comparison of these structures, throughout this review we have numbered GspD secondary structure based on the full sequence, which begins at the N^0 domain. The N^0 domain shares the same fold as the signaling domain of TonB-dependent outer membrane receptors [70]. An anti-parallel two α-helical core (α1-α2) is packed between an anti-parallel β1-β3 sheet on one side and an anti-parallel β2-β5-β4 sheet on the other (**Fig. 2C**) [71, 72]. The N^1, N^2 and N^3 domains on the other hand share structural homology with the KH-domain motif and are composed of a three stranded anti-parallel β-sheet (N^1:β6-β8-β7; N^2:β9-β11-β10; N^3:β12-β14-β13) packed against two α-helices (N^1:α3-α4; N^2:α5-α6; N^3:α7-α8) (**Fig. 2C**) [39, 49, 72, 73]. KH-domains usually mediate binding of DNA/RNA [74], as does the N^1 domain of the HofQ secretin involved in uptake of external DNA [75]. However, the GxxG motif, which is essential for the nucleotide binding, is not conserved in GspD and other secretins.

The cryo-EM structures of *E. coli* and *V. cholerae* GspD, coupled with secondary structure predictions [76, 77], highlight significant intrinsic disorder that is inherent within GspD across different T2SSs (**Fig. 2A**). The first disordered region is localized to an approximate ten-residue linker connecting N^0 and N^1. In the absence of an inner-membrane platform to dock with, in the structures of *E. coli* and *V. cholerae* pentadecameric GspD the N^0 domain is disordered and could not be modeled [49]. Linkers that connect the N^1/N^2 and N^2/N^3 domains, albeit shorter, also provide some flexibility between rings of N-domains [78]. Within these rings the β-sheet of one domain packs against the α-helices of an adjacent identical domain, with a buried dimer interface of ~500 A^2 (N^3 domains) and ~250 A^2 (N^1 domains).

Disorder of the N^0 domain in pentadecameric GspD structures suggest that the N^0 domain is highly dynamic when not docked to the inner-membrane platform. It may

also adopt multiple conformations during secretion, a suggestion supported by the crystal structures of isolated N-domains. For example, in both *P. aeruginosa* XcpQ N^{012} and *E. coli* N^{012} crystal structures, the β-3 strand of N^0 forms parallel interactions with the β-6 strand of their N^1 domain (**Fig. 2C**) [72, 73]. However, this interface is not compatible with the arrangement of N^1-domains in the pentadecameric GspD structures and would lead to clashes [49]. Furthermore, in the crystal structure of an isolated *E. coli* N^0 domain, the β-3 strand from one subunit forms antiparallel interactions with the β-2 strand of another, which results in formation of a helical dodecameric ring-like structure that runs throughout the crystal lattice (**Fig. 2C**) [71]. However, structural studies of T3SS secretins resulted in a different model for the arrangement of N^0/N^1 domains in the T3SS (**Fig. 2C**) [50, 79, 80] and a particular dodecameric-ring structure was formed by N-domains of the HofQ secretin that involves a domain swapping mechanism [75]. Therefore, flexibility seems to be an inherent property of the chain of secretin N-domains.

In the crystal structure of *P. aeruginosa* XcpQ N^{012} the alignment of N^1/N^2 is again inconsistent with the pentadecameric GspD structures [49, 72], with the β-sheet of N^2 packing against the α-helices of N^1 (**Fig. 2C**). This forms a face-to-face dimer in the crystal lattice, which has also been observed *in vivo* with cross-linking studies in the *D. dadantii* Out system [68]. On the other hand, in the *E. coli* N^{012} structure these two domains adopt orientations similar to that observed in full-length pentadecameric GspD (**Fig. 2C**) [49, 73]. Looking across all N-domain structures, the interaction between domains is mainly the hydrophobic burial of a relatively small contact surface. It is presumably these numerous small hydrophobic regions that enable GspD to adopt several conformations and allow access to large cargo (**Fig. 1B**). Disulfide-bonding analysis in a functional *D. dadantii* T2SS established multiple *in vivo* interactions involving N^0-N^3 domains and showed that the same sites of N^0 are involved in self-interactions (N^0-N^0) but also interactions with GspC [68]. The dynamics of these transient contacts was inverted by secreted substrates and by inner membrane components GspE, L and M. Therefore, it may be that upon periplasmic recognition of substrates the N-domains can unzip, rotate and change their association with adjacent chain; analogous to a curtain opening. This would open up the entire periplasmic face of the T2SS, which would close up again once the cargo has entered.

The second disordered region of GspD is located within the homologous α5-β10 and α7-β13 loops of the N^2 and N^3 domains, respectively. Whilst in the N^1 domain this loop is only a few residues long and well ordered, in N^2 there are up to 15 residues, which are disordered in all available structures [49, 72, 73] (**Fig. 2A, C**). Moreover, in the N^3 domain this loop is longer still and in *D. dadantii* and *L. pneumophila* it contains up to 80 residues of serine-glycine rich sequence (**Fig. 2A, C**). The cryo-EM structure reveals this disordered region to be a flexible weak N^3 constriction site, which is anticipated to be more appreciable in *D. dadantii* and *L. pneumophila*.

3.1.2 Structure and gating mechanism of the secretin

The structure of the secretin domain is a unique double β-barrel assembly composed of mainly β-sheet secondary structure. It is arranged in two distinct regions: a predominantly β-sheet outer-barrel (six β-strands and two α-helices) and a four-stranded β-sheet forming inner barrel and internal gate (**Fig. 2B,C**) [49, 50]. In the *V. cholerae* EpsD secretin there is also an additional feature; an external gated cap (two β-sheets and two α-helices) that extends into the extracellular space (**Fig. 2B**). However, many other T2SS secretins, e.g. *E. coli* K12 GspD, *D. dadantii* OutD, *L. pneumophila* LspD and *P. aeruginosa* XcpQ, do not possess such an external gated cap and instead their exit channel is in a constitutively open conformation (**Fig. 2B**) [49]. The functional significance of this external gate for species-specific type II secretion is not clear. In the *Salmonella* T3SS secretin, this region contains just a single short helix, presumably functioning as an interface for its needle filament [50].

Although the overall structure of secretins appear to be tremendously stable [49, 50] and in some systems they require extreme conditions to denature [81], the gate regions must undergo significant conformational change to allow substrates to exit through the pore. Three gate regions have been observed in the T2SS GspD structures, the N^3 constriction site, the internal gate and the external gate discussed above [43, 49]. The flexible N^3 constriction site (unstructured loop between α7-β13) corresponds to the N^3 variable loop in T3SS secretins and was not observed in the structure [49, 50]; its length varies significantly in both T2SS and T3SS secretins. The intervening loop of the internal gate β-strands, β16-β17 [49], is also flexible and was not seen [49], however, in the T3SS secretin structure, the equivalent loops of the

internal gate hairpin pack against one another and could therefore be modeled [50]. The cholera toxin is the archetypal substrate of the *V. cholerae* T2SS and its orientation within the closed secretin vestibule has been determined by cryo-EM [43]. In this conformation, the cholera toxin is too large to pass through these gates, but the growing pseudopilus may simply push the gates open, which have glycine pivots, during active transport of its substrates [49, 50].

3.1.3 Biogenesis of GspD

The best-characterized intrinsically disordered region of GspD is its C-terminal 60 residues termed; the S-domain. Evidence from NMR spectroscopy reveals that the C-terminal region of the secretin protomer (incipient α14) is disordered before binding to the pilotin (**Fig. 2A**) [26, 68, 82]. However, upon binding of *D. dadantii* OutD to its OutS pilotin, this region adopts an α-helical conformation and forms a high affinity complex [68, 82]. As will be discussed below, the formation of the secretin oligomeric pore also orders this region of the secretin, yet to a different extent in the secretins from *V. cholerae* and *E. coli* K12 (Yan, Yin et al. 2017).

During T2SS biogenesis pilotins bind their cognate secretin S-domain as the secretin emerges from the Sec translocon in the inner-membrane and they are transported to the inner-leaflet of the outer-membrane probably via the Lol system [83]. The absence of a lipidated pilotin results in the degradation and mis-location of the assembled secretin to the inner-membrane, which suggests that the pilotin has a major role in transport and targeting of the secretin to the outer-membrane [25, 27, 62].

In the *V. cholerae* EpsD cryo-EM structure, the S-domain reaches across two adjacent protomers and interacts with them via two helices (α13/α14) separated by a linker [49]. This provides significant stability to the final quaternary structure but importantly, the location of the α14-helix is such that it could also still bind the pilotin in its folded state [49]. In the *E. coli* GspD structure, the S-domain provides a similar function in stabilizing the mature structure [49]. However, the equivalent α14-region has less defined secondary structure and it is not as apparent how it can interact with its pilotin without some rearrangement in the complex. The *E. coli* and *V. cholerae* pilotins belong to two structurally dissimilar groups [29, 84] suggesting that the mode of binding to their cognate secretins could vary. Nonetheless, this disorder

to order transition appears to be important for regulating secretin oligomerization in addition to transport. It is also possible that the ordering of unstructured regions is a similarly important step in the assembly of other components of the T2SS, such as assembly of the inner-membrane platform.

3.2 Substrate recognition and entry

3.2.1 Overall structure of GspC

GspC proteins from different bacteria range in mass from approximately 20 to 35 kDa. They are inserted into the inner membrane through an N-terminal transmembrane (TM) helix, which is followed by an intervening flexible linker (TMHR) and a structured homology region (HR) (**Fig. 3A**). The sequence of HR domains, a 7-stranded β sandwich fold [69, 85], is relatively well conserved across bacterial species, whereas the TMHR shares very little sequence homology. Intrinsic disorder and secondary structure predictions [76, 77] suggest that the TMHR linker is not fully unstructured though and contains a single α-helix. Moreover, this has been confirmed by NMR, at least for the *D. dadantii* GspC protein OutC [85] (**Fig. 3B**).

The C-terminal regions of GspC proteins, however, can vary between different T2SSs. For example, *Vibrio*- and *Dickeya*-like T2SSs possess a PDZ domain at their extreme C-terminus (**Fig. 3C**) [86], while in *P. aeruginosa* XcpP, this region comprises a coiled-coil domain [87] and in *L. pneumophila* LspC there is no additional sequence. The C-terminal domains of the *D. dadantii* Out and *P. aeruginosa* Xcp GspC proteins have both been shown to mediate homomeric interactions but whilst these domains are superfluous for correct biogenesis of the Out system (an *outC* PDZ-domain deletion mutant affects secretion specificity but does not totally compromise function), they are essential for correct functioning of Xcp [65, 87].

3.2.2 Substrate recognition

There is significant evidence from *in vivo* experiments that the OutC PDZ domain is involved in recruiting pectate lyase type cargo from the *D. dadantii* periplasm [60, 64]. However, for secretion of some other exoproteins of the Out system, the PDZ

domain is dispensable [64]. PDZ domains are relatively promiscuous protein-protein interaction modules and commonly recognize short peptide ligands [88]. The crystal structure of the *V. cholerae* EpsC PDZ domain contains 4 α-helices and 6 β-strands and the putative binding site between the β1 strand and α1 helix accommodates the α4 helix from an adjacent molecule in the lattice (**Fig. 3C**) [86]. This is noteworthy because interactions between the OutC PDZ domain and its cargo are mediated through a conserved partially-helical/loop region within the substrate [56, 60]. However, this could also represent a mechanism for oligomerization or a dynamic combination of the two.

In *P. aeruginosa* GspC, XcpP, the TMHR linker has been assumed to be involved in the capture of its cargo **(Fig. 3A)** [65] yet the entire XcpP periplasmic region was used to demonstrate such interactions *in vitro* [63]. In *D. dadantii*, both HR and PDZ domains of OutC have been shown to interact with the secreted substrates [60], suggesting that substrate recruitment should involve multiple contacts with GspC. Interactions between substrates and GspD N-domains have also been observed in several T2SSs but it is unclear whether these may represent contacts made within the vestibule after substrate entry or actual periplasmic recruitment [42, 60, 62, 63]. It has been shown that OutD does play a role in selecting *D. dadantii* substrates for secretion [60, 62, 64] and this has also been confirmed in *P. aeruginosa* [63] and *V. cholerae* [42]. Furthermore, direct interaction was observed between the substrate and the pseudopilus tip [63] but this would correspond to the final step of the secretion process prior to substrate release from the cells. Recently, structural and computational approaches employed with *K. oxytoca* pullulanase PulA revealed the significance of inner membrane association of T2SS substrates, and showed that structurally dynamic regions and subdomains are important for T2SS-mediated protein transport [61]. Therefore, it is unclear whether there is a universal core mechanism for substrate capture (e.g. the TMHR linker) that can be augmented with additional recognition processes (e.g. PDZ, GspD N-domains) or whether there are multiple mechanisms that have evolved for different T2SSs and for the selection of different types of cargo.

3.2.3 Substrate entry into the T2SS vestibule

Another major function of GspC is bridging the inner membrane platform and the secretin through interactions within its HR domain and the periplasmic N-domain(s) of GspD. However, it is expected that many substrates will be too large to enter the interior of the T2SS without disruption of this hetero-dimer and so GspC or GspC together with GspD can be considered as the gatekeepers for entry into the vestibule. Although we do not know exactly how substrate recognition is coupled to changes within the system, several different dimer orientations of GspC and GspD have been suggested from structural studies and/or trapped with *in vivo* cross-linking [39, 68, 72, 85, 86].

In a crystal structure of the *E. coli* GspD-N^{01}/GspC-HR complex, the dimer interface is a β-sheet augmentation created by the β1 strands of both components (**Fig. 3D**) [39]. However, these domain orientations are not compatible with the full length 15-fold symmetry GspD structure [49], but are compatible with the N^0 domain 12-fold structure [71]. On the other hand, solution structural studies with the isolated *D. dadantii* OutD-N^0 and OutC-HR domains show an alternative arrangement, where the β1 strand of OutC-HR augments the β3 strand of OutD-N^0 (**Fig. 3E**) [85]. In the *E. coli* GspD-N^{01}/GspC-HR structure [39], the GspD β3 strand forms the interface with the adjacent N^1 domain, although in the dodecameric N^0 structure [71] the GspD β3 strand forms the interface with the β2 strand of the adjacent N^0 domain. Therefore, one of these models may embody an "open" state while the other a "closed" arrangement of the secretion system, made available through disruption of the GspD-N^0/N^1 or N^0/N^0 interface.

In vivo cross-linking studies in *D. dadantii* and *in vitro* binding assays also suggest that the β7 strand of OutC-HR may form additional interfaces with the β2 strand of OutD-N^0 and the β10 strand of OutD-N^2 [68, 89]. This work also indicates that there could be local rearrangements within the HR structure caused by secreted substrates and the inner membrane components GspE, L and M to accommodate these displacements, although there is currently no structural data to corroborate these findings. Finally an interaction has also been proposed between the OutC-TMHR linker and OutD-N^0 but the implications of this are not understood [89]. In all it is clear that complexes formed between GspC and GspD are transient and highly

dynamic and they must be related to selection and passage of cargo into the interior of the secretion device.

3.3 Conformational signaling through the inner-membrane platform

In addition to substrate recognition "opening" the T2SS to allow entry, it must also stimulate a signal cascade that initiates the active transport of the cargo from the cell; this is the role of the inner membrane platform. Along with GspC, GspL and GspM form integral components of this sub-complex, which are thought to encircle a membrane embedded GspF [90]. Fluorescent microscopy studies suggested that during biogenesis of the T2SS, the secretin is first inserted into the outer membrane and then, components of the inner membrane platform are co-assembled using GspD as a template [91-93]. The transmembrane helix of OutC has been shown to self-associate in *D. dadantii* and it has been suggested that this drives the formation of the platform complex [94]. The transmembrane helix of *V. cholerae* EpsM has also been shown to homo-dimerize *in vivo* [95] and interact with the equivalent region of EpsL in a species-specific manner [78]. More comprehensive analysis showed that the transmembrane regions of OutC, OutL and OutM form together a dynamic network in the inner-membrane and so it seems likely that inter-TM helix interactions are not maintained throughout the secretion process but are later displaced by other components [96].

GspL and GspM have a similar architecture: a transmembrane helix followed by a C-terminal periplasmic domain; however, GspL is bitopic and contains an additional N-terminal cytoplasmic region. The periplasmic domain of both GspL and GspM adopts a ferredoxin-like fold, which may indicate a common evolutionary origin [97, 98]. They exist as homo-dimers in solution and these states have been trapped in the crystal lattices of *V. parahaemolyticus* EpsL and *V. cholerae* EpsM [97, 98] (**Fig. 4A**), where they are formed through interactions between the $^{c}\beta$A strand or the β3 strand and α2 helix, respectively. Furthermore, *in vivo* cross-linking studies in the *D. dadantii* Out system has detected both homo- and hetero-dimerization of these proteins and here the $^{c}\beta$C and $^{c}\alpha$2 helix of OutL and again the β3 strand and α2 helix of OutM are implicated in mediating both dimerization states [96] (**Fig. 4A**). These

dynamic *in vivo* homo- and heterodimers of GspL and GspM are formed via a process called partner switching and imply that there are large rotations of both core periplasmic interfaces but also in the cognate TM domains [96]. This mechanism was proposed to signal between the cytoplasmic and periplasmic portions of the T2SS machinery. Recent structure-based mutagenesis and cross-linking analysis in the related T4PS has provided a similar picture [99]. PilN and PilO (equivalent to periplasmic GspL and M) form both homo- and heterodimers *in vivo* via equivalent interfaces and their dynamic rearrangement is necessary for T4PS function [99]. In another study of the *V. cholerae* Eps system, it was reported that the β1 strand of EpsM could be important for homo-dimerization, while the α1 helix helped to stabilize the EpsL/EpsM complex [100]. Therefore, several potential GspL/GspM interfaces have been identified in different studies, yet it is unclear which are important in the context of the assembled secretin system.

Displacement of the periplasmic domain of GspM has been shown to propagate changes in the orientation and/or conformation of the cognate transmembrane region [96]. Since the TM helices of GspL, M and C interact, this therefore represents a potential mechanism for signalling between the periplasmic and cytoplasmic portions of the T2SS. The cytoplasmic region of GspL is ~25 kDa, composed of three domains [101] and has structural homology with actin like ATPases (**Fig. 4B**) but ATPase activity has not been detected. It forms a ring on the cytosolic face of the inner membrane and upon activation of the T2SS it is anticipated that the GspL intra-domain and/or inter-GspL orientations are altered, which allows recruitment of the GspE ATPase [102, 103].

3.4 Recruitment of the GspE motor

Pseudopilin assembly by the inner-membrane complex requires proton motive force, and energy from the hydrolysis of ATP by the ATPase, GspE [104]. This hexameric AAA+ ATPase motor is structurally related to the assembly and disassembly ATPases of the T4PS, PilB and PilT [105, 106]. ATP hydrolysis requires contact between the N- and C-terminal domains of GspE with large displacements occurring in the ATP and ADP bound forms of these motor domains [107]. GspE has an extended N-terminal domain compared to other AAA+ ATPases which forms a stable complex with the cytoplasmic domain of GspL and the first cytoplasmic domain of GspF,

anchoring the ATPase to the rest of the inner-membrane platform (**Fig. 4B**) [90, 102, 103, 108]. It has been shown that activation of the ATPase GspE in *V. cholerae* necessitates an interaction between the GspL segment adjacent to the TM region and membrane lipids [109]. This is consistent with EpsL displacement up onto the inner membrane to activate the ATPase. Therefore, there is the possibility that coordinated displacements of the periplasmic and TM domains of GspC, GspL and GspM drive these dynamics resulting in ATPase activation.

It has to be noted that GspE is a reluctant hexamer, preferring to crystallize in different oligomeric states [51, 110]. When fused to a hexameric protein, Hcp1, a quasi-C6 GspE hexamer can be produced showing increased ATPase activity [51]. The oligomeric structures resolved in this study show considerable inter-domain flexibility within the GspE subunit. Rotation could also be involved in the pilus assembly mechanism, by analogy with the bacterial flagella rotation [111], although in the T2SS the rotating element is probably the assembly machinery rather than the pilus. If GspL acts as an anchor for the ATPase in the inner-membrane then GspF would be a candidate for the rotating component. Moreover, cross linking experiments in *V. cholerae* suggest that GspL may act as a scaffold connecting GspE with the pseudopilus [112].

GspF is an integral membrane protein with three transmembrane helices, short connecting loops on the periplasmic side of the inner membrane and two helical bundle cytoplasmic domains. In addition, its N-terminal 75 residues are predicted to be significantly disordered, although the relevance of this is not clear **(Fig. 4C**) [76, 77]. The first and N-terminal cytoplasmic domain crystallizes and forms dimers, but the second cytoplasmic domain which follows the second transmembrane helix has resisted crystallization despite being of similar architecture according to sequence identity [113]. The first cytoplasmic domain of GspF interacts with GspL and GspE, and the full-length GspF binds pseudopilin subunits in two-hybrid assays suggesting it plays a central role in pilus assembly [90, 114]. The oligomeric state of GspF in the context of the assembled secretion machine is uncertain; it is probably a dimer and possibly a tetramer. Based on the EM tomography model of the *M. xanthus* T4PS [47], GspF undoubtedly forms the central part of the inner membrane assembly

platform. However, whether it does rotate or whether it simply provides a platform for pseudopilus assembly has yet to be elucidated.

3.5 The final push

The T2SS pilus is built up from a major component (GspG) and four minor components (GspH to GspK) [66]. These pilin subunits share a positively charged N-terminal cytoplasmic tail within their signal peptide, a conserved inner membrane embedded α-helix and a unique C-terminal periplasmic globular domain (**Fig. 4D**). Upon cleavage of the charged tail at a conserved glycine residue and then methylation of the new N-terminus by a dedicated peptidase (GspO), these pilin domains are able to enter the inner membrane platform and polymerize via their hydrophobic α-helical stems [115, 116]. This results in a pilus forming that extends into the periplasm with a core composed of helically arranged α-helices, decorated by solvent exposed globular domains [115] (**Fig. 4E**). During the biogenesis of the T2SS it is thought that the minor pilin subunits form first within the inner membrane platform [117, 118] and initiation of pilus assembly requires minor pilins GspH-I-J-K [119]. Upon activation, GspG is then recruited and its incorporation into the pilus allows the fiber to extend [120]. Assembly of the pilus has been proposed to occur in a rotation-driven mechanism and involve the ATPase GspE together with the inner membrane rotor, GspF [114, 119]. This is an attractive idea allowing the ATPase to control the assembly process. It might involve rotation, or some more subtle level of molecular control; but again this remains to be elucidated in detail.

In the *P. aeruginosa* Xcp system, the substrate LasB has been shown to interact with GspH, GspI and GspK but not GspG or GspJ [63]. Notably, LasB bound with a higher affinity to GspH-I-J-K complex than to each of them alone. The GspI-J-K complex adopts an architecture compatible with localisation at the pilus tip and possesses a conserved area that might interact with secreted substrates [117]. It therefore seems likely that upon entry of substrates into the T2SS vestibule they may orientate themselves on the pilus tip, which could then activate pilus elongation. The T2SS pilus is usually referred to as a pseudopilus because although it shares high homology with the T4PS, it is not extended on the bacterial surface under physiological conditions. Nonetheless, through overexpression of GspG it can grow substantially

longer and be observed protruding from the cell [121, 122]. As well as minor subunits taking part in substrate recognition it is thought that GspK may modulate the extent of the fiber growth [123], while GspI and GspJ initiate and therefore control assembly of the pilus [120]. However, as the T2SS seemingly lacks the ability to retract its pilus after substrate translocation, the mechanism of pilus disassembly remains to be established.

4. Conclusion and perspective

Since the first structures approximately fifteen years ago, structural biology has gone a long way in aiding our understanding of type II dependent secretion in Gram-negative bacteria. The majority of structures have been determined as sub-domains using X-ray crystallography and it is likely that we are now approaching the limit of what this technique can offer here. On the other hand, recent near-atomic resolution structures of the T2SS and T3SS secretins highlight the important role that cryo-EM will play in the coming years [49, 50]. The secretin is a very stable complex but we also anticipate cryo-EM structures of the more fragile sub-complexes of the secretion system to emerge over the next few years. Cryo-ET can also be applied *in vivo* under native conditions and as it has now broken the 10 Å resolution barrier [124]. Recently, high-resolution cryo-ET has allowed the *in situ* visualization of the assembled T4PS and T3SS nanomachines in their native state and has shown their functional conformational dynamics [47, 125]. Again, this technique will play a significant role in determining unambiguous snapshots of intact type II systems.

Solution state nuclear magnetic resonance (NMR) spectroscopy is also now widely used to investigate large complexes and may be particularly useful for probing the inherent dynamics within this system including disorder to order transitions encountered during substrate secretion. Furthermore, *in vivo* solid state NMR has been used to study the type IV secretion system [126] and this work indicates that in-cell analysis of T2SS function and dynamics may also be possible. The T2SS has received renewed interest in recent years and with the implementation of cutting-edge complementary high-resolution techniques, we can be sure that further exciting discoveries in this field are on the horizon.

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References

[1] J. Haiko, B. Westerlund-Wikstrom, The role of the bacterial flagellum in adhesion and virulence, Biology (Basel), 2 (2013) 1242-1267.
[2] J.A. Garnett, S. Matthews, Interactions in bacterial biofilm development: a structural perspective, Curr Protein Pept Sci, 13 (2012) 739-755.
[3] M.K. Hospenthal, T.R.D. Costa, G. Waksman, A comprehensive guide to pilus biogenesis in Gram-negative bacteria, Nat Rev Microbiol, 15 (2017) 365-379.
[4] C. Dong, K. Beis, J. Nesper, A.L. Brunkan-Lamontagne, B.R. Clarke, C. Whitfield, J.H. Naismith, Wza the translocon for E. coli capsular polysaccharides defines a new class of membrane protein, Nature, 444 (2006) 226-229.
[5] T.R. Costa, C. Felisberto-Rodrigues, A. Meir, M.S. Prevost, A. Redzej, M. Trokter, G. Waksman, Secretion systems in Gram-negative bacteria: structural and mechanistic insights, Nat Rev Microbiol, 13 (2015) 343-359.
[6] K. Nakayama, Porphyromonas gingivalis and related bacteria: from colonial pigmentation to the type IX secretion system and gliding motility, J Periodontal Res, 50 (2015) 1-8.
[7] R. Liu, H. Ochman, Stepwise formation of the bacterial flagellar system, Proc Natl Acad Sci U S A, 104 (2007) 7116-7121.
[8] N. Noinaj, J.C. Gumbart, S.K. Buchanan, The beta-barrel assembly machinery in motion, Nat Rev Microbiol, 15 (2017) 197-204.
[9] S. Okuda, D.J. Sherman, T.J. Silhavy, N. Ruiz, D. Kahne, Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model, Nat Rev Microbiol, 14 (2016) 337-345.
[10] M. Juhas, J.R. van der Meer, M. Gaillard, R.M. Harding, D.W. Hood, D.W. Crook, Genomic islands: tools of bacterial horizontal gene transfer and evolution, FEMS Microbiol Rev, 33 (2009) 376-393.

[11] J.P. Demers, B. Habenstein, A. Loquet, S. Kumar Vasa, K. Giller, S. Becker, D. Baker, A. Lange, N.G. Sgourakis, High-resolution structure of the Shigella type-III secretion needle by solid-state NMR and cryo-electron microscopy, Nat Commun, 5 (2014) 4976.
[12] E.A. Merritt, T.K. Sixma, K.H. Kalk, B.A. van Zanten, W.G. Hol, Galactose-binding site in Escherichia coli heat-labile enterotoxin (LT) and cholera toxin (CT), Mol Microbiol, 13 (1994) 745-753.
[13] D.J. Richardson, J.N. Butt, J.K. Fredrickson, J.M. Zachara, L. Shi, M.J. Edwards, G. White, N. Baiden, A.J. Gates, S.J. Marritt, T.A. Clarke, The 'porin-cytochrome' model for microbe-to-mineral electron transfer, Mol Microbiol, 85 (2012) 201-212.
[14] M. Fries, J. Ihrig, K. Brocklehurst, V.E. Shevchik, R.W. Pickersgill, Molecular basis of the activity of the phytopathogen pectin methylesterase, EMBO J, 26 (2007) 3879-3887.
[15] P. Natale, T. Bruser, A.J. Driessen, Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane--distinct translocases and mechanisms, Biochim Biophys Acta, 1778 (2008) 1735-1756.
[16] R. Voulhoux, G. Ball, B. Ize, M.L. Vasil, A. Lazdunski, L.F. Wu, A. Filloux, Involvement of the twin-arginine translocation system in protein secretion via the type II pathway, EMBO J, 20 (2001) 6735-6741.
[17] S.S. Abby, J. Cury, J. Guglielmini, B. Neron, M. Touchon, E.P. Rocha, Identification of protein secretion systems in bacterial genomes, Sci Rep, 6 (2016) 23080.
[18] L.M. Hales, H.A. Shuman, Legionella pneumophila contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease, Infect Immun, 67 (1999) 3662-3666.
[19] Y. Ferrandez, G. Condemine, Novel mechanism of outer membrane targeting of proteins in Gram-negative bacteria, Mol Microbiol, 69 (2008) 1349-1357.
[20] T.G. Strozen, G. Li, S.P. Howard, YghG (GspSbeta) is a novel pilot protein required for localization of the GspSbeta type II secretion system secretin of enterotoxigenic Escherichia coli, Infect Immun, 80 (2012) 2608-2622.
[21] S. DebRoy, J. Dao, M. Soderberg, O. Rossier, N.P. Cianciotto, Legionella pneumophila type II secretome reveals unique exoproteins and a chitinase that promotes bacterial persistence in the lung, Proc Natl Acad Sci U S A, 103 (2006) 19146-19151.

[22] A.E. Sikora, R.A. Zielke, D.A. Lawrence, P.C. Andrews, M. Sandkvist, Proteomic analysis of the Vibrio cholerae type II secretome reveals new proteins, including three related serine proteases, J Biol Chem, 286 (2011) 16555-16566.
[23] N. Kazemi-Pour, G. Condemine, N. Hugouvieux-Cotte-Pattat, The secretome of the plant pathogenic bacterium Erwinia chrysanthemi, Proteomics, 4 (2004) 3177-3186.
[24] A.P. Pugsley, M.G. Kornacker, Secretion of the cell surface lipoprotein pullulanase in Escherichia coli. Cooperation or competition between the specific secretion pathway and the lipoprotein sorting pathway, J Biol Chem, 266 (1991) 13640-13645.
[25] S. Daefler, I. Guilvout, K.R. Hardie, A.P. Pugsley, M. Russel, The C-terminal domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pIVf1 function, Mol Microbiol, 24 (1997) 465-475.
[26] N.N. Nickerson, T. Tosi, A. Dessen, B. Baron, B. Raynal, P. England, A.P. Pugsley, Outer membrane targeting of secretin PulD protein relies on disordered domain recognition by a dedicated chaperone, J Biol Chem, 286 (2011) 38833-38843.
[27] V.E. Shevchik, G. Condemine, Functional characterization of the Erwinia chrysanthemi OutS protein, an element of a type II secretion system, Microbiology, 144 (Pt 11) (1998) 3219-3228.
[28] S. Gu, S. Rehman, X. Wang, V.E. Shevchik, R.W. Pickersgill, Structural and functional insights into the pilotin-secretin complex of the type II secretion system, PLoS Pathog, 8 (2012) e1002531.
[29] R.A. Dunstan, E. Heinz, L.C. Wijeyewickrema, R.N. Pike, A.W. Purcell, T.J. Evans, J. Praszkier, R.M. Robins-Browne, R.A. Strugnell, K.V. Korotkov, T. Lithgow, Assembly of the type II secretion system such as found in Vibrio cholerae depends on the novel Pilotin AspS, PLoS Pathog, 9 (2013) e1003117.
[30] V. Viarre, E. Cascales, G. Ball, G.P. Michel, A. Filloux, R. Voulhoux, HxcQ liposecretin is self-piloted to the outer membrane by its N-terminal lipid anchor, J Biol Chem, 284 (2009) 33815-33823.
[31] J. Seo, A. Brencic, A.J. Darwin, Analysis of secretin-induced stress in Pseudomonas aeruginosa suggests prevention rather than response and identifies a novel protein involved in secretin function, J Bacteriol, 191 (2009) 898-908.
[32] V.M. Ast, I.C. Schoenhofen, G.R. Langen, C.W. Stratilo, M.D. Chamberlain, S.P. Howard, Expression of the ExeAB complex of Aeromonas hydrophila is required

for the localization and assembly of the ExeD secretion port multimer, Mol Microbiol, 44 (2002) 217-231.
[33] G. Li, S.P. Howard, ExeA binds to peptidoglycan and forms a multimer for assembly of the type II secretion apparatus in Aeromonas hydrophila, Mol Microbiol, 76 (2010) 772-781.
[34] T.G. Strozen, H. Stanley, Y. Gu, J. Boyd, M. Bagdasarian, M. Sandkvist, S.P. Howard, Involvement of the GspAB complex in assembly of the type II secretion system secretin of Aeromonas and Vibrio species, J Bacteriol, 193 (2011) 2322-2331.
[35] E.M. Vanderlinde, S. Zhong, G. Li, D. Martynowski, P. Grochulski, S.P. Howard, Assembly of the type two secretion system in Aeromonas hydrophila involves direct interaction between the periplasmic domains of the assembly factor ExeB and the secretin ExeD, PLoS One, 9 (2014) e102038.
[36] D. Martynowski, P. Grochulski, P.S. Howard, Structure of a periplasmic domain of the EpsAB fusion protein of the Vibrio vulnificus type II secretion system, Acta Crystallogr D Biol Crystallogr, 69 (2013) 142-149.
[37] G. Condemine, V.E. Shevchik, Overproduction of the secretin OutD suppresses the secretion defect of an Erwinia chrysanthemi outB mutant, Microbiology, 146 (Pt 3) (2000) 639-647.
[38] L.A. Kelley, S. Mezulis, C.M. Yates, M.N. Wass, M.J. Sternberg, The Phyre2 web portal for protein modeling, prediction and analysis, Nat Protoc, 10 (2015) 845-858.
[39] K.V. Korotkov, T. Gonen, W.G. Hol, Secretins: dynamic channels for protein transport across membranes, Trends Biochem Sci, 36 (2011) 433-443.
[40] B.M. Davis, E.H. Lawson, M. Sandkvist, A. Ali, S. Sozhamannan, M.K. Waldor, Convergence of the secretory pathways for cholera toxin and the filamentous phage, CTXphi, Science, 288 (2000) 333-335.
[41] C.R. Peabody, Y.J. Chung, M.R. Yen, D. Vidal-Ingigliardi, A.P. Pugsley, M.H. Saier, Jr., Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella, Microbiology, 149 (2003) 3051-3072.
[42] S.L. Reichow, K.V. Korotkov, W.G. Hol, T. Gonen, Structure of the cholera toxin secretion channel in its closed state, Nat Struct Mol Biol, 17 (2010) 1226-1232.
[43] S.L. Reichow, K.V. Korotkov, M. Gonen, J. Sun, J.R. Delarosa, W.G. Hol, T. Gonen, The binding of cholera toxin to the periplasmic vestibule of the type II secretion channel, Channels (Austin), 5 (2011) 215-218.

[44] M. Chami, I. Guilvout, M. Gregorini, H.W. Remigy, S.A. Muller, M. Valerio, A. Engel, A.P. Pugsley, N. Bayan, Structural insights into the secretin PulD and its trypsin-resistant core, J Biol Chem, 280 (2005) 37732-37741.
[45] N. Nouwen, H. Stahlberg, A.P. Pugsley, A. Engel, Domain structure of secretin PulD revealed by limited proteolysis and electron microscopy, EMBO J, 19 (2000) 2229-2236.
[46] J.L. Berry, M.M. Phelan, R.F. Collins, T. Adomavicius, T. Tonjum, S.A. Frye, L. Bird, R. Owens, R.C. Ford, L.Y. Lian, J.P. Derrick, Structure and assembly of a trans-periplasmic channel for type IV pili in Neisseria meningitidis, PLoS Pathog, 8 (2012) e1002923.
[47] Y.W. Chang, L.A. Rettberg, A. Treuner-Lange, J. Iwasa, L. Sogaard-Andersen, G.J. Jensen, Architecture of the type IVa pilus machine, Science, 351 (2016) aad2001.
[48] J. Koo, R.P. Lamers, J.L. Rubinstein, L.L. Burrows, P.L. Howell, Structure of the Pseudomonas aeruginosa Type IVa Pilus Secretin at 7.4 A, Structure, 24 (2016) 1778-1787.
[49] Z. Yan, M. Yin, D. Xu, Y. Zhu, X. Li, Structural insights into the secretin translocation channel in the type II secretion system, Nat Struct Mol Biol, (2017).
[50] L.J. Worrall, C. Hong, M. Vuckovic, W. Deng, J.R. Bergeron, D.D. Majewski, R.K. Huang, T. Spreter, B.B. Finlay, Z. Yu, N.C. Strynadka, Near-atomic-resolution cryo-EM analysis of the Salmonella T3S injectisome basal body, Nature, (2016).
[51] C. Lu, S. Turley, S.T. Marionni, Y.J. Park, K.K. Lee, M. Patrick, R. Shah, M. Sandkvist, M.F. Bush, W.G. Hol, Hexamers of the type II secretion ATPase GspE from Vibrio cholerae with increased ATPase activity, Structure, 21 (2013) 1707-1717.
[52] A. Filloux, Secretion signal and protein targeting in bacteria: a biological puzzle, J Bacteriol, 192 (2010) 3847-3849.
[53] K.V. Korotkov, M. Sandkvist, W.G. Hol, The type II secretion system: biogenesis, molecular architecture and mechanism, Nat Rev Microbiol, 10 (2012) 336-351.
[54] T. Palomaki, R. Pickersgill, R. Riekki, M. Romantschuk, H.T. Saarilahti, A putative three-dimensional targeting motif of polygalacturonase (PehA), a protein secreted through the type II (GSP) pathway in Erwinia carotovora, Mol Microbiol, 43 (2002) 585-596.

[55] N. Sauvonnet, A.P. Pugsley, Identification of two regions of Klebsiella oxytoca pullulanase that together are capable of promoting beta-lactamase secretion by the general secretory pathway, Mol Microbiol, 22 (1996) 1-7.
[56] M. Lindeberg, C.M. Boyd, N.T. Keen, A. Collmer, External loops at the C terminus of Erwinia chrysanthemi pectate lyase C are required for species-specific secretion through the out type II pathway, J Bacteriol, 180 (1998) 1431-1437.
[57] R. Voulhoux, M.P. Taupiac, M. Czjzek, B. Beaumelle, A. Filloux, Influence of deletions within domain II of exotoxin A on its extracellular secretion from Pseudomonas aeruginosa, J Bacteriol, 182 (2000) 4051-4058.
[58] V. Chapon, M. Czjzek, M. El Hassouni, B. Py, M. Juy, F. Barras, Type II protein secretion in gram-negative pathogenic bacteria: the study of the structure/secretion relationships of the cellulase Cel5 (formerly EGZ) from Erwinia chrysanthemi, J Mol Biol, 310 (2001) 1055-1066.
[59] O. Francetic, A.P. Pugsley, Towards the identification of type II secretion signals in a nonacylated variant of pullulanase from Klebsiella oxytoca, J Bacteriol, 187 (2005) 7045-7055.
[60] C. Pineau, N. Guschinskaya, X. Robert, P. Gouet, L. Ballut, V.E. Shevchik, Substrate recognition by the bacterial type II secretion system: more than a simple interaction, Mol Microbiol, 94 (2014) 126-140.
[61] A. East, A.E. Mechaly, G.H. Huysmans, C. Bernarde, D. Tello-Manigne, N. Nadeau, A.P. Pugsley, A. Buschiazzo, P.M. Alzari, P.J. Bond, O. Francetic, Structural Basis of Pullulanase Membrane Binding and Secretion Revealed by X-Ray Crystallography, Molecular Dynamics and Biochemical Analysis, Structure, 24 (2016) 92-104.
[62] V.E. Shevchik, J. Robert-Baudouy, G. Condemine, Specific interaction between OutD, an Erwinia chrysanthemi outer membrane protein of the general secretory pathway, and secreted proteins, EMBO J, 16 (1997) 3007-3016.
[63] B. Douzi, G. Ball, C. Cambillau, M. Tegoni, R. Voulhoux, Deciphering the Xcp Pseudomonas aeruginosa type II secretion machinery through multiple interactions with substrates, J Biol Chem, 286 (2011) 40792-40801.
[64] J. Bouley, G. Condemine, V.E. Shevchik, The PDZ domain of OutC and the N-terminal region of OutD determine the secretion specificity of the type II out pathway of Erwinia chrysanthemi, J Mol Biol, 308 (2001) 205-219.

[65] M. Gerard-Vincent, V. Robert, G. Ball, S. Bleves, G.P. Michel, A. Lazdunski, A. Filloux, Identification of XcpP domains that confer functionality and specificity to the Pseudomonas aeruginosa type II secretion apparatus, Mol Microbiol, 44 (2002) 1651-1665.
[66] M. Campos, D.A. Cisneros, M. Nivaskumar, O. Francetic, The type II secretion system - a dynamic fiber assembly nanomachine, Res Microbiol, 164 (2013) 545-555.
[67] A. Rondelet, G. Condemine, Type II secretion: the substrates that won't go away, Res Microbiol, 164 (2013) 556-561.
[68] X. Wang, C. Pineau, S. Gu, N. Guschinskaya, R.W. Pickersgill, V.E. Shevchik, Cysteine scanning mutagenesis and disulfide mapping analysis of arrangement of GspC and GspD protomers within the type 2 secretion system, J Biol Chem, 287 (2012) 19082-19093.
[69] K.V. Korotkov, T.L. Johnson, M.G. Jobling, J. Pruneda, E. Pardon, A. Heroux, S. Turley, J. Steyaert, R.K. Holmes, M. Sandkvist, W.G. Hol, Structural and functional studies on the interaction of GspC and GspD in the type II secretion system, PLoS Pathog, 7 (2011) e1002228.
[70] A.D. Ferguson, E. Hofmann, J.W. Coulton, K. Diederichs, W. Welte, Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide, Science, 282 (1998) 2215-2220.
[71] K.V. Korotkov, J.R. Delarosa, W.G. Hol, A dodecameric ring-like structure of the N0 domain of the type II secretin from enterotoxigenic Escherichia coli, J Struct Biol, 183 (2013) 354-362.
[72] R. Van der Meeren, Y. Wen, P. Van Gelder, J. Tommassen, B. Devreese, S.N. Savvides, New insights into the assembly of bacterial secretins: structural studies of the periplasmic domain of XcpQ from Pseudomonas aeruginosa, J Biol Chem, 288 (2013) 1214-1225.
[73] K.V. Korotkov, E. Pardon, J. Steyaert, W.G. Hol, Crystal structure of the N-terminal domain of the secretin GspD from ETEC determined with the assistance of a nanobody, Structure, 17 (2009) 255-265.
[74] R. Valverde, L. Edwards, L. Regan, Structure and function of KH domains, FEBS J, 275 (2008) 2712-2726.
[75] M. Tarry, M. Jaaskelainen, A. Paino, H. Tuominen, R. Ihalin, M. Hogbom, The extra-membranous domains of the competence protein HofQ show DNA binding, flexibility and a shared fold with type I KH domains, J Mol Biol, 409 (2011) 642-653.

[76] J.J. Ward, L.J. McGuffin, K. Bryson, B.F. Buxton, D.T. Jones, The DISOPRED server for the prediction of protein disorder, Bioinformatics, 20 (2004) 2138-2139.
[77] L.J. McGuffin, K. Bryson, D.T. Jones, The PSIPRED protein structure prediction server, Bioinformatics, 16 (2000) 404-405.
[78] M. Sandkvist, L.P. Hough, M.M. Bagdasarian, M. Bagdasarian, Direct interaction of the EpsL and EpsM proteins of the general secretion apparatus in Vibrio cholerae, J Bacteriol, 181 (1999) 3129-3135.
[79] J.R. Bergeron, L.J. Worrall, N.G. Sgourakis, F. DiMaio, R.A. Pfuetzner, H.B. Felise, M. Vuckovic, A.C. Yu, S.I. Miller, D. Baker, N.C. Strynadka, A refined model of the prototypical Salmonella SPI-1 T3SS basal body reveals the molecular basis for its assembly, PLoS Pathog, 9 (2013) e1003307.
[80] T. Spreter, C.K. Yip, S. Sanowar, I. Andre, T.G. Kimbrough, M. Vuckovic, R.A. Pfuetzner, W. Deng, A.C. Yu, B.B. Finlay, D. Baker, S.I. Miller, N.C. Strynadka, A conserved structural motif mediates formation of the periplasmic rings in the type III secretion system, Nat Struct Mol Biol, 16 (2009) 468-476.
[81] N.A. Linderoth, P. Model, M. Russel, Essential role of a sodium dodecyl sulfate-resistant protein IV multimer in assembly-export of filamentous phage, J Bacteriol, 178 (1996) 1962-1970.
[82] S. Rehman, S. Gu, V.E. Shevchik, R.W. Pickersgill, Anatomy of secretin binding to the Dickeya dadantii type II secretion system pilotin, Acta Crystallogr D Biol Crystallogr, 69 (2013) 1381-1386.
[83] S. Collin, I. Guilvout, N.N. Nickerson, A.P. Pugsley, Sorting of an integral outer membrane protein via the lipoprotein-specific Lol pathway and a dedicated lipoprotein pilotin, Mol Microbiol, 80 (2011) 655-665.
[84] K.V. Korotkov, W.G. Hol, Crystal structure of the pilotin from the enterohemorrhagic Escherichia coli type II secretion system, J Struct Biol, 182 (2013) 186-191.
[85] S. Gu, G. Kelly, X. Wang, T. Frenkiel, V.E. Shevchik, R.W. Pickersgill, Solution structure of homology region (HR) domain of type II secretion system, J Biol Chem, 287 (2012) 9072-9080.
[86] K.V. Korotkov, B. Krumm, M. Bagdasarian, W.G. Hol, Structural and functional studies of EpsC, a crucial component of the type 2 secretion system from Vibrio cholerae, J Mol Biol, 363 (2006) 311-321.

[87] S. Bleves, M. Gerard-Vincent, A. Lazdunski, A. Filloux, Structure-function analysis of XcpP, a component involved in general secretory pathway-dependent protein secretion in Pseudomonas aeruginosa, J Bacteriol, 181 (1999) 4012-4019.
[88] Y. Ivarsson, Plasticity of PDZ domains in ligand recognition and signaling, FEBS Lett, 586 (2012) 2638-2647.
[89] F.H. Login, M. Fries, X. Wang, R.W. Pickersgill, V.E. Shevchik, A 20-residue peptide of the inner membrane protein OutC mediates interaction with two distinct sites of the outer membrane secretin OutD and is essential for the functional type II secretion system in Erwinia chrysanthemi, Mol Microbiol, 76 (2010) 944-955.
[90] B. Py, L. Loiseau, F. Barras, An inner membrane platform in the type II secretion machinery of Gram-negative bacteria, Embo Rep, 2 (2001) 244-248.
[91] S.R. Lybarger, T.L. Johnson, M.D. Gray, A.E. Sikora, M. Sandkvist, Docking and assembly of the type II secretion complex of Vibrio cholerae, J Bacteriol, 191 (2009) 3149-3161.
[92] N. Buddelmeijer, M. Krehenbrink, F. Pecorari, A.P. Pugsley, Type II secretion system secretin PulD localizes in clusters in the Escherichia coli outer membrane, J Bacteriol, 191 (2009) 161-168.
[93] N. Buddelmeijer, O. Francetic, A.P. Pugsley, Green fluorescent chimeras indicate nonpolar localization of pullulanase secreton components PulL and PulM, J Bacteriol, 188 (2006) 2928-2935.
[94] F.H. Login, V.E. Shevchik, The single transmembrane segment drives self-assembly of OutC and the formation of a functional type II secretion system in Erwinia chrysanthemi, J Biol Chem, 281 (2006) 33152-33162.
[95] N. Sal-Man, D. Gerber, I. Bloch, Y. Shai, Specificity in transmembrane helix-helix interactions mediated by aromatic residues, J Biol Chem, 282 (2007) 19753-19761.
[96] M. Lallemand, F.H. Login, N. Guschinskaya, C. Pineau, G. Effantin, X. Robert, V.E. Shevchik, Dynamic interplay between the periplasmic and transmembrane domains of GspL and GspM in the type II secretion system, PLoS One, 8 (2013) e79562.
[97] J. Abendroth, A.E. Rice, K. McLuskey, M. Bagdasarian, W.G. Hol, The crystal structure of the periplasmic domain of the type II secretion system protein EpsM from Vibrio cholerae: the simplest version of the ferredoxin fold, J Mol Biol, 338 (2004) 585-596.

[98] J. Abendroth, A.C. Kreger, W.G. Hol, The dimer formed by the periplasmic domain of EpsL from the Type 2 Secretion System of Vibrio parahaemolyticus, J Struct Biol, 168 (2009) 313-322.
[99] T.L. Leighton, D.H. Yong, P.L. Howell, L.L. Burrows, Type IV Pilus Alignment Subcomplex Proteins PilN and PilO Form Homo- and Heterodimers in Vivo, J Biol Chem, 291 (2016) 19923-19938.
[100] T.L. Johnson, M.E. Scott, M. Sandkvist, Mapping critical interactive sites within the periplasmic domain of the Vibrio cholerae type II secretion protein EpsM, J Bacteriol, 189 (2007) 9082-9089.
[101] J. Abendroth, M. Bagdasarian, M. Sandkvist, W.G. Hol, The structure of the cytoplasmic domain of EpsL, an inner membrane component of the type II secretion system of Vibrio cholerae: an unusual member of the actin-like ATPase superfamily, J Mol Biol, 344 (2004) 619-633.
[102] C. Lu, K.V. Korotkov, W.G. Hol, Crystal structure of the full-length ATPase GspE from the Vibrio vulnificus type II secretion system in complex with the cytoplasmic domain of GspL, J Struct Biol, 187 (2014) 223-235.
[103] J. Abendroth, P. Murphy, M. Sandkvist, M. Bagdasarian, W.G. Hol, The X-ray structure of the type II secretion system complex formed by the N-terminal domain of EpsE and the cytoplasmic domain of EpsL of Vibrio cholerae, J Mol Biol, 348 (2005) 845-855.
[104] J.L. Camberg, M. Sandkvist, Molecular analysis of the Vibrio cholerae type II secretion ATPase EpsE, J Bacteriol, 187 (2005) 249-256.
[105] J.M. Mancl, W.P. Black, H. Robinson, Z. Yang, F.D. Schubot, Crystal Structure of a Type IV Pilus Assembly ATPase: Insights into the Molecular Mechanism of PilB from Thermus thermophilus, Structure, 24 (2016) 1886-1897.
[106] K.A. Satyshur, G.A. Worzalla, L.S. Meyer, E.K. Heiniger, K.G. Aukema, A.M. Misic, K.T. Forest, Crystal structures of the pilus retraction motor PilT suggest large domain movements and subunit cooperation drive motility, Structure, 15 (2007) 363-376.
[107] S. Reindl, A. Ghosh, G.J. Williams, K. Lassak, T. Neiner, A.L. Henche, S.V. Albers, J.A. Tainer, Insights into FlaI functions in archaeal motor assembly and motility from structures, conformations, and genetics, Mol Cell, 49 (2013) 1069-1082.

[108] S.J. Shiue, K.M. Kao, W.M. Leu, L.Y. Chen, N.L. Chan, N.T. Hu, XpsE oligomerization triggered by ATP binding, not hydrolysis, leads to its association with XpsL, EMBO J, 25 (2006) 1426-1435.
[109] J.L. Camberg, T.L. Johnson, M. Patrick, J. Abendroth, W.G. Hol, M. Sandkvist, Synergistic stimulation of EpsE ATP hydrolysis by EpsL and acidic phospholipids, EMBO J, 26 (2007) 19-27.
[110] M.A. Robien, B.E. Krumm, M. Sandkvist, W.G. Hol, Crystal structure of the extracellular protein secretion NTPase EpsE of Vibrio cholerae, J Mol Biol, 333 (2003) 657-674.
[111] T. Mora, H. Yu, Y. Sowa, N.S. Wingreen, Steps in the bacterial flagellar motor, PLoS Comput Biol, 5 (2009) e1000540.
[112] M.D. Gray, M. Bagdasarian, W.G. Hol, M. Sandkvist, In vivo cross-linking of EpsG to EpsL suggests a role for EpsL as an ATPase-pseudopilin coupling protein in the Type II secretion system of Vibrio cholerae, Mol Microbiol, 79 (2011) 786-798.
[113] J. Abendroth, D.D. Mitchell, K.V. Korotkov, T.L. Johnson, A. Kreger, M. Sandkvist, W.G. Hol, The three-dimensional structure of the cytoplasmic domains of EpsF from the type 2 secretion system of Vibrio cholerae, J Struct Biol, 166 (2009) 303-315.
[114] M. Nivaskumar, J. Santos-Moreno, C. Malosse, N. Nadeau, J. Chamot-Rooke, G. Tran Van Nhieu, O. Francetic, Pseudopilin residue E5 is essential for recruitment by the type 2 secretion system assembly platform, Mol Microbiol, 101 (2016) 924-941.
[115] M. Campos, M. Nilges, D.A. Cisneros, O. Francetic, Detailed structural and assembly model of the type II secretion pilus from sparse data, Proc Natl Acad Sci U S A, 107 (2010) 13081-13086.
[116] D.N. Nunn, S. Lory, Cleavage, methylation, and localization of the Pseudomonas aeruginosa export proteins XcpT, -U, -V, and -W, J Bacteriol, 175 (1993) 4375-4382.
[117] K.V. Korotkov, W.G. Hol, Structure of the GspK-GspI-GspJ complex from the enterotoxigenic Escherichia coli type 2 secretion system, Nat Struct Mol Biol, 15 (2008) 462-468.
[118] B. Douzi, E. Durand, C. Bernard, S. Alphonse, C. Cambillau, A. Filloux, M. Tegoni, R. Voulhoux, The XcpV/GspI pseudopilin has a central role in the assembly

of a quaternary complex within the T2SS pseudopilus, J Biol Chem, 284 (2009) 34580-34589.
[119] M. Nivaskumar, G. Bouvier, M. Campos, N. Nadeau, X. Yu, E.H. Egelman, M. Nilges, O. Francetic, Distinct docking and stabilization steps of the Pseudopilus conformational transition path suggest rotational assembly of type IV pilus-like fibers, Structure, 22 (2014) 685-696.
[120] D.A. Cisneros, P.J. Bond, A.P. Pugsley, M. Campos, O. Francetic, Minor pseudopilin self-assembly primes type II secretion pseudopilus elongation, EMBO J, 31 (2012) 1041-1053.
[121] E. Durand, A. Bernadac, G. Ball, A. Lazdunski, J.N. Sturgis, A. Filloux, Type II protein secretion in Pseudomonas aeruginosa: the pseudopilus is a multifibrillar and adhesive structure, J Bacteriol, 185 (2003) 2749-2758.
[122] N. Sauvonnet, G. Vignon, A.P. Pugsley, P. Gounon, Pilus formation and protein secretion by the same machinery in Escherichia coli, EMBO J, 19 (2000) 2221-2228.
[123] E. Durand, G. Michel, R. Voulhoux, J. Kurner, A. Bernadac, A. Filloux, XcpX controls biogenesis of the Pseudomonas aeruginosa XcpT-containing pseudopilus, J Biol Chem, 280 (2005) 31378-31389.
[124] F.K. Schur, W.J. Hagen, A. de Marco, J.A. Briggs, Determination of protein structure at 8.5A resolution using cryo-electron tomography and sub-tomogram averaging, J Struct Biol, 184 (2013) 394-400.
[125] B. Hu, M. Lara-Tejero, Q. Kong, J.E. Galan, J. Liu, In Situ Molecular Architecture of the Salmonella Type III Secretion Machine, Cell, 168 (2017) 1065-1074 e1010.
[126] M. Kaplan, A. Cukkemane, G.C. van Zundert, S. Narasimhan, M. Daniels, D. Mance, G. Waksman, A.M. Bonvin, R. Fronzes, G.E. Folkers, M. Baldus, Probing a cell-embedded megadalton protein complex by DNP-supported solid-state NMR, Nat Methods, 12 (2015) 649-652.
[127] M.E. Yanez, K.V. Korotkov, J. Abendroth, W.G. Hol, Structure of the minor pseudopilin EpsH from the Type 2 secretion system of Vibrio cholerae, J Mol Biol, 377 (2008) 91-103.
[128] K.V. Korotkov, M.D. Gray, A. Kreger, S. Turley, M. Sandkvist, W.G. Hol, Calcium is essential for the major pseudopilin in the type 2 secretion system, J Biol Chem, 284 (2009) 25466-25470.

[129] R.G. Zhang, D.L. Scott, M.L. Westbrook, S. Nance, B.D. Spangler, G.G. Shipley, E.M. Westbrook, The three-dimensional crystal structure of cholera toxin, J Mol Biol, 251 (1995) 563-573.
[130] S. Kolappan, M. Coureuil, X. Yu, X. Nassif, E.H. Egelman, L. Craig, Structure of the Neisseria meningitidis Type IV pilus, Nat Commun, 7 (2016) 13015.

Figure Legends

Figure 1. Model of T2SS architecture and secretion. (A) Genetic organization of T2SSs from *V. cholerae*, *E. coli*, *D. dadantii*, *P. aeruginosa* and *L. pneumophila*. Genes are labelled as single letters based on the Gsp nomenclature except for *P. aeruginosa* Xcp (XcpP-XcpZ/GspC-GspM; XcpA/GspO). Genes encoding GspC proteins are coloured brown, the secretins are coloured blue, the inner membrane platform proteins are orange, the pseudo-pilins are coloured green, pre-pilin peptidases are yellow and accessory components are grey. Pilotins are coloured magenta. Operons are separated by double lines. (B) Structural model of the *V. cholerae* Eps T2SS in its resting state. All T2SS components are represented as cartoons and intact protomers were modelled starting from either *V. cholerae* structures (EpsD, pdb 5wq8; EpsE, pdb 2bh1, 4ksr; EpsF, pdb 2vma; EpsG, pdb 3fu1; EpsH, pdb 2qv8; EpsL, pdb 1yf5; EpsM, pdb 1uv7) [49, 51, 97, 103, 113, 127, 128] or homologous structures (*E. coli* GspC/GspD, pdb 3oss; *V. parahaemolyticus* EpsL, pdb 2w7v; *E. coli* GspI/GspJ/GspK, pdb 3ci0; *M. xanthus* T4PS, pdb 3jc9) [47, 69, 98, 117] using the Phyre2 server [38]. Structures were assembled using the EM model of the type IVa pilus system as a guide [47]. The cholera toxin (CT) is also shown as a grey surface and to the same scale as the T2SS model [129]. (C) Current model of T2SS translocation pathway. T2SS proteins are labelled and coloured as in (A). Domains of GspC and GspD are annotated. Numbered pentagons represent the path of a substrate during its export.

Figure 2. Structure and function of GspD. (A) Intrinsic disorder plots of *V. cholerae* EpsD and *D. dadantii* OutD with domain boundaries annotated above [76]. Coloured stars represent significantly disordered regions: black, N^0/N^1 loop; cyan, N^2 α5-β10 loop; N^3 α7-β13 loop; blue, external cap/gate. Numbering of GspD secondary structure throughout this review is based on the full sequence and begins from the N^0 domain. (B) Cryo-EM structures of the *V. cholerae* and *E. coli* full length GspD proteins showing the external and internal features. Coloured stars showing disordered regions are as in (A) [49]. (C) Representative T2SS N-domain and *S. typhimurium* InvG T3SS N^0N^1 domain structures [49, 71-73, 79].

Figure 3. Substrate recognition by the T2SS. (A) Intrinsic disorder plots of *D. dadantii* OutC, *P. aeruginosa* XcpP and *L. pneumophila* LspC with domain

boundaries annotated above [76]. Black star represents the helical region in the TMHR. (B) NMR structure of the *D. dadantii* OutC HR domain including its TMHR [85]. (C) Crystal structure of the *V. cholerae* EpsC PDZ domain [86]. Regions involved in substrate recognition are colored lighter. (D) Crystal structure of the *E. coli* GspC HR/ GspD N^{01} complex [39]. (E) NMR derived model of the *D. dadantii* OutC HR/ OutD N^{0} complex [85].

Figure 4. T2SS inner membrane platform and pseudopilus assembly. (A) Crystal structures of *V. parahaemolyticus* EpsL [98] and *V. cholerae* EpsM [97] periplasmic domains highlighting homo-dimer formation within these crystal lattices. In one chain of each, helices are red and sheets are blue, whilst the other chain is coloured yellow (EpsL) or brown (EpsM). Secondary structure elements in *D. dadantii* OutL and OutM that have been shown to mediate both homo- and hetero-dimer formation *in vivo* are coloured cyan and green, respectively. Secondary structure elements in *V. cholerae* EpsM that have been highlighted to mediate either homo- or hetero-dimerization *in vivo* are coloured purple and grey, respectively. (B) Crystal structure of hexameric *V. cholerae* EpsE [51]. (C) Intrinsic disorder plots of *E. coli* GspF with domain boundaries annotated above [76]. Black stars represent two regions with significant disorder within the initial 75 residues. (D) Crystal structure of the *V. cholerae* EpsG major domain [128]. The signal peptide and membrane embedded helix of this domain are absent. (E) Cryo-EM structure of the *N. meningitides* type IV pilus highlighting helical bundle formation [130].

FIGURES

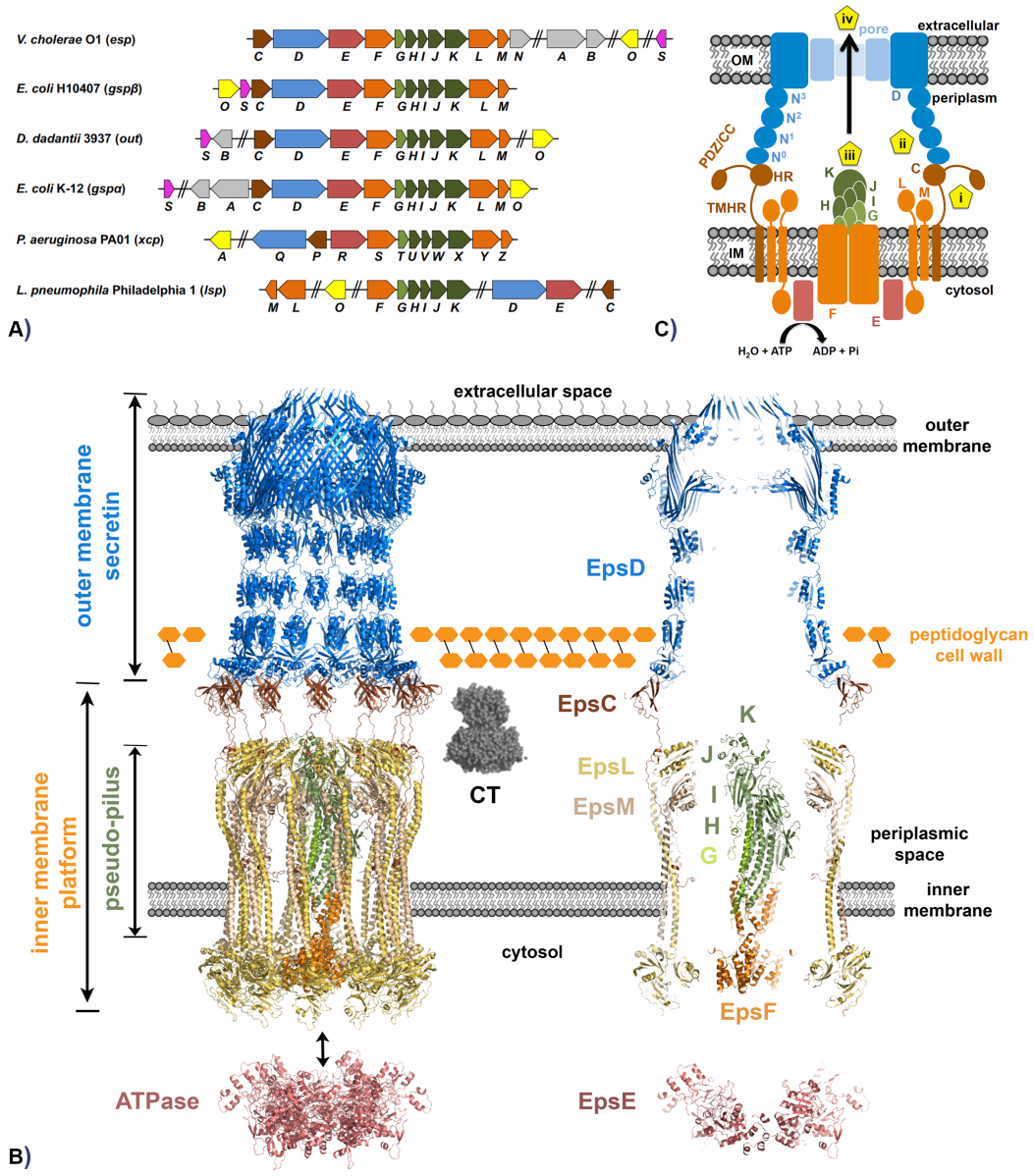
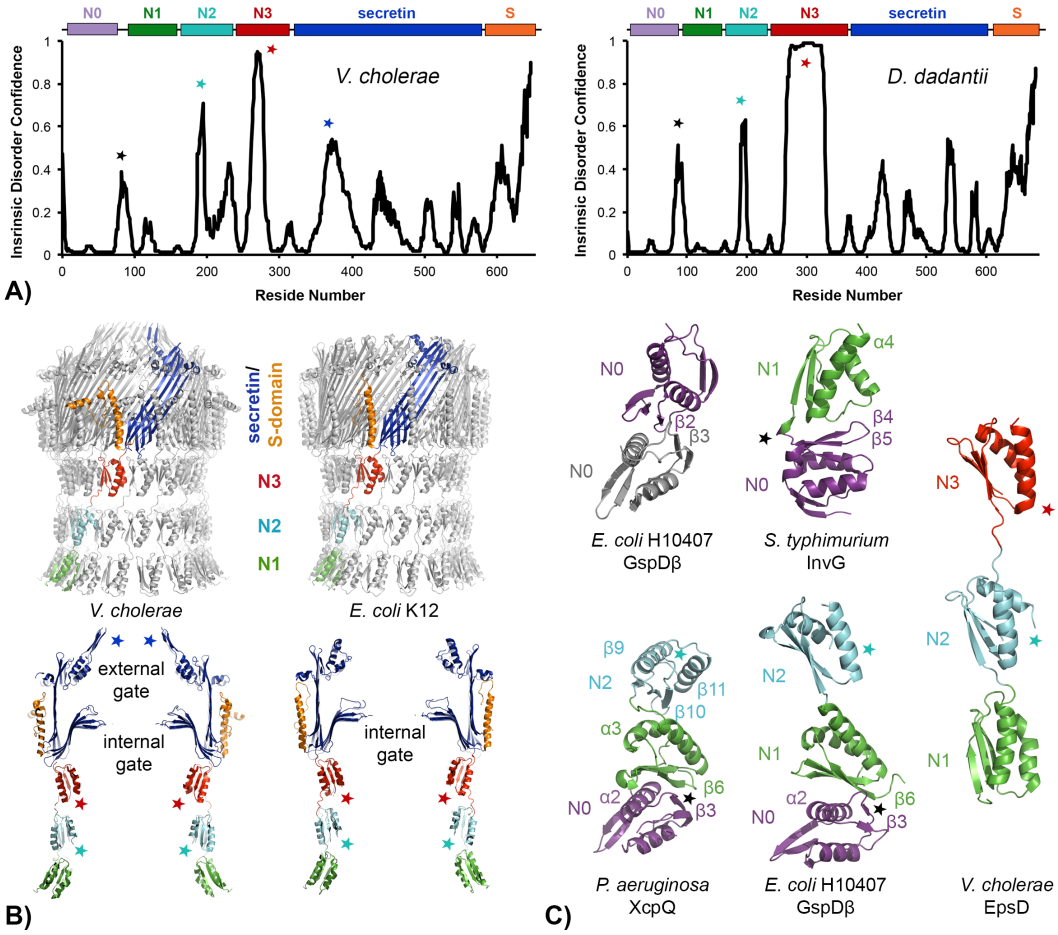


Figure 1


A)
N0
N1
N2
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secretin
S
V. cholerae
D. dadantii
Insrinsic Disorder Confidence
Reside Number
B)
secretin/
S-domain
V. cholerae
E. coli K12
external
gate
internal
gate
C)
E. coli H10407
GspDβ
S. typhimurium
InvG
P. aeruginosa
XcpQ
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GspDβ
V. cholerae
EpsD

Figure 2

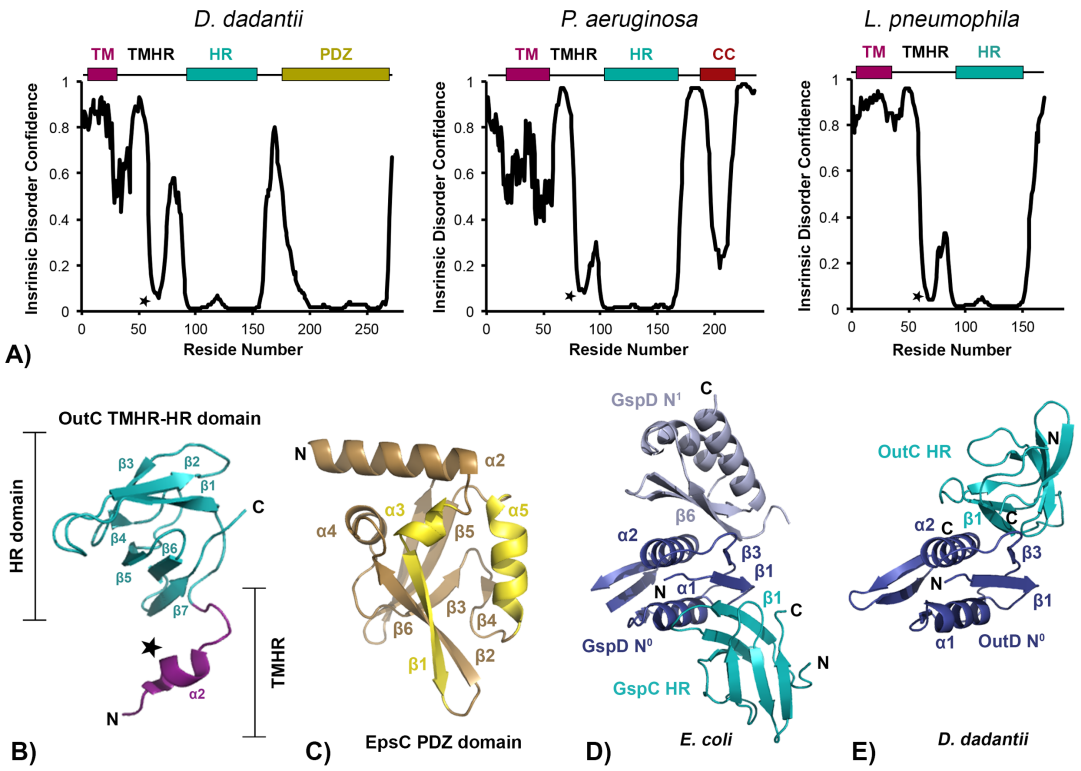

Figure 3

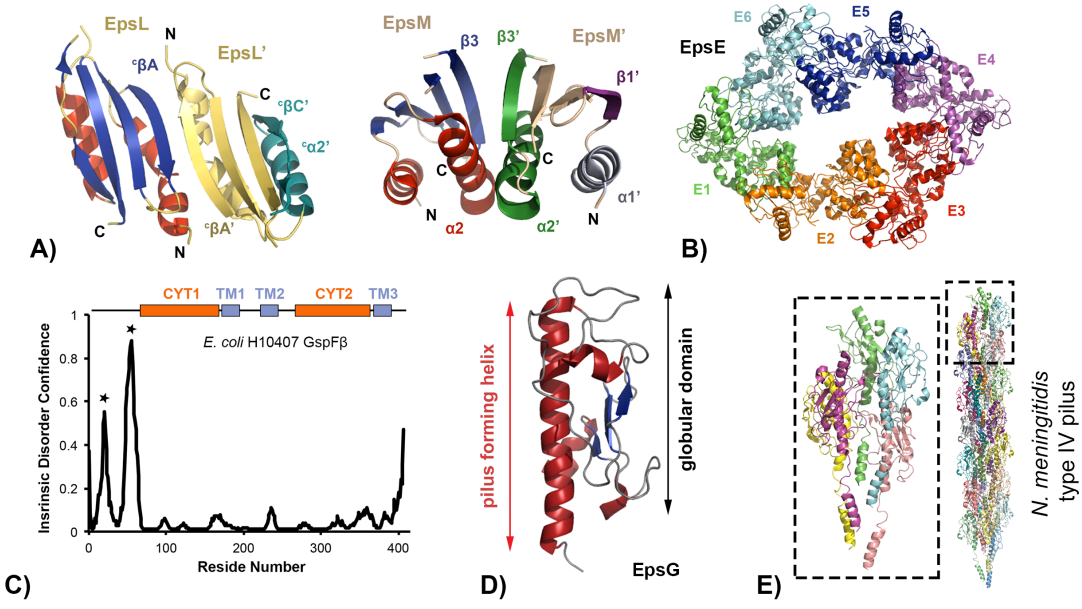

Figure 4