MINIREVIEW

Type II secretion: from structure to function

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Abstract

Gram-negative bacteria use the type II secretion system to transport a large number of secreted proteins from the periplasmic space into the extracellular environment. Many of the secreted proteins are major virulence factors in plants and animals. The components of the type II secretion system are located in both the inner and outer membranes where they assemble into a multi-protein, cell-envelope spanning, complex. This review discusses recent progress, particularly newly published structures obtained by X-ray crystallography and electron microscopy that have increased our understanding of how the type II secretion apparatus functions and the role that individual proteins play in this complex system.

Introduction

Extracellular secretion in gram-negative bacteria requires complex transport systems to move secreted proteins from the cytoplasm into the extracellular environment. Several highly specialized pathways have evolved for this purpose, including the type II secretion (T2S) system. Secretion via the T2S pathway occurs in two distinct steps. First, the proteins to be secreted are expressed with signal peptides that target them to either the Sec or Tat machinery for transport across the cytoplasmic membrane into the periplasm (Voulhoux *et al*., 2001; de Keyzer *et al*., 2003; Palmer *et al*., 2005). This is followed by removal of the signal peptides and recognition and translocation of the fully folded proteins across the outer membrane by components of the T2S system. The T2S pathway was first discovered in *Klebsiella oxytoca*, where it was found to be required for secretion of the starch-hydrolyzing lipoprotein, pullulanase (d'Enfert *et al*., 1987). Since then, *Vibrio cholerae*, Shiga-toxin producing *Escherichia coli*, *Legionella pneumophila*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Erwinia chrysanthemi* and *Xanthomonas campestris* have been shown to make use of this pathway for secretion and targeted delivery of toxins, proteases, cellulases and lipases (Dow *et al*., 1989; Filloux *et al*., 1990; Reeves *et al*., 1993; Sandkvist *et al*., 1997; DeShazer *et al*., 1999; Lathem *et al*., 2002; Iwobi *et al*., 2003; Rossier *et al*., 2004). Functional T2S pathways are also likely present in many additional species, as sequencing of bacterial genomes has identified T2S-like genes in numerous species (Cianciotto, 2005).

The number of genes identified as being essential for T2S is species-dependent and varies from 12 to 15. The homologous genes and their subsequent proteins have been designated in most systems by the letters A–O and S. *P. aeruginosa* is an exception, however, as the letters P–Z and A have been used instead. To indicate homology, in this review we will use the term T2S followed by the letter(s) of the gene product. For example, T2S:E_R will be used to indicate that *P. aeruginosa* XcpR is the homolog to the T2S family protein E. For clarity with respect to previously published reviews, the general term T2S is equivalent to Gsp (Desvaux *et al*., 2004).

Based on data from many different experimental approaches, including subcellular localization and protein-protein interactions between individual components of the T2S system, it has been proposed that the T2S proteins

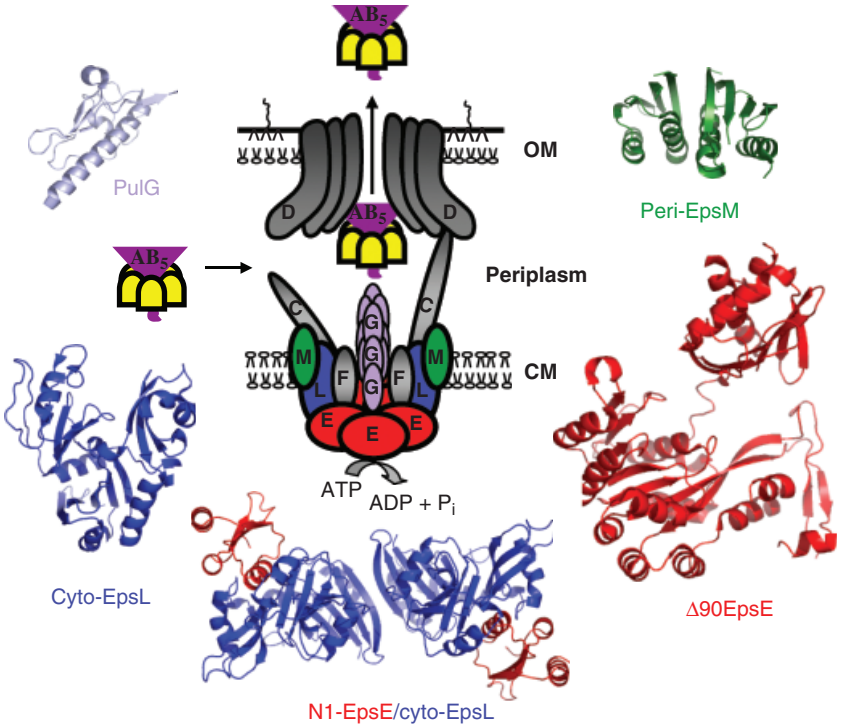

Fig. 1. Model of pilus-mediated secretion via the type II secretion (T2S) system in *Vibrio cholerae* with structures of EpsE (red), EpsL (blue), EpsM (green) and the T2S:G_T homolog PulG (light purple). Once assembled in the periplasmic compartment, cholera toxin (AB_5, dark purple and yellow; Protein Data Bank ID 1S5E) is targeted to the T2S machinery and transported across the outer membrane to the extracellular environment. The ATPase EpsE (red) is shown in this model as a hexameric ring associating with the inner membrane via its interaction with EpsL (blue). A ribbon structure of N-terminally truncated, monomeric, EpsE alone is shown in red (PDB:1P9R, 1P9W), and the structure of the β-sheet-rich cytoplasmic domain of EpsL is shown in blue (PDB:1YF5). The X-ray structure of the cytoplasmic domain of EpsL co-purified and crystallized with the N-terminal 96 residues of EpsE is also depicted in blue and red (PDB:2BH1), respectively. EpsM (green, PDB:1UV7) crystallized as a dimer and is capable of interacting with and localizing EpsL to the cell poles in *V. cholerae*. The X-ray structure of the T2S:G_T homolog from *Klebsiella oxytoca*, PulG (light purple, PDB:1T92), shows that PulG has a structure similar to the type IV pilins; a conserved α-helix followed by four β-strands arranged in a globular domain. Once the PulG monomers assemble to form a pilus-like structure, they may act as a piston to push the toxin and other secreted proteins out of the periplasm through the outer membrane secretin, T2S:D_Q. T2S:C_P interacts with T2S:D_Q in the outer membrane and has been shown to stabilize T2S:L_Y and T2S:M_Z in the inner membrane. Only the core components of the T2S system are shown in this model as the T2S:A, T2S:B and T2S:S proteins are not present in every species. The prepilin peptidase T2S:O_A and the pseudopilins T2S:H_U, I_V, J_W, and K_X are also not depicted as they likely interact transiently with T2S:G_T, and no structural information about these proteins is available.

assemble to form a complex that spans the entire gram-negative cell envelope. This multi-protein assembly includes a component in the cytoplasm, an inner membrane sub-complex that reaches into the periplasmic compartment and a secretion pore in the outer membrane (Fig. 1). The exact configuration of this apparatus and the mechanism, or order, of its assembly in the cell envelope is still unclear. In this review, we will highlight the very recent advances, particularly in structural data from X-ray crystallography and electron microscopy, which have increased our understanding of the complex machinery that makes up the T2S system. For broader reviews of T2S, see references Russel (1998), Sandkvist (2001a), Filloux (2004), and for reviews that highlight the involvement of T2S in pathogenesis and other bacterial processes, see references Sandkvist (2001b) and Cianciotto (2005).

The inner membrane complex

A functional T2S transport system requires the presence of T2S:E_R, a cytoplasmic protein that is associated with the cytoplasmic membrane via the membrane proteins T2S:L_Y and T2S:M_Z. T2S:E_R proteins are members of a large family of secretion nucleoside triphosphates, which are thought to power bacterial protein secretion through NTP hydrolysis (Planet *et al.*, 2001). T2S:E_R proteins are multi-domain proteins (Fig. 2a), and while they all contain domains N1 to C2, domain N0 is only present in a few homologs including XpsE and XcpR in *Xanthomonas campestris* and *Pseudomonas aeruginosa*, respectively. All T2S:E_R proteins have typical ATP-binding sites, called Walker A boxes, commonly present in ATPases, ATP synthases and kinases. Recently it was demonstrated that purified EpsE,

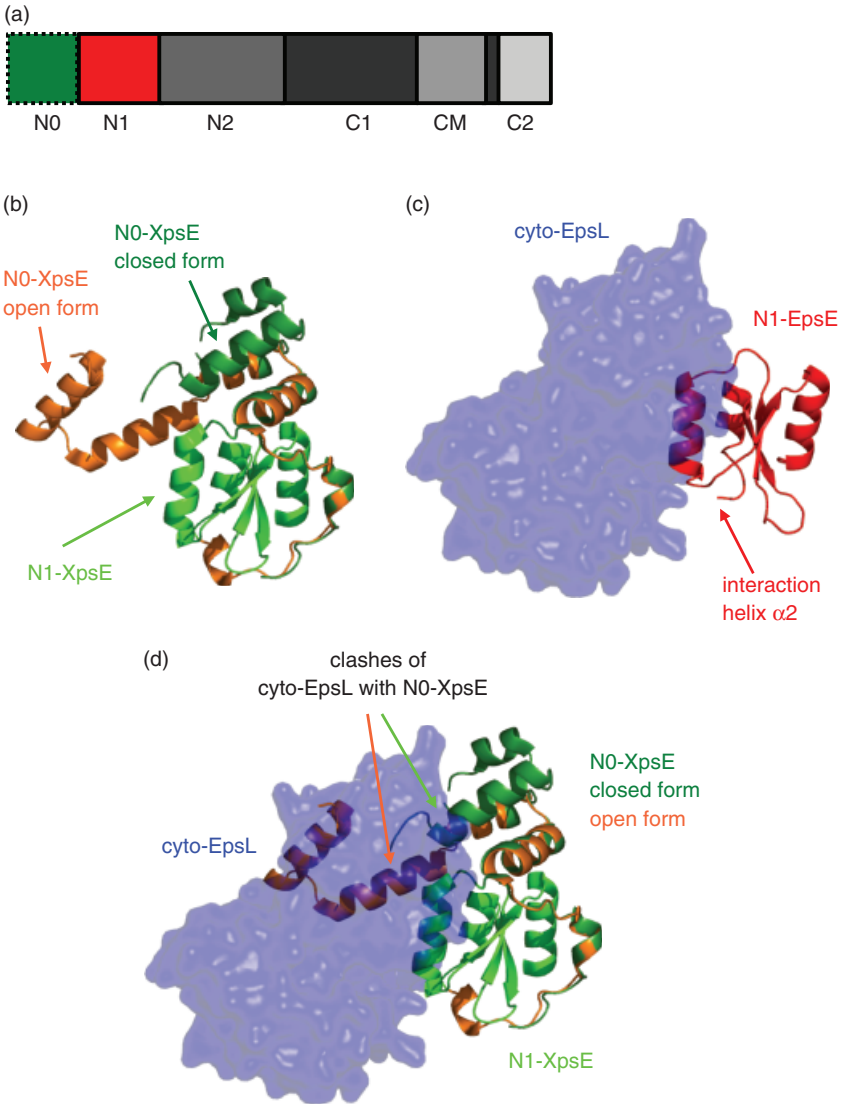

Fig. 2. Type II secretion (T2S):E_R domain organization and X-ray crystal structures of the N-terminal subdomains of EpsE and XpsE. (a) Structural domains of T2S:E_R with the subdomains N0 (dark green), N1 (red), N2, C1, CM and C2 (gray shades). The N0 subdomain is restricted to a subset of T2S:E_R family members. (b) Superposition of the two structures of XpsE N0–N1 with domain N1 colored light green and domain N0 in its closed and open conformations colored in dark green and orange, respectively. For clarity, only one chain is shown for domain N1. (c) The N1 domains of EpsE (red ribbon) and XpsE (b) have very similar structures. Helix α2 of N1-EpsE is responsible for the majority of interactions with cyto-EpsL (blue surface). (d) A superposition of the N1 domains of EpsE and XpsE leads to sterical clashes between cyto-EpsL and the N0 domain of XpsE in both the open and closed conformation. Colors and orientations are the same in (b–d.)

the T2S:E_R family member from *Vibrio cholerae*, is a Mg^{2+}-dependent ATPase (Camberg & Sandkvist, 2005). A mutation introduced in the Walker A ATP-binding motif reduces the specific ATPase activity *in vitro* by several fold and results in a protein that is unable to support secretion *in vivo* (Sandkvist *et al.*, 1995; Camberg & Sandkvist, 2005). The rate of ATP hydrolysis by purified EpsE is similar to those observed for other secretion ATPases, which range from approximately 0.5 to 15 $nmol^{-1}\,min^{-1}\,mg^{-1}$ protein.

Interestingly, EpsE displays cooperative ATPase activity, and purified preparations of EpsE contain a small fraction of oligomers, specifically hexamers, with increased specific activity (Camberg & Sandkvist, 2005). This may suggest that the functional form of T2S:E_R proteins *in vivo* is hexameric. Several members of the type IV secretion ATPase family, such as HP0525 from *Helicobacter pylori* and the type IV pilus retraction ATPase, PilT, have been characterized as hexameric ATPases (Yeo *et al.*, 2000; Forest *et al.*, 2004). The X-ray structure of an N-terminally truncated form of EpsE, containing the N2 domain and the complete C-terminal domain with its three subdomains C1, C_M and C2, was also recently solved (Figs 1 and 2a) (Robien *et al.*, 2003). The truncated EpsE crystallized as anti-parallel helical filaments, however a hexameric ring model was proposed, as 8 of the 10 closest structural homologs assemble into multi-subunit rings. Structural data of EpsE also uncovered a metal ion tetrahedrally coordinated by a tetracysteine motif, protruding from the rest of the protein in an extended hairpin-like loop. Additional biochemical analysis revealed that EpsE incorporates 1 mol of zinc per mol of EpsE (Camberg & Sandkvist, 2005).

T2S:E_R is dependent on the bitopic cytoplasmic membrane protein T2S:L_Y for membrane association (Sandkvist *et al.*, 1995; Ball *et al.*, 1999; Py *et al.*, 1999; Possot *et al.*,

2000). T2S:L_Y consists of a large cytoplasmic domain, a single transmembrane helix and a smaller periplasmic domain. The cytoplasmic domain binds to and localizes T2S:E_R to the cytoplasmic membrane, whereas either the transmembrane helix or the periplasmic domain, or both, interact with T2S:M_Z (Sandkvist *et al.*, 2000). This interaction results in mutual protection of T2S:L_Y and T2S:M_Z against proteolysis (Michel *et al.*, 1998; Sandkvist *et al.*, 1999), as well as localization of T2S:L_Y to the cell poles of *V. cholerae* (Scott *et al.*, 2001). The recently published X-ray structure of the cytoplasmic domain of the *V. cholerae* TS2:L_Y homolog, cyto-EpsL, unexpectedly revealed structural homology with the actin-like ATPase super family (Fig. 1) (Abendroth *et al.*, 2004a). Cyto-EpsL consists of three β-sheet-rich domains, with domains I and III of cyto-EspL corresponding to the conserved domains 1A and 2A of the actin-like ATPases. Cyto-EpsL does not contain the non-conserved 1B and 2B domains of the actin-like proteins, and as a consequence, cyto-EpsL is incapable of forming actin-like filaments. Domain II of cyto-EspL has only been observed in one other member of the actin-like ATPase super family: the cell division protein FtsA, where it likely participates in intramolecular interactions that result in recruitment of other proteins essential for cell division to the bacterial midcell (Rico *et al.*, 2004). Structural and sequence analysis suggested that domain II of cyto-EpsL is also involved in protein-protein interactions (Abendroth *et al.*, 2004a). The absence of domains 1B and 2B of the actin-like proteins, as well as the presence of the flexible domain II, makes cyto-EpsL unique among known members of the actin-like ATPase super family. Additionally, unlike the vast majority of the actin-like proteins, neither the sequence nor the X-ray crystal structure of cyto-EpsL reveal a nucleotide-binding site (Abendroth *et al.*, 2004a). Instead, EspL likely participates indirectly in ATP hydrolysis through its interaction with the T2S ATPase EpsE.

Mutational studies have shown that the interface between EpsE and EpsL is formed by the N-terminal 90 residues of EpsE (Sandkvist *et al.*, 1995) and a region of the cytoplasmic portion of EpsL that has been designated as domains II and III in the X-ray structure of cyto-EpsL (Sandkvist *et al.*, 2000; Abendroth *et al.*, 2004a). The first structural view of these interacting partners completes the structural picture of EpsE (which could not be done in the earlier X-ray structure of the N-terminally truncated EpsE) and confirms that domains II and III of cyto-EspL are involved in the interaction with the N1 subdomain of EpsE, which is folded into a compact α+β structure composed of three α-helices and β-strands each (Fig. 1) (Abendroth *et al.*, 2005). One of the N1 α-helices appears to be wedged in a cleft between domains II and III of cyto-EspL. Given that this α-helix only partially fills the groove between the cyto-EpsL domains II and III, it is possible that other residues of EpsE or another member of the T2S machinery, such as T2S:F_S, may also bind in this pronounced cleft.

The X-ray structure of subdomains N0 (helical region) and N1 (core domain) of XpsE from *X. campestris* has very recently been determined in two crystal forms (Fig. 2b) (Chen *et al.*, 2005). In both forms, subdomain N1 adopts the same α+β fold as seen for N1 of EpsE in complex with the cytoplasmic domain of EpsL (Fig. 2c) (Abendroth *et al.*, 2005) and shows some structural homologies with a NifU-like domain (PDB ID: 1veh). Of note, another member of the NifU-like family, IscU, is known to bind to and stimulate the activity of an ATPase (Silberg *et al.*, 2001; Chen *et al.*, 2005); an intriguing finding that may suggest that the N1 subdomain similarly regulates the activity of the T2S:E_R ATPase. Consistent with this suggestion is the finding that removal of the N-terminal 90 residues of EpsE results in increased ATPase activity (Camberg & Sandkvist, unpublished data). In the two crystal forms of N0–N1 of XpsE, the 62-residue helical N0 subdomain adopts two vastly different conformations (Fig. 2b), but in spite of this conformational variability, the very N-terminal 36 residues appear indispensable for the functioning of the T2S system in *X. campestris*. Chen *et al.* hypothesized that these residues participate in binding to XpsL and that the two conformations might represent the bound and unbound states of the XpsE N-terminus; a hypothesis that would be consistent with the finding that EpsE may exist in two different forms in *V. cholerae* (Sandkvist *et al.*, 1995). However, the structural superposition of the N1 subdomain of XpsE onto that of EpsE in the EpsE–EpsL complex leads to severe sterical clashes between subdomain N0 of XpsE in both open and closed conformation and the cytoplasmic domain of EpsL (Fig. 2d). This could mean that the N0 domain of XpsE adopts yet another conformation when bound to XpsL. Alternatively, the observed sterical clashes may support the suggestion of Chen *et al.* that XpsE and EpsE belong to two slightly distinct families of T2S:E_R proteins and differ in the presence of domain N0, which may result in different T2S:L_Y binding properties.

The three T2S components T2S:M_Z, T2S:L_Y and T2S:E_R form a stable complex at the cytoplasmic membrane. T2S:M_Z is a small inner membrane protein with a short cytoplasmic segment, one transmembrane helix and a periplasmic C-domain. The *V. cholerae* homolog, EpsM, inherently localizes to the cell pole, independently of other Eps proteins (Scott *et al.*, 2001). It is also capable of directing EpsL to the bacterial cell pole. Besides localization and stabilization of T2S:L_Y, little has been reported about the role of T2S:M_Z in the complex. All functional T2S systems contain a T2S:M_Z homolog that is essential for secretion, however. The sequence homology between the T2S:M_Z family members is concentrated in the predicted transmembrane helix and two blocks of conserved residues present in

the periplasmic region of the polypeptide chain (Abendroth *et al*, 2004b). The short, N-terminal, portion of the protein shows very little homology with other family members.

The X-ray structure of a major portion of the periplasmic domain of EpsM has recently been solved (Fig. 1) (Abendroth *et al*., 2004b). Full-length EpsM and the soluble periplasmic domain are capable of forming dimers in solution, and two monomers of periplasmic EpsM are present in each asymmetric unit of the crystal. In the model of the dimer, subunit-subunit contact occurs mostly through interactions between C-terminal residues 123 through 135 in each EpsM monomer. Residues that make up the dimer interface are conserved only among the closest homologs, however.

Interestingly, when two EpsM monomers come together a 10 Å deep cleft that contains a hydrophobic bottom and a hydrophilic rim is formed at the interface (Abendroth *et al*., 2004b). The majority of hydrophobic residues in the bottom of the cleft are highly conserved between T2S:M_Z family members, whereas the hydrophilic residues that line the rim show very little conservation. The periplasmic domain of EpsM represents a ferredoxin-like fold with a unique αββ–αββ permutation, and like other ferredoxin-like proteins which tend to bind substrates at the β-strand side (Russell *et al*., 1998), the EpsM dimer interface might also form a ligand-binding domain. Interestingly, additional electron density was found in the cleft between the subunits (Abendroth *et al*., 2004b). The chemical identity of the small molecular ligand could not be determined by X-ray crystallography. The shape of the additional density and possible molecular interactions are compatible with a short peptide, however (J. Abendroth & W. G. J. Hol, unpublished data).

A third cytoplasmic membrane protein, T2S:F_S, has also been implicated in the cytoplasmic platform of the T2S machinery. Co-immunoprecipiation of OutE, OutL and OutF as well as co-purification of $XcpR_E$, $XcpS_F$ and $XcpY_L$ with histidine-tagged $XcpZ_M$ by metal affinity chromatography have demonstrated interactions between the T2S components E_R, L_Y, M_Z and F_S (Py *et al*., 2001; Robert *et al*., 2005b). Similar interactions have also recently been demonstrated for the two T2S:E_R and T2S:F_S homologs BfpD and BfpE, which are required for biogenesis of the bundle-forming pilus of enteropathogenic *Escherichia coli* (Crowther *et al*., 2004). Furthermore, the ATPase activity of BfpD was found to be slightly stimulated by a fragment of the N-terminal cytoplasmic domain of BfpE (Crowther *et al*., 2005).

The pseudopilins

Sequence analysis of the T2S components has revealed homology with several components involved in type IV pilus biogenesis, suggesting that the T2S apparatus may function similarly to the type IV pilus machinery (Nunn, 1999; Burrows, 2005). The T2S:G_T, H_U, I_V, J_W and K_X proteins display some similarity to the subunits of type IV pili and, like the pilin subunits, are translated with a specialized leader peptide required for export across the cytoplasmic membrane. Furthermore, T2S:O_A shares a high degree of identity with the prepilin peptidase (PilD). PilD is a novel bifunctional aspartic acid protease that cleaves the leader peptides of type IV pilin subunits and *N*-methylates the newly generated N-terminus (Nunn & Lory, 1993; LaPointe & Taylor, 2000). T2S:O_A performs the same function for the proteins T2S:G_T, H_U, I_V, J_W and K_X in the T2S system. In some species no gene encoding T2S:O_A is linked to the rest of the T2S genes; in these cases, PilD acts on both the precursor forms of type IV pilin subunits and the related proteins of the T2S system (Nunn & Lory, 1992).

The notable similarity with type IV pilus components has led to T2S:G_T, H_U, I_V, J_W and K_X proteins being termed *pseudopilins*. It has recently been shown that certain T2S pseudopilins are indeed capable of forming a pilus-like, helical, fiber that spans the cell envelope and is exposed on the cell surface upon overexpression (Fig. 3) (Sauvonnet *et al*., 2000; Durand *et al*., 2003; Köhler *et al*., 2004). In agreement with the finding that T2S:G_T is expressed at much higher levels than the other pseudopilins, immunogold-labeling and overexpression studies show that the pilus-like structure may be comprised of only T2S:G_T subunits (Nunn & Lory, 1993; Durand *et al*., 2003; Vignon *et al*., 2003). Although the formation of the surface exposed polymer interferes with secretion, possibly by occluding the channel in the outer membrane, it suggests that the pseudopilin does assemble into a pilus-like structure that may span the periplasmic compartment and participate in protein secretion across the outer membrane (Possot *et al*., 2000; Hu *et al*., 2002; Durand *et al*., 2003).

The recently published crystal structure of a truncated form of T2S:G_T, PulG from *Klebsiella oxytoca*, confirms the suspected structural parallels between T2S:G_T proteins and type IV pilins (Fig. 1) (Köhler *et al*., 2004). Overall, PulG and the type IV pilins display a similar structure made up of an N-terminal hydrophobic α-helix followed by four β-strands arranged in a globular domain. PulG lacks, however, a highly variable loop region containing a disulphide bond found in all type IV pilins. Electron microscopy and modeling predict that PulG most likely assembles into a left-handed helical pilus with the α-helix of each subunit packing into the core of the pilus structure (Fig. 3). This helix in the type IV pilin monomer is proposed to be critically involved in subunit–subunit interactions that facilitate pilus formation. When the pilus is not assembled, the same region of the protein would anchor the pilin subunits in the membrane.

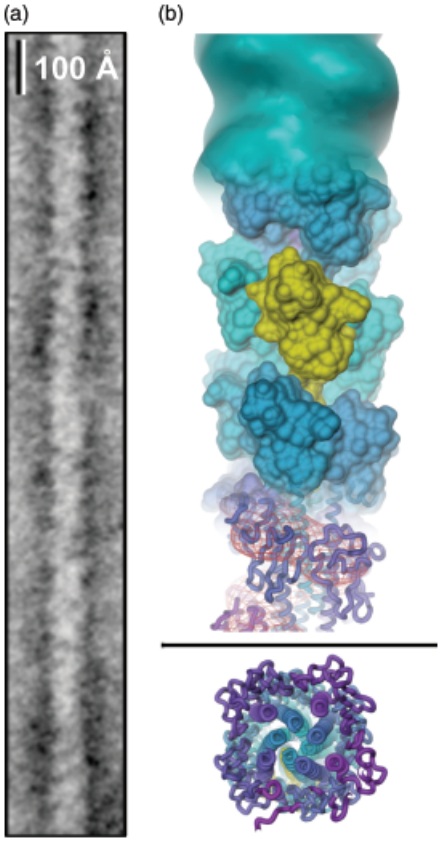

Fig. 3. The type II secretion (T2S) pseudopilus. (a) Computationally straightened scanning transmission electron microscope image of the pseudopilus obtained following overexpression of the *Klebsiella oxytoca* T2S:G_T homolog, PulG. (b) Left-handed helical model of the PulG pseudopilus derived from (a) and the X-ray crystal structure in Fig. 1 showing the arrangement of PulG monomers in the pseudopilus structure. A view up the axis of the pseudopilus is displayed at the bottom. Reprinted with slight modification with permission from Köhler *et al.* (2004).

Pseudopilins and type IV pilins have similar overall structures and share some sequence similarity; however, there are key differences between the two groups of proteins. PulG appears to be more compactly folded than type IV pilin proteins (Köhler *et al.*, 2004). Additionally, it is proposed that at least two of the type IV pilus proteins pack into right-handed helices (Parge *et al.*, 1995; Craig *et al.*, 2003), rather than the left-handed helix observed for PulG by electron microscopy (Köhler *et al.*, 2004). As further demonstration of the differences between these two groups of proteins, Köhler *et al.* (2004) showed using chimeric proteins generated between PulG and the type IV pilins PilE or PilA that the type IV pilins can be assembled into pili by the T2S system only when fused to the first 17 or 21 amino acids of the N-terminal helical domain of PulG. Even then, efficiency of the assembly was reduced. Therefore, despite their N-terminal sequence and structural similarity, this region of the pseudopilins and type IV pilins appears to not be functionally interchangeable.

Although interactions between the pseudopilins have been demonstrated, the function of the minor pilins T2S:H_U, I_V, J_W and K_X is not known (Nunn & Lory, 1993; Hu *et al.*, 2002; Kuo *et al.*, 2005). While all of them are essential for T2S mediated transport of proteins across the outer membrane, only T2S:I_V appears to be required for the formation of the surface exposed pilus when the major pseudopilin T2S:G_T is overexpressed (Sauvonnet *et al.*, 2000). T2S:I_V may be necessary for initiation of T2S:G_T assembly or it may perhaps anchor the T2S:G_T pilus to the cell envelope, as no pilus-like structure is visualized on the cell surface in its absence (Sauvonnet *et al.*, 2000; Vignon *et al.*, 2003; Durand *et al.*, 2005). T2S:K_X, on the other hand, may possibly terminate pilus assembly as overexpression of PulK from *K. oxytoca* has been shown to abolish the formation of the PulG pseudopilus (Vignon *et al.*, 2003), and overproduction of the *P. aeruginosa* protein XcpX interfered with the assembly of XcpT, the T2S:G_T homolog, into a pseudopilus (Durand *et al.*, 2005). Conversely, the absence of XcpX resulted in a dramatic increase in the length and number of pseudopili observed. These findings collectively suggest that T2S:K_X controls the length and possibly also the number of assembled pili. T2S:G_T and T2S:K_X likely interact directly, as XcpX can be cross-linked with XcpT and also appears to render XcpT more susceptible to proteolytic degradation when the two proteins are incubated together *in vitro* (Durand *et al.*, 2005). As T2S:K_X is an atypical pseudopilin in that it is longer than T2S:G_T, H_U, I_V or J_W, its incorporation into the growing filament could terminate pseudopilus elongation by disrupting packing of the helix or perhaps by interfering with recognition of next subunit extending the fiber (Parge *et al.*, 1995). Given that XcpX also appears to destabilize XcpT *in vitro*, it is also possible that the presence of XcpX acts as a regulator or signal for disassembly of the pseudopilus subunits (Durand *et al.*, 2005). It is likely that regulation of pilus assembly termination or disassembly is kinetically and stoichiometrically well controlled and may be, in part, governed by the affinity of T2S:K_X for T2S:G_T. Interestingly, T2S:K_X differs from the other pseudopilins and pilins in that it lacks the highly conserved glutamate residue found at position +5; a residue that has been proposed to aid in the helical assembly of type IV pilins, as mutant pilins with E+5 substitutions are incapable of self-assembly (Pasloske *et al.*, 1989; Strom & Lory, 1991; Parge *et al.*, 1995; Craig *et al.*, 2003). The lack of E+5 in T2S:K_X may reduce the affinity for T2S:G_T to prevent the premature termination of pilus assembly. In agreement with this suggestion, the T+5E substitution in XcpX displayed a stronger negative effect on XcpT assembly, as its overexpression completely prevented the formation of surface exposed XcpT pili (Durand *et al.*, 2005). In contrast,

a similar mutation in the homologous PulK had no deleterious consequence on secretion or pilus assembly (Vignon *et al.*, 2003).

Reports that connect the pseudopilins to other components of the T2S system are fewer and may be due to their limited, and perhaps transient, interactions. Nevertheless, interactions between the major pseudopilin XpsG and the outer membrane protein XpsD and the T2S:C_P-like XpsN in *Xanthomonas campestris* have been revealed by chemical cross-linking (Lee *et al.*, 2005). Furthermore, the minor T2S:J_W pseudopilin OutJ from *Erwinia chrysanthemi* was recently reported to interact with subdomains of the outer membrane protein OutD, the inner membrane protein OutL and the pilin-like protein OutI in a yeast-two hybrid system (Douet *et al.*, 2004). These interactions await confirmation in *E. chrysanthemi*.

The outer membrane complex

T2S:C_P interacts with T2S:L_Y and T2S:M_Z present in the inner membrane and T2S:D_Q in the outer membrane (Possot *et al.*, 1999; Sauvonnet *et al.*, 2000; Gerard-Vincent *et al.*, 2002; Robert *et al.*, 2002; Lee *et al.*, 2004). Specifically, a region of the periplasmic domain near the membrane-spanning helix has been shown to contribute to interactions with the T2S:L_Y–T2S:M_Z subcomplex (Gerard-Vincent *et al.*, 2002; Lee *et al.*, 2004), and the C-terminal region has been shown in many species to be important for interaction with T2S:D_Q (Bleves *et al.*, 1999; Possot *et al.*, 1999). These interactions allude to a role for T2S:C_P in linking the two membranes, where it may act as a energy transducer between the ATP-binding T2S:E_R in the cytoplasm and the secretion pore in the outer membrane. As mentioned earlier, cross-linking between the T2S:C_P-like protein, XpsN, in *Xanthomonas campestris* and pseudopilin protein XpsG has also recently been shown (Lee *et al.*, 2005), suggesting that T2S:C_P may play a direct role during pseudopilus assembly or activity as well.

Although T2S systems are generally well conserved, the components of the system have been found to be highly species-specific. T2S:C_P and T2S:D_Q, in particular, cannot be replaced by homologs even from closely related species (de Groot *et al.*, 1991; Lindeberg *et al.*, 1996). This suggests that these two proteins may be able to determine specificity for the substrates transported by the T2S system and/or perhaps for the assembly of the secretion complex. Domain swapping between closely and distantly related T2S:C_P, as well as T2S:D_Q, homologs have identified several regions that may contribute to the species-specificity. The results from the analysis of the T2S:C_P and T2S:D_Q chimeras are at times difficult to interpret, however, most likely because these components interact with more than one other protein. For instance, at the same time as T2S:C_P may interact with both T2S:L_Y and T2S:M_Z in the cytoplasmic membrane and T2S:D_Q in the outer membrane, there is also the possibility that it interacts with T2S:G_T and the proteins to be secreted. Sorting out the function of particular domains and establishing their contribution to species-specificity by swapping domains may therefore be challenging. For example, the PDZ, or coiled–coiled (CC region), motif in T2S:C_P has, on the one hand, been suggested to participate in protein-protein interactions to promote T2S:C_P homo-oligomerization (Gerard-Vincent *et al.*, 2002), and on the other, to directly participate in the recognition of secreted proteins and confer secretion specificity to a subgroup of secreted proteins (Bouley *et al.*, 2001). In the latter study, it was suggested that there are two sets of secreted proteins in *Erwinia* that are recognized and secreted by different mechanisms; one set is directly interacting with the PDZ domain of OutC and the other is not (Bouley *et al.*, 2001). It is also possible, however, that the PDZ domain participates indirectly by promoting oligomerization and presentation of a recognition motif located elsewhere in T2S:C_P. A subset of the secreted proteins may recognize this motif in the monomer, and as T2S:C_P protein oligomerizes, the affinity increases. For the weaker interacting proteins in the other subgroup of secreted proteins, oligomerization may be required for binding.

T2S:D_Q proteins are members of the secretin family of outer membrane proteins required for T2S, type IV pilus biogenesis, type III secretion, and filamentous phage extrusion. Several secretins including two T2S:D_Q family members, as well as pIV, which is required for f1 phage secretion in *Escherichia coli*, have been purified and well characterized. They form stable, heat- and detergent-resistant ring-like assemblies of 12–14 subunits with internal pores that range from 50 to 100 Å in diameter that are occluded with a centrally located plug (Bitter *et al.*, 1998; Nouwen *et al.*, 2000; Opalka *et al.*, 2003; Chami *et al.*, 2005). Precisely how opening and closing the pore is regulated and what components are involved is not known. It has been hypothesized, however, that the very C-terminal region of T2S:C_P may control the gating, as deletion or modification of this region results in increased levels of secreted proteins released to the extracellular environment (Robert *et al.*, 2005a). Additionally, the suggestion that docking of secreted proteins may displace the centrally located plug, thus creating an uninterrupted channel through which the proteins can be secreted, has also been discussed (Chami *et al.*, 2005).

Sequence comparisons between T2S:D_Q family members have shown that the N-terminal domain is not well conserved, while the C-terminal domain is highly conserved (Genin & Boucher, 1994; Peabody *et al.*, 2003). The conserved C-terminus is also protease-resistant and contains several putative amphipathic transmembrane β-strands (Bitter *et al.*, 1998). Deletion and limited proteolysis studies

as well as circular dichroism spectral analysis, nano-gold labeling and cryo-electron microscopy have strengthened the suggestion that the C-terminal and N-terminal regions are two structurally separate domains; the C-terminal domain is necessary for outer membrane insertion and multimerization, and the N-terminus may be free to interact with other T2S components and exoproteins in the periplasmic space (Chen *et al.*, 1996; Shevchik *et al.*, 1997; Bouley *et al.*, 2001; Chami *et al.*, 2005). 3D reconstruction of PulD revealed a three stacked ring structure with a cup and saucer appearance that is similar to the structure of pIV (Opalka *et al.*, 2003; Chami *et al.*, 2005), but for unknown reasons, quite different from the EM structure of PilQ, the T2S:D_Q-like secretin involved in type IV pilus biogenesis in *Neisseria meningitides* (Collins *et al.*, 2005). When modeled into an outer membrane environment, the PulD saucer is anchored in the outer membrane with some exposure to the cell surface, while the majority of the cup formed by the N-terminus is exposed to the periplasmic compartment. The chamber of the cup is likely the site of insertion of secreted proteins prior to their outer membrane translocation (Chami *et al.*, 2005).

Exactly how T2S:D_Q inserts into the outer membrane and assembles into a oligomeric ring is not known, however, the general outer membrane assembly factor Omp85 (YaeT) may be one of the factors that participates in these processes (Voulhoux *et al.*, 2003). In addition, specific T2S components such as the lipoprotein T2S:S and the ATPase T2S:A in complex with T2S:B play important roles in promoting outer membrane insertion and stabilization of the T2S:D_Q oligomer in some species (Shevchik *et al.*, 1997; Condemine & Shevchik, 2000; Schoenhofen *et al.*, 2005). To date, genes encoding these proteins have not been found in all T2S systems, however. This does not rule out the existence of other, non-homologous, proteins that could function in a similar capacity in systems where these proteins are not found.

It is assumed that T2S:D_Q acts as the export channel for T2S proteins, and although exoprotein secretion through this channel has not been directly visualized yet, several secreted proteins have been shown to interact with the T2S:D_Q homolog in *Erwinia chrysanthemi* (Shevchik *et al.*, 1997). Furthermore, accumulation of assembled type IV pili in the periplasm of *Neisseria gonorrhoeae* in the absence of PilQ suggests that PilQ forms the outer membrane conduit through which type IV pili are transported to the cell surface (Wolfgang *et al.*, 2000). Additional support for secretins acting as transport channels comes from studies showing that actively transported phage can block pIV-mediated uptake of oligosaccharides into *E. coli* cells (Marciano *et al.*, 2001).

The *N. meningitides* PilQ has recently been shown to interact directly with the type IV pilus (Collins *et al.*, 2005). Following separation by SDS-PAGE, purified type IV pili and full-length and truncated forms of PilQ proteins were subjected to far-Western analysis. Interaction of the type IV pilus protein with full-length, as well as with N- and C-terminal fragments of PilQ was observed. Transmission electron microscopy on preparations of purified pili incubated with PilQ oligomers revealed that the PilQ oligomer localized to only one end of the pilus, resulting in the formation of a 'mallet-type' structure (Fig. 4). A view of a section through the center of the pilin/secretin complex reveals that, in contrast to the previous structure of the PilQ oligomer alone, the interior of the PilQ/pilus complex is inaccessible to stains and therefore apparently filled; suggesting that the PilQ oligomer is capable of dynamic behavior previously predicted, but unobserved (Fig. 4).

Concluding remarks and future directions

The individual components that make up the T2S system are largely well defined. The challenge for future years is to reveal how these proteins come together to create a large, highly organized, multiprotein complex capable of secreting a diverse array of fully folded exoproteins across the outer membrane of gram-negative bacteria. Recent structural characterizations noted above are an important first step toward completing our picture of how the T2S system assembles and functions, however numerous interactions between T2S proteins still need to be uncovered to better understand these processes. Particularly exciting is the work showing the predicted, and unpredicted, similarities between T2S and other cellular systems. For instance, as EpsL and FtsA show unexpected structural similarities, FtsA's role in cell division will perhaps give us more clues to the function of EpsL and possibly other members of the T2S complex. The two different structures of the N-terminal portion of XpsE is another potentially very interesting finding that may suggest that T2S:E_R cycles between two functional forms with different ATPase activity.

Another multiprotein secretion system, the type III secretion needle complex, has been successfully extracted from bacterial membranes (Kubori *et al.*, 1998; Marlovits *et al.*, 2004); an important achievement that, if repeatable with the T2S system, would greatly add to our knowledge of T2S. Whether it is ultimately possible to purify the entire T2S complex or whether we will have to continue to express, purify and crystallize domains of the proteins and, like a puzzle, piece them together remains an open question. A promising avenue seems to be to separate the T2S system into larger sub-assemblies for the studies of inner membrane, pseudopilus and outer membrane subcomplexes. This has been initiated in recent years, but much remains to be done to obtain higher resolution and to discover how these subcomplexes interact with one another.

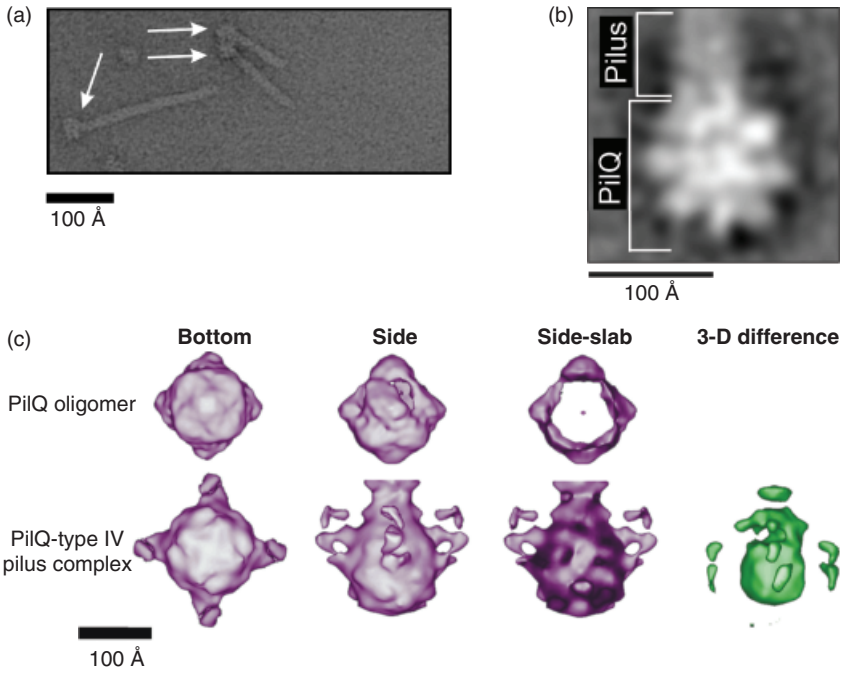

Fig. 4. The *Neisseria meningitides* PilQ-type IV pilus interaction. (a) Transmission electron microscope image showing PilQ and the type IV pilus interacting to form a complex with a 'mallet-like' structure indicated by arrows. (b) A 2-D projection map of the interaction obtained by averaging several hundred particles. The map shows the 140 Å wide and 150 Å high PilQ oligomeric structure at the end of the pilus. (c) Surface rendered bottom and side views, and a side view of a central section of the PilQ oligomer and the PilQ-type IV pilus complex are shown from left to right in purple. The 3-D difference map (green), calculated by subtracting the PilQ oligomer structure alone from that of the PilQ-type IV pilus complex, shows that the interior of the PilQ/pilus complex is filled. Reprinted with slight modification with permission from Collins *et al*. (2005).

Many additional challenges will have to be overcome to characterize this dual membrane complex, not the least of which is the possibility that the secretion apparatus is a dynamic structure constantly assembling and disassembling. The mechanism and energy requirement for the function of the putative periplasmic-spanning pilus-like piston in the extrusion of proteins through the outer membrane needs to be addressed. The type IV pilus system contains two ATPases with opposing functions, one for polymerization of pilin and the other for pilus retraction or disassembly, while the T2S system may only contain one ATPase. T2S:A proteins, such as ExeA, may fulfil the energy requirement for the retraction function, as ExeA has been shown to exhibit ATPase activity (Schoenhofen *et al*., 2005). T2S:A is not present in every T2S system, however. Filloux and colleagues have put forth another hypothesis that suggests that incorporation of the minor pseudopilin T2S:K_X could drive pilus retraction by inducing pilus disassembly (Durand *et al*., 2005). Incorporation of T2S:K_X into the growing pilus would not only bring the T2S:G_T polymerization to a halt, but also initiate pilus disassembly, because binding of T2S:K_X results in a conformational change in T2S:G_T that makes it more susceptible to proteolytic degradation (Durand *et al*., 2005). Understanding how proteins to be secreted are sorted from other resident periplasmic proteins and determining how the peptidoglycan layer is negotiated during assembly of the T2S machinery and/or during transport are also of great importance. Elucidation of the atomic resolution architecture and dynamics of the T2S system in a variety of functional states is an immense challenge, albeit important goal, which in the future may lead to the identification of novel ways to block secretion of virulence factors in a large number of pathogens.

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