The type II secretion system: biogenesis, molecular architecture and mechanism

Konstantin V. Korotkov[1,2], Maria Sandkvist[3] and Wim G. J. Hol[1]*

Abstract | Many Gram-negative bacteria use the sophisticated type II secretion system (T2SS) to translocate a wide range of proteins from the periplasm across the outer membrane. The inner-membrane platform of the T2SS is the nexus of the system and orchestrates the secretion process through its interactions with the periplasmic filamentous pseudopilus, the dodecameric outer-membrane complex and a cytoplasmic secretion ATPase. Here, recent structural and biochemical information is reviewed to describe our current knowledge of the biogenesis and architecture of the T2SS and its mechanism of action.

General secretory pathway
A traditional name for the type II secretion system (T2SS), in which substrates are transported through the inner membrane via the Sec or Tat pathways. Historically, the T2SS was found to rely on the Sec pathway, hence the use of 'general' in the name. However, many other systems also use the Sec pathway, so the term general secretory pathway is considered to be inaccurate by some scientists.

[1]Department of Biochemistry and Biomolecular Structure Center, University of Washington, Seattle, Washington 98195, USA.
[2]Department of Molecular & Cellular Biochemistry, University of Kentucky, Lexington, Kentucky 40536, USA.
[3]Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, Michigan 48109, USA.
**Present address.*
Correspondence to W.G.J.H.
e-mail: wghol@u.washington.edu
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The past few decades have seen the discovery of a wide range of systems that enable bacteria to export proteins across one or two membranes into the extracellular milieu[1]. Many Gram-negative bacteria use the type II secretion system (T2SS) to translocate folded proteins from the periplasm through the outer membrane and into the extracellular milieu. The T2SS is important for pathogenic and non-pathogenic species alike. Human pathogens with one or more T2SSs include *Vibrio cholerae*[2], enterotoxigenic and enterohaemorrhagic *Escherichia coli* (ETEC and EHEC, respectively)[3–5], *Pseudomonas aeruginosa*[6,7], *Klebsiella* spp.[8,9], *Legionella pneumophila*[10] and *Yersinia enterocolitica*[11]. *Aeromonas hydrophila*, a pathogen of fish and amphibia, also contains a T2SS[12]. Plant pathogens that contain a T2SS include *Dickeya dadantii* (formerly part of the *Erwinia chrysanthemi* complex; the causative agent of fire blight), *Erwinia carotovora* (which is responsible for soft rot disease in potato and other crops[13]) and *Xanthomonas campestris* (which causes black rot in crusifers), among others[14]. Non-pathogens with a T2SS gene cluster include metal-reducing bacteria such as *Shewanella oneidensis*[15]. It has also become apparent that the T2SS, the type IV pilus system (T4PS), archaeal flagella and the transformation system are evolutionarily related and share several structural and functional features[16–19] (BOX 1).

The T2SS is a sophisticated multiprotein machinery containing 12–15 different proteins (FIG. 1; TABLE 1) that are generally encoded in a single operon[20]. The T2SS is also the main terminal branch of the general secretory pathway[21]. Hence, a Gsp prefix followed by a capital letter has been proposed for the names of all T2SS proteins, but this nomenclature is not universally used. Here, we use a Gsp-based nomenclature with the species-and-system-specific name in superscript text (TABLE 1).

It has been suggested that the T2SS apparatus spans both the inner and outer membranes, although the fully assembled machinery has not yet been visualized in a purified form and perhaps may never be captured for analysis, as it is likely to be dynamic in nature. Four T2SS subassemblies can be distinguished: the pseudopilus, the outer-membrane complex, the inner-membrane platform and the secretion ATPase. The pseudopilus is a fibrous periplasmic structure formed by five different pseudopilins, with multiple copies of the major pseudopilin[22–24]. These proteins received their name owing to their amino-terminal sequence homology to type IV pilins and their dependence on prepilin peptidase. The outer-membrane complex is formed mainly by a multimeric protein known as the T2SS secretin[25–27]. The inner-membrane platform contains multiple copies of at least four core membrane proteins[28,29], and although the secretion ATPase in the cytoplasm is tightly associated with the inner-membrane complex, it is considered as a separate subassembly here. The inner-membrane platform has a central role in the mechanism of action of T2SS, as it communicates with all other elements of the system (it is responsible for contacting the outer-membrane complex in the periplasm, the ATPase in the cytoplasm and the major pseudopilin). The inner-membrane complex might have a key role in converting conformational changes in the ATPase (caused by ATP hydrolysis) into an extension of the pseudopilus, which possibly acts as a piston that pushes exoproteins through the outer-membrane channel.

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In this Review we focus on the biogenesis of the T2SS subassemblies and the entire machinery, the architecture of the fully assembled system and the mechanism of action for this system. We try to integrate the large number of currently known three-dimensional structures (see Supplementary information S1 (table)) with recent biochemical studies to illustrate our current understanding of the T2SS.

Biogenesis of the T2SS

During T2SS biogenesis, approximately 40–70 proteins of 12–15 different types have to come together to form the final machinery. Owing to this complexity, many questions regarding the biogenesis of the T2SS are still unanswered, but recent results make it possible to outline several of the steps involved (FIG. 1).

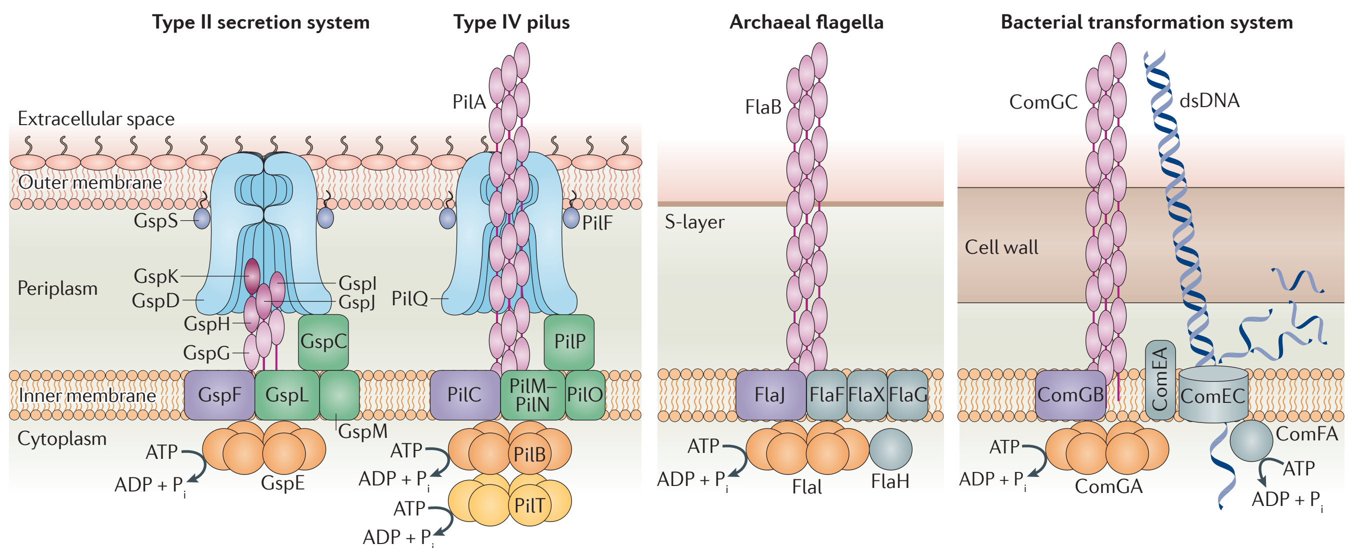
Pseudopilus biogenesis. Studies on *P. aeruginosa* GspG^{XcpT} and *Klebsiella oxytoca* GspG^{PulG} have shown that the major pseudopilin, GspG, uses the Sec translocon for insertion into the inner membrane[30,31]. Insertion occurs co-translationally through the signal recognition particle (SRP) pathway, which recognizes the N terminus of GspG and targets it to the Sec translocon[30,31]. On the basis of sequence similarity in the N-terminal sequences of all five pseudopilins, the insertion of the minor pseudopilins (GspH, GspI, GspJ, and GspK) also probably involves the SRP pathway.

The N-terminal region of amino acid sequence homology between pilins and pseudopilins includes a short positively charged sequence followed by a stretch of hydrophobic residues. After transport, the segment with the positively charged residues is cleaved by a specific aspartic protease called prepilin peptidase[6,32], a bifunctional enzyme that also methylates the N-terminal residue after proteolytic processing[33]. The gene for this peptidase can be either part of the T2SS operon or elsewhere in the genome. Interestingly, in some species the same prepilin peptidase is shared by the T2SS and the T4PS, as it processes both pilins and pseudopilins[34]. The prepilin peptidase contains eight putative transmembrane helices[35], and the Asp residues that have been implicated in the proteolysis activity are located in the cytoplasmic loops, consistent with the location

Sec translocon
A universal pathway for transport of proteins through the cytoplasmic membrane in bacteria and archaea and the endoplasmic reticulum membrane in eukaryotes. The bacterial and archaeal Sec translocon is composed of SecYEG (the integral membrane channel), SecA (the peripheral membrane ATPase), and SecD and SecF (the auxiliary release proteins). Targeting to the Sec translocon relies on the signal recognition particle for co-translational transport or on the cytoplasmic chaperone SecB for post-translational transport.

Box 1 | **The T2SS, the T4PS, archaeal flagella and the transformation system of Gram-positive bacteria**

There are four bacterial and archaeal secretion systems — the type II secretion system (T2SS) of Gram-negative bacteria, the type IV pilus system (T4PS) of Gram-positive and Gram-negative bacteria[140], the archaeal flagellum synthesis system and the transformation system of Gram-positive bacteria — which between them contain various related proteins (see the figure; the ATPase in each system is in orange, the inner-membrane protein with multiple transmembrane helices is in purple, the inner-membrane proteins with a single transmembrane helix are in green, the inner-membrane proteins with multiple transmembrane helices are in purple, the outer-membrane proteins are in blue, and the pilins, pseudopilins and flagellins are in pink; unrelated system-specific proteins are shown in grey). The pseudopilins, pilins and flagellins in all four systems are processed by a related inner-membrane peptidase (not shown). For T2SS protein names, the general secretory pathway (Gsp) nomenclature is used, followed by a capital letter. The similarities between the T2SS and the T4PS have been recognized for many years. Indeed, the systems have similar outer-membrane conduits (secretins), filamentous structures (the pseudopilus and pilus, respectively), prepilin peptidases and cytoplasmic ATPases, and also have homologous membrane proteins that span the membrane multiple times (GspF and PilC, respectively). The similarities between other proteins of the inner-membrane platform became apparent only when crystal structures of several domains were solved[113,118,141]. Moreover, it has been shown that T4PSs in some cases function as secretion systems. Interestingly, the archaeal flagellum synthesis system has more similarities with the T4PS and T2SS than with the bacterial flagellum synthesis system[18]. Archaeal flagellins are processed by a preflagellin peptidase, an aspartic membrane protease that belongs to the same class as the prepilin peptidase. After cleavage by the preflagellin peptidase, the flagellin subunits (FlaB) are assembled into the flagellum. Almost all archaea lack an outer membrane, so a secretin is lacking in this system. Similarly, the transformation system that occurs in many Gram-positive bacteria has no secretin. The cytoplasmic ATPase, the inner-membrane protein with multiple transmembrane helices, the pilin or pseudopilin or flagellin, and the specific membrane protease (not shown) are common components of these four systems. P_i, inorganic phosphate.



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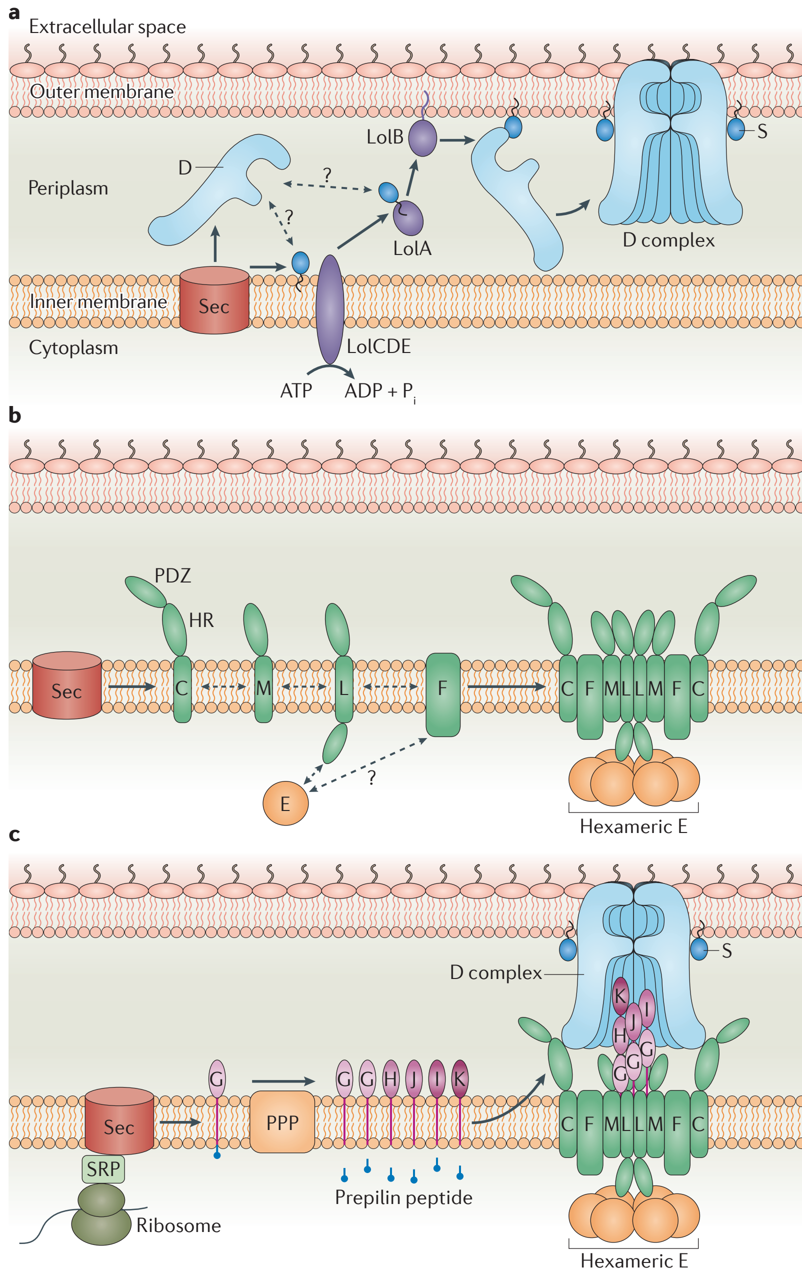

Figure 1 | **T2SS subassemblies and biogenesis.** Schematics of the type II secretion system (T2SS) subassemblies and their biogenesis, with hypotheses shown for several unknown steps. For T2SS proteins, only the generic capital letter using the general secretory pathway (Gsp) nomenclature is shown (the Gsp prefix is omitted, and species-specific names are not shown). Proteins of the outer-membrane complex are shown in blue, pseudopilins are shown in pink, proteins of the inner-membrane platform are in green and the secretion ATPase is in orange. The order of assembly for T2SS components is still largely unknown. For instance, although the figure suggests that pseudopilins are added to the inner membrane after the inner-membrane complex and secretion ATPase are assembled, it is possible that one or more pseudopilins are co-assembled with the inner-membrane complex. **a** | The outer-membrane complex and its biogenesis. The pilotin, GspS, is a lipoprotein; the secretin, GspD, is a protein dodecamer (according to electron microscopy studies). The pilotin is transported to the outer membrane by the Lol sorting pathway, which consists of an ABC (ATP-binding cassette) transporter, LolCDE, a periplasmic shuttle chaperone, LolA, and an outer-membrane chaperone, LolB[159]. **b** | The inner-membrane platform and its biogenesis. The inner-membrane platform is shown in complex with the secretion ATPase[69,72,107,113,160]. Although it has not yet been directly demonstrated, the Sec translocon or YidC insertase are likely to be involved in the biogenesis of the inner-membrane platform. It has also been suggested that the secretin is involved, but this still needs to be established. **c** | Pseudopilin processing and the almost-assembled T2SS. Shown is an assembly with the tip of the pseudopilus added to the inner- and outer-membrane complexes, although it is not known at which stage pseudopilins are actually added to the system. Here, it is also assumed that the presumed tip of the pseudopilus[39,41], consisting of GspKIJ and possibly also GspH, is present when the complex is in a state that permits the addition of more GspG subunits during exoprotein secretion (see FIG. 5). In some species an additional T2SS protein, GspN, is present; GspN has a predicted amino-terminal transmembrane helix, the function and location of which has not yet been determined[161,162]. HR, homology region; P_i, inorganic phosphate; PPP, prepilin peptidase; SRP, signal recognition particle.

of the cleavage site in pseudopilins after transport[30,31]. The crystal structure of the homologous *Methanococcus maripaludis* preflagellin peptidase, FlaK, has been solved recently[36], but details of the catalytic mechanism for these peptidases are still not completely elucidated, especially for the bifunctional T2SS–T4PS prepilin peptidases. There are currently no data available about whether the prepilin peptidase is an integral part of the T2SS machinery.

After cleavage by the prepilin peptidase, the pseudopilins may reside transiently in the inner membrane with their hydrophilic domains facing the periplasmic compartment[30,31]. Apart from GspK, which was not initially recognized as a pseudopilin[37], most pseudopilins and T4PS pilins have a Glu5 residue that is thought to be involved in interactions with the N-terminal amino group of a neighbouring subunit[38–40]. The replacement of this residue in GspK by an aliphatic or Thr side chain in all species is probably related to assembly or packing requirements, but this remains to be formally established. GspK assembles into a trimer with the pseudopilins GspI and GspJ, and this trimer is likely to be the tip of the pseudopilus[39,41]. When and how GspH[41] and the multiple copies of GspG are added to the putative pseudopilus tip also remains an open question.

Signal recognition particle (SRP). A universally conserved protein–RNA complex that is involved in targeting secreted proteins to the Sec translocon for co-translational transport. The bacterial and archaeal SRP is formed by the protein Ffh and 4.5S RNA.

Biogenesis of the outer-membrane complex. The major T2SS outer-membrane protein, GspD (often referred to as secretin), belongs to a family of multimeric channels that also include secretins from the T3SS, the T4PS and the filamentous-phage assembly system[27,42–46]. The T2SS secretin carries an N-terminal signal sequence that

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Table 1 | **Composition and species-specific nomenclature* of the T2SS**

Protein function	**ETEC**	***Vibrio cholerae***	***Aeromonas hydrophila***	***Dickeya dadantii*** [‡]	***Klebsiella oxytoca***	***Pseudomonas aeruginosa*** [§]	***Xanthomonas campestris***
Peptidoglycan binding		EpsA	ExeA				
Unknown function		EpsB	ExeB	OutB	PulB		
Inner-membrane platform protein, interaction with secretin	GspC	EpsC	ExeC	OutC	PulC	XcpP	XpsC[‖]
Outer-membrane secretin	GspD	EpsD	ExeD	OutD	PulD	XcpQ	XpsD
Secretion ATPase	GspE	EpsE	ExeE	OutE	PulE	XcpR	XpsE
Inner-membrane platform protein	GspF	EpsF	ExeF	OutF	PulF	XcpS	XpsF
Major pseudopilin	GspG	EpsG	ExeG	OutG	PulG	XcpT	XpsG
Minor pseudopilin	GspH	EpsH	ExeH	OutH	PulH	XcpU	XpsH
Minor pseudopilin	GspI	EpsI	ExeI	OutI	PulI	XcpV	XpsI
Minor pseudopilin	GspJ	EpsJ	ExeJ	OutJ	PulJ	XcpW	XpsJ
Minor pseudopilin	GspK	EpsK	ExeK	OutK	PulK	XcpX	XpsK
Inner-membrane platform protein	GspL	EpsL	ExeL	OutL	PulL	XcpY	XpsL
Inner-membrane platform protein	GspM	EpsM	ExeM	OutM	PulM	XcpZ	XpsM
Unknown function		EpsN	ExeN		PulN		
Prepilin peptidase	GspO	VcpD	TapD	OutO	PulO	XcpA (also known as PilD)	XpsO
Pilotin	YghG[¶]			OutS	PulS		

ETEC, enterotoxigenic *Escherichia coli*. *The general secretory pathway (Gsp) prefix has been suggested for use in all type II secretion systems (T2SSs), but this nomenclature is not universally used. To denote all homologues with the same name but maintain a link with species-specific names used in the literature, a Gsp-based nomenclature is used throughout this Review, with the species-and-system-specific name in superscript. This table gives the species-and-system-specific names only. ‡Formerly part of the *Erwinia chrysanthemi* complex. *D. dadantii* also contains a second T2SS cluster, Stt[165]. §*P. aeruginosa* also contains a second T2SS cluster, and proteins from this cluster have a designated Hxc prefix and capital letters corresponding to the Xcp cluster (for example, HxcQ for the secretin)[55]. ‖Initially named XpsN and later renamed XpsC. ¶A small lipoprotein is located in the T2SS cluster of ETEC and several other *E. coli* strains; however, it is not homologous to other T2SS pilotins. The pilotin function of this protein has not yet been experimentally confirmed.

targets this protein to the periplasm via the Sec pathway. Many bacterial outer-membrane proteins rely on the β-barrel assembly machinery (BAM) complex for efficient insertion into the outer membrane[47,48]. However, the multimerization and outer-membrane insertion of the *K. oxytoca* T2SS secretin, $GspD^{PulD}$, does not seem to depend on the BAM complex[49], although it should be noted that dependence on BAM proteins has been shown for the homologous *Neisseria meningitidis* T4PS secretin, PilQ[47]. In some cases the T2SS secretin relies on a small lipoprotein known as GspS (also commonly referred to as the T2SS pilotin) for targeting to the outer membrane[50,51]. The pilotin interacts with the carboxy-terminal S-domain of the secretin[50,52]. Recent studies on *K. oxytoca* show that the S-domain of $GspD^{PulD}$ is disordered but adopts a folded structure on binding to its pilotin, $GspS^{PulS}$ (REF. 53). In order to reach the outer membrane, the pilotin itself uses the Lol pathway[54].

Intriguingly, some species do not have a gene encoding a T2SS pilotin homologue. In a few of these cases, the secretin itself is a lipoprotein and therefore does not depend on a pilotin for correct outer-membrane targeting. For instance, a recently identified secretin, $GspD^{HxcQ}$, from the second *P. aeruginosa* T2SS, has a long N-terminal extension and a lipidated N-terminal Cys. The extension presumably connects the lipid group in the outer membrane to the N0 domain (which is usually the N-terminal domain of the T2SS secretin) in the periplasm[55]. However, the importance of secretin lipidation varies among species. On the one hand, a non-lipidated mutant form of $GspD^{HxcQ}$ is not functional; on the other hand, lipid modification is not necessary for the function of *X. campestris* $GspD^{XpsD}$ (REF. 56). In other cases it is not immediately clear how the secretin is targeted to the outer membrane in the absence of the pilotin and/or direct secretin lipidation. These bacteria may carry genes outside of the *gsp* operon that encode functional homologues of the pilotin which are not related in sequence to the known homologues; alternatively, the secretin in these species may use a different mechanism for assembly and targeting. In fact, other proteins in addition to the pilotin have been implicated in assembly of the secretin multimer in the outer membrane. For example, the peptidoglycan-binding complex ExeAB of *A. hydrophila* supports $GspD^{ExeD}$ oligomerization, but the requirement for this complex can be bypassed through overexpression of $GspD^{ExeD}$ (REFS 57–59). Moreover, a soluble periplasmic protein has recently been identified that is important for targeting the *P. aeruginosa* secretin $GspD^{XcpQ}$ to the outer membrane[60].

β-barrel assembly machinery
A machinery for the correct folding and insertion of outer-membrane proteins which have a β-barrel structure.

Lol pathway
A machinery for the transport of outer-membrane lipoproteins from the inner to the outer membrane. The Lol pathway consists of LolCDE (the ABC (ATP-binding cassette) transporter), LolA (the periplasmic chaperone) and LolB (the outer-membrane receptor and release assistant).

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Biogenesis of the secretion ATPase and the inner-membrane platform. The cytoplasmic T2SS ATPase, GspE, belongs to the type II/IV secretion ATPase family[61]. GspE is a Zn-containing protein, unlike most other members of this ATPase family[62–64]. Retraction ATPases from the T4SS and T4aPS (a subclass of the T4PS) readily form hexamers[65,66]. By contrast, the evidence for the hexameric nature of GspE or the T4aPS assembly ATPase has so far been mainly indirect[67,68]. Whether inner-membrane components or other factors are needed for proper GspE assembly remains to be determined, although it has been established that the association of GspE with the cytoplasmic membrane requires the inner-membrane protein GspL[69].

The inner-membrane platform proteins GspC, GspL and GspM span the membrane once, whereas GspF has three transmembrane helices[29]. Each protein probably uses the Sec machinery for membrane insertion. Several studies have shown that GspC, GspL and GspM protect each other from proteolysis through protein–protein interactions[70–73]. Whether the different proteins assemble spontaneously into the inner-membrane platform or instead require interactions with the pseudopilus and/or the ATPase remains to be established. It has been suggested that the outer-membrane complex supports assembly of the inner-membrane platform, as GspC–GFP and GspM–GFP form fluorescent foci in the *V. cholerae* cell envelope in the presence but not in the absence of the T2SS secretin[73].

Combining subassemblies. Limited information is available regarding how and when the subassemblies interact with each other. The cytoplasmic secretion ATPase might be permanently associated with the inner-membrane platform[69]; however, exactly when the various pseudopilins interact with the platform remains a mystery, as does the number of pseudopilins associated with the inner-membrane complex before an exoprotein is encountered. An interaction between the major pseudopilin, $GspG^{EpsG}$, and the inner-membrane protein $GspL^{EpsL}$ of *V. cholerae* has been reported and might have a key role in biogenesis of the pseudopilus[74]. The outer-membrane complex contacts the inner-membrane platform through GspC[51,75–78], and, as mentioned above, the outer-membrane complex may assist assembly of the inner-membrane platform[73]. This leads to a possible biogenesis pathway for the T2SS machinery. First, the pilotin and the Lol system enable the secretin multimer to form in the outer membrane (FIG. 1a), and processed pseudopilins accumulate in the inner membrane with their hydrophilic domains facing the periplasm. Then, the secretin assists the formation of the inner-membrane platform[73] and associated ATPase (FIG. 1b), and finally the inner-membrane platform assembles the minor pseudopilins into the tip of the pseudopilus (FIG. 1c). At some point, the pseudopilus is extended by the addition of several major pseudopilin subunits to the tip — specifically, by GspL, with the help of ATP hydrolysis by the secretion ATPase. The addition of GspH and multiple GspG subunits to an initial GspKIJ or GspKIJH tip might occur after an exoprotein interacts with the outer-membrane complex.

Architecture of the T2SS subassemblies

The pseudopilus. The three-dimensional structures of all five pseudopilins exhibit a remarkable diversity in their globular domains, but these domains nonetheless have several common features: a long N-terminal α-helix followed by a variable region and a quasi-conserved β-sheet (FIG. 2a). The first half of the N-terminal α-helix is hydrophobic, although all pseudopilins except GspK contain a Glu5 residue. Three crystal structures of GspG homologues reveal a Ca^{2+}-binding site that seems to be present throughout the major pseudopilin family despite the differences in the coordinating Asp residues[79]. Substitution of the coordinating residues in the Ca^{2+}-binding site in *V. cholerae* $GspG^{EpsG}$ impairs the function of the T2SS[79], perhaps by affecting subunit contacts in the pseudopilus or interactions with other T2SS proteins. The minor pseudopilins GspH and GspJ have more extended variable regions, whereas GspI and GspK have a single β-strand in the variable region[39,80–82] (FIG. 2a). These differences might serve as determinants of the pseudopilin assembly order. Interestingly, GspK possesses a unique α-domain that is composed of two structurally similar units[39], contains an essential disulphide bond[83] and has a binuclear Ca^{2+}-binding site of unknown function formed by several conserved amino acid residues.

The crystal structure of the ETEC GspKIJ heterotrimer[39] revealed a right-handed pseudohelical subunit arrangement that had been previously inferred from the GspIJ heterodimer structure[81] (FIG. 2b). In the heterotrimer, the subunits form a triangular arrangement with GspK at the top, and GspI and GspJ at the bottom. The N-terminal α-helices of the three pseudopilins interact at the centre of the trimer, resembling the interactions in models of the T2SS pseudopilus and the type IV pilus (see below). GspK is probably the tip of the pseudopilus, as its large globular domain extends above the smaller globular domains of GspI and GspJ[39] (FIG. 2b). This is consistent with a role of GspK in preventing pseudopilus extension beyond the cell envelope. Indeed, *P. aeruginosa* $GspK^{XcpX}$ deletion mutants display long surface-exposed fibres, whereas strains with elevated levels of $GspK^{XcpX}$ display shorter fibres when $GspG^{XcpT}$ is artificially overproduced[84,85]. *In vitro* studies of *P. aeruginosa* pseudopilins have shown that $GspH^{XcpU}$ interacts with the $GspK^{XcpX}$–$GspI^{XcpV}$–$GspJ^{XcpW}$ trimer[41]. Therefore, it has been suggested that GspI acts as an initiator of pseudopilus assembly by binding GspJ and GspK, followed by GspH recruitment[41]. This suggestion is in agreement with the finding that when $GspG^{PulG}$ is overproduced in a *K. oxytoca* mutant lacking the gene encoding $GspI^{PulI}$, the production of surface-exposed pseudopili is severely hampered[22]. Furthermore, it has recently been shown that *K. oxytoca* $GspI^{PulI}$–$GspJ^{PulJ}$ self-assembles in the membrane, recruits $GspK^{PulK}$ and initiates pseudopilus assembly[86].

The GspKIJ crystal structure and the electron microscopy-based pilus model for the T4aPS pilin PilE have several common features, including the right-handed nature of the fibre with the central arrangement of the N-terminal helices, an ~10 Å shift between subunits along the fibre axis and an ~100° rotation about

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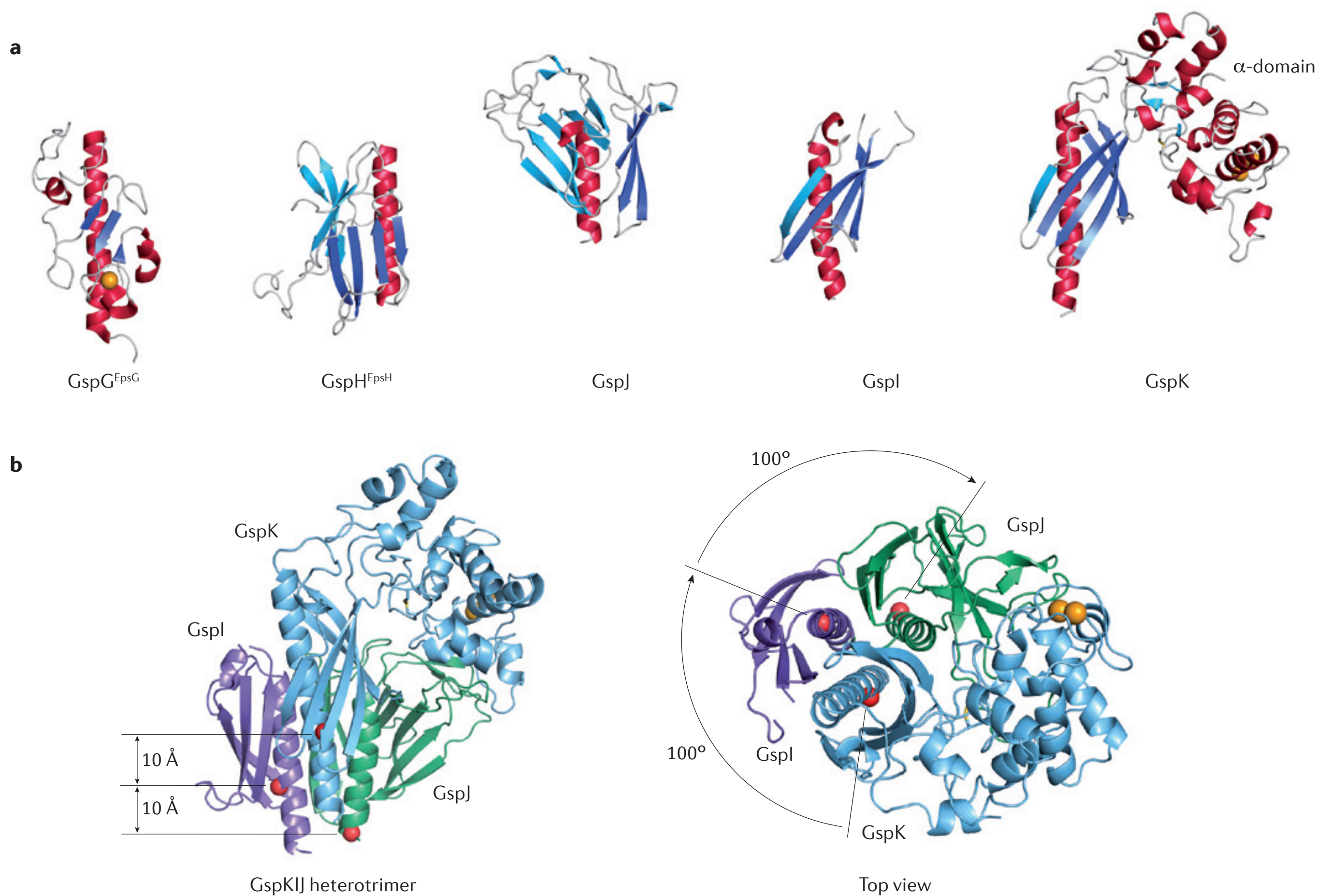

Figure 2 | **Structures of the T2SS pseudopilins.** For protein names, the general secretory pathway (Gsp) nomenclature is used, followed by a capital letter. **a** | Ribbon diagrams of type II secretion system (T2SS) pseudopilin monomers: *Vibrio cholerae* GspG^{EpsG} (Protein Data Bank (PDB) accession 3FU1)[79], *V. cholerae* GspH^{EpsH} (PDB accession 2QV8)[80], and enterotoxigenic *Escherichia coli* (ETEC) GspJ, GspI and GspK (PDB accession 3CI0)[39]. The amino-terminal α-helix is red, the conserved β-sheet is dark blue and the variable region is cyan; the Ca^{2+} ions in GspG and GspK are shown as orange spheres. These crystal structures did not include the ~20 N-terminal residues that form a hydrophobic α-helix. All structures are shown in the same orientation, after the N-terminal α-helices were superimposed. The structures of *Klebsiella oxytoca* GspG^{PulG} (REF. 87), *Vibrio vulnificus* GspI^{EpsI}–GspJ^{EpsJ} (REFS 81,163), and *Pseudomonas aeruginosa* GspG^{XcpT} (REF. 164) and GspJ^{XcpW} (REF. 82) are also available. **b** | The quasihelical ETEC GspKIJ heterotrimer structure (PDB accession 3CI0)[39]. Cα atoms of equivalent residues (Ala40 of GspK^{EpsK}, Ala40 of GspI^{EpsI} and Lys40 of GspJ^{EpsJ}) are shown as red spheres. The vertical distance between those atoms is ~10 Å. The top view along the pseudohelical axis (right) shows the right-handed arrangement of GspK, GspI and GspJ subunits, with a helical rotation angle of ~100°. Part **b** image is modified, with permission, from REF. 39 © (2008) Macmillan Publishers Ltd. All rights reserved.

the fibre axis[38,39]. Therefore, it has been suggested that the T2SS pseudopilus assembles with the same helical parameters as the type IVa pilus[79,80]. By contrast, a second T2SS pseudopilus model with different helical parameters has been suggested on the basis of earlier electron microscopy data from fibres obtained by GspG^{PulG} overproduction and a novel molecular modelling procedure[40,87,88]. In this model, the pseudopilin subunits are arranged in a right-handed fibre with a 10 Å helical rise, similar to the type IVa pilus model, but with a different subunit-to-subunit helical turn of 84.5° (REFS 40,88). The model has been tested using double-Cys substitutions in the hydrophobic region of the N-terminal helix followed by crosslinking and charge-altering substitutions in putative salt bridges[40]. However, the substitutions in the hydrophobic region of the N-terminal helix lie close to the centre of the fibre and might be insensitive indicators of the helical-turn angle. Higher-resolution electron microscopy data or a crystal structure of a complex of several consecutive pseudopilin subunits might establish the T2SS pseudopilus structure. Alternatively, the pseudopilus could adopt several conformations depending on the functional state, similar to type IV pili and type I fimbriae[89–91].

The outer-membrane complex. The major T2SS outer-membrane protein, the secretin (GspD), forms dodecamers[25,26]. Individual T2SS secretin subunits have a modular multidomain nature with a conserved C-terminal domain and four N-terminal domains[27].

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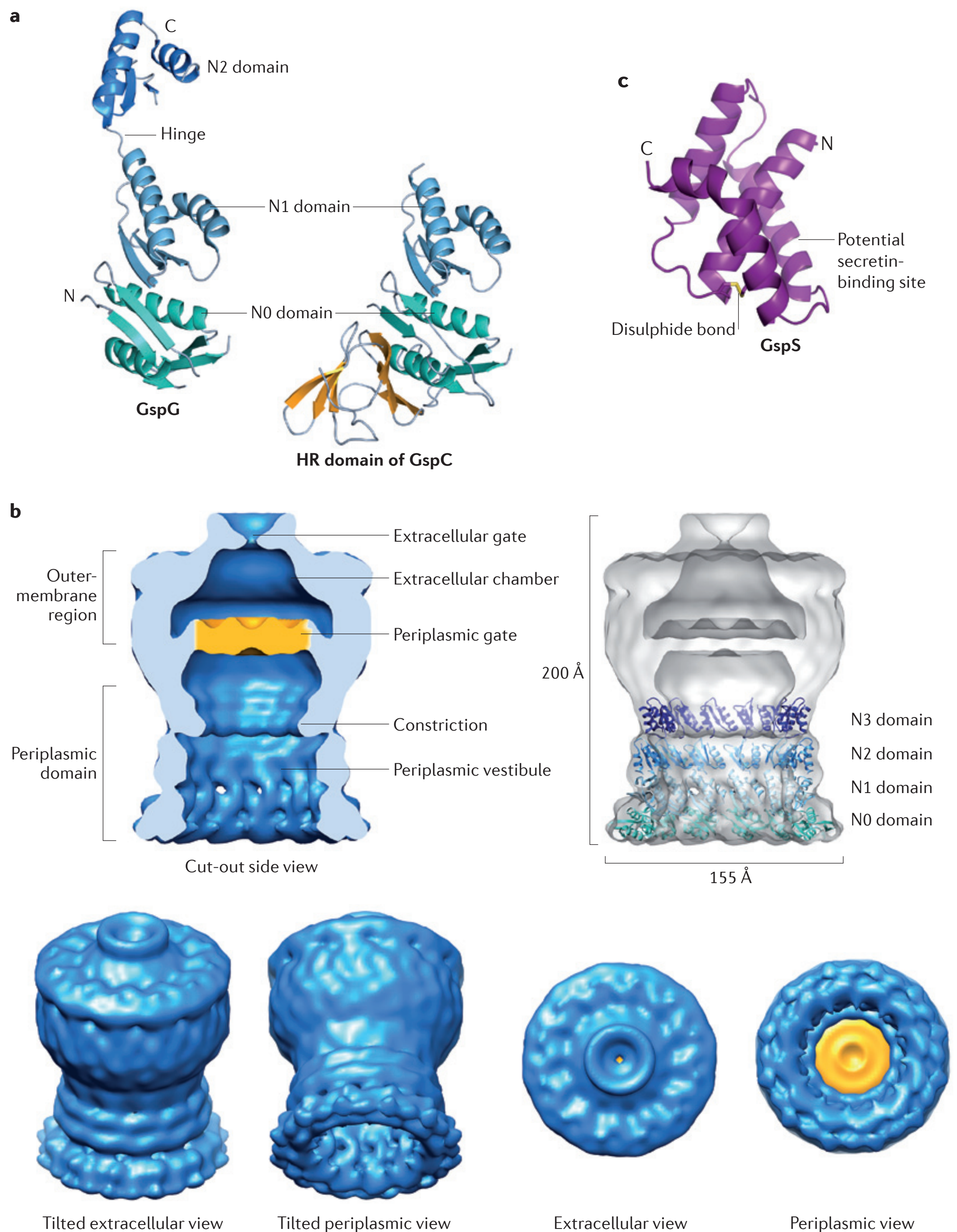

Figure 3 | **Structures of the T2SS secretin and pilotin.** For protein names, the general secretory pathway (Gsp) nomenclature is used, followed by a capital letter. **a** | The left panel shows the crystal structure of the first three amino-terminal domains of the enterotoxigenic *Escherichia coli* (ETEC) type II secretion system (T2SS) secretin, GspD (Protein Data Bank (PDB) accession 3EZJ)[92]. The N2 domain connects to the lobe formed by the N0 and N1 domains via a potentially flexible linker; the N0–N1 linker is also flexible. The right panel shows the structure of the ETEC T2SS secretin N0–N1 domains in complex with the homology region (HR) domain of GspC, a component of the T2SS inner-membrane platform (PDB accession 3OSS)[78]. **b** | The cryo-electron microscopy structure of the *Vibrio cholerae* T2SS secretin (Electron Microscopy Data Bank accession 1763)[26]. The cut-out side view in the top left panel reveals the periplasmic vestibule, a constriction, the periplasmic gate, an extracellular chamber and an extracellular gate. The top right panel shows how ring models of the secretin N-terminal domains fit into this structure. **c** | The crystal structure of the enterohaemorrhagic *E. coli* (EHEC) T2SS pilotin, GspS (PDB accession 3SOL). The location of a potential secretin-binding site, as suggested by extra electron density in the crystal structure, and of a disulphide bond are indicated. The structure of *Klebsiella oxytoca* GspS^{PulS} is also available[99]. C, carboxyl terminus. Part **b** image is modified, with permission, from REF. 26 © (2010) Macmillan Publishers Ltd. All rights reserved.

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The structure of an ETEC secretin fragment, which includes the N-terminal N0, N1 and N2 domains, has been solved using nanobodies as crystallization chaperones[92] (FIG. 3a). The structure revealed two lobes, one composed of the N0–N1 domains and the other of the N2 domain, connected by a potentially flexible linker. The N0 domain adopts a fold that is also seen in the signalling domain of the TonB-dependent receptor[93], the *L. pneumophila* T4SS protein DotD[94], the *E. coli* T4SS protein VgrG[95] and the bacteriophage T4 protein gp27 (REF. 96). The N1 and N2 domains share the heterogeneous nuclear ribonucleoprotein K homology fold[97], which is also adopted by the N3 domain (according to sequence similarities). Although a high-resolution structure of the T2SS secretin C-terminal domain is still lacking, it is thought to form a mostly β-strand assembly in the outer membrane[25,98].

The structure of the *V. cholerae* T2SS secretin, $GspD^{EpsD}$, was determined at 19 Å resolution using cryo-electron microscopy[26] (FIG. 3b). The cylindrical structure with 12-fold-symmetrical features is shaped like an inverted cup with a diameter of 155 Å and length of 200 Å. The surface of the periplasmic domain is segmented, with three distinguishable concentric rings, whereas the surface of the outer-membrane domain seems to be relatively smooth. The cross-section of the secretin channel reveals a large periplasmic vestibule and a smaller extracellular chamber that are separated by a continuous periplasmic gate. The periplasmic vestibule has a wide opening of 70 Å that narrows down to a 55 Å constriction approximately two-thirds of the way into the channel. By contrast, the extracellular chamber is closed from the top by an extracellular gate that has a small opening of 10 Å.

The N0–N1–N2 crystal structures could be mapped into the density of the wall of the periplasmic chamber[26] (FIG. 3b). A dodecameric ring of the N0–N1 domains fits into the wider bottom part of the wall, whereas rings of the N2 and N3 domains can be accommodated above. According to this model, the N3 domain ring occupies the constriction site of the wall and may have a role in initiating conformational changes during protein secretion. Despite having differences in sequence, symmetry and biological function, secretins from other transport systems also share some of these features, including the periplasmic gate formed by a part of the secretin domain, and a constriction site possibly formed by the N3 domain[27].

The T2SS pilotin is essential for secretin biogenesis in some species. The crystal structure of the putative EHEC T2SS pilotin, GspS, reveals an arrangement of four α-helices, with helix 4 bending around helix 1, and with a stabilizing disulphide bond (FIG. 3c), as has been observed in *K. oxytoca* $GspS^{PulS}$ (REF. 99). Despite its stabilizing function, the intramolecular disulphide bond seems to be dispensable for $GspS^{PulS}$ function[83]. The groove formed by helices 1, 3 and 4 hints at a possible secretin-binding site. Notably, the structure of the T2SS pilotin is not related to the previously determined structures of T3SS and T4PS pilotins[100–104]. It remains unclear whether the pilotin is part of the final T2SS machinery or disengages after the secretin is multimerized and inserted into the outer membrane, although in some instances the pilotin has been shown to be a component of the assembled outer-membrane complex[105].

The secretion ATPase. All T2SSs have a single ATPase, named GspE, that is thought to provide the mechanical force for the secretion process. GspE contains Walker A and B motifs that are essential for the secretion and ATPase activity of the T2SS[9,64,69,106,107]. The crystal structure of N-terminally truncated *V. cholerae* $GspE^{EpsE}$ revealed a two-domain fold consisting of the N2 and C domains of the ATPase[63] (FIG. 4a), the C domain containing a Zn^{2+} that is coordinated by four Cys residues[64]. The Zn^{2+}-binding motif is also present in the T4aPS extension ATPase, PilB, but not in other secretion ATPases. Interestingly, N-terminally truncated $GspE^{EpsE}$ forms a helical filament with 6_1 symmetry in the crystal, which suggests that the protein has an oligomeric nature. Indeed, several homologous secretion ATPases have been shown to form hexamers that can adopt diverse regular and irregular arrangements of six subunits in a ring[65,66,108–111]. This is also evident in models of *V. cholerae* $GspE^{EpsE}$ that are based on the structure of the *P. aeruginosa* T4aPS retraction ATPase, PilT[66,111] (FIG. 4a). Although purified $GspE^{EpsE}$ is mostly a monomer, a higher-molecular-mass oligomer with increased ATPase activity (relative to the monomer) is also present in small amounts[64,67]. The effects of site-directed mutagenesis studies targetting interface residues in the putative $GspE^{EpsE}$ hexamer further support the suggestion that the functional form of GspE is hexameric[68]. Interestingly, the ATPase activity of *V. cholerae* $GspE^{EpsE}$ is greatly increased by the cytoplasmic domain of the inner-membrane platform protein $GspL^{EpsL}$ and acidic phospholipids[67].

Two crystal structures of the N0–N1 domains from *X. campestris* $GspE^{XpsE}$ have been elucidated[112], and in both structures the N1 domain adopts the same helical fold as the N1 domain of *V. cholerae* $GspE^{EpsE}$ (REFS 113,114) (FIG. 4a). The extra N-terminal domain in the *X. campestris* protein, N0, occurs in a subfamily of the T2SS ATPases only and seems to be able to adopt different conformations[112].

The inner-membrane platform. The inner-membrane platform of the T2SS comprises multiple copies of at least four different membrane proteins: GspM, GspL, GspF and GspC. GspM has a short cytoplasmic sequence, a transmembrane helix (TMH) and a periplasmic domain (FIG. 4b). The crystal structure of the periplasmic domain of *V. cholerae* $GspM^{EpsM}$ consists of two αββ repeats that form a circular permutation of the ferredoxin fold[115]. Interestingly, the structure contains an extra peptide-like electron density in the cleft between the subunits of the $GspM^{EpsM}$ dimer, indicating that GspM might bind a partner protein at this site.

GspL consists of a cytosolic domain, a TMH and a periplasmic domain (FIG. 4b). The crystal structure of the cytoplasmic domain of *V. cholerae* $GspL^{EpsL}$ revealed

Nanobodies
The smallest antigen-binding fragments of the heavy-chain-only antibodies from camelids. Nanobodies have a single immunoglobulin fold domain and three antigen-binding loops.

Crystallization chaperones
Proteins that are used in co-crystallization because they bind a particular target. Examples of crystallization chaperones include antibody fragments and designed scaffold proteins. These chaperones may reduce the conformational heterogeneity of the target protein and/or form favourable crystal contacts.

Walker A and B motifs
Two protein motifs. The Walker A motif, also known as the P-loop (phosphate-binding loop), is a GXXXGK(T/S) motif that is found in many nucleotide-binding proteins and interacts with phosphate groups of the bound nucleotide. The Walker B motif, XXXXD, coordinates Mg^{2+} and is essential for ATP hydrolysis.

Circular permutation
A change in the protein sequence that leads to a similar three-dimensional structure to that of the original sequence but with a different connectivity.

Ferredoxin fold
A common protein fold with a βαββαβ secondary structure.

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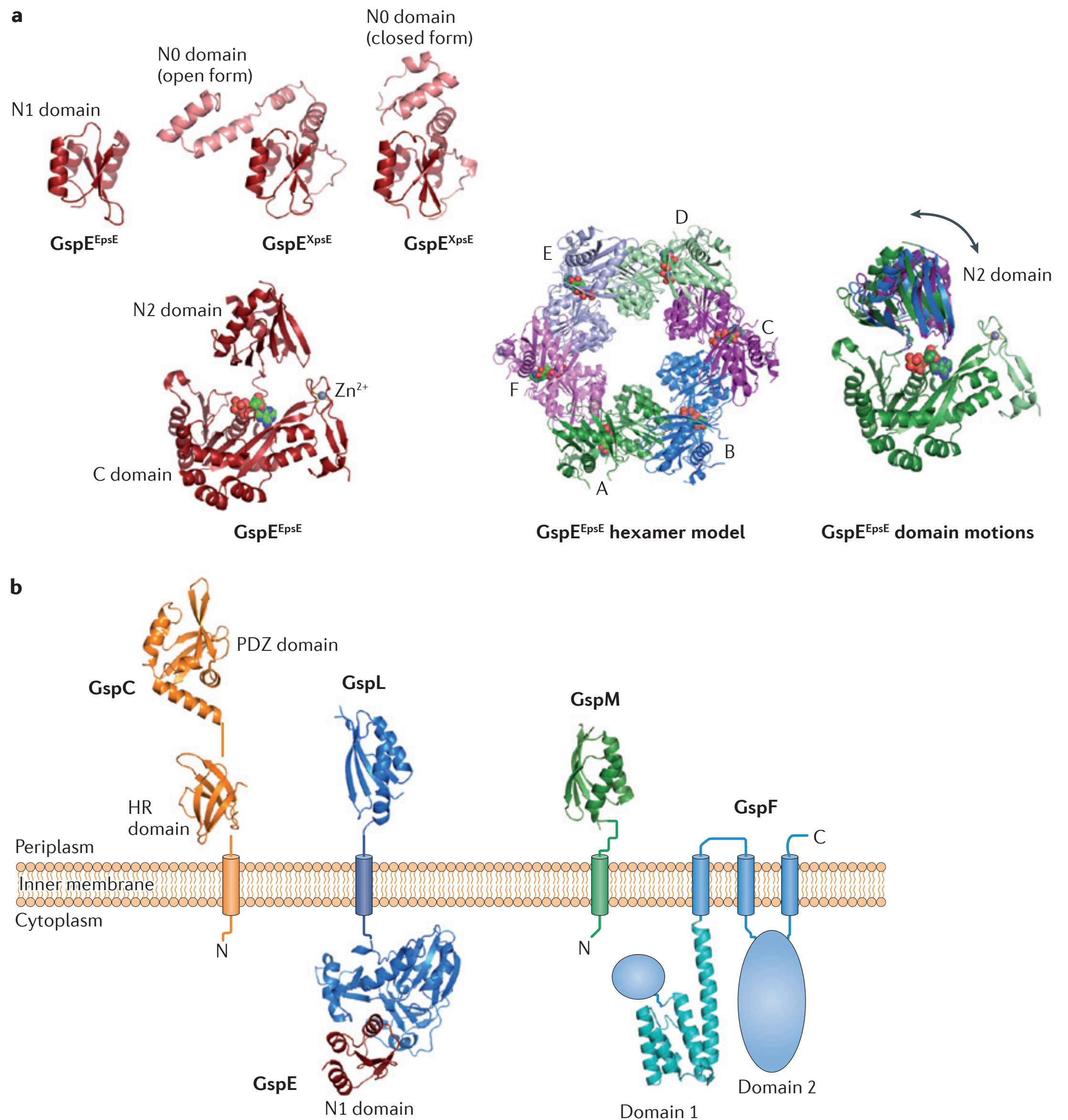

Figure 4 | **Structures and topologies of the secretion ATPase and the inner-membrane components of the T2SS.** For protein names, the general secretory pathway (Gsp) nomenclature is used, followed by a capital letter. **a** | Structures of the type II secretion system (T2SS) secretion ATPase, GspE. The top panel shows the N1 domain of the *Vibrio cholerae* secretion ATPase, $GspE^{EpsE}$ (Protein Data Bank (PDB) accession 2BH1)[113], to the left, and the *Xanthomonas campestris* $GspE^{XpsE}$ N0 and N1 domains in the open and closed conformations (PDB accessions 2D27 and 2D28, respectively)[112] to the right. The bottom left panel shows the N2 and C domains of $GspE^{EpsE}$ (PDB accession 1P9W)[63]. The bottom middle panel shows a hexameric model of $GspE^{EpsE}$ (REF. 68) based on the crystal structure of PilT (PDB accession 3JVV)[66], the *Pseudomonas aeruginosa* retraction ATPase of the type IVa pilus system. The bottom right panel shows the different conformations of the N2 domain in a hexamer model of $GspE^{EpsE}$ (REF. 68). **b** | Structures of the inner-membrane components of the T2SS: the homology region (HR) domain of enterotoxigenic *Escherichia coli* GspC (PDB accession 3OSS)[78] and the PDZ domain of *V. cholerae* $GspC^{EpsC}$ (PDB accession 2I4S)[76]; the periplasmic domain of *Vibrio parahaemolyticus* $GspL^{EpsL}$ (PDB accession 2W7V)[118]; the complex between the cytoplasmic domain of $GspL^{EpsL}$ and the N1 domain of *V. cholerae* $GspE^{EpsE}$ (PDB accession 2BH1)[113]; the periplasmic domain of *V. cholerae* $GspM^{EpsM}$ (PDB accession 1UV7)[115]; and the first cytoplasmic domain of *V. cholerae* $GspF^{EpsF}$ (PDB accession 3C1Q)[119]. Known structures are shown in ribbon representation; domains without known structure are shown as ovals. C, carboxyl; N, amino.

distant homology to the actin-like ATPase family[113,116]. However, no ATPase-binding site has been found in the structure. This is in contrast to a T4PS protein, *Thermus thermophilus* PilM, which is homologous to the cytoplasmic domain of GspL and has a site that is capable of binding ATP[117]. Remarkably, the chain of the periplasmic domain of *Vibrio parahaemolyticus* $GspL^{EpsL}$ adopts the same circular permutation of the ferredoxin fold that is observed in the periplasmic domain of $GspM^{EpsM}$ (REF. 118) (FIG. 4b). In the crystals, two of these $GspL^{EpsL}$

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periplasmic domains form a dimer with an extensive interface. However, the site of subunit–subunit interaction in the periplasmic $GspL^{EpsL}$ dimer differs from that of the $GspM^{EpsM}$ dimer.

GspF is the only polytopic inner-membrane protein of the T2SS to have three TMHs, and has two homologous cytoplasmic domains with ~ 28% sequence identity[118] (FIG. 4b). The N-terminal cytoplasmic domain of *V. cholerae* $GspF^{EpsF}$ consists of a bundle of six antiparallel helices[119]. In the crystals, the N-terminal domains form a dimer with two Ca^{2+}-binding sites in the interface. However, the metal-coordinating residues are not well conserved, and the physiological relevance of metal binding is not yet established.

GspC consists of a short cytoplasmic segment, a TMH and two periplasmic domains, the homology region (HR) domain and a PDZ domain (FIG. 4b). In some GspC homologues, the PDZ domain is absent or replaced by a coiled-coil domain[120]. The structure of the PDZ domain of *V. cholerae* $GspC^{EpsC}$ reveals a circular permutation of the canonical PDZ domain[76]. The peptide-binding groove in the PDZ domain appears to be wider in GspC than in other PDZ domain structures and might be able to accommodate an α-helical binding moiety rather than a peptide in an extended conformation[76]. Although the PDZ and coiled-coil domains can be swapped without loss of function[121], in *D. dadantii* deletion of the PDZ domain abolishes secretion of all proteins but one, so the PDZ domain might be involved in the regulation of secretion specificity[122].

The recent crystal structures of the ETEC GspC HR domain in complex with the secretin N0–N1 domains show that the HR domain is composed of six β-strands forming two antiparallel β-sheets[78] (FIGS 3a,4b). The surface of this domain is highly irregular with respect to charge distribution and contains a deep pocket of unknown function.

The global T2SS architecture

Interactions of the inner-membrane platform and the secretion ATPase. The cytoplasmic domain of *V. cholerae* $GspL^{EpsL}$ contains a cleft between domains II and III which forms the binding site for the N1 domain of the ATPase $GspE^{EpsE}$ (REF. 111) (FIG. 4b). This extensive interface confirms the role of GspL as a tethering protein for the recruitment of the ATPase to the inner-membrane platform[69]. This interface is conserved across many species, suggesting that the motions that the ATPase undergoes during ATP hydrolysis (FIG. 4a) are conveyed to the rest of the secretion system by GspL[68].

Although GspE is likely to be a hexamer (FIG. 4a), little direct evidence is available for the number of inner-membrane proteins in the T2SS machinery. However, it has been speculated[78] that an equal number of subunits of GspL, GspM and GspC are present. In addition, a 1:1 ratio of GspE and GspL is likely according to the crystal structure, in which one N1 domain of GspE interacts with one cytoplasmic domain of GspL[113]. Taking these observations together, GspE, GspL, GspM and GspC might be present in an equimolar ratio in the inner-membrane platform. As GspE is probably a hexamer, there would be six subunits of each of these four proteins. The number of subunits of the inner-membrane protein GspF remains unknown.

Intriguingly, the crystal structures of the first cytoplasmic domain of GspF, the cytoplasmic domain of GspM, the cytoplasmic domain of GspL and the periplasmic domain of GspL all contain dimers with C2 symmetry[113,115,116,118]. Combining dimers of GpsL, GspM and GspF with a GspE hexamer of C6 symmetry is non-trivial, however[118]. It is therefore possible that one, some or all of the GspM, GspL and GspF dimers that are seen in the four crystal structures represent an intermediate state in the assembly process, rather than dimers that occur in the assembled T2SS. Alternatively, some of the dimers seen in the crystals might occur transiently when the system is secreting folded exoproteins or might be crystallization artefacts.

Interactions of the inner-membrane platform and the pseudopilus. Although genetic data suggest that the major pseudopilin, GspG, interacts with the secretion ATPase, GspE[123], biochemical and crosslinking studies have shown that GspG interacts with the inner-membrane platform protein GspL[74]. As GspL also interacts with GspE, it has been suggested that GspL has a crucial role in the conversion of energy from ATP hydrolysis into the assembly, or at least the elongation, of the pseudopilus. Although the site of interaction remains to be identified, processing of GspG by prepilin peptidase is a prerequisite for the GspG–GspL interaction[44]. Minor pseudopilins may also associate with GspL, as truncated forms of *D. dadantii* $GspJ^{OutJ}$ and $GspL^{OutL}$ were found to interact in the yeast two-hybrid system[124].

Interactions of the inner-membrane platform and the outer-membrane complex. The periplasmic HR domain of GspC has been implicated in binding to the T2SS secretin, GspD[75–77,125]. A recent crystal structure of the complex between the HR domain of ETEC GspC and the N0–N1 fragment of the secretin shows that the HR domain interacts with the N0 domain[78] (FIG. 3a). Furthermore, the functional relevance of the interface has been confirmed in *V. cholerae* by analysis of interface residue substitutions in the bacterial two-hybrid system, by an *in vivo* functional assay and by fluorescent localization studies[78]. Recent surface plasmon resonance studies indicate that in *P. aeruginosa* the N3 domain of $GspD^{XcpQ}$ is needed for the interaction between the periplasmic domains of $GspD^{XcpQ}$ and $GspC^{XcpP}$ (REF. 125). The periplasmic domains of $GspC^{XcpP}$ consist of an HR domain followed by a predicted coiled-coil domain. This coiled-coil domain has no sequence similarity with the PDZ domain that follows the HR domain of GspC in most other species, including ETEC and *V. cholerae*[76]. The importance of the N3 domain for the periplasmic $GspD^{XcpQ}$–$GspC^{XcpP}$ interaction in *P. aeruginosa* might reflect a difference between species that is due to the unique domain structure of $GspC^{XcpP}$. Alternatively, it may be that

PDZ domain
A ubiquitous protein domain of approximately 90 amino acids that is typically involved in protein–protein interactions or signalling. It is commonly found in eukaryotic proteins, but is relatively rare in bacterial proteins. The acronym is derived from the first proteins found to share this domain: postsynaptic density protein 95 (PSD95; also known as DLG4), Disks large 1 (DLG1) and zona occludens 1 (ZO1).

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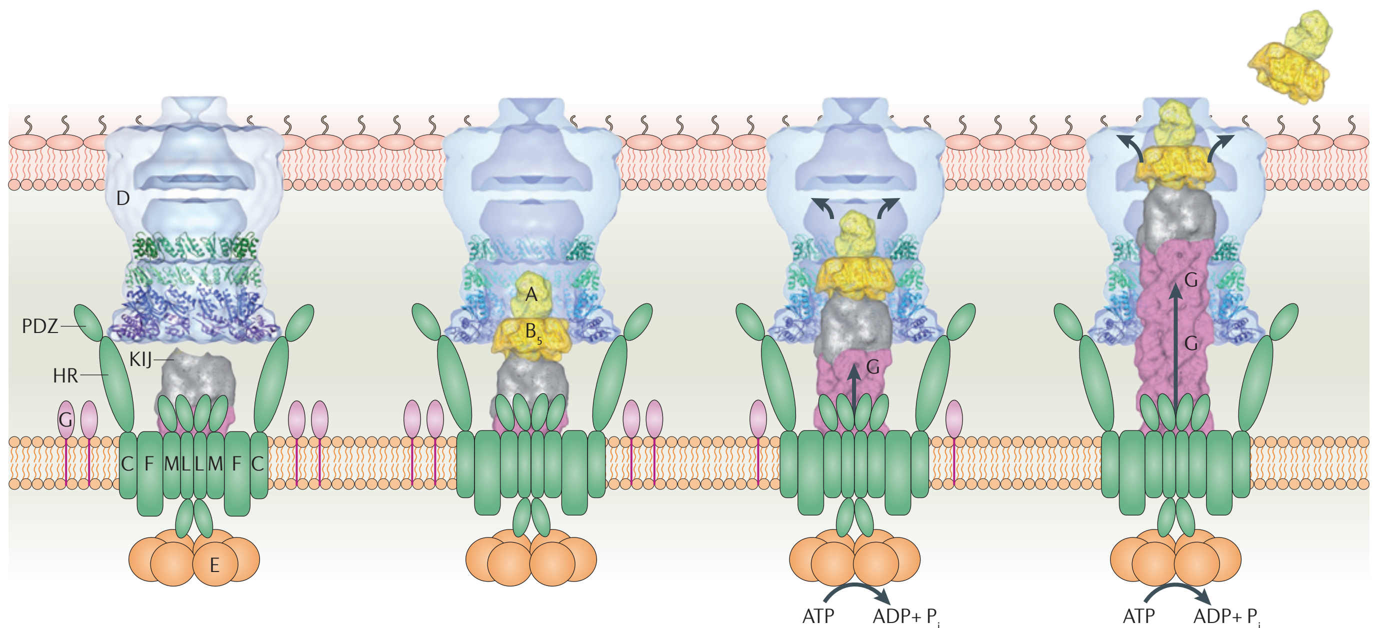

Figure 5 | **A possible mode of action of the T2SS.** The hypothetical mechanism of action for the type II secretion system (T2SS) is based on numerous biochemical and structural studies (see main text and Supplementary information S1 (table) for details). General secretory pathway (Gsp) proteins are labelled with their capital-letter identifiers (the Gsp prefix and the species-specific names are omitted). The secretion ATPase (GspE) is in orange, the inner-membrane platform proteins are in green, the trimeric tip of the pseudopilus (GspKIJ) is in silver, the major pseudopilin (GspG) is in pink and the outer-membrane secretin (GspD) channel is in blue, cyan and purple. The possible architecture of the T2SS before binding an exoprotein is shown on the left (see also FIG. 1). The stoichiometry of many protein components of the system is still uncertain or unknown (see main text for details). The second structure shows the T2SS with an exoprotein (in this case, the AB_5 cholera toxin) in the periplasmic vestibule of the T2SS secretin. On exoprotein recognition by the secretin and/or GspC, a signal is possibly transmitted to the ATPase to induce ATP hydrolysis. One might speculate that a dynamic nature of the periplasmic part of the T2SS secretin, as deduced from proteolytic and electron microscopy studies[25], may assist in or be essential for allowing exoproteins to reach the periplasmic vestibule of the secretin. Exoproteins might subsequently become sequestered in this vestibule by the secretin interacting with GspC of the inner-membrane platform. ATP hydrolysis leads to conformational changes in the GspE hexamer, of which we have only a glimpse from homologues[65,66,108–111]. These motions are transferred by the ATPase-to-pseudopilin coupling protein GspL, which may be involved in adding pseudopilins, such as GspH and multiple copies of GspG, to the still-short pseudopilus on the periplasmic side of the inner membrane. At some stage the exoprotein and/or the tip of the pseudopilus contact the constriction in the periplasmic vestibule of the secretin. The size of the GspKIJ tip is such that it can enter the periplasmic vestibule but not pass the constriction site without alterations in the structure of the secretin[26]. Further addition of GspG subunits to the pseudopilus leads to additional contacts of the exoproteins and/or the pseudopilus with the secretin, resulting in conformational changes (arrows) and expulsion of the exoprotein via the open periplasmic and extracellular gates. HR, homology region; P_i, inorganic phosphate. Figure is modified, with permission, from REF. 26 © (2010) Macmillan Publishers Ltd. All rights reserved.

$GspC^{XcpP}$ binds the N0 domain of $GspD^{XcpQ}$, but the N3 domain stabilizes the complex by either providing a second site of interaction or inducing a conformational change in the N0 domain to strengthen the interaction.

The first crystal structure of a complex between domains from an outer- and an inner-membrane protein of the T2SS[78] raises questions about the stoichiometry of the T2SS. The 1:1 ratio of the secretin–GspC complex (FIG. 3a), combined with electron microscopy evidence that the secretin forms a dodecamer[25,26], suggests that there are 12 copies of GspC interacting with the dodecameric ring of secretin domains in the periplasm. However, when a model of a secretin(N0–N1) dodecameric ring, based on the electron microscopy reconstruction of *V. cholerae* $GspD^{EpsD}$, is compared with 12 copies of the ETEC secretin(N0–N1)–GspC(HR) complex, slight clashes occur between the HR domains. Possibly, minor adjustment in either the N0–N1 ring or the HR ring would alleviate these clashes. Alternatively, the dodecameric ring of periplasmic secretin might only have space for alternating HR domains, resulting in six GspC molecules per T2SS[78].

This second option would be compatible with the hexameric nature of the secretion ATPase and suggests that there would be six copies of GspC, GspL, GspM and GspE in the inner-membrane platform (with the number of GspF molecules still unknown). This arrangement might have an approximate overall cyclic C6 symmetry for the combined inner- and outer-membrane complex. The helical symmetry of the pseudopilus does not follow this possible C6 symmetry, and clearly the organization of the entire T2SS still has numerous unanswered questions.

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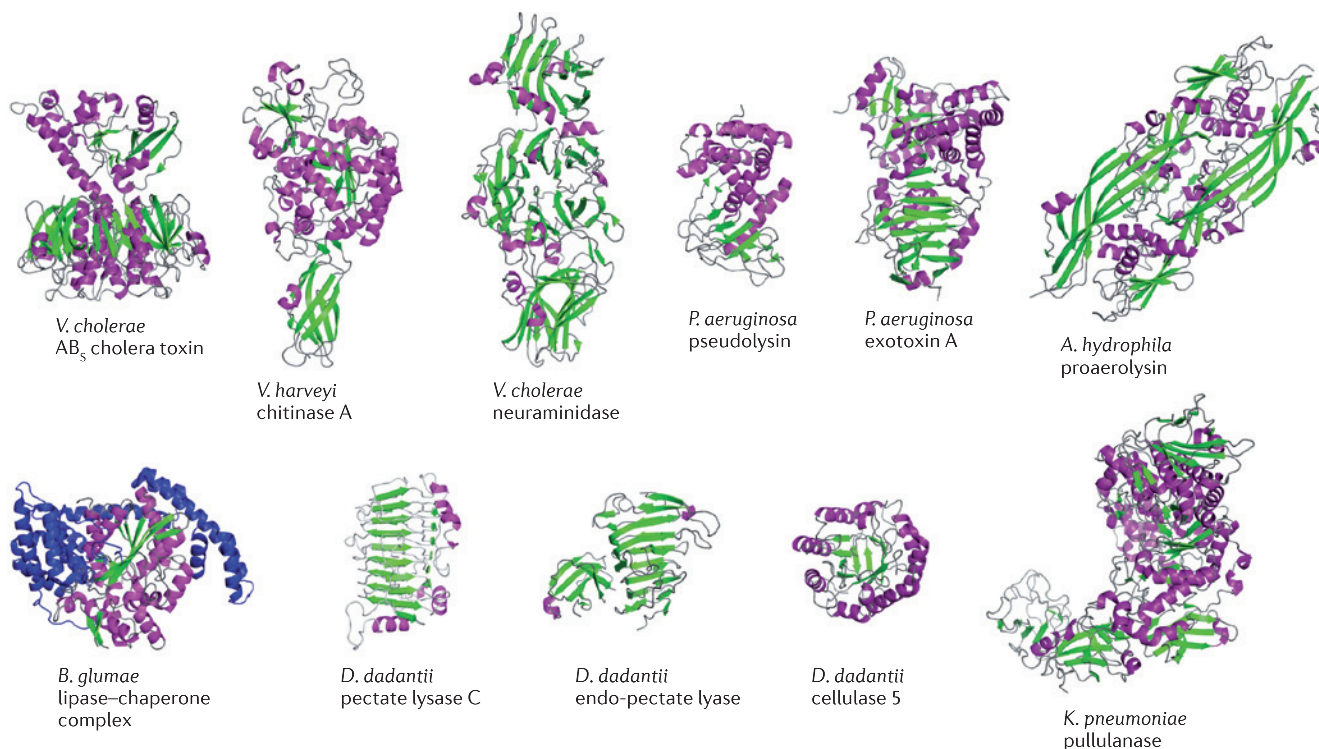
Mechanism of T2SS exoprotein secretion

Initially, exoproteins are synthesized with N-terminal signal peptides that target them for inner-membrane translocation through either the Sec translocon or the Tat complex[126]. Following removal of the signal peptides and release from the cytoplasmic membrane, the exoproteins transiently reside in the periplasmic compartment before outer-membrane translocation, as shown by pulse–chase experiments[127]. The periplasmic intermediate is generally stable and remains secretion competent even under conditions that uncouple the inner- and outer-membrane translocation events[128]. Our current understanding of the mechanism by which the T2SS functions remains limited. The prevalent idea is that exoprotein binding to periplasmic domains of secretin, GspC and/or the pseudopilus tip stimulates the ATPase activity of GspE so that pseudopilin subunits are added to the pseudopilus, and the growing pseudopilus functions

Box 2 | Characteristics of T2SS exoproteins

The type II secretion system (T2SS) is capable of transporting a wide range of substrates. (For T2SS protein names, the general secretory pathway (Gsp) nomenclature is used, followed by a capital letter.) Although it may secrete a single protein in some species, it transports between 15 (*Pseudomonas aeruginosa* and *Erwinia carotovora*) and more than 20 (*Vibrio cholerae* and *Legionella pneumophila*) different proteins in other species[20]. These proteins have a range of biological functions, but they are generally enzymes. They include proteases, lipases, phosphatases and several enzymes that process complex carbohydrates, and — with the exception of toxins, which act inside eukaryotic cells — their site of action is primarily extracellular. Folding into a secretion-competent conformation in the periplasm seems to be a prerequisite for secretion[127,131–134]. What this conformation presents to the T2SS for successful recognition and transport is unknown at present, but the secretion-competent conformation may be identical or similar to the conformation of the fully secreted protein, as it was recently shown that active pseudolysin (LasB) from *P. aeruginosa* can bind to the *P. aeruginosa* homologues of GspC, GspD (also called the T2SS secretin) and the pseudopilus tip components[125]. Many studies, most often using chimeric genes encoding easily detectable reporter proteins, have identified domains or regions that carry information for secretion; however, these domains are large and have not been narrowed down to a common secretion signal[142–146]. The ability of some reporter proteins to contribute to the secretion of their chimeric forms has also complicated the interpretation of some results. The secretion motif may consist of residues from different parts of the protein and be generated only after folding[146] or assembly, as is the case for oligomeric toxins[127]. Although the crystal structures for a limited set of substrates suggest that a β-strand-rich content may be common to proteins that are transported through the T2SS (see the figure for examples; helices and β-strands are shown in magenta and green, respectively), the T2SS signal has yet to be identified. The structures displayed in the figure are: *Vibrio cholerae* AB_5 cholera toxin[147] (Protein Data Bank (PDB) accession 1S5E); *Vibrio harveyi* chitinase A (PDB accession 3B8S)[148], which has 82% sequence identity to *V. cholerae* chitinase; *V. cholerae* neuraminidase[149] (also known as sialidase; PDB accession 1W0P); *Pseudomonas aeruginosa* pseudolysin[150] (also known as elastase; PDB accession 1EZM); *P. aeruginosa* exotoxin A[151] (PDB accession 1IKQ); *Aeromonas hydrophila* proaerolysin[152] (PDB accession 1PRE); the *Burkholderia glumae* lipase–chaperone complex[153], with the chaperone in blue (PDB accession 2ES4); *Dickeya dadantii* pectate lyase C[154] (PDB accession 2PEC); *D. dadantii* endo-pectate lyase[155] (PDB accession 3B4N); *D. dadantii* cellulase 5[156] (also known as endoglucanase Z; PDB accession 1EGZ); and *Klebsiella pneumoniae* pullulanase[157] (PDB accession 2FHB), which has 92% sequence identity to *Klebsiella oxytoca* pullulanase. Several of these secreted proteins contain disulphide bridges that are formed in the periplasm[133,158]. The lipase–chaperone complex[153] is so far a unique example of a chaperone involved in the T2SS. Several exoproteins, such as cholera toxin, are multimeric proteins. Others, such as pullulanase, are large single-chain proteins. How this wide range of proteins is recognized by the T2SS and subsequently translocated across the outer membrane is a fascinating outstanding question.



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Tat complex
(Twin-Arg translocation complex). A system for the transport of folded proteins via the cytoplasmic membrane of bacteria and archaea or the thylakoid membrane of the plant chloroplast. A conserved twin-Arg motif is present in the amino-terminal signal sequence of substrate proteins.

as a piston, pushing exoproteins through the secretin channel[16,51] (FIG. 5). This model is supported by recent electron microscopy studies in *V. cholerae* on exoprotein binding by $GspD^{EpsD}$ (REF. 129) and by surface plasmon resonance studies in *P. aeruginosa* on exoprotein interactions with the periplasmic domains of $GspD^{XcpQ}$ and $GspC^{XcpP}$ as well as with the components of the pseudopilus tip[125]. Numerous aspects of this model still need to be confirmed, in particular the structure and dynamics of the inner-membrane platform and the structure of the open outer-membrane channel. Growth and, in particular, retraction of the pseudopilus are other key aspects of the model that are still to be confirmed or disproved. Retraction might not be well orchestrated given, for example, the absence of a second ATPase, which in other systems is responsible for retraction[130].

Several studies have shown that the T2SS translocates exoproteins in a folded form across the outer membrane[127,131–134]. Using mass spectrometry approaches, the secretomes of *L. pneumophila, E. carotovora* and *V. cholerae* have been reported recently[135–137], but the T2SS secretion signal remains a mystery[138]. In general, exoproteins tend to be rich in β-strands[139], and this is also true of the exoproteins that are secreted by the T2SS, according to the currently available structures (BOX 2).

The similarity in structure of the secretin N0 domain and the signalling domain of the *P. aeruginosa* TonB-dependent outer-membrane receptor FpvA, combined with the fact that a β-strand of the signalling domain binds to a β-strand from a region elsewhere in FpvA, led to the suggestion that the exposed strand β2 of the N0 domain is involved in exoprotein binding as well as in interacting with other T2SS proteins[92]. In the recent crystal structure of the GspC HR domain interacting with the secretin N0 domain, strand β2 of the N0 domain is also accessible for potential interactions with exoproteins[78]. Although these observations have not yet revealed a precise T2SS secretion signal, they indicate that β-strand complementation might be important in the early steps of the secretion process and that the secretin N0 domain and the GspC HR domain are key players in the initial stages of exoprotein binding.

Final conclusions

Despite the considerable recent progress regarding the structural biology of the T2SS, there are still major gaps in our understanding of the biogenesis and architecture of this system. In particular, the inner-membrane complex — the nexus of interactions with the other components of the system — is still poorly understood. The same holds for interactions of the T2SS with exoproteins. Structural studies are required to reveal the nature of these interactions in cases for which the variation in function and structure of the exoproteins is intriguing (BOX 2). In addition, the substantial conformational changes that need to occur in the secretin to open the periplasmic gate and enlarge the extracellular gate, and thus allow the folded exoproteins to reach the extracellular milieu, remain to be clarified. The inner-membrane platform probably has to change conformation in various places and at specific moments during the secretion process. For example, the platform is most probably responsible for the transfer of a signal to the ATPase to indicate that exoprotein binding has been directly or indirectly sensed by GspC, and, subsequently, for converting the conformational changes of the ATPase into a pseudopilus extension process. Most puzzling are the steps leading to disassembly and retraction of the pseudopilus after expulsion of the exoprotein. The possibility exists that in the case of the T2SS, for which no retraction mechanism is required to import molecules, a rather disorderly disassembly of the pseudopilus occurs, after which most pseudopilins end up in the outer leaflet of the inner membrane, ready to be used for a next pseudopilus extension event.

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Competing interests statement
The authors declare no competing financial interests.

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