Review

Secretins: dynamic channels for protein transport across membranes

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Secretins form megadalton bacterial-membrane channels in at least four sophisticated multiprotein systems that are crucial for translocation of proteins and assembled fibers across the outer membrane of many species of bacteria. Secretin subunits contain multiple domains, which interact with numerous other proteins, including pilotins, secretion-system partner proteins, and exoproteins. Our understanding of the structure of secretins is rapidly progressing, and it is now recognized that features common to all secretins include a cylindrical arrangement of 12–15 subunits, a large periplasmic vestibule with a wide opening at one end and a periplasmic gate at the other. Secretins might also play a key role in the biogenesis of their cognate secretion systems.

Secretins: functions and characteristics

Life as we know it requires busy traffic across cellular membranes. This includes transport of large and small molecules in either direction across bacterial envelopes. In Gram-negative bacteria, multiple systems are involved in the secretion of proteins to the extracellular space, and in assembly of fiber structures on the cell surface [1]. Three of these systems feature large, multimeric, outer-membrane channels formed by membrane proteins called secretins: the type II secretion system (T2SS), the type IV pili system (T4PS) and the type III secretion system (T3SS) (Figure 1). Secretins also participate in the assembly and extrusion of filamentous bacteriophages. In plant, animal and human bacterial pathogens, many of the proteins secreted by these systems are important virulence factors.

The T2SS is responsible for secreting toxins and hydrolytic enzymes from the periplasm to the extracellular environment in many Gram-negative bacteria. In *Vibrio cholerae* and enterotoxigenic *Escherichia coli* (ETEC), the T2SS secretes cholera toxin and heat-labile enterotoxin, the hallmark virulence factors of the two diseases caused by these bacteria: cholera and children's diarrhea, respectively. The T2SS consists of multiple copies of 12–15 different proteins distributed over three subassemblies: the outer-membrane complex, a filamentous pseudopilus that remains in the periplasm, and the inner-membrane platform [2].

The T4PS assembles and disassembles long extracellular polymeric fibers on the surfaces of many pathogenic and environmental bacteria, including *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* and *V. cholerae* [3]. The individual pilus is composed of multiple type IV pilin (T4P) subunits from two subclasses: the T4aP and T4bP. The T4P systems are responsible for a wide variety of functions as diverse as host-cell attachment, twitching motility, biofilm formation and DNA uptake; some T4PSs are also capable of secreting specific exoproteins [4–6]. The T4PS is an assembly of more than 12 different proteins [7] and shares many functional and structural features with the T2SS [8].

The T3SS, also called the injectisome, is a protein-transport pathway that delivers virulence factors from the bacterial cytoplasm directly into the membrane or cytosol of the target animal or plant cell [9]. Several important pathogens, including enteropathogenic *E. coli* (EPEC), *Salmonella typhimurium*, *Shigella flexneri* and *Yersinia enterocolitica*, use the T3SS to transport multiple proteins called effectors, which modulate a variety of crucial biochemical functions in the host cell, such as inflammatory responses, cytoskeleton remodeling, apoptosis and phagocytosis. The injectisome consists of a multiprotein basal body spanning both membranes, with an attached hollow needle or pilus [10].

Filamentous bacteriophages use a relatively simple system composed of a secretin and two additional proteins to assemble into particles and exit the bacteria without causing lysis [11].

The widespread emergence of antibiotic resistance in pathogenic bacteria requires the exploration of novel classes of antibacterial compounds. Because the T2SS, T3SS and T4PS are important for the virulence of many human, animal and plant pathogens, targeting bacterial virulence functions – and secretin-containing systems in particular – has been suggested as an attractive strategy for the development of new drugs [12].

In these four families of secretion and assembly systems, secretins provide the outer-membrane pores, 50–80 Å in width, needed for translocation of folded proteins, assembly of oligomeric fibers or DNA uptake. During secretin function, the opening and closing of the pore must be carefully regulated to maintain the integrity of the outer membrane and the periplasmic content. All secretins are large

Glossary

Effectors: proteins secreted by the T3SS into host target cells.
Exoproteins: proteins secreted across the outer membrane.
Needle complex: a large multiprotein complex, spanning the inner and outer membrane from bacteria, involved in delivering proteins from bacteria to the membrane and the cytosol of target host cells via an extended hollow needle.
Pilotin: small lipoprotein involved in the assembly of the secretin multimer.
Secretin: large outer-membrane protein, part of the T2SS, T4PS, T3SS and phage-assembly systems.

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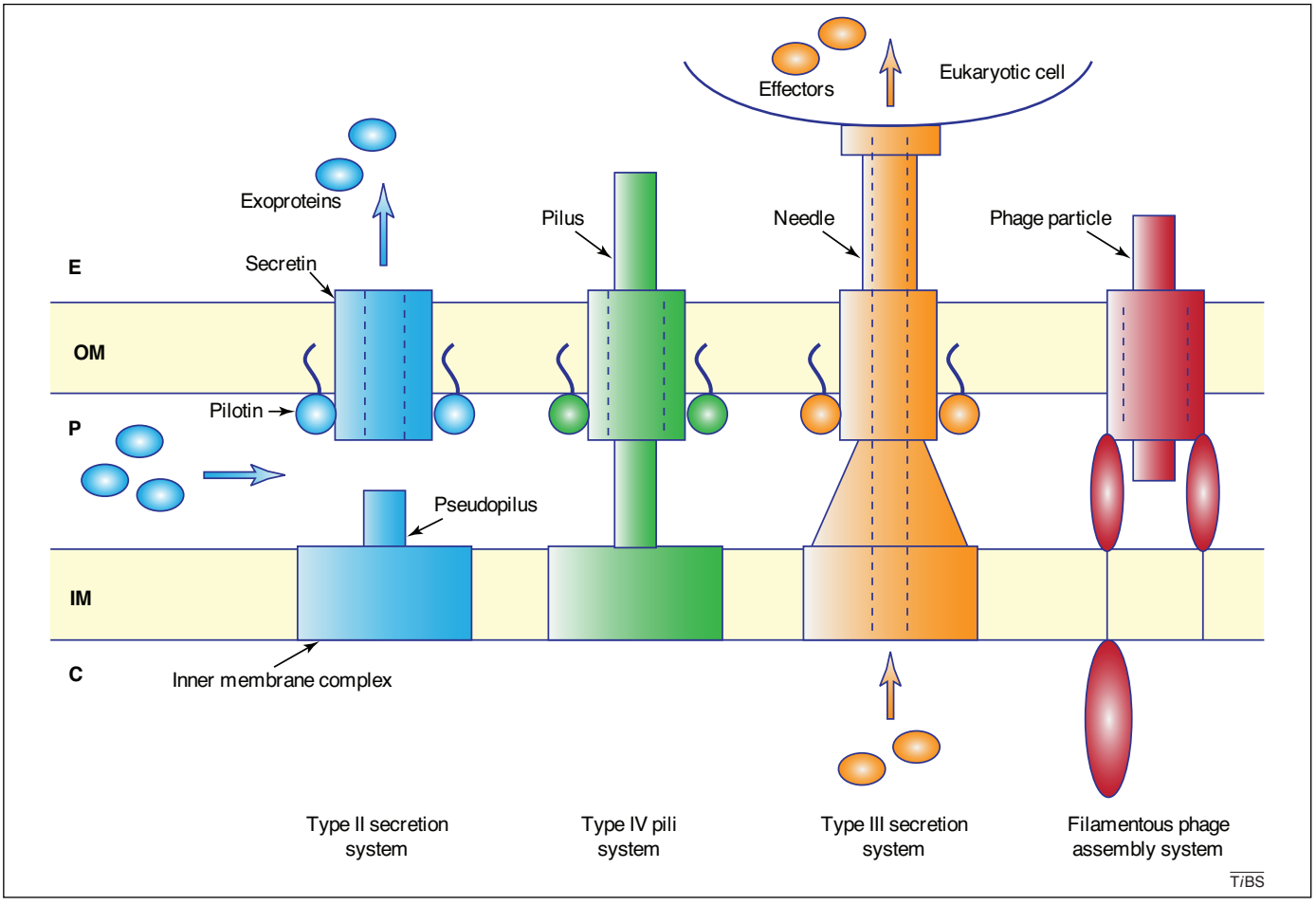

Figure 1. Secretins in Gram-negative bacteria. Schematic view of the type II and type III secretion systems, type IV pili system, and bacteriophage assembly system. The secretin is the major outer-membrane component of all these systems. The insertion of secretins into the outer membrane is often assisted by specific lipoproteins called pilotins. The T2SS secretes exoproteins from the periplasm to the extracellular space in the folded form. The T2SS pseudopilus is formed by multiple pseudopilin subunits, and the pseudopilus is thought to act as a piston and/or plug during the secretion process (Box 1). The T4PS is related to the T2SS in several architectural and functional aspects, but a key difference is that the pilus extends outside the bacterial surface. The T3SS transport effectors directly to the eukaryotic cytoplasm or membrane via a hollow needle. The inner-membrane complexes of the T2SS, T4PS and the T3SS are composed of multiple proteins that include at least one ATPase, which is involved in providing energy for secretion or pilus extension/retraction processes. The filamentous phage-assembly system is composed of a secretin and two inner-membrane proteins. C, cytoplasm; E, extracellular space; IM, inner membrane; OM, outer membrane; P, periplasm.

multidomain proteins with a canonical secretin domain at or near the C-terminus [13,14] (Figure 2a). Secretin assembly is a multistep process, which is often carried out with the help of small lipoproteins, called pilotins. In this review, we examine recent studies of secretins and pilotins, with an emphasis on their overall architecture and domain structure (Table 1). Rapid progress is being made in unraveling the symmetries of these proteins and understanding the functions of their different domains. In particular, the crystal structures of domains now can be placed into electron microscopy maps of increasingly high resolution. The assembly process remains a mystery in many ways, but the role of pilotins in this process are being unraveled for some cases. Exciting times are ahead, in view of e.g. (i) the first views of the conformational changes that secretins must undergo to perform their functions; (ii) the first discernable images of an exoprotein in the periplasmic vestibule of a secretin; and (iii) the preliminary steps undertaken to inhibit these sophisticated outer-membrane proteins.

Crystallographic studies of secretin domains

The crystal structure of the first three N-terminal domains N0–N1–N2 of the T2SS secretin GspD (General Secretion Pathway) from ETEC was solved with the assistance of a nanobody, a camelid antibody fragment (Figure 2b) [15]. The structure shows that the three periplasmic GspD domains are arranged into two lobes: a compact N-terminal lobe containing the N0 and N1 domains, and a second lobe containing the N2 domain. The GspD domains N1 and N2 share the same fold, which is different from that of the N0 domain.

A study on periplasmic domains from the EscC secretin from the EPEC T3SS shows two N-terminal domains that are connected by a linker (Figure 2b) [16]. These two N-terminal domains adopt folds similar to those observed in the N0 and N1 domains of the ETEC GspD T2SS secretin; however, the mutual orientation of these domains with respect to each other in the T2SS and T3SS secretins is unexpectedly different. When the N1 domains of T2SS and T3SS are superimposed, the N0 domains are rotated by not less than 143 degrees (Figure 2b). Further studies are needed to establish whether these structural differences (i) simply indicate that the first two domains are differently organized in these two secretins; or (ii) represent different conformations of a flexible unit that can be adopted by both secretins during secretion or assembly. Interestingly, both

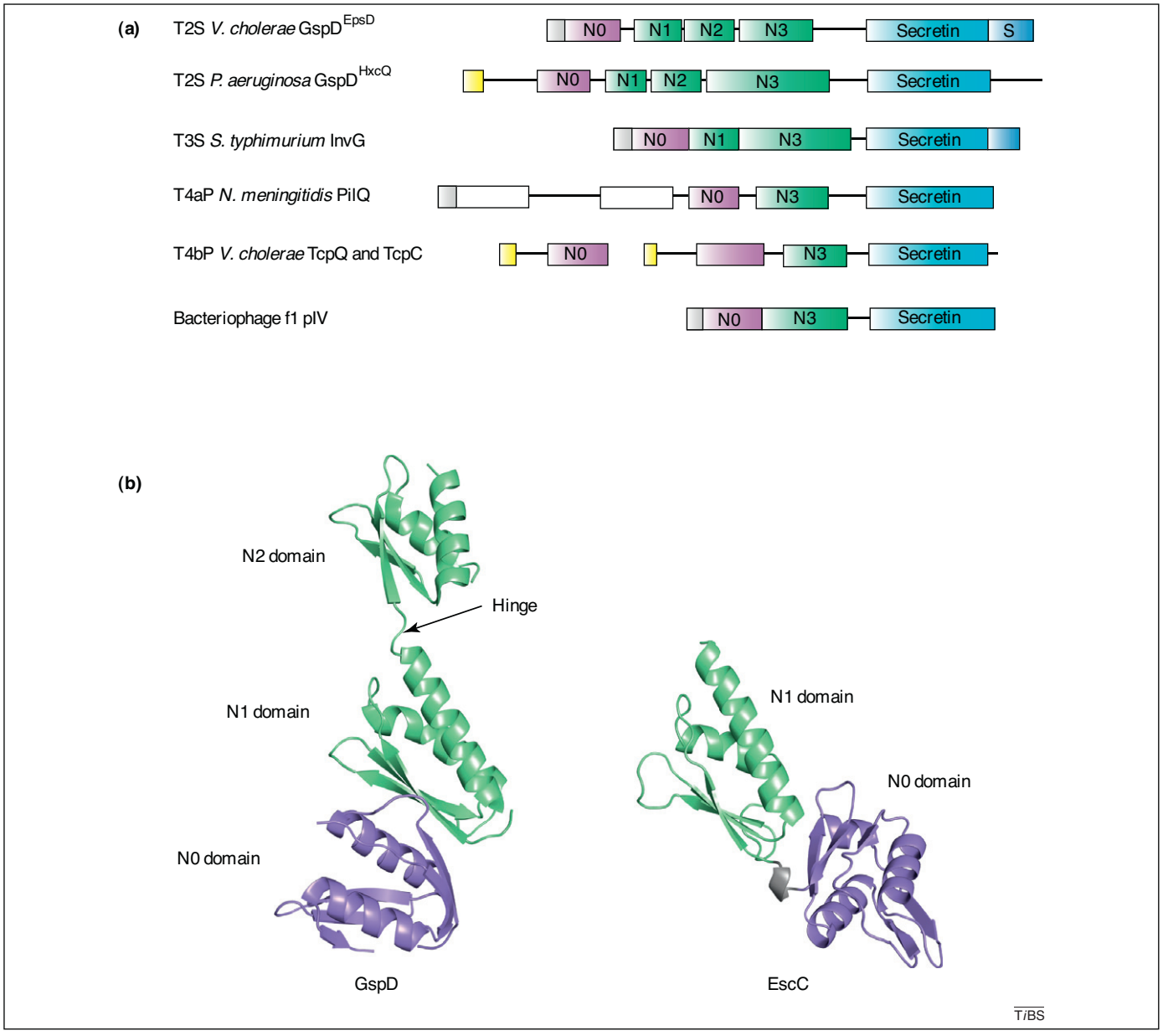

Figure 2. Secretin domains: modular organization and structures. **(a)** Domain composition of secretins. Secretins are synthesized as precursors with N-terminal signal sequences recognized and cleaved off by a signal peptidase (gray) or a prolipoprotein signal peptidase (yellow). In the latter case, secretins are lipoproteins with acylated N-terminal Cys residues. The C-terminal secretin core homology domain (light blue; Pfam family number PF00263 [75]) contains putative amphipathic transmembrane β-strands. The N-terminal N0 domain (purple; Pfam PF07660) is followed by one or several homologous repeat domains (light green; Pfam PF03958) termed N1–N3, depending on the number of repeats. Some N3 domains have long loop insertions. The T4bPS secretins require small periplasmic proteins for stability and multimerization [65,66], and these also have putative N0 domains. Some secretins require specific lipoproteins, known as pilotins, for correct outer-membrane targeting, and contain C-terminal pilotin-interaction domains (dark blue), called S domains in the T2SS secretins. The pilotin-interaction domains are not related in sequence, which reflects the diversity of cognate pilotins. Some secretins contain domains of unknown topology (white). **(b)** Crystal structures of secretin domains. The N0–N1–N2 domains structure of ETEC GspD [15] (PDB 3EZJ) and the N0–N1 domains structure of EPEC EscC [16] (PDB 3GR5) are superimposed and shown in the same orientation relative to the N1 domains. The core of the N0 domain (purple) has a βαββαββ fold that is structurally related to the signaling domain of the TonB-dependent outer-membrane receptors [18], a lipoprotein DotD from the *Legionella pneumophila* type IVb secretion system [20], a domain of protein VgrG from the *E. coli* type VI secretion system [19], and a domain of protein gp27 from T4-related bacteriophages [17]. As expected from sequence homology, the repeat N1 and N2 domains (light green) have similar βαββα folds; the first helix in the N1 domain is a tandem of 3_{10} and α helices. The fold of the N1 domain is different from N0, but structurally related to the eukaryotic type I KH [heterogeneous nuclear ribonucleoprotein K (hnRNP K) homology] domain [76], which is also found in several ring-forming inner-membrane T3SS proteins: EPEC EscJ [21], *S. typhimurium* PrgH [16] and InvA [22,23]. Although the structures of individual N0 and N1 domains of GspD and EscC superimpose well, the relative orientation of these domains and the N0–N1 contact interface is different in the T2SS and T3SS secretins. In the ETEC GspD structure, the N2 domain connects to the N0–N1 lobe via a potentially flexible linker. The relative orientation of the N2 domain with respect to the N0–N1 lobe is stabilized by crystal contacts and interactions with a nanobody (not shown), and is presumed to differ from the orientation in the secretin multimer [15].

the N0 and N1 folds also occur in several other, non-secretin, bacterial-membrane proteins [16–23].

Electron microscopy studies of secretins

A number of electron microscopy (EM) studies on secretins have been published, including several 3D cryo-EM reconstructions (Table 1). Cryo-EM studies on the T2SS secretin $GspD^{PulD}$ (in this review we use the standard T2SS nomenclature followed by a species-specific name in superscript) from *Klebsiella oxytoca* show a cylindrical dodecameric arrangement of secretin subunits with the N-terminal domains located in the periplasm [24]. An open

Table 1. Structural studies of secretins

X-ray crystallography				
Protein name	**System**	**Domains**	**Refs.**	**PDB**
ETEC GspD	T2SS	N0-N1-N2	[15]	3EZJ
EPEC EscC	T3SS	N0-N1	[16]	3GR5
Electron microscopy				
Protein name	**System and sample**	**Symmetry and state**	**Refs.**	**EMDB[a]**
K. oxytoca $GspD^{PulD}$	T2SS secretin[b]	C12	[48]	
K. oxytoca $GspD^{PulD}$	T2SS secretin		[84]	
K. oxytoca $GspD^{PulD}$	T2SS secretin[c]	C12, closed	[24]	
V. cholerae $GspD^{EpsD}$	T2SS secretin	C12, closed	[25]	1763
Y. enterocolitica YscC	T3SS secretin	C13	[85]	
S. typhimurium InvG	T3SS complex[d]	C20	[30]	1100, 1224
S. typhimurium InvG	T3SS complex[e]	C20	[86]	1214, 1215
S. typhimurium InvG	T3SS complex	C15	[32]	1871, 1874, 1875
S. flexneri MxiD	T3SS complex		[87]	1422
S. flexneri MxiD	T3SS complex	C12	[31]	1617
N. meningitidis PilQ	T4PS secretin	C12	[88]	
N. meningitidis PilQ	T4PS secretin	C12	[26]	
N. meningitidis PilQ	T4PS secretin	C4 (quasi C12)	[27]	
N. meningitidis PilQ	T4PS secretin–pilus complex	C4 (quasi C12)	[89]	
N. meningitidis PilQ	T4PS complex[f]	C14	[28]	
N. gonnorrhoea PilQ	T4PS complex[f]	C14	[28]	
T. thermophilus PilQ	T4PS secretin		[29]	
bacteriophage *f1* pIV	Phage-assembly system secretin	C14 (D14), closed	[34]	

[a]Available deposition numbers are indicated.

[b]Secretin–pilotin complex.

[c]Secretin–pilotin complex and trypsinized complex reconstructions.

[d]Needle and base complexes.

[e]T3SS complexes from Δ*invJ* and Δ*prgJ* mutants.

[f]Complexes in isolated membranes.

ring is present in the outer leaflet of the outer membrane, with weak connections to a second ring in the periplasm and the inner leaflet of the outer membrane. The periplasmic entrance of the cylinder is wide open, but a continuous density closes off the periplasmic part of the structure from the outer-membrane region. The most recent T2SS cryo-EM structure is that of the secretin $GspD^{EpsD}$ from *V. cholerae* [25]. In this bacterium, the 12-fold symmetric multimeric channel has the shape of an inverted cup of 200 Å in length, with an outer diameter of 155 Å (Figure 3a). The bottom periplasmic part of the channel appears to be highly convoluted. Above, the smooth outer-membrane part of the channel is followed by a narrower extracellular gate with an opening 10 Å in width. Furthermore, the cross-section of the map reveals a cylindrical periplasmic vestibule, 125 Å in length, which is closed off at one end by the periplasmic gate. This periplasmic vestibule has an opening to the periplasm with an inner diameter of 75 Å (Figure 3a). The internal diameter of the periplasmic vestibule narrows to a constriction 55 Å wide, located 70 Å from the opening. The periplasmic gate appears as a continuous density, and closes off the periplasmic vestibule from an extracellular chamber that is 100 Å in diameter.

The structure of the T4PS secretin PilQ from *Neisseria meningitidis* was initially described as a doughnut-like channel with one open end and C12 symmetry [26]. Subsequent cryo-EM and 2D crystal analyses led to a C4 (quasi-C12) symmetric model that is closed at both ends but lacks the periplasmic gate found in all other secretin reconstructions [27]. The T4PS secretin PilQ from *N. meningitidis*, like its *N. gonorrhoeae* homolog, has also been studied in isolated membranes [28]. The axial views show a double-ring structure with 14–15-fold symmetry for the central ring, which is assumed to be the secretin. Furthermore, a recent study on the T4PS PilQ from *Thermus thermophilus* [29] showed in class-averaged side views a particle with a width of 150 Å and a length of 340 Å. The reconstruction revealed six rings in the periplasm, thought to be formed by the N-terminal 500 residues, underneath a cup structure in the membrane (Figure 3e). The views along the cylinder axis indicate an assembly of approximately 12–14 subunits.

The T3SS secretins have been studied by EM as part of the needle complex that spans both the outer and inner membranes, and includes inner-membrane, needle and shaft proteins (Figure 3b). Initial reconstructions of the *S. typhimurium* needle and base complexes were obtained using 20-fold averaging [30]. However, the reconstruction of the *S. flexneri* needle complex indicates a 12-fold symmetry for the outer-membrane secretin-containing region, and 24-fold symmetry for the inner-membrane region [31]. Unexpectedly, a very recent, higher-resolution reconstruction of the *S. typhimurium* needle complex revealed a 15-fold symmetry for the secretin and a 24-fold symmetry for the inner-membrane ring, yielding a three-fold symmetry for the overall complex [32]. By comparing 3D reconstructions of the T3SS complexes with available secretin

structures from other systems, it has been suggested that the secretin occupies most of the outer-membrane rings [16,31]. The actual location of the secretin in the complex has been experimentally shown by comparing basal complexes isolated from wild-type and secretin-deficient strains [33] (Figure 3d). Thus, the available data suggest that the secretin occupies the top three ring structures in the needle and also in the base complexes of T3SS (Figure 3b). Importantly, in the base structure without needle components, several major T3SS secretin features strongly resemble those of the T2SS GspD secretin. Indeed, it is possible to identify a periplasmic vestibule, a periplasmic gate (or septum), and an extracellular chamber in both the T3SS and the T2SS secretins (Figure 3b).

The pIV secretin of the phage-assembly system that has been studied by cryo-EM reveals a barrel-like structure composed of two multimeric secretin channels with D14 symmetry [34]. A single multimer channel has a diameter of 135 Å and length of 120 Å. A large pore with variable diameter from 60 to 88 Å is blocked by a feature similar to the periplasmic gate seen in the GspD^{EpsD} secretin structure [25]. Interestingly, the pore diameter in the pIV structure is smaller than the diameter of a phage particle, pointing to a similar mechanism of gating as in the T2SS, which might involve a constriction site that initiates considerable conformational changes (Box 1).

The range of 12–15 subunits represented by secretins from different systems and species is intriguing, yet in other cases, homologous proteins have also been observed to form assemblies with dramatically different symmetries [35,36]. Indeed, ATPases have been reported to change symmetry in response to nucleotide binding [37]. Moreover, detergent versus lipid environments can alter the symmetry of membrane proteins [38]. Also, in virus capsids, hexamers and pentamers of identical or similar subunits often occur side by side. Hence, a diversity of symmetries is also a possibility in the case of the secretins.

Combining crystallographic and EM structural data

With high-resolution domain crystal structures and EM reconstructions of secretins in hand, several groups have reported combining the results of these studies. The

Box 1. Possible mode of action of the secretin from the T2SS

A model for the mechanism of action of the T2SS has been proposed [25], continuing with the idea of a pseudopilus piston mechanism (Figure I) [46,77,78]. The model is based on a wealth of data [2,79], of which only recent results can be mentioned here. Surface plasmon resonance (SPR) studies showed that the N0–N1–N2 domains of ETEC GspD interact with an exoprotein, the B-pentamer of heat-labile enterotoxin [25]. More recently, EM studies have indicated a binding site of the B-pentamer of cholera toxin at the entrance to the periplasmic vestibule of *V. cholerae* GspD^{EpsD} [39]. The fact that the N-terminal part of the secretin is (by sequence) the most divergent domain of this protein is consistent with the notion that its N terminus is involved in the recognition of exoproteins. Other studies have reported that the HR domain of the inner-membrane protein GspC interacts with the N-terminal domains of GspD [71,72]. In addition, it has been shown that the periplasmic domains of the T2SS secretin from ETEC interacts with the tip of the T2SS pseudopilus formed by the pseudopilins GspK, GspI and GspJ [25].

Comparing the diameter of the constriction site in *V. cholerae* GspD^{EpsD} with the dimensions of secreted proteins and the tip of the pseudopilus [80] led to the hypothesis that interactions with the constriction site of the secretin might be important for the opening and closing of the periplasmic gate[25]. A possible sequence of events is as follows. Once the inner-membrane protein GspC has contacted N0 and sensed the arrival of an exoprotein in the periplasmic vestibule of GspD, it transmits a signal to the secretion ATPase, whereupon the latter starts expending energy to elongate the pseudopilus. The inner-membrane protein GspL^{EpsL} interacts with both the secretion ATPase GspE^{EpsE} [81,82] and the major pseudopilin GspG^{EpsG} [83], and probably plays a key role in adding subunits to the pseudopilus. The growing pseudopilus pushes the exoprotein from the secretin vestibule further upwards until the constriction site is reached. This contact induces large conformational changes in the T2SS secretin, which results in opening of the periplasmic gate and eventually to release of the exoprotein into extracellular space. The nature and extent of the conformational changes that the T2SS secretin undergoes still have to be determined, but a comparison of the large structural differences in the T3SS secretin with and without the needle (Figure 3b) indicates that secretins can adopt very different conformations.

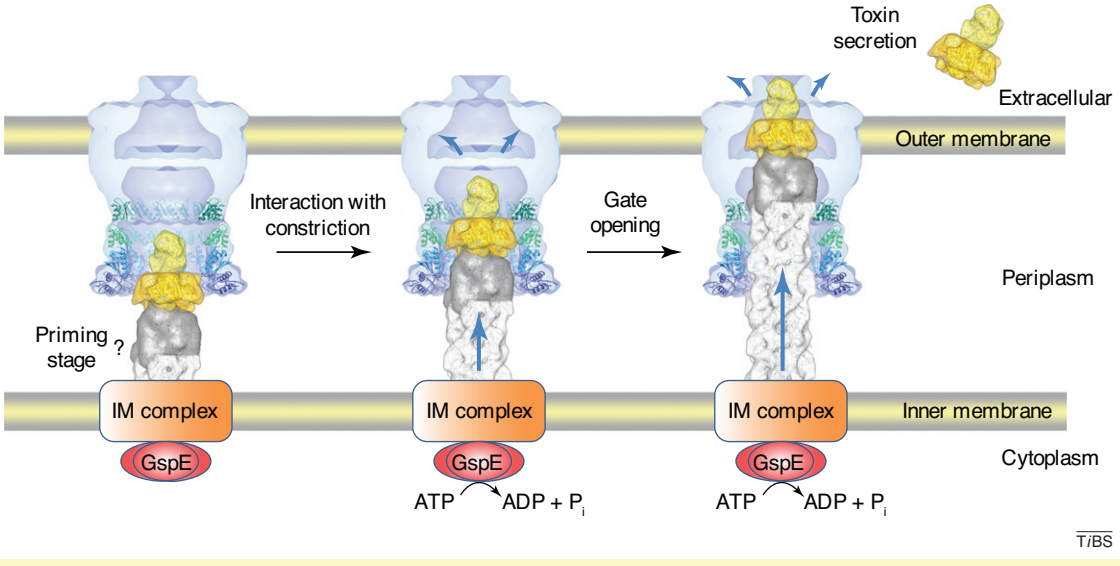

Figure I. Possible mechanism of the T2SS. Modified and reproduced with permission from [25]. Copyright: © 2010 Reichow *et al.*

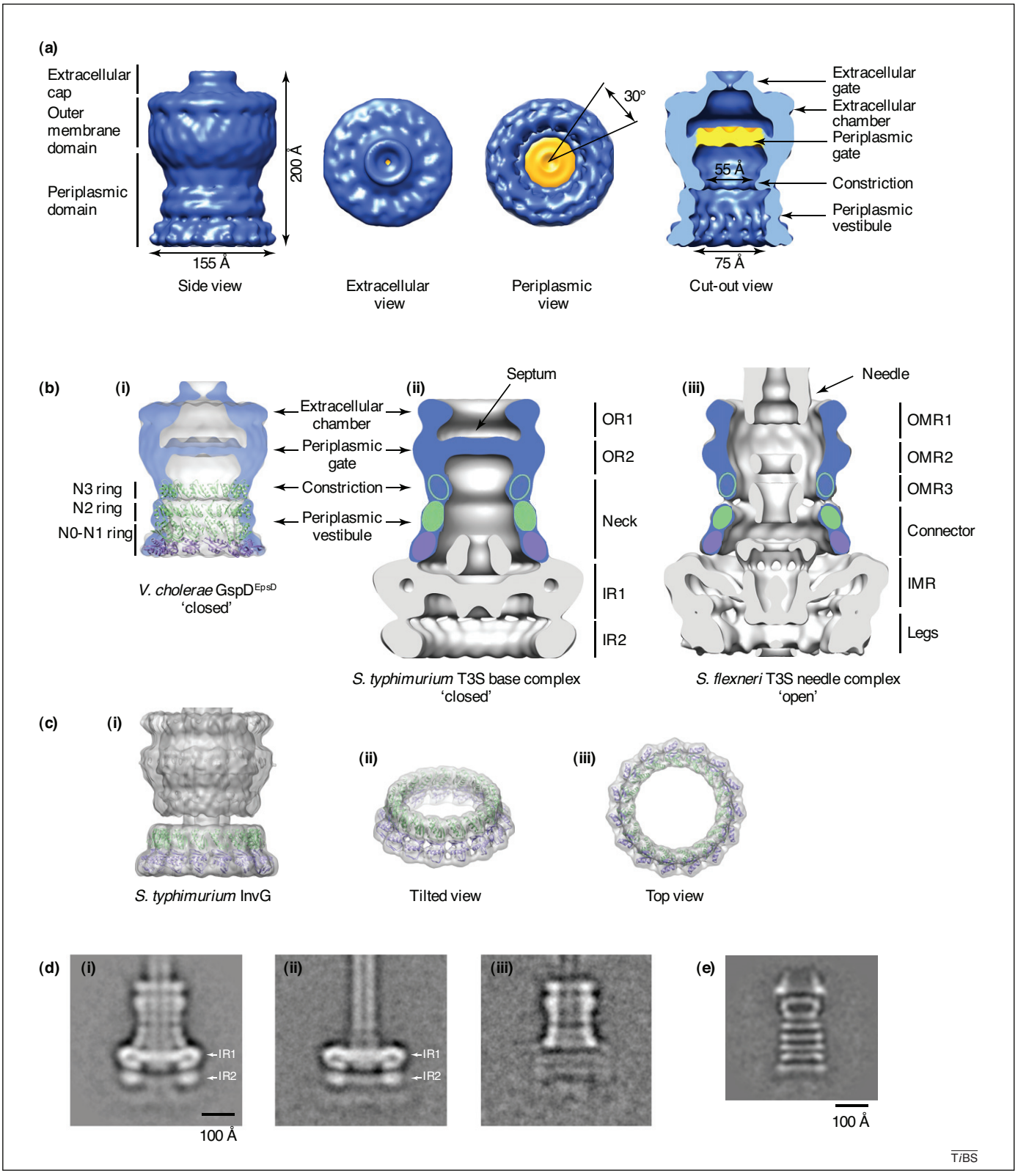

Figure 3. Electron microscopy structures of secretins.
(a) Cryo-EM reconstruction of *V. cholerae* T2SS secretin GspD^{EpsD} [25] (EMDB 1763). In side view, three domains are identified from top to bottom: the extracellular cap, the outer-membrane domain and the periplasmic domain. In a cross-section, the channel contains an extracellular gate, an extracellular chamber, a periplasmic gate, and a periplasmic vestibule with a constriction (yellow). **(b)** The secretin architecture is conserved in different secretion systems. **(i)** Fitting of 12-fold symmetric ring models of the N-terminal periplasmic domains (N0 domain in purple, N1–N3 domains in light green) into the GspD^{EpsD} density map [25]. The N0 and N1 domain ring is anchored at the bottom of the map. The N2 domain ring fits into the central periplasmic domain density and the N3 domain ring into the periplasmic constriction. **(ii)** EM reconstruction of *S. typhimurium* T3SS base complex in the closed state [30] (EMDB 1224). OR, outer ring; IR, inner ring. (iii) EM reconstruction of the *S. flexneri* T3SS needle complex in the open state [31] (EMDB 1617). OMR, outer-membrane ring; IMR, inner-membrane ring. The secretin (blue) occupies the top part of the T3SS reconstructions above the inner membrane complex: **(ii)** and **(iii)**. *V. cholerae* GspD^{EpsD} secretin is in a closed state; compare **(i)** and **(ii)**. The modeled locations of N0 (purple filled ovals) and N1 (light-green filled ovals) domain rings in the T3SS secretins are shown [33,41], as well as the putative locations of the N3 (light-green open ovals) domain ring in the constriction site (upper neck or OMR3) based on the corresponding N3 domain ring fit in the T2SS secretin. **(c)** Cryo-EM reconstruction of *S. typhimurium* T3SS secretin InvG as part of the

structures of the N0–N1–N2 domains of GspD have been used to generate 12-fold symmetrical rings [15]. Although the crystal structure of the N3 domain has not yet been solved, it is predicted to have a structure similar to the N1 and N2 domains, based on the high sequence homology between them. Dodecameric ring models composed of N0–N1, of N2 and of N3 domains have been placed in the density map of *V. cholerae* $GspD^{EpsD}$ [25]. The N0–N1 ring fits into the widest area at the bottom of the periplasmic part of the secretin, and the N2 and N3 rings can be positioned directly above the N0–N1 ring in the map (Figure 3b). In this model, the N3 domain ring fits to the constriction site of the cryo-EM map, suggesting an important function for this domain (Box 1). Interestingly, a recent study showed that the B-pentamer of cholera toxin binds in the lower part of the periplasmic vestibule of the *V. cholerae* $GspD^{EpsD}$ secretin [39].

For the T3SS, the structures of the N0 and N1 domains of EPEC EscC have been used to generate ring models with C12 and C14 symmetry of secretin domains, using a novel procedure for modeling symmetrical protein assemblies [40]. These ring models, with symmetries ranging from 12-fold to 14-fold, have been fitted into cryo-EM reconstructions of *S. typhimurium* and *S. flexneri* T3SS complexes [16,41]. The 14-fold symmetric models fit the density best when placed in the lower part of the secretin or neck region (Figure 3b). The N0 domain is located below the N1 domain in this model – close to the inner-membrane rings – consistent with biochemical data that show interactions between the N0 domain of the secretin InvG and the C-terminal domain of the inner-membrane protein PrgH [33,41]. In another study, the secretin InvG was reported to occupy the outer rings and 'neck' regions of the *S. typhimurium* needle complex, but the symmetry of the secretin has not been determined [33]. Most recently, a homology model of the N0 and N1 domains of the InvG secretin was found to fit in the neck region of the cryo-EM map of the *S. typhimurium* needle complex with 15-fold symmetry for the secretin[32] (Figure 3c), consistent with previous crosslinking and modeling data [33,41].

It appears that the general placements of ring models of the N0 and N1 domains of the T3SS secretin are presently in agreement with each other, and also with the model for the T2SS secretin. The N3 domain has not been formally placed in the density maps of T3SS; however, based on the high degree of homology between T3SS and T2SS secretins, we can tentatively place the N3 ring in the narrower part of the connector region of the injectisome complex, corresponding to the constriction site of the T2SS secretin in the periplasmic vestibule (Figure 3b).

Another striking common feature of both the T2SS and T3SS secretin cryo-EM reconstructions is the periplasmic gate (Figure 3b). However, the importance of this feature for the mechanism of action of these two secretins might be very different, given that the inner wall of the T3SS secretin dodecamer becomes, to a large degree, covered internally by rings of other proteins (Figure 3b), in contrast to the same region of the T2SS and T4PS secretins. A recent study on the bacteriophage secretin pIV identified two regions, called GATE1 and GATE2, within the C-terminal secretin domain, in which amino acid substitutions resulted in a 'leaky' channel phenotype, indicating destabilization of the closed state [42]. Interestingly, several mutations resulting in a similar phenotype have also been identified in the N3 domain, in agreement with a possible triggering role of this domain in secretin gate opening (Box 1).

Pilotins assist in the assembly of secretins

In many cases, the assembly of secretins in the outer membrane requires a specific small outer-membrane lipoprotein, the pilotin [43]. These proteins might assist their cognate secretins in targeting to the outer membrane, and in formation or stabilization of the secretin multimer [44]. The C-terminal region in the T2SS and T3SS secretins interacts with their corresponding T2SS and T3SS pilotins (Figure 2a) [45–47]. A pilotin might or might not form a stable complex with the secretin [48]; moreover, the ionic detergents typically required for secretin solubilization could affect the complex stability during *in vitro* studies. Intriguingly, pilotins from different systems appear to be entirely unrelated in sequence and structure, yet they play similar functional roles in the biogenesis of their secretins.

The lipoprotein MxiM from the *S. flexneri* T3SS is required for the stability, outer-membrane targeting, and multimerization of the secretin MxiD [49]. The crystal structure of the pilotin MxiM reveals a conical shape β-barrel structure interrupted by an α-helix (Figure 4a) [50]. The hydrophobic cleft at the top of the β-barrel serves as a binding site for either lipids or the C-terminal domain of MxiD [50,51]. The ability of MxiM to bind lipids and secretin at the same site prompted a model for pilotin-mediated targeting of secretins to the outer membrane [51]. It has been suggested that as a first step, MxiM binds its own N-terminal lipid. Subsequent binding of the C-terminal region of MxiD substitutes the lipid moiety and makes it available to engage the Lol sorting pathway [52]. As a result, the MixM–MxiD complex is targeted to the outer membrane, where formation of the secretin multimer can occur. Interestingly, the characterized T3SS pilotins from *S. typhimurium* InvH [47,53] and *Yersinia enterocolitica* YscW [54] are not homologous in sequence to *S. flexneri* MxiM or to each other.

needle complex [32]. **(i)** Fitting of the periplasmic domains of InvG (N0 domain in purple, N1 domain in light green) into the density map (EMDB 1875). The inner-membrane part of the needle complex is omitted for clarity. **(ii, iii)** Tilted and top views of the C15 map (EMDB 1871) corresponding to the neck region of InvG secretin with fitting of the periplasmic domains (PDB 2Y9K). **(d)** Experimental visualization of the secretin in the T3SS complex. Class averages of the *S. typhimurium* T3SS needle complexes purified from **(i)** wild-type and **(ii)** *ΔinvG* secretin mutant strains. **(iii)** The density difference between averaged images of wild-type and *ΔinvG* complexes [**(i)** – **(ii)** = **(iii)**] indicates the position of the secretin in the complex. Reproduced from [33] under the terms of the Creative Commons Attribution License. Copyright: © 2010 Schraidt *et al.* **(e)** EM analysis of the *T. thermophilus* T4PS secretin PilQ. A class average of purified PilQ shows a particle 150 Å wide and 340 Å long with features similar to the T2SS and T3SS secretins (compare with the side view in (a) and (bii)) [29]. The upper (outer-membrane) part of the PilQ particle reveals a bisecting density corresponding to the periplasmic gate. The conical lower (periplasmic) part of PilQ consists of six concentric rings that probably correspond to the N-terminal secretin domains. Sequence and fold-recognition analysis of PilQ by the Pcons server [63] indicates the presence of an N0 domain and five putative N1-like domains. Reproduced with permission from [29]. Copyright: © 2011 the American Society for Biochemistry and Molecular Biology.

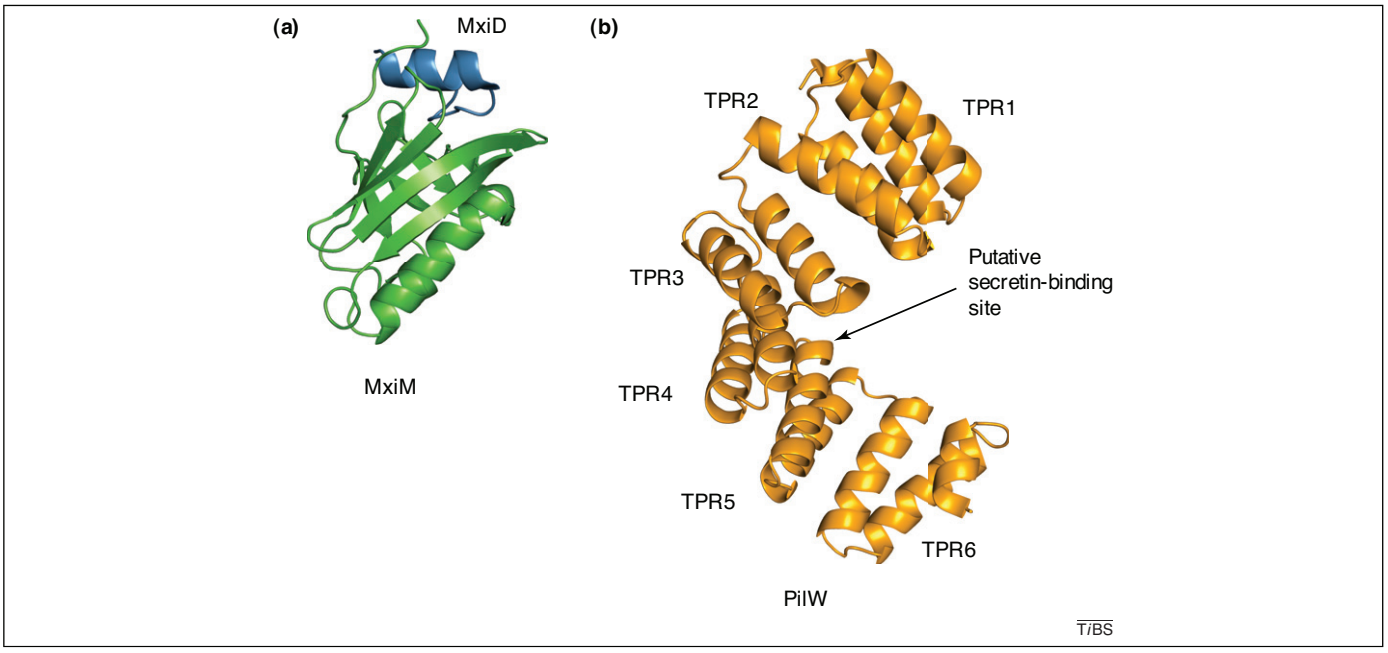

Figure 4. Structures of pilotins. These structures are remarkably dissimilar, despite having a common function. **(a)** The structure of the *S. flexneri* T3SS pilotin MxiM (green) in complex with the C-terminal fragment of the secretin MxiD (dark blue) [51] (PDB 2JW1). The 'cracked β barrel' of MxiM forms a cleft at the top for binding of a short α helix of MxiD. **(b)** The structure of the *N. meningitidis* T4PS pilotin PilW (orange) [59] (PDB 2VQ2) is composed of six TPR motifs arranged as a superhelix. Structures of a homologous *P. aeruginosa* pilotin PilF (26% sequence identity with *N. meningitidis* PilW) show a similar arrangement of TPR motifs [56,58].

The pilotin PilF from *P. aeruginosa* is required for T4aPS biogenesis [55] and is involved in the outer-membrane targeting and multimerization of the T4PS secretin PilQ [56]. A homologous pilotin PilW from *N. meningitidis* appears to be essential for secretin multimer formation, but not for targeting to the outer membrane [57]. Both *P. aeruginosa* PilF and *N. meningitidis* PilW are composed of six tetratricopeptide repeats (TPRs) arranged as a superhelix (Figure 4b) [56,58,59]. The orientation of TPR motifs 5 and 6 is different in the structures of PilF and PilW, with the N- and C-terminal parts of PilW being closer to each other compared with the corresponding parts of PilF [56]. It is not clear whether this structural flexibility is important for function or is a result of crystal packing. The concave surfaces on the PilF and PilW structures have been suggested as potential binding sites for the T4aP secretin. However, which part of T4aP secretin interacts with a pilotin is still unknown. Indeed, T4aP secretins lack the C-terminal domain that is responsible for pilotin binding in T2SS and T3SS secretins (Figure 2a). Notably, *N. meningitidis* lipoprotein PilP was initially described as a pilotin because of its role in assembly and stabilization of the T4aPS secretin PilQ [60], but subsequent studies showed that it is in fact an inner-membrane lipoprotein [61] and part of the inner-membrane complex of the T4PS [62]. Therefore, PilP should not be referred to as a pilotin, as it is instead a functional homolog of the inner-membrane T2SS protein GspC, and also a predicted structural homolog of the homology region (HR) domain of GspC [63].

Some secretins are lipoproteins, and do not depend on separate pilotins for correct outer-membrane targeting. A recently identified lipoprotein secretin, $GspD^{HxcQ}$, from the second T2SS of *P. aeruginosa*, has a long N-terminal linker that presumably extends from the outer membrane to the bottom part of the secretin multimer (Figure 2a) [64]. The lipid moiety is essential for $GspD^{HxcQ}$ function, given that a non-lipidated mutant secretin is incorrectly targeted to the inner membrane. The lipoprotein secretins BfpB and TcpC, from the T4bP systems of EPEC and *V. cholerae*, respectively, require the periplasmic proteins BfpG and TcpQ for multimerization and stability [65,66]. However, fold-recognition analysis by the Pcons server [63] indicates that BfpG and TcpQ have an N0 secretin-like fold, and therefore could function as integral parts of the secretin multimeric channel, not as pilotins (Figure 2a).

Role of secretins in the assembly of secretion systems

In the T2SS, T3SS and T4P systems, secretins are connected to multiprotein complexes in the inner membrane (Figure 1). Does the secretin multimer form first and the inner-membrane platform assembles later; is it the other way around; or do they assemble independently, and interact when needed? Several recent studies have provided some clues, but the answer is complicated. In the *K. oxytoca* T2SS, the fusion of the secretin $GspD^{PulD}$ with a monomeric red fluorescent protein, mCherry, was found to form distinct fluorescent foci at the cell periphery [67]. Furthermore, in the *V. cholerae* T2SS, the green fluorescent protein (GFP) fusions of two proteins of the inner-membrane complex, $GspC^{EpsC}$ and $GspM^{EpsM}$, localize in punctate fluorescent foci along the full length of the bacterial membrane [68]. Those fluorescent foci, which probably represent fully assembled functional T2SS complexes, have not been observed in a *V. cholerae* strain that has the secretin gene deleted. Expression of the $GspD^{EpsD}$ secretin from a plasmid in secretin-deletion mutants restores the punctate fluorescence of GFP-$GspC^{EpsC}$ and GFP-$GspM^{EpsM}$, as well as secretion activity. Therefore, the secretin $GspD^{EpsD}$ appears to be crucial for proper localization of the inner-membrane proteins $GspC^{EpsC}$ and $GspM^{EpsM}$, and also for

assembly of the complete T2SS complex. A study of the *Y. enterocolitica* T3SS assembly using fluorescent protein fusions with secretin YscC, and also with the inner-membrane proteins YscD and YscJ, revealed a similar pattern of isolated fluorescent foci on the cell surface [69]. Moreover, the formation of fluorescent foci by YscD or YscJ was found to be dependent on the presence of the secretin YscC, whereas the assembly of YscC was independent of YscD or YscJ. It has been suggested that the assembly of the *Y. enterocolitica* T3SS injectisome follows an outside-in process that starts with secretin ring formation [69], analogous to the proposed order in *V. cholerae* T2SS assembly [68]. However, the assembly of the *S. typhimurium* T3SS does not appear to depend on the presence of a secretin. It has also been shown that the inner-membrane proteins PrgH and PrgK, homologs of *Y. enterocolitica* YscD and YscJ, can form a ring-like structure in the absence of other proteins [70]. Even more remarkably, an incomplete *S. typhimurium* needle complex has been isolated from a mutant strain that lacks the secretin (Figure 3d) [33]. Taken together, the studies on *Y. enterocolitica* and *S. typhimurium* injectisomes indicate that T3SS biogenesis might have evolved to follow different pathways in these species. Therefore, the role of secretins in assembly of cognate secretion complexes appears to be system- and possibly even species-specific.

Concluding remarks

Secretins are key components of several complex transport systems present in many bacteria, including a number of major bacterial pathogens. The structures of secretins, in isolation or as parts of larger assemblies, have been studied by EM methods. It is only recently that high-resolution crystal structures of the soluble secretin domains of the T2SS and T3SS, which are localized in the periplasmic regions of the EM maps, have become available. Secretins assemble into 12- to 15-meric cylindrical channels, with the C-terminal domain embedded into the outer membrane and the N-terminal domains arranged into rings that form a large periplasmic vestibule. This vestibule is generally wide open towards the periplasm, and closed off by the periplasmic gate, except in the assembled injectisome. This gate probably undergoes conformational changes after interacting with exoproteins and/or partner proteins of the secretion system. The functions of several domains of the secretins are emerging: the N-terminal domains interact with domains from inner-membrane proteins [33,41,71,72] and, at least in some cases, with exoproteins [25,39,46], while the N3 domains might contain a constriction site that could play a role in triggering conformational changes [25,42]. The secretin domain is inserted into the outer membrane, and the S-domain of the T2SS and a functional homologous C-terminal domain of the T3SS interacts with a pilotin, an assembly assistant. A proposed mechanism for the transport of cholera toxin across the outer membrane by the T2SS of *V. cholerae* combines multiple functional and structural results (Box 1).

However, we still need to obtain a large amount of additional information – both structural and biochemical – to fully understand how secretins function to secrete proteins, take up DNA, or assemble fibrous filaments. Establishing the symmetry of secretins across species and systems will be an important area of future research. Future studies of the secretin family of proteins in different conformational states are needed for a better understanding of transmembrane transport in bacteria, and this understanding could assist in the development of novel drugs to interfere with the translocation process.

The recent progress in our understanding of the architecture and assembly of secretins, and the beginning of our insight into their interactions with pilotins, is especially important because secretins and pilotins represent attractive targets for the development of novel antibacterial compounds. The possibility to inhibit secretion was demonstrated by engineering specific binding proteins against the *K. oxytoca* secretin $GspD^{PulD}$ [73]. Furthermore, in a functional screen for T3SS inhibitors, a compound was identified that interfered with needle-complex assembly in *S. typhimurium* [74]. Most intriguingly, the same compound also inhibited the function of *Y. enterocolitica* T3SS, *P. aeruginosa* T2SS and *P. aeruginosa* T4PS. These studies might represent the very first steps in what could become a fruitful translation of our understanding of these impressive outer-membrane proteins into compounds of relevance for preventing and curing several major infectious diseases.

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