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REVIEW

Bacterial secretins: Mechanisms of assembly and membrane targeting

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Abstract

Secretion systems are employed by bacteria to transport macromolecules across membranes without compromising their integrities. Processes including virulence, colonization, and motility are highly dependent on the secretion of effector molecules toward the immediate cellular environment, and in some cases, into the host cytoplasm. In Type II and Type III secretion systems, as well as in Type IV pili, homomultimeric complexes known as secretins form large pores in the outer bacterial membrane, and the localization and assembly of such 1 MDa molecules often relies on pilotins or accessory proteins. Significant progress has been made toward understanding details of interactions between secretins and their partner proteins using approaches ranging from bacterial genetics to cryo electron microscopy. This review provides an overview of the mode of action of pilotins and accessory proteins for T2SS, T3SS, and T4PS secretins, highlighting recent near-atomic resolution cryo-EM secretin complex structures and underlining the importance of these interactions for secretin functionality.

KEYWORDS

bacterial virulence, protein–protein interactions, secretin, toxin secretion, Type IV pilus system, Types II and III secretion systems

1 | INTRODUCTION

Bacteria depend on the transport of molecules, toxins, and macromolecules to (and from) the external environment in order to survive and proliferate. In Gram-negative bacteria, such transport events require the crossing of the cytoplasmic membrane, the periplasmic space and the outer bilayer. In order to ensure substrate transport while still maintaining cell wall integrity, bacteria have developed secretion systems (T1SS–T9SS) and competence machineries that play key roles in competition with other microbes, virulence, surface attachment, and gene transfer processes. In addition, the various transported substrates include proteins (such as toxins and effectors), DNA, and protein-DNA complexes.[1–4]

One of the main challenges in the secretion process is the trespassing of the outer membrane without causing membrane damage and eventual cell rupture. Secretins, key members of the outer membrane complex in several systems, are essential for this stability. These homomultimeric complexes are key elements of the Type II and Type III secretion systems (T2SS and T3SS, respectively) as well as the Type IV pilus system (T4PS).[5–7] Secretins are also present in bacteriophage extrusion systems[8,9] but these will not be discussed here. Other

secretion systems employ different strategies to translocate molecules through the outer membrane, such as the formation of a pore by the secreted protein or a dedicated translocation partner in the T5SS,[10] employment of the TolC oligomer by the T1SS,[11] and transport through the 36-stranded β-barrel structures in the T8SS and the T9SS.[12–14]

Primary sequence analyses and electron microscopy studies of secretins from different transport systems and strains have revealed similarities in domain organization and overall structure. Secretin monomers display over 600 residues (Figure 1) and associate into oligomers of >1 MDa. The exact stoichiometry of secretin pores has been a matter of discussion. While assemblies of 12 protomers have been reported,[18–20] more recent higher resolution cryo-EM structures have indicated that the pentadecameric assembly is the most stable and predominant arrangement, at least for the T2SS and T3SS.[15,16,21–25] Notably, structures of T4PS secretins have been reported to carry 12–14 subunits.[17,26–29] The assembled secretin pores present two major domains: a C-terminal, conserved core that folds into a 60-stranded β-barrel composed of inner and outer walls, and an N-terminal region composed of a variable number of small α/β domains separated by flexible linkers (Figures 1 and 2). The latter are structurally similar to each other and may interact with the inner membrane (IM) platform, secreted substrates as well as internal structures.[6,25,30–32] Secretins from the T2SS and T3SS also carry C-terminal S-domains, involved in localization, assembly and membrane stability[15,21,25,33–38] (red in Figures 1 and 2). T4PS secretins, on the other hand, in some cases can present amidase N-terminal (AMIN) domains, involved in peptidoglycan binding and secretin localization,[27,39,40] or β-domains that can act as a periplasmic gate.[41]

Recent developments in cryo-EM methods, associated to elegant genetic, biochemical, and microbiological studies have been essential not only for the understanding of secretin structures, but also of complete secretion machineries, as highlighted above. Importantly, mechanistic details of secretin targeting, assembly, and stability in the membrane, which are all essential for secretion system functionality, have also started to emerge. These events can be dependent on different classes of partner proteins or mechanisms (or a combination thereof): pilotins, ancillary molecules, and self-piloting systems. This review will summarize the most recent evidence regarding such mechanisms for the T2SS, T3SS, and T4PS secretins, with particular emphasis on structural details regarding interactions between secretins and their partner molecules and their importance for substrate secretion and virulence.

2 | ASSEMBLY OF T2SS SECRETINS

The T2S apparatus is used by at least 32 genera of Proteobacteria to secrete folded proteins from the periplasm to the outer milieu or to the cell surface. Secreted substrates are involved in survival and growth in the environment and inside hosts, and are essential for virulence in the case of pathogens. The T2SS translocates folded substrates from the periplasm toward the outside of the cell, and well-studied substrates secreted by the T2SS include the heat labile toxin of enterotoxigenic *Escherichia coli*,[42] cholera toxin of *Vibrio cholerae*[43] and exotoxin A of *Pseudomonas aeruginosa*.[44] The T2SS apparatus is composed of four major components:

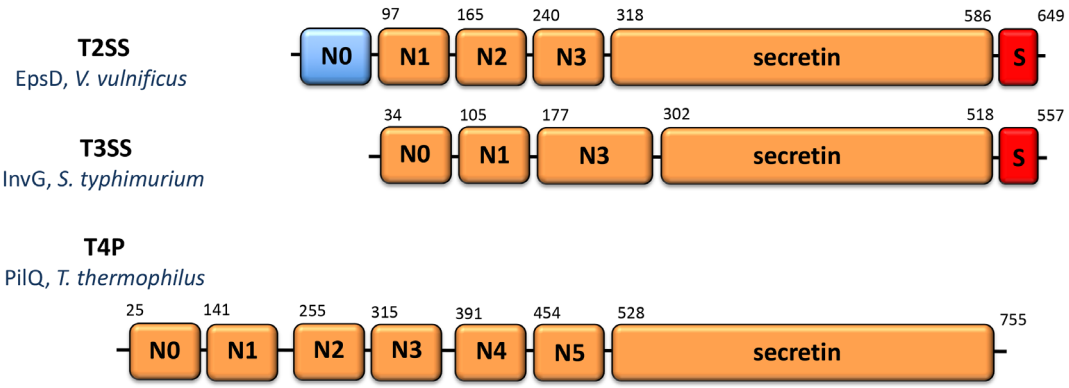

FIGURE 1 Schematic diagrams of secretin domains of the T2SS, T3SS, and T4PS. The number of N-terminal domains can be variable, and T3SS do not carry N2 domains. N0 in EpsD from the T2SS of *V. vulnificus* was not traceable in the cryo-EM map due to flexibility[15] and is thus indicated in blue. Numbers indicate domain delimitations, as indicated in the publications describing the structures in Figure 2[15–17]

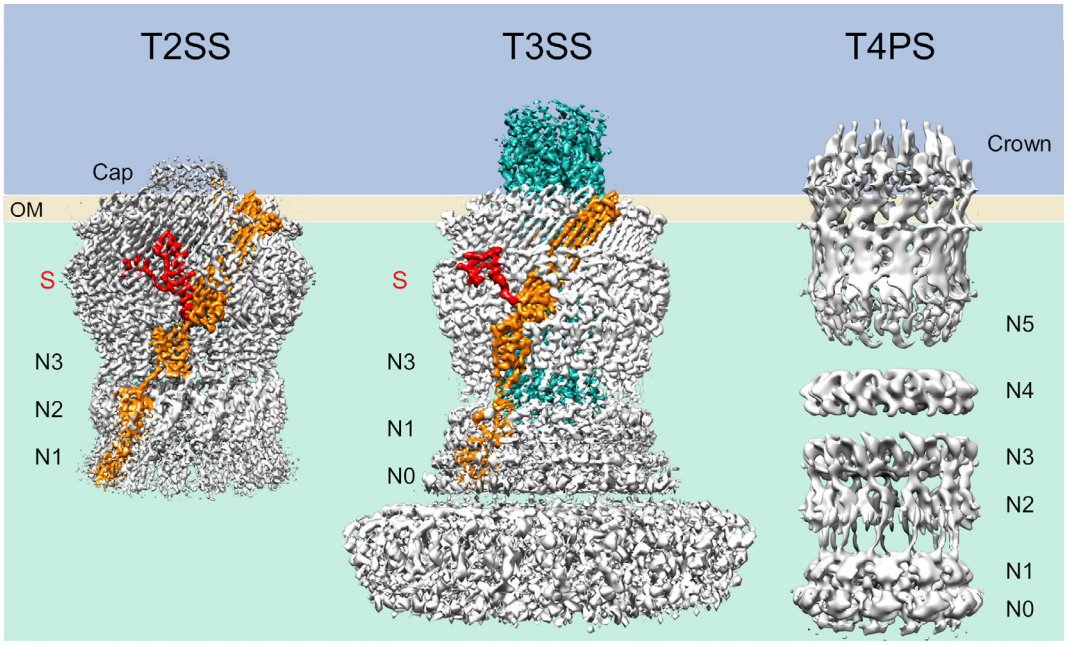

FIGURE 2 Cryo-EM structures of secretins (T2SS, T4PS) and injectisome needle complex (T3SS). The resolution of the maps for the T2SS (EpsD from *V. vulnificus*, 3.4 Å[15]) and T3SS (InvG, PrgH, PrgK, PrgI from *S. typhimurium*; ~3.6 Å[16]) allowed for the positioning of the monomer (orange) including the S-domain (red). The T3SS needle is indicated in cyan. The cryo-EM structure of the T4PS secretin from *T. thermophilus* (right[17]) displays a large number of traceable N-terminal domains, which is unusual due to their well-documented flexibility in absence of the base components. OM, outer membrane; S, S-domain. The figure was generated with PDB files and EM maps corresponding to: 6i1y and EMD-0327 (T2SS); 6duz/6dwb/6dv3 and EMD-8913/EMD-8914/EMD-8924 (T3SS); EMD-3995/EMD-3996/EMD-3997/EMD-3998 (T4PS)

an IM platform which, together with the OM secretin channel, forms a passage that accommodates substrates to be secreted; the pseudopilus, which polymerizes and pushes substrates through the interior of the secretin pore; and an ATPase, which provides energy for pseudopilus polymerization.[5,25,45]

Most full-length secretin structures published to date are from the T2SS, and include those from pathogens such as *V. cholerae*, *E. coli*,[21,23,36] *Vibrio vulnificus*, *Aeromonas hydrophila*,[15] *Klebsiella pneumoniae*,[25] and *Klebsiella oxytoca*.[20] In addition, the structural characterization of the periplasmic domains of T2SS secretins as well as inner T2SS components have been instrumental in providing insight toward a mechanistic understanding of the system as well as the development of targeted inhibitors.[46–48]

Several T2SS secretins depend on pilotins for their assembly in the membrane. Pilotins are small lipoproteins that target the secretin monomers to the inner leaflet of the outer membrane via the Lol pathway.[49,50] The pilotin-dependent assembly mechanism was first described by Hardie and co-workers for PulD from *K. oxytoca*,[51] which was shown to be dependent on the outer membrane-anchored protein PulS for assembly in the leaflet. Studies of the PulS-PulD interaction showed that the role of the pilotin is to protect the secretin from degradation in the periplasm; in its absence, secretin assembly can occur within the IM, leading to the initiation of the phage shock response.[52,53] More recent secretin assembly studies have confirmed the role of pilotins in T2SS secretin stability in the outer membrane.[15,36,38] It is of interest that T2SS secretins have been classified as *Vibrio*-type, *Klebsiella*-type or *Pseudomonas*-type based not only on sequence homologies, but also on the identity of their cognate pilotins.[38]

Two families of T2SS pilotins have been identified and structurally characterized by X-ray crystallography: the OutS-PulS pilotins, that interact with *Klebsiella*-type secretins, and the AspS-GspS$_\beta$ pilotins, present in strains expressing *Vibrio*-type secretins.[38,54] Interestingly, representatives from the two pilotin families are distinct both at the sequence and the structural levels (Figure 3, Table 1). Pilotins from the OutS-PulS family fold into a bundle of 4 α-helices that assemble with a concave hydrophobic groove at the center.[33,61] In the case of interaction studies performed with PulS and the S-domain of PulD, all four helices were shown to interact with a disordered segment of the S-domain that undergoes a disorder-to-order transition and folds into a helix upon binding.[33] However, pilotins from the AspS/GspS$_\beta$ family display a completely different fold, consisting of a 5-stranded

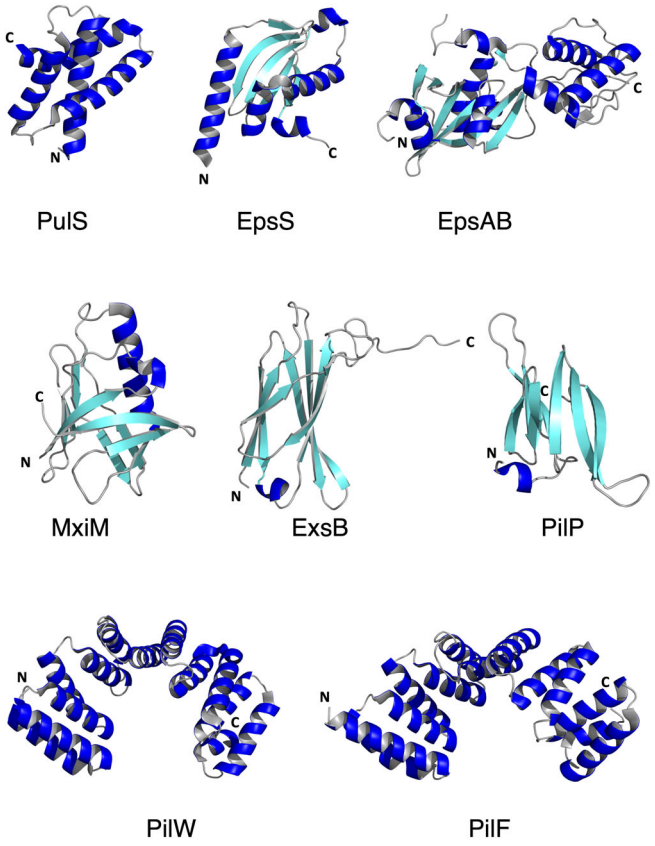

FIGURE 3 Gallery of X-ray structures of pilotins and accessory molecules. The PDB codes for the different molecules include: T2SS: 4A56 (PulS[33]), 6I2V (EpsS[15]); 4G54 (EpsAB[55]); T3SS: 1Y9I (MxiM[56]), 2YJL (ExsB[57]); T4PS: 4AV2 (PilP[58]), 2VQ2 (PilW[59]), 2HO1 (PilF[60])

β-sheet flanked by 4 α-helices.[15,36,38] This fold is reminiscent of a cupped hand, where the central region of the β-sheet, which is highly hydrophobic, could represent the palm (EpsS in Figure 3).

Structural details showing the interaction between the latter family of pilotins and their cognate secretins have recently become available (*E. coli* GspD-AspS, *V. vulnificus* EpsD-EspS, and *K. pneumoniae* PulD-PulS).[15,25,36] A major player in the interaction is the S-domain that forms a "belt" around the secretins and contains the two C-terminal α-helices (α11 and α12, in the case of the *E. coli* and *V. vulnificus* secretins) and the interconnecting loops. The cryo-EM structure of the GspD-AspS complex reveals that in order for the pilotin to bind, α12 must undergo an outward movement and place itself away from the body of the structure, after which recognition of AspS is possible through the hydrophobic platform formed by the central β-sheet (Figure 4). In the structure of the GspD-AspS complex, however, the lower resolution (approx. 5 Å) in the AspS binding region did not allow the visualization of details regarding the pilotin. Nevertheless, modeling of the interaction between *V. vulnificus* EpsD and its pilotin EpsS (a member of the AspS pilotin family) onto the GpsD-AspS complex, where the structure of EpsS was at 1.7 Å resolution, shed more light on the issue. This analysis indicated that upon binding to α12, EpsS could undergo a minor conformational modification, as if "closing" its hand on the C-terminal helix of EpsD (Figures 3 and 4). Mutational studies performed on *E. coli* and *V. cholerae* highlighted the importance of hydrophobic regions of both the S-domain and the pilotins for secretin stability in the outer membrane and bacterial toxicity.[15,36]

Some bacteria also present accessory proteins that may either substitute or work together with pilotins. ExeD from *A. hydrophila*, for example, requires the IM complex ExeA/ExeB for piloting and assembly. ExeA is an ATPase that can bind to peptidoglycan and form large multimers in the periplasm,[62] while ExeB binds directly to the N0/N1 domains of ExeD.[64] Notably, in the absence of the complex, ExeD is inserted in the IM,[63] as in the case of PulS deletion mutants in *K. oxytoca*.[53] It is of note that genomic analyses of *A. hydrophila* have not revealed the presence of a pilotin-encoding gene, underlining the relevance of pilotin-independent assembly mechanisms. Interestingly, ExeA/ExeB homologs have been identified as EpsA and EpsB in *Vibrio* spp, and are represented as a fusion protein in *V. vulnificus*[55] (Figure 3).

The T2SS secretin from *P. aeruginosa*, HxcQ, is an exception to all of the cases described above. Viarre and co-workers showed that in addition to forming stable multimers within the outer membrane, HxcQ is also a lipoprotein, and carries a fatty acid at its extreme N-terminus that plays a key role in its oligomerization in the membrane.[79] Surprisingly, it was later identified that PA3611, a conserved T2SS protein of unknown function in *P. aeruginosa*, presents high structural similarity to AspS, but also has no lipoprotein signature sequence.[38] This observation led authors to suggest that the function of PA3611 could involve recognition of the S-domain of its cognate secretin HxcQ but could be limited to protecting it from proteolysis, which is plausible in the case of an autopiloting secretin like HxcQ. Interestingly, the T4PS also has examples of secretins capable of self-piloting (below), indicating the importance of there being multiple mechanisms that guarantee the assembly of these important proteins.

3 | ASSEMBLY OF T3SS SECRETINS

The T3SS, whose main structural element is the injectisome, is a complex machinery of more than 20 proteins that plays a key role in the secretion of substrates with the goal of modulating eukaryotic host cell

TABLE 1 Pilotins and accessory molecules of the T2SS, T3SS, and T4PS

System	Pilotin	Accessory molecule	Cognate secretin	Bacterial species	Main references
T2SS	EpsS	EpsA EpsB	EpsD	*Vibrio vulnificus*	Howard et al. [15] Strozen et al.[55]
	AspS		GspD	*Escherichia coli* (EPEC)	Dunstan et al.[38] Yin et al.[36]
	AspS		GspD	*Vibrio cholerae*	Dunstan et al.[38]
	OutS		OutD	*Erwinia chrysanthemi*	Rehman et al.[61]
	PulS		PulD	*Klebsiella oxytoca*	Tosi et al.[33] Nickerson et al.[34]
		ExeA ExeB	ExeD	*Aeromonas hydrophila*	Li and Howard[62] Ast et al.[63] Vanderlinde et al.[64]
T3SS	MxiM	MxiJ	MxiD	*Shigella flexneri*	Schuch and Maurelli[65] Lario et al.[56] Okon et al.[66]
	ExsB		PscC	*Pseudomonas aeruginosa*	Izoré et al.[57] Perdu et al.[67]
	YscW		YscC	*Yersinia enterocolitica*	Burghout et al.[68] Ross and Plano[132]
	InvH		InvG	*Salmonella enterica*	Daefler and Russell[69] Craig and Koronakis[70]
	YsaP		YsaC	*Yersinia enterocolitica*	Rau and Darwin[71]
T4aPS	PilW	PilP	PilQ	*Neisseria meningitidis*	Trindade et al.[59] Carbonnelle et al.[72] Golovanov et al.[58]
	PilF		PilQ	*Pseudomonas aeruginosa*	Koo et al.[60]
	Tgl		PilQ	*Myxococcus xanthus*	Nudleman et al.[73]
T4bPS		BfpG	BfpB	*Escherichia coli*	Lieberman et al.[74]
		TcpQ	TcpC	*Vibrio cholerae*	Chang et al.[75]
		FimV	PilQ	*Pseudomonas aeruginosa*	Wehbi et al.[76]
		CpaE	CpaC	*Caulobacter crescentus*	Viollier et al.[77]
	TadD		RcpA	*Aggregatibacter actinomycetemcomitans*	Clock et al.[78]

function.[80,81] It is evolutionarily related to the flagellum assembly system and is widely used by bacteria to establish relations of mutualism or pathogenicity with eukaryotic organisms, including animals, plants, and fungi.[81–83] A remarkable characteristic of this system is the needle complex that encompasses both inner and outer membrane rings and is completed by a protruding hollow needle. This apparatus allows the passage of substrates in semiunfolded form into the eukaryotic cytoplasm through a pore formed directly on the target cell membrane, thus forming a direct channel between the bacterial cytoplasm and that of the host.[16,24,84–90]

As is the case for the T2SS, T3SS secretin assembly can also be guided by partner proteins (Figure 3, Table 1). The first identified T3SS pilotin was YscW (formerly known as VirG) from *Yersinia enterocolitica*, a lipoprotein responsible for targeting the secretin YscC to the outer membrane, facilitating its oligomerization.[68] Soon other pilotins were identified, such as InvH, responsible for localization and functionality of InvG in the outer membrane of *Salmonella enterica*,[69,70,91] and MxiM, involved in stability, localization, and assembly of MxiD in *Shigella flexneri*.[65] Other examples of T3SS pilotins include YsaP, involved in localization of the YsaC secretin in *Y. enterocolitica*,[71] and ExsB, a YscW homologue shown to be critical for PscC targeting and assembly in *P. aeruginosa*[57,67] (Figure 3, Table 1).

Structures of MxiM[56,66] and ExsB[57] reveal folds that are different from T2SS pilotins. Both proteins are predominantly β-stranded, but while the seven β-strands of ExsB form an antiparallel sandwich with a short α-helix between β4 and β5, MxiM presents eight β-strands that

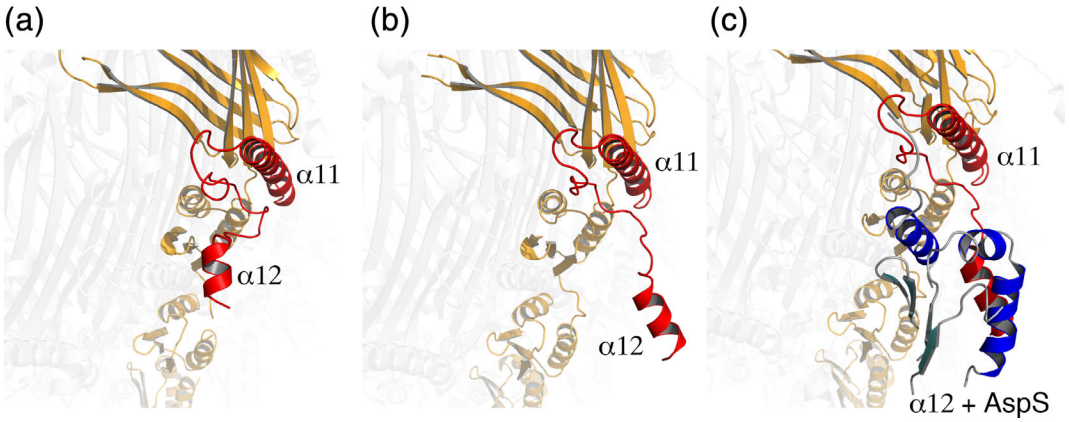

FIGURE 4 Pilotin recognition requires a conformational modification within the S-domain of T2SS secretins. One monomer is indicated in orange, with the S domain in red, while the rest of the GspD secretin is indicated in light gray. (a) In the absence of the AspS pilotin, α12 is positioned close to the body of the secretin. (b) Pilotin recognition of α12 requires that it move away from the homo-oligomer. (c) AspS (in blue) recognizes the "open" location of α12[36]

form a pseudo-barrel that is interrupted by a long α-helix (Figure 3). NMR and ITC studies of the interaction of MxiM and InvH with the S-domains of their cognate secretins revealed that central residues of these domains become structured upon binding of the pilotins, as was observed in the PulS-PulD system described above.[33,66] Interestingly, in the case of the MxiM-MxiD interaction, the cavity within the S-domain of the secretin was shown to bind either lipid or the pilotin, suggesting a mechanism in which the pilotin only becomes lipid-free in the presence of the secretin.[66]

Lastly, the presence of other accessory proteins that participate in secretin localization and assembly is not exclusive of T2S systems. In *S. flexneri*, one of the IM ring-forming proteins, MxiJ, works synergistically with the pilotin MxiM, and the presence of either protein can stabilize the secretin.[65] In enteropathogenic *E. coli* (EPEC), the IM ring protein EscD assists the oligomerization of EscC by interacting with its C-terminus.[92]

4 | ASSEMBLY OF T4PS SECRETINS

Many bacterial strains display surface pili, which are long (1–5 μM), fiber-like appendages. In Gram-negative organisms, these include the Type IV and chaperone-usher pili.[93] Type IV pili (T4P) are highly dynamic, and possess the remarkable ability to quickly extend and retract repeatedly. This ability is crucial for multiple functions, including adherence, motility, DNA uptake, and protein secretion.[94–100]

T4PS assembly is coordinated by a complex machinery that encompasses both inner and outer bacterial membranes, and the pilus itself is formed by polymerized pilin subunits arranged helically.[101] The core T4PS machinery includes four main components: an ATPase, located at the base of the system and which provides energy for pilus extension; an IM platform; the pilus filament itself; and an outer membrane secretin.[93] T4PS pilins are synthesized as prepilins in the cytoplasm and translocated toward the periplasmic side of the IM by the Sec system, as is the case of T2SS pseudopilins.[102,103] Prepilins are then processed by dedicated peptidases into mature pilins.[104] Energy transduced from the cytoplasmic ATPase through an IM platform guides polymerization and depolymerization of the filament[105,106] that emerges in the outer membrane through the secretin channel.[4,107–109] Interestingly, Gram-positive bacteria[110] and archaea[111] also present homologous T4P systems, though the secretin is mostly absent.

The most widely accepted classification of Type IV pili was generated based on sequence similarities of the pilin-coding genes.[112] Type IVa pili (T4aP) include those of *Pseudomonas*, *Neisseria*, *Dichelobacter*, *Thermus*, *Myxococcus*, and *Deinococcus* spp, among others, while Type IVb pili (T4bP) are represented in enteropathogenic, enterohemorrhagic, and enterotoxigenic *E. coli*, *S. enterica*, *Caulobacter crescentus*, and *V. cholerae*. In what relates to secretin assembly, outer-membrane pilotin-like accessory lipoproteins have been identified for T4aP,[59,60,72,73,113] but T4bP secretins require other stability factors (Table 1) and often undergo auto-assembly.[74,114,115]

4.1 | T4aP

Stability and assembly of *Neisseria* spp, *P. aeruginosa* and *Myxococcus xanthus* T4aP secretins (PilQ) are dependent on the presence of the lipoproteins PilW, PilF, and Tgl, respectively, since in their absence only the monomeric form of

PilQ can be detected.[72,73,116] However, outer membrane targeting of these secretins seems to depend on different pathways: while in *N. meningitidis* deletion of *pilW* does not affect localization[72] and PilQ is probably inserted by the BAM system,[117] *P. aeruginosa* PilF pilots PilQ to the outer membrane in a Lol-dependent manner.[60,118] It is interesting to note that Tgl can be transferred from *tgl*+ cells to *tgl*− mutants through a contact-dependent mechanism, inducing the formation of PilQ multimers.[73]

Crystal structures of PilW and PilF reveal similar superhelical folds, with 13 anti-parallel α-helices that fold into six TPR (tetratricopeptide repeat) motifs.[59,60,119] A similar number of TPRs is predicted for Tgl.[73] TPRs are thermostable motifs that mediate protein–protein interactions and are often critical parts of large complexes.[120] The TPR superhelix fold of PilW and PilF differs clearly from the α/β folds of the classical pilot proteins described above, indicating that their function could be distinct from the "piloting" of secretin monomers. Rather, their highly charged convex region could interact with other T4PS proteins or with the negatively charged outer membrane, while the concave groove could be involved in partner protein recognition.[59,120] Interestingly, TPR motifs found in chaperones involved in T3SS needle assembly do employ their conserved concave interface to allow partner binding.[121–123] In PilW, a disulfide bond interconnects the two parts of the TPR superhelix, and plays a role in its functionality.[59,113]

Other factors that may be involved in PilQ assembly and stabilization are the accessory factors FimV, PilP, and TsaP. FimV is a peptidoglycan-binding factor found in *P. aeruginosa* that participates in IM subcomplex formation and ensures efficient multimerization of the secretin, in a similar manner to ExeAB in the T2SS of *A. hydrophila*, although the two proteins do not present sequence similarities.[76,124] In fact, FimV has multiple binding partners and possibly different functions, including a role in Type II secretion and regulation of cAMP production,[125,126] functions in which its TPR domains could play a key role.[127]

PilP is an inner-membrane anchored lipoprotein present in *N. meningitidis*, *N. gonorrhoeae*, and *P. aeruginosa*. It binds directly to PilQ and is essential for T4PS formation.[72,128] Following the N-terminal lipid attachment site, PilP presents an unstructured region and a C-terminal globular domain that folds as a 7-stranded β-sandwich[58] and presents structural homology with the T2SS IM protein GspC.[41] Structural studies of the PilQ:PilP interaction involving cryo-EM, NMR, and modeling indicate that PilP interacts at the interface between the central and peripheral rings of PilQ, with a potential role in stabilizing PilQ during the secretion process, in order to prevent channel disruption.[116,129]

In the pathogen *N. gonorrhoeae*, absence of TsaP (T4PS secretin-associated protein) results in formation of multiple pili in membrane protrusions instead of on the surface of the cell, indicating its function in extrusion of pili from the periplasm. This protein is in fact part of the peripheral ring observed in cryo-EM maps of *M. xanthus* T4aP systems, and also plays roles in peptidoglycan attachment and T4PS localization to cell poles.[28,29]

4.2 | T4bP

Many T4bP secretins are lipoproteins.[74,115] In the EPEC bundle-forming pilus (BFP), BfpB has been shown to be recognized by the Lol pathway,[74] which can be involved in its outer membrane targeting, as is the case for HxcQ in the T2SS.[79] In addition, the accessory protein BfpG is critical for assembly of a functional BfpB in the outer membrane, but only at the multimerization step.[114] Likewise, in the toxin-coregulated pilus (TCP) of *V. cholerae*, despite the fact that the TcpC secretin presents a lipidation signal, it also requires the periplasmic protein TcpQ for outer membrane localization and stability, and in its absence TcpC is degraded.[115] Recent electron cryotomography studies reveal that TcpC appears as a ring around the periplasmic domain of TcpQ.[75] Insight into the fold of TcpC was obtained by homology modeling using the Type 4 secretion system (T4SS) protein VirB7, which displays a compact globular structure that is reminiscent of the N0 domain of secretins.[75]

The Flp pilus or Tad pilus is a distinct subclass of T4bP[130] and is sometimes called the Type IVc pilus.[97] Unlike other T4bPs, its secretins (CpaC from *C. crescentus* and RcpA from *Aggregatibacter actinomycetemcomitans*) are not lipidated and in fact are very similar to those of the T2SS.[78] CpaC requires CpaE for correct polar localization and for multimer assembly,[77] and outer membrane insertion may be assisted by the BAM machinery.[131] Meanwhile, in *A. actinomycetemcomitans*, the TPR-containing TadD lipoprotein appears to be involved in RcpA stabilization, assembly, protection from proteolysis and targeting to the outer membrane,[78] performing a role that is similar to that of pilotins.

5 | CONCLUDING REMARKS

The combination of structural biology, biochemistry, and microbiology approaches have been instrumental in the comprehension of the assembly and functionality of secretins and of the systems in which they are involved. Nevertheless, there are still multiple questions that should be addressed in the future. One of the most

interesting points involves the precise orchestration of secretin regulatory steps, as well as their potential interaction with the peptidoglycan and secreted substrates. Advances in single particle electron microscopy, cryo-electron tomography, and the application of high-resolution fluorescence microscopy strategies to the study of secretins and their complexes will undoubtedly shed light on these questions both from *in vitro* and *in situ* perspectives.

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