Multiple structures disclose the Secretins Secrets

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\textbf{ABSTRACT}

Bacterial secretins are outer membrane proteins that provide a path for secreted proteins to access the cell exterior/surface. They are one of the core components of secretion machines and are found in type II and type III secretion systems (T2SS and T3SS). The secretins comprise giant ring-shaped homo-oligomers whose precise atomic organization was only recently deciphered thanks to the spectacular developments in cryo-electron microscopy (cryo-EM) imaging techniques.

\textbf{KEYWORDS:} T2SS, Secretin, GspD
Gram-negative bacteria have evolved highly complex molecular nanomachines which span the whole cell envelope to ensure access of proteins/enzymes to the extracellular medium or to inject toxins into other target cells. This mechanism of protein secretion is important to ensure bacterial survival and pathogenesis. The secretion systems have been classified over the years according to the nature of the different proteins that make the building blocks. There are thus differences but overall the idea is to form a channel in the membrane that will funnel proteins to be secreted. Each set and each genuine machine was then coined a type, which add up now from type I secretion system (T1SS) to type IX secretion system (T9SS). The nomenclature has slightly gone wild but experts in the field would recognize their favorites. The complexity of these supramolecular assemblies and their membrane localization has long been an issue to obtaining high-resolution structure of these nanomachines.

One of the first key structures in protein secretion systems to be resolved was the TolC trimer from the T1SS (1), which did not differ too much from classical monomeric outer membrane proteins (Figure 1 left part). However, despite forming a beta-barrel, in this case the TolC barrel takes shape only through assembly of a homo-trimer while a large coiled-coiled domain protrudes in the periplasm and makes a hermetic conduit connecting the inside of the cell with the outside world. Thanks to recent outstanding improvements in structural biology and particularly in cryo-electron microscopy (Cryo-EM), acknowledge by the 2017 Nobel prize of Chemistry, the pace at which larger secretion systems could be seen with the naked eye and at molecular resolution has dramatically accelerated. We can now for example visualize the whole T3SS (2, 3) or exquisite details of the T4SS (4). In the latter case, it came with a surprise since what makes the outer membrane channel has no resemblance with our standard belief, and particularly the portion of the protein which plugged the system in the outer membrane is not a β-barrel but a series of amphipatic α-helices. This was also telling us that we still know very little about the molecular options bacteria have evolved to shape an outer membrane channel. Secretins are one of these options and were always suspected to form giant pores (5). They are found in the T2SS to allow the release of large folded virulence factors, or in type 4 pilus assembly systems (T4PS) and T3SS for the assembly and the movement of cell surface appendages. A paper in this issue, by Hay and colleagues in the laboratory of Trevor Lithgow at Monash University (6), describes the 3D high resolution structure of a T2SS secretin from
Enteropathogenic *Escherichia coli* (EPEC), which together with previously published structures is illuminating our understanding of these astounding objects.

There has been a long series of papers which built our understanding of secretin structure that probably originated from the first observation of very low-resolution EM images of ring-shaped structure for YscC (T3SS), XcpQ (T2SS) and PilQ (T4PS) (7, 8). Slowly but convincingly, it was shown that secretins are large homo-oligomeric assemblies of 12 to 16 monomers forming a distinctive bipartite ring-shaped and cylindrical structure on their C- and N-terminal sides, respectively. They comprise a well-conserved membrane embedded C-terminal domain and a modular periplasmic N-terminal domain constituted by the piling up of structurally independent sub-domains labeled N0 to N3 forming a cylindrical structure. In contrast to the conserved C-domain, the number of N-domains and structural organization is variable and dependent on the nanomachine-type. While four so called N sub-domains, N0, N1, N2 and N3, are found in T2SS secretins, T4PS and T3SS secretins are shorter due to the absence of N1-N2 and N2 sub-domains respectively. This might reflect a specificity associated with these domains and could depend on the kind of secretion performed. In all cases, the N-domain is thought to protrude deep into the periplasm, just like the TolC funnel did. However, it has a totally different organization, and in this case, it likely plays an important role in effector recognition in addition to connecting outer and inner membranes.

During two decades of extensive trials the atomic organization of the C-domain (also called pore forming or β-domain) of the secretins was hunted. Combining biochemistry, imaging and re-projections, low-resolution cryo-EM images produced for PulD from *Klebsiella* (9), GspD from *Vibrio* (10) or PilQ from *Neisseria* at 12 Å (11) contributed to provide a rough but sensible model of a saucer-like complex with gated properties which would sit in the outer membrane. However, the long awaited higher resolution at 7.4 Å was first glimpsed to the impatient scientific community only in 2016 with a study on the *Pseudomonas aeruginosa* PilQ T4P secretin (12). A few months later the 3.6 Å structure of the *Salmonella enterica* InvG T3SS secretin was published (PDB code 5TCQ) (figure 1) (3). As much as when the T4SS structure was first published, it was for our viewing pleasure to realize that many novel unpredictable features could be found in the secretin organization and how much ingenuity bacteria demonstrated in varying structures on a same theme (Figure 1). The secretin C-domain is indeed unique and appears to be formed by a giant pentadecameric double-layered β-barrel with an
outer 60-stranded anti-parallel β-sheet constituted by the 15 4-stranded β-sheets of each subunits. The high-resolution structure also showed an additional inner barrel whose beta strands extend into the channel lumen and form the periplasmic gate that separates the internal periplasmic chamber and the top lumen. This C-domain is believed to form the OM portal through which secreted substrates or cell surface appendages transit to the cell surface. The C-terminal S domain has been shown to be involved in secretin localization, assembly and outer-membrane insertion by interacting with cognate chaperones known as pilotins (13). In the high-resolution structure, the S-domain is well defined and forms a helix-turn-helix motif that extends laterally across neighboring protomers on the exterior of the β-barrel thus providing structural arguments that in addition to pilotin-binding, S domains have a key role in efficient secretin assembly and stability (Figure 1).

In the meantime, the high-resolution cryo-EM structures of two Klebsiella- and Vibrio-types (14) T2SS secretins, from *Escherichia coli* K12 (PDB code 5WQ7) and *Vibrio cholerae* (PDB code 5WQ8) (Vibrio-type), were reported at ~3 Å resolution (figure 1) (15). While confirming the global 3D structure organization observed in the T3SS secretin’s β-domains this dual presentation highlights interesting differences between the two T2SS secretins (Figure 1). The major difference is the presence of a gate cap in the Vibrio-type which is absent in the Klebsiella-type as can be clearly seen in Figure 1. The cap gate of Vibrio-type channel has a cone-like structure formed by 15 pairs of anti-parallel β12–β13 strands. The linker loops between outer β-barrel and cap gate (β12 and β13 strands) are relatively loose which suggests that the cap gate of Vibrio-type is probably independent of other structural parts therefore constituting a signature distinguishing two different classes of secretins (15). The internal diameters of the cap and central gates of the secretins are not compatible with the diameters of the secreted substrates and therefore suggest that purified full length secretins are in a “closed state” and must undergo conformational changes during the secretion process. Yan and colleagues (15) proposed that the flexible linker loops on the bottom of the cap gate may act as pivots to allow β-strands in the cap gate to rotate outward during substrate secretion. Regarding the central gate, formed by a four β-strands extension from the inner β-barrel, site directed mutagenesis of the highly conserved glycine residues located at the bend points in those β-strands, revealed their involvement as pivot to allow the conformational changes of β-strands in the central gate. The authors therefore proposed that upon substrate loading into the lumen of the Vibrio-type channel, it reaches the
central gate and triggers the upwards rotation of the \( \beta \) strands around the glycine pivots. This also involves the force provided by a distinct element in the T2SS, the periplasmic pseudopilus, to allow substrate entry into the top lumen. With the uninterrupted drive of the periplasmic pseudopilus, \( \beta 12 \) and \( \beta 13 \) of the cap gate are forced to move outward to open the cap gate and release the substrate. After substrate release, strands of the cap and central gates revert to their closed-state conformations to start a new translocation cycle.

All together the high-resolution structures of the three secretins revealed that the N3 sub-domain, which is common to all secretins, structurally belongs to the C-domain (3, 15) (Figure 1). It was moreover shown that the N2 domain is linked to the N3 domain through a long linker loop inducing a large flexibility between N0-N1-N2 sub-domains and the upper N3/C-domain. This bipartite structural organization is supported by previous studies showing that the N3 sub-domain plays an important role in secretin prepore stabilization by forming a thermodynamic seal (16). These observations suggest that secretins are structurally organized into two independent domains, the N-domain composed by N0, N1 and N2 sub-domains and the C-domain constituted by the rest of the protein (N3 and former C-domain).

In the paper published in this issue by Hay and colleagues (6), it seems obvious that secretin structures are likely to adopt slight variations that may accommodate specific function even with secretin from a same subfamily. The study presents the cryo-EM high-resolution 3D structure of the T2SS secretin from Enteropathogenic E. coli, EPEC O127:H6 str. E2348/69 (PDB code 5W68), at 3.3 Å (Figure 1) and compare the structure with the other available high-resolution secretin 3D structures. The upper chamber, inner and cap gates are clearly resolved. The N-domain was less well resolved than the upper secretin part suggesting that there is a relatively high flexibility/movement among the N-domains. Based on sequence analysis, the authors previously proposed that the EPEC secretin belongs to the Vibrio-type sub-family (14). In agreement with sequence similarities, a similar structural architecture can be seen between the two Vibrio-type secretins, i.e. the EPEC structure from Hay and colleagues in the present issue (6) and the Vibrio structure from Yan and colleagues (15), with loops extending from the extracellular side to form the typical gate-cap (Figure 1). It is noticeable that the EPEC and K12 E. coli secretins diverge and obvious differences are apparent in the cap structure which is longer in the EPEC or Vibrio-type secretins, and protrudes outside the outer membrane in a closed state. Interestingly, the electrostatic charge of the interior of the cavities are also dissimilar, the EPEC
secretin contains alternating positively and negatively charged bands whereas the \textit{E. coli} K12 
\textit{Klebsiella}-type secretin is largely negatively charged. This would be in agreement with the 
different shape and electrostatic properties of their respective secreted substrates \textit{i.e.}, mucinases 
StcE and SslE for EPEC and the pullulanase PulA for \textit{K. oxytoca}, thus constituting a new 
playground to investigate substrate specificity in the T2SS. In their study, Hay and colleagues 
clarify secretion topography by defining in the EPEC T2SS secretin the hydrophobic belt and 
aromatic girdle signature that they found highly conserved among the sequences of 581 \textit{Vibrio}-
type secretins.

Overall, with their structure-based blast experiment, Hay and colleagues (6) revealed that 
the previous T2SS secretins classification, \textit{i.e.} \textit{Vibrio} and \textit{Klebsiella}-types (14), is definitely 
structurally relevant. Moreover, in providing an additional high-resolution 3D structure of a 
secretin, these authors are giving us a booster shot on the structural diversity existing among 
T2SS secretins and are lending support to the existence of subtle functional differences. This 
should be seen as an encouragement for the resolution of additional structures which is essential 
to pinpoint singularities of significant common features.

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**FIGURE LEGEND:**

**Figure 1. 3D high-resolution structures of secretion systems channels and their membrane localization.** Shown here to scale in the bacterial envelope, from left to right, are the typical monomeric β-barrel outer membrane protein, OMPLA (1FW2), the T1SS channel TolC (PDB 1TQQ) (1) and four bacterial secretins: the T3SS (5TCQ) (3), T2SS *Klebsiella*-type (5WQ7) and T2SS *Vibrio*-types (5WQ8) (15) and (6W68) (6) all sharing cylindrical structures. Such cryo-EM near atomic 3D structures reveal the more complicated structural organization of bacterial secretins adopting a novel double-β-barrel architecture involving the N3 sub-domain (N3), a partial β-barrel transmembrane and showing the stabilizing property of the S domain (S). Such resolution also highlights subtle differences among members of bacterial secretins including the optional cap gate and different mode of outer membrane (OM) insertion including a possible curvature of the OM for T3SS secretins. IM stands for Inner membrane.
Figure 1.

OMPLA 1FW2  T1SS TolC 1TQQ  T3SS Secretin 5TCQ  T2SS Klebsiella-type Secretin 5WQ7  T2SS Vibrio-types Secretins 5WQ8 1FW2

Periplasmic space

Cap gate 100 Å