PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2012) **367**, 1059–1072
doi:10.1098/rstb.2011.0204

Review

On the path to uncover the bacterial type II secretion system

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Gram-negative bacteria have evolved several secretory pathways to release enzymes or toxins into the surrounding environment or into the target cells. The type II secretion system (T2SS) is conserved in Gram-negative bacteria and involves a set of 12 to 16 different proteins. Components of the T2SS are located in both the inner and outer membranes where they assemble into a supramolecular complex spanning the bacterial envelope, also called the secreton. The T2SS substrates transiently go through the periplasm before they are translocated across the outer membrane and exposed to the extracellular milieu. The T2SS is unique in its ability to promote secretion of large and sometimes multimeric proteins that are folded in the periplasm. The present review describes recently identified protein–protein interactions together with structural and functional advances in the field that have contributed to improve our understanding on how the type II secretion apparatus assembles and on the role played by individual proteins of this highly sophisticated system.

Keywords: type II secretion system (T2SS); secreton; pseudopilus; secretin; protein–protein interaction

1. INTRODUCTION

Gram-negative bacteria are surrounded by a dual membrane structure establishing an interface between the environment and the interior of the cells. The two membranes are separated by an aqueous periplasmic space containing a rigid peptidoglycan layer. This cell envelope constitutes a highly selective barrier for uptake and release of various compounds. Gram-negative bacteria have evolved several highly specialized secretory pathways to release proteins into their surrounding environment. Among them, the type II secretion pathway is a two-step process dedicated to the secretion of folded and/or oligomeric exoproteins. This ability to secrete large molecules is extremely valuable and is achieved by a sophisticated molecular nano-machine embedded in the bacterial envelope called the secreton.

The type II secretion pathway is conserved in Gram-negative bacteria [1] with prevalence in bacterial pathogens of plants (*Pseudomonas fluorescens, Erwinia* or *Xanthomonas* species), animals (*Aeromonas hydrophila*) and humans (*Klebsiella oxytoca, Pseudomonas aeruginosa, Vibrio cholerae* or *Legionella pneumophila*) [2–5]. The number of proteins secreted via the T2SS by any given organism is variable and ranges from one, in the case of *K. oxytoca* [6], to more than ten in *P. aeruginosa* [7], *V. cholerae* [8] or *L. pneumophila* [9]. The functions of these proteins are extremely diverse and include toxins [10,11], surface-associated virulence factors [12,13], cytochromes [14] and a broad range of enzymes that hydrolyse macromolecules such as lipids, polysaccharides and proteins [15].

(a) *Genetic organization*

Typical type II secretion systems (T2SSs) are encoded by a set of 12 to 16 *gsp* (general secretion pathway) genes organized into large operons including the conserved 'core' genes denoted $gspC_P$ to O_A and in some bacterial species extra *gsp* genes such as *gspAB*, *gspN* or *gspS* (figure 1). Because a different nomenclature is used for *Pseudomonas* and non-*Pseudomonas* T2SSs, the alternative gene or protein nomenclature is indicated throughout the review. For example, in $GspE_R$ the 'R' refers to the *Pseudomonas* XcpR T2SS component, which is reciprocally called $XcpR_E$. Apart from rare exceptions, mutation in any *gsp* gene prevents secretion and causes accumulation of the exoproteins in the periplasm. The genetic organization of the T2SS clusters is remarkably conserved. However, in some species, the position of the $gspC_PD_Q$ genes is peculiar (figure 1). In the *P. aeruginosa xcp* cluster, these two genes form an operon divergent from the operon containing the $gspE_R$-M_Z genes. In *Xanthomonas*

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One contribution of 11 to a Theme issue 'Bacterial protein secretion comes of age'.

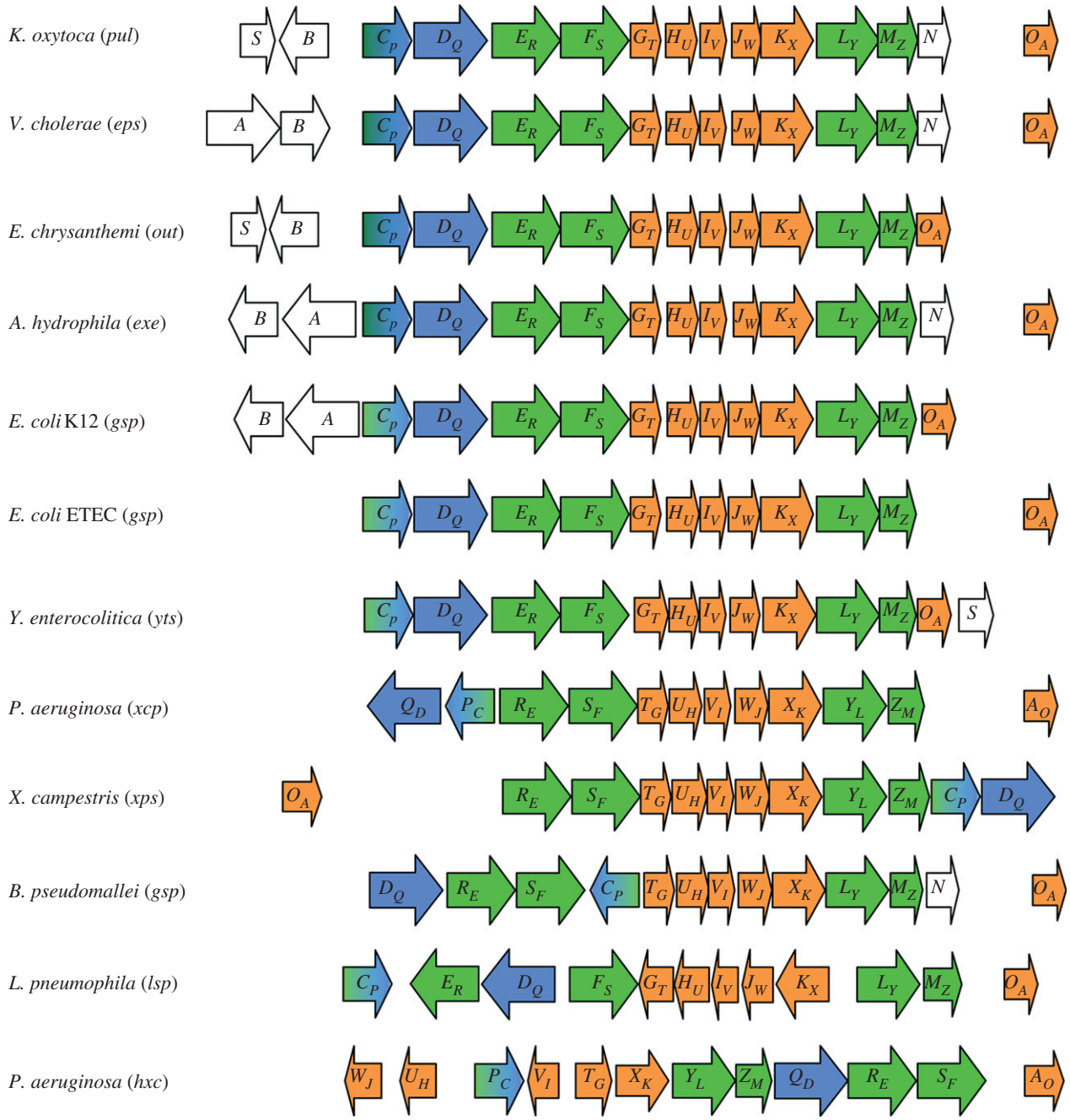

Figure 1. Genetic organization of the T2SS clusters. The name of each T2SS gene cluster is shown in brackets beside the name of the bacterial species. Each gene is represented by an arrow and 'core' genes present in all T2SS clusters are represented in colour. The *gspE*$_R$, *F*$_S$, *L*$_Y$ and *M*$_Z$ genes encoding components of the inner membrane platform are shown in green; the *gspG*$_T$, *H*$_U$, *I*$_V$, *J*$_W$ and *K*$_X$ genes encoding pseudopilins and *gspO*$_A$ gene encoding the prepilin peptidase are shown in orange; the *gspD*$_Q$ gene encoding the secretin is shown in blue; the *gspC*$_P$ gene encoding the trans-periplasmic protein is represented in shaded green and blue tones because GspC$_P$ is a component of the inner membrane surface interacting with secretin. The *gspA*, *B*, *N* and *S* genes that are not considered to be core components of the T2SS are represented in white.

campestris, the *gspC*$_P$*D*$_Q$ genes are found after *gspM*$_Z$ at the end of the *gsp* operon (figure 1). Exceptions are with the T2SS genes in *Burkholderia pseudomallei* and *L. pneumophila*, where *gspC*$_P$ and *gspD*$_Q$ are not next to each other. Finally, it should be noted that the whole *P. aeruginosa hxc* (for homologous to *xcp*) cluster [16] has a radically different organization of its genes. This T2SS is used by *P. aeruginosa* for the secretion of a single exoprotein, the alkaline phosphatase LapA, and the *hxc* genes are expressed in phosphate-limited growth conditions [16]. The specificity of the *P. aeruginosa* Hxc system versus the more general Xcp pathway is probably not linked with growth in phosphate starvation conditions since phosphate-regulated phospholipases (PlcH, –N and –B) [17,18], or alkaline phosphatase, PhoA [19], are all secreted via the Xcp machinery. Interestingly, Durand *et al.* [20] recently identified specific Hxc phenotypes suggesting the existence of two T2SS subtypes called T2aSS and T2bSS to which, Xcp and Hxc, respectively, belong. Indeed, the authors propose that the secretion process of the Hxc T2bSS of *P. aeruginosa* involves a pseudopilus whose structure and stability may differ from the one commonly found in Xcp and other known T2aSSs. A

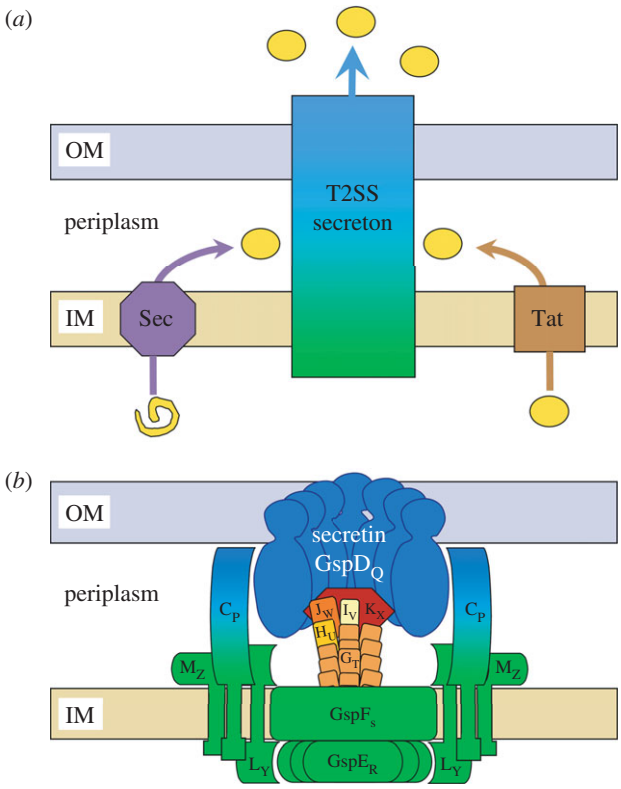

Figure 2. Model of the type II secretion pathway in Gram-negative bacteria. (*a*) The T2SS-dependent exoproteins, shown as yellow circles, are first exported across the IM via the Sec (purple) or Tat (brown) machineries. The exoproteins are subsequently recognized and transported across the OM by the secreton (blue/green). (*b*) Schematic of the secreton divided into three sub-complexes. Secretin $GspD_Q$ (blue) forms a dodecameric pore in the OM through its C-terminal domain whilst its N-terminal part protrudes in the periplasm. The IM surface, composed of T2SS proteins F_S, L_Y and M_Z, and the traffic ATPase $GspE_R$ are coloured green. Secretin is connected to the IM surface through the transperiplasmic protein $GspC_P$ (blue/green). The pseudopilus, mostly constituted by the $GspG_T$ major pseudopilin and capped by the minor pseudopilins $GspH_U$, I_V, J_W and K_X quaternary complex, is shown in orange and red. Gsp proteins are indicated by their corresponding letter.

second T2SS called Stt has also recently been identified in *Erwinia chrysanthemi* (now called *Dickeya dadantii*) where it involves cell-surface targeting of a non-conventional T2SS substrate, PnlH, possessing a non-cleavable Tat-dependant amino-terminal targeting signal [21].

(b) *The type II secretion pathway*

Exoproteins that use the T2SS are secreted into the extracellular medium by a two-step process in which the proteins are exported across the cytoplasmic membrane and released into the periplasm before being transported across the outer membrane (OM) (figure 2*a*). Exoproteins requiring cytoplasmic folding are exported through the inner membrane (IM) by the Tat export pathway, while translocation of unfolded protein precursors through the IM goes via the Sec export system [5,17]. In a second step, the folded exoproteins, transitorily localized in the periplasm, are translocated across the OM in a T2SS-dependent manner, thus involving the trans-envelope supramolecular complex, called the secreton, made of the different Gsp proteins.

(c) *Structural organization of the secreton*

Based on data obtained by many different experimental approaches, including subcellular localization, protein–protein interactions between individual components of the T2SS and resolution of protein structure, the current model for the secreton is represented by three functional sub-complexes (figure 2*b*). An inner membrane platform (IMP) (figure 2*b*, green) is composed of the $GspC_P$, F_S, L_Y and M_Z IM proteins; the cytoplasmic traffic ATPase $GspE_R$ is associated with this through an interaction with the bitopic protein $GspL_Y$ [22–25]. The secreton also contains five proteins that display homologies with the type IV pilin PilA and are designated pseudopilins [26–28]. These proteins have been proposed to be involved in the formation of a fibrillar piston-like structure, the pseudopilus (figure 2*b*, orange/red) [29–33]. Finally, $GspD_Q$, the OM component of the system, belongs to the secretin family and likely constitutes the channel giving T2SS substrates access to the extracellular medium (figure 2*b*, blue) [34,35]. Whereas the proton motive force has been shown to be involved in the translocation of T2SS substrates across the OM [36,37], $GspE_R$, which contains motifs characteristic of traffic ATPases, also contributes to energize the T2SS-dependent process [38,39] and could drive the pseudopilus through the $GspD_Q$ channel, pushing out exoproteins to the external medium [15,40,41].

(d) *Cellular localization of the secreton*

In *P. aeruginosa* the number of assembled secreton machines is thought to be relatively low and has been estimated at 50–100 secretons per cell [34]. Moreover, while results obtained in *K. oxytoca* and *V. cholerae* [42–44] with GFP-fused Gsp proteins indicate a circumferential distribution of the machinery into foci, the *P. aeruginosa* Xcp secreton was proposed to be polar. This was shown by adding a Lumino tag onto XcpR or XcpS or by the visualization of protease secretion with an intramolecularly quenched casein conjugate [45]. Such discrepant results could be due to artefacts related to the artificial production of the reporters used, as clearly demonstrated by Lybarger *et al.* [42]. Alternatively, it cannot be ruled out that cellular localization of T2SSs might vary from one species to another. Interestingly, further localization experiments of the secreton, which were performed in various *gsp* backgrounds, indicate that in contrast to other Gsp proteins, secretin does not need other secreton components for correct localization in the bacterial envelope, thus suggesting an assembly of the secreton from the OM [42]. This relatively new concept of molecular machines assembly from their OM secretins was also recently proposed for the type III secretion machinery [46].

In this review, we will summarize what is currently known about the individual organization of the three secreton sub-complexes briefly outlined in this introduction. We will particularly highlight new findings on solved protein structures and protein–protein interactions among and between the three sub-

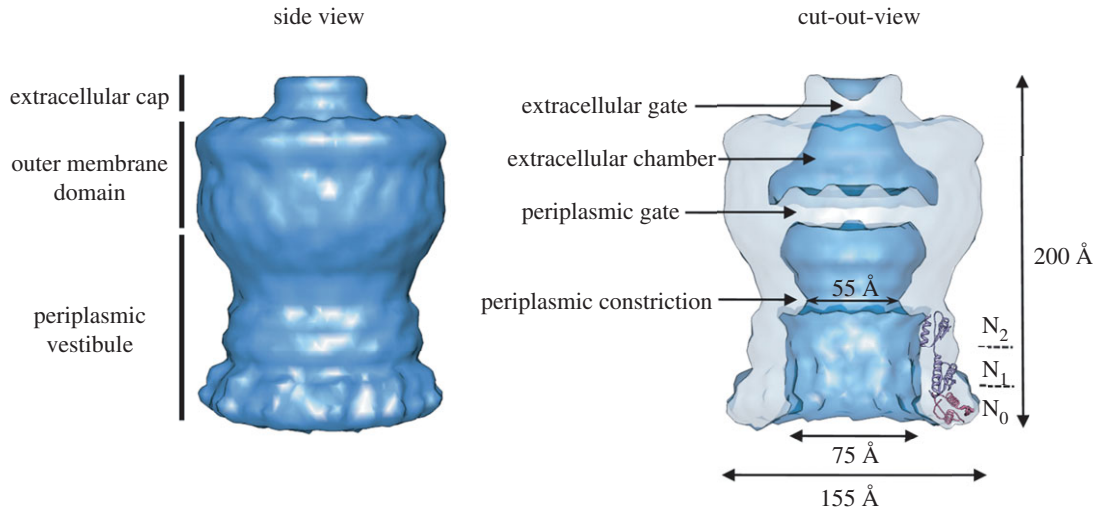

Figure 3. Electron microscopy structure of T2SS secretin. Cryo-EM reconstitution of *V. cholerae* T2SS secretin $GspD_Q$ at 19 Å resolution (EMDB1763 and adapted from [61] by permission from Nature Publishing Group). The $GspD_Q$ cryo-EM density reveals a cylindrical channel assembly 155 Å in diameter and 200 Å in length. In side view, three domains are identified from top to bottom: the extracellular cap, the outer-membrane domain and the periplasmic vestibule domain. In a cutout view, secretin contains an extracellular chamber limited by an extracellular gate and a periplasmic gate. The vestibule domain shows a constriction which results in a narrowing of the channel diameter from 75 to 55 Å. The crystal structure of the N-terminal periplasmic subdomains N_0–N_1–N_2 from ETEC [62] is fitted into the $GspD_Q$ periplasmic vestibule (adapted from [47] by permission from Elsevier).

complexes. Finally, we will propose an integrative model for T2SS assembly and mechanism.

2. THE OUTER MEMBRANE SECRETIN

Secretins are members of a protein superfamily [47] involved not only in T2SS, but also in type III secretion system [48], type IV pilus assembly [49], DNA uptake and extrusion of the filamentous phage [50]. These proteins form large homo-multimers of 12–15 subunits assembled in the OM [51]. They form a ring-shaped structure with a central cavity 50–80 Å in diameter [47].

In *K. oxytoca* and *E. chrysanthemi*, the insertion of the secretin in the OM has been shown to depend on the presence of a small OM lipoprotein, the pilotin GspS [41,52]. This protein has chaperone-like properties since it is involved in the protection of the secretin from proteolytic degradation. PulS is also involved in secretin transport since in its absence, *K. oxytoca* secretin PulD mislocalizes to the IM [53], indicating that a lipid-anchored chaperone is required for efficient and correct insertion of the secretin into the OM. To date, genes encoding GspS members have not been found in all T2SSs (figure 1). Therefore, it cannot be ruled out that genes with low homologies, and which are not associated with the *gsp* cluster, could encode proteins playing the same function as GspS [54]. Alternatively, secretin transport is assisted in some species by the non-core components GspAB. Since the *X. campestris* ExeA directly interacts with the peptidoglycan layer, the complex may contribute to create space in the peptidoglycan mesh to allow the transport and assembly of the megadalton-sized secretin multimer in the OM [55]. Finally, some T2SS secretins do not require any specific assistance for their transport to the OM. This is the case for the liposecretin HxcQ of *P. aeruginosa*, which is targeted to the OM by its N-terminal lipid anchor [56]. Interestingly, it has been shown that transport to the OM of the T2SS secretin PulD is not dependent on the general Bam OM protein transport pathway [57]. It is therefore possible that secretins use the Lol lipoprotein transport route for their transport to the OM either directly for liposecretin [56] or via their pilotin [58]. Alternatively, it cannot be excluded that some secretins use the Bam pathway as was demonstrated for the *Neisseria* type IV pilus secretin PilQ [59].

Secretin monomers are bipartite proteins ranging from 50 to 70 kDa in size. Homology among members of the secretin family resides in the C-terminal half of the protein that is important for oligomerization, whereas the N terminus is conserved only within subgroups from related transport pathways and thought to be involved in system-specific interactions [60]. Recently, Reichow *et al.* [61] have solved the low-resolution structure of the *V. cholerae* full-length $GspD_Q$ ($EspD_Q$) secretin using cryo-electron microscopy (figure 3). The cryo-EM reconstitution of the *V. cholerae* secretin at 19 Å resolution suggests a dodecameric structure reminiscent of a barrel with a large internal channel containing two compartments separated by a closed periplasmic gate, a periplasmic vestibule and an extracellular chamber located in the OM (figure 3).

The N terminus of T2SS secretins comprises four structurally independent domains, N_0–N_3 (figure 4). The three-dimensional structure of the N_0–N_1–N_2 region of the enterotoxigenic *Escherichia coli* (ETEC) $GspD_Q$ secretin has been solved at 2.8 Å by X-ray crystallography [62]. This structure has been used to generate a 12-fold symmetrical ring, which was fitted into the density map of the full length *V. cholerae* $EpsD_Q$ obtained by cryo-EM [61] (figure 3). The reconstitution confirmed that the C-terminal and N-terminal regions are two structurally separate domains located in the OM and the periplasm, respectively. Since only low-resolution structures have been obtained for the

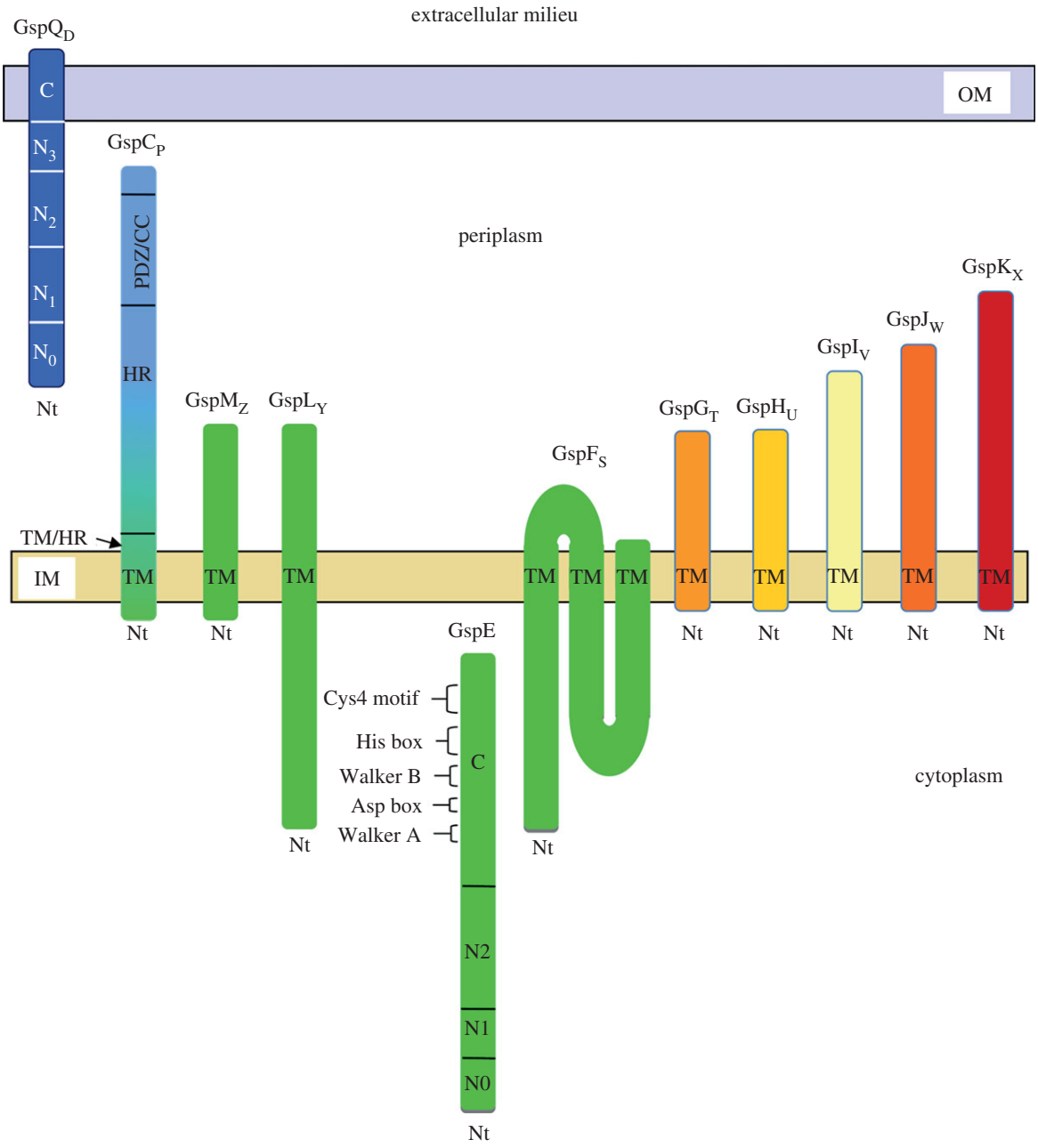

Figure 4. Topology of the Gsp proteins. Schematic of the topology of each individual component of the secreton. The domains of the Gsp proteins discussed in the review are mentioned. The colour code used is the one generally used in all figures, i.e. blue for OM secretin, green for IMP components and orange/red for pseudopilins. The N terminus (Nt) and the transmembrane (TM) domain of each Gsp protein are indicated where appropriate. CC, coiled coil.

pore-forming part of the secretin, the precise structural folding of this domain remains unknown. Like the majority of the bacterial OM proteins, secretins are predicted to adopt a β-barrel structure [63]. For example, the topology of the $XcpQ_D$ secretin together with predictions for β-strands in the primary amino acid sequence has previously been assessed [64]. If this is the case, whether this domain is formed by one large single homomultimeric β-barrel or by the assembly of 12 individual β-barrels remains an open question. Alternatively, it is still a likely possibility that secretin pores do not form β-barrels but adopt alternative α-helical folds. Such an original fold was first identified in the *E. coli* capsular polysaccharides OM pore Wza [65], but is also required for insertion of the type IV secretion OM protein VirB10 in *Agrobacterium tumefaciens* [66] or the *P. aeruginosa* PelC protein [67]. The hypothesis of an α-helical fold in T2SS secretins is supported by the observation that transport of this group of proteins does not involve the Bam complex [57] which is required for insertion of OM proteins forming β-barrels [59,68].

A recent cryo-EM study showed that the T2SS-dependent cholera toxin binds in the lower part of the periplasmic vestibule of the *V. cholerae* $EpsD_Q$ secretin [69]. This observation is in agreement with the previous interaction found between the T2SS secretin $OutD_Q$ and its cognate substrate PelB [41] and confirms the role of the secretin in substrate binding. How the substrate further travels through the pore is still unknown but the closed state of the channel suggests that several conformational changes might occur in the secretin core to accommodate the

substrate and to trigger communication between the different chambers. In addition, and as we shall see in §3–7, other components of the secreton such as the transperiplasmic protein $GspC_P$ and the pseudopilus are also involved in this process since they also interact with the substrate [70].

3. THE TRANS-PERIPLASMIC PROTEIN $GSPC_P$

$GspC_P$ proteins are bitopic IM proteins that are the least conserved of the T2SS components. Proteins of this family are organized into several domains including an N-terminal cytoplasmic region, a transmembrane (TM) domain, a highly conserved central periplasmic domain (homology region, HR) and a C-terminal part containing specific secondary structures such as coiled-coil domains (in *P. aeruginosa* and *Pseudomonas alcaligenes*, for instance) or PDZ domains (in *K. oxytoca* and *E. chrysanthemi*) (figure 4) [71]. $GspC_P$ was shown to be active as a dimer and self-associates by its TM domain, which is not a simple membrane anchor but plays an active role in the function of the protein [72]. The HR domain of *V. cholerae* $GspC_P$ ($EpsC_P$) was shown to interact directly with the periplasmic N_0 domain of the secretin $EpsD_Q$ [44,73]. This interaction was also seen in *E. chrysanthemi* where the interaction site of $OutC_P$ on $OutD_Q$ was localized between residues 139 and 158 of the HR domain. This peptide called $OutC_{P\text{-}sip}$ '*secretin interacting peptide*' recognizes two different sites on the $OutD_Q$ secretin. The first one is localized on domain N_0, whilst the second one sits astride domains N_2 and N_3 [74]. Furthermore, mapping of the interaction between $XcpP_C$ and $XcpQ_D$ in *P. aeruginosa* showed that the N_3 domain is essential for this interaction [70]. These different interaction sites between secretins and GspCs suggest that $GspC_P/D_Q$ partners have evolved various strategies to interact with each other.

Several genetic data indicate that $GspC_P$ and $GspD_Q$ form a functional couple determining the specificity of the machinery. For example, in the very closely related *E. chrysanthemi* and *E. carotovora* Out systems, all genes are individually exchangeable except for $outC_P$ and $outD_Q$ [75]. Similar investigations performed comparing *P. aeruginosa* and *P. alcaligenes* also indicate that $XcpP_C$ and $XcpQ_D$ are determinants of substrate specificity [76]. In order to localize the $GspC_P$ domain directly or indirectly involved in substrate specificity, chimeras between *E. chrysanthemi* $OutC_P$ and *P. aeruginosa* $XcpP_C$ domains have been generated and their ability to support secretion in *P. aeruginosa* tested [71]. Interestingly, $XcpP_C$ chimeras containing the TM, HR or C-terminal domain of $OutC_P$ remain functional, indicating that none of these domains play a role in substrate specificity. However, the replacement of the intermediary domain between TM and HR called TM/HR (figure 4) leads to secretion defect, suggesting that this domain plays an essential role in specificity and potentially in substrate recognition. Recently, a set of *in vitro* experiments has revealed direct interactions between purified $XcpP_C$ and two substrates of the Xcp T2SS, the elastase (LasB) and the lipase (LipA) [70]. Importantly, no interaction was detected using the substrate of the second *P. aeruginosa* T2SS (Hxc), i.e. the alkaline phosphatase (LapA). These observations revealed that the species-specificity of the T2SS mechanism is largely contributed by the exoproteins and involves $GspC_P$ and $GspD_Q$, which directly interact with cognate substrates.

4. THE ATPASE OF THE SYSTEM: $GSPE_R$

A functional T2SS requires the presence of a traffic ATPase, $GspE_R$. Traffic ATPases are involved in several other transport machines such as type IV secretion, conjugation and type IV piliation systems. Structural analysis on the type IV secretion and type IV piliation ATPases indicated that they may function as dynamic hexamers [77,78]. The members of the traffic ATPase superfamily are characterized by two nucleotide-binding motifs designated Walker A and B boxes and also His and Asp boxes (figure 4) [79]. $GspE_R$ proteins have a characteristic Walker box A containing the P-loop of an NTP-binding motif, and a less well-defined Walker B box in which the second conserved aspartate residue is replaced by either a glycine or an alanine. Mutation of a conserved glycine residue within the Walker A motif of $GspE_R$ from *P. aeruginosa*, *K. oxytoca*, *E. chrysanthemi* or *V. cholerae* causes the bacteria to be secretion-defective, showing the important role played by this protein in the secretory process [80–83]. Mutations in the less conserved Walker B box have little or no effect on the secretion process [83]. The T2SS traffic ATPase family is distinct from other ATPases in three additional conserved regions: first the aspartate box 'Asp Box' between the Walker A and B boxes consisting of two short aspartate-rich motifs (figure 4), which is required for the function of $GspE_R$ in the secretion process and may be involved in the formation and stabilization of the nucleotide-binding fold by interacting with Mg^{2+}[83]; second, the His box, including two histidine residues, which is located downstream of the Walker B box, although the role of the His box in $GspE_R$ function is still unknown; last, a tetracysteine (Cys4) motif that appears to be essential for function, since replacement of any of the cysteine residues by a serine within the *K. oxytoca* $GspE_R$ leads to a large decrease in pullulanase secretion (figure 4) [15].

$GspE_R$ traffic ATPases lack hydrophobic domains and exhibit the general characteristics of a cytoplasmic protein (figure 4). However, they were found to be associated with the IM through an interaction with the bitopic protein $GspL_Y$ [80,84]. Results obtained from *V. cholerae* indicate that ATP hydrolysis by the $EpsE_R/EpsL_Y$ complex is stimulated by acidic phospholipids, whereas the activity of $EpsE_R$ alone is unaffected [85]. Further mutagenesis revealed that the membrane-proximal region of the cytoplasmic domain of $EpsL_Y$ subtly controls the interaction of $EpsE_R$ with the cytoplasmic membrane and influences its oligomerization, thereby stimulating its ATPase activity [85]. Other results from *X. campestris* have shown that $XpsE_R$ oligomerization, as well as its association with $XpsL_Y$, requires ATP binding but not ATP hydrolysis, thus indicating that association between $XpsE_R$ and $XpsL_Y$ is needed for ATPase activity [86].

The crystal structure of a truncated *V. cholerae* $EpsE_R$ protein lacking the N-terminal 90 residues was determined with or without the nucleotide bound [87].

These structures reveal a two-domain architecture with the five characteristic motifs of the $GspE_R$ subfamily clustering around the nucleotide-binding site in the C-terminal domain. The $EpsE_R$ subunits form a right-handed helical arrangement in the crystal with extensive and conserved contacts between the C and N domains of neighbouring subunits, thus suggesting that $EpsE_R$ is organized as a hexameric structure. The hexameric state of $GspE_R$ is confirmed by results obtained in *V. cholerae* and *X. campestris* showing that optimal ATPase activity is obtained with hexameric $GspE_R$ [38,86]. The crystal structure of the N-terminal part of *V. cholerae* $EpsE_R$ in complex with the cytoplasmic domain of *V. cholerae* $EpsL_Y$ showed that these two proteins form a hetero-tetramer in which $EpsL_Y$ forms a central dimer and $EpsE_R$ binds at the periphery [88].

Amino acid sequence alignments have shown that $XpsE_R$ of *X. campestris* contains an additional N-terminal extension not found in most other $GspE_R$s. This additional domain appears to be essential for $XpsL_Y$ binding, therefore indicating that a more sophisticated interaction process between $GspE_R$ and $GspL_Y$ might occur within the Xps secreton of *X. campestris* [89]. To date, the structure of a full-length $GspE_R$ has not been reported and this would provide key and definite structural information about the architecture of $GspE_R$ monomer and multimer.

5. GSPF$_S$, L$_Y$ AND M$_Z$: THE IMP STABILIZERS

$GspF_S$ is a polytopic integral membrane protein with a small periplasmic loop and two large cytoplasmic domains connected by three TM regions (figure 4) [90,91]. Two-hybrid studies have shown that the N-terminal domain of the *E. chrysanthemi* $OutF_S$ protein interacts both with $OutE_R$ and $OutL_Y$ [24] suggesting that $OutF_S$ could participate in the stability of the IMP [92]. Construction of a chimera between *P. aeruginosa* and *P. putida* $XcpS_F$ has shown that interaction with other T2SS components is mediated by the cytoplasmic domains [25].

$GspL_Y$ is a bitopic IM protein organized in three domains, the C-terminal domain localized in the cytoplasmic compartment, the TM domain, and the periplasmic domain (figure 4) [22]. The structures of both cytoplasmic and periplasmic domains of EpsL in *V. cholerae* have been solved at 2.7 and 2.3 Å, respectively [93,94]. The cytoplasmic part is composed of subdomains I, II and III and was shown to interact with the N-terminal part of $GspE_R$ through subdomains II and III [88].

$GspM_Z$ is a bitopic protein [22] with a short cytoplasmic domain, a TM domain and a periplasmic domain (figure 4) involved in homo-dimerization [24,95,96]. $GspM_Z$ was shown to be required for $GspL_Y$ stability since the amount of the former is greatly dependent on the presence of the latter [23]. Studies on $GspM_Z$ variants in *P. aeruginosa* revealed that three periplasmic domains of the protein were found to be important for interaction with $GspL_Y$. Two distinct stabilizing domains were localized, respectively, at the beginning and at the end of the periplasmic part of the protein whereas the third one, localized next to the TM domain, also required the presence of the transperiplasmic protein $GspC_P$ to promote $GspL_Y$ stabilization [97]. The influence of $GspC_P$ on the stability of the $GspL_Y/GspM_Z$ complex was also observed in *X. campestris* since $GspL_Y$ dissociates faster from the $GspL_Y/GspM_Z$ complex than from the $GspC_P/L_Y/M_Z$ one [98]. In addition, antibodies against $GspM_Z$ co-immunoprecipitated $GspL_Y$, $GspC_P$, and $GspE_R$ from detergent-solubilized cell extracts, confirming the existence of a complex containing these four proteins [99].

6. THE PSEUDOPILUS: A CENTRAL STRUCTURE OF THE T2SS MACHINE

Six of the 12 conserved *gsp* genes are dedicated to the formation of a periplasmic pilus-like structure called the pseudopilus (figure 1). Five of those genes, $gspG_TK_X$ encode the pseudopilins which are the constitutive elements of the pseudopilus, whereas $gspO_A$ encodes the prepilin peptidase involved in their maturation [26–28,100,101]. Like the closely related type IV pilins involved in type IV pilus formation, the five pseudopilins are synthesized as precursors with a short leader peptide of 6–7 mostly charged residues that is cleaved off by the prepilin peptidase $PilD/GspO_A$. Mature pilins and pseudopilins are characterized by a highly conserved N-terminal hydrophobic domain of about 20 residues followed by a C-terminal extension specific for each pilin and pseudopilin [15]. Topology studies have shown that pilins and pseudopilins are bitopic IM proteins with a single N-terminal trans-membrane domain segment and a periplasmic C-terminal globular domain (figure 4) [27,101]. Similar to typical IM proteins, pseudopilins use the Sec/SRP pathway for their membrane targeting and insertion [90,102]. While it is an essential step, the significance and role of pseudopilin maturation by the peptidase is unknown. Nevertheless, the removal of cytoplasmic positive charges may facilitate extraction of the protein from the membrane. Interestingly, it was shown that pseudopilins co-fractionate with both IM and OM fractions [28,103], suggesting either a re-localization of these proteins to the OM after processing or more likely the formation of a supramolecular complex.

Among the five pseudopilins, $GspG_T$ is the most abundant and is therefore called the major pseudopilin [27], in contrast to $GspH_U$, I_V, J_W and K_X, which are named minor pseudopilins. Biochemical data obtained in *X. campestris* revealed the presence of the major pseudopilin, $XpsG_T$, within a large complex of about 440 kDa [103]. This observation clearly favoured the formation of a pilus-like structure spanning the bacterial envelope. In agreement with this hypothesis, it was shown that when $GspG_T$ is overproduced it is able to assemble into an unusually long fibrillar structure protruding out of the cell, which closely resembles the type IV pilus [32,33,104,105]. Such a structure, also called a hyper pseudopilus (HPP), is only obtained upon overproduction of $GspG_T$ pseudopilins and probably represents an uncontrolled elongation of what could be a physiologically relevant pseudopilus. Based on crystallographic and electron microscopy data, an assembly model of $GspG_T$ into HPP has been generated by a molecular modelling approach. This pseudo-atomic model was

experimentally validated and showed how protomers of $GspG_T$ interact with each other to form the pseudopilus [31,106,107]. The model proposes a right-handed helical organization of the T2SS pseudopilus, consistent with the type IV pilus structure [108].

The four minor pseudopilins $GspH_U$–K_X are unable to assemble into an HPP when overproduced [105], but their role in pseudopilus formation is undeniable. Results obtained with *K. oxytoca* or *P. aeruginosa* pseudopilins have shown that $GspI_V$ is essential for HPP formation and could play a major role during the initiation step of fibre formation [33,105]. It was also proposed that HPP elongation is controlled by $GspK_X$ [101] since its length varied depending on the number of $GspK_X$ subunits produced [104,105]. While none of the four minor pseudopilins have ever been detected in HPP structures [32,33,104], their presence in a native pseudopilus became clear in light of identified protein–protein interactions and resolved three-dimensional structures. The four minor pseudopilins share the typical αβ-fold commonly found in major pilins and pseudopilins with a long N-terminal α-helix involved in their helical oligomerization [29,107,109–118]. Three of the four minor pseudopilins ($GspI_V$, J_W and K_X) have been co-crystallized and shown to form a ternary complex through their globular domains [29]. Recently, a quaternary complex containing the globular domains of the four minor pseudopilins was identified, indicating that the fourth minor pseudopilin, $GspH_U$, also belongs to the minor pseudoplin complex [30]. Structural prediction using the entire amino acid sequence of the minor pseudopilins, i.e. including also the N-terminal hydrophobic domain, suggests a similar helical assembly as the one observed for $GspG_T$. Since extra major pseudopilins could only be added beneath the minor pseudopilins complex [29], it was proposed that the tetrameric complex was localized at the tip of the $GspG_T$ pseudopilus. Given that $GspH_U$ was previously shown to interact with the major pseudopilin $GspG_T$ [103,119], $GspH_U$ could constitute the hinge between the tip and the core of the pseudopilus [30]. Taking into account the resemblance to the type IV piliation system, the phenotypic, structural and interaction network data lead to a reasonable model for pseudopilus architecture and biogenesis which consists of the prior assembly of the quaternary tip complex in the IM. The tip complex could then be driven to the secretin by the addition of major pseudopilin subunits underneath. Given that $GspK_X$ has a large globular domain and is located at the very end of the pseudopilus, its involvement in pseudopilus length control could consist of stopping pseudopilus growth when contacting the secretin. Therefore, when the physiological stoichiometry between $GspK_X$ and $GspG_T$ is respected, the length of the pseudopilus is likely to be restricted to the periplasmic space. Only when a large excess of $GspG_T$ is produced, may the pseudopilus grow without a tip and therefore pass through the secretin channel. This model, which requires experimental validation, is nevertheless supported by the direct interaction observed between minor pseudopilins and secretin [61,96].

The pseudopilus assembly likely occurs as in the type IV piliation process with the energetic assistance of the $GspE_R$ and PilB traffic ATPases, respectively. If the retraction process exists in type II secretion, it could be associated with a piston-like mechanism of the pseudopilus, but probably involves a different mechanism since there is no counterpart in the T2SS for the PilT ATPase that disassembles type IV pili [120,121]. Interestingly, the minor pseudopilin $GspK_X$ was found to interact with $GspG_T$, and this interaction triggers a destabilization of $GspG_T$ [105]. An alternative retraction process can therefore be proposed for the T2SS pseudopilus, i.e. upon contact with the secretin pore, $GspK_X$ acquires, possibly upon conformational changes, the capacity to interact with $GspG_T$, thus leading to pseudopilus collapse. An ATPase-free retraction event might thus be sufficient to support the disassembly of a short trans-periplasmic pseudopilus.

7. SUBSTRATE RECOGNITION AND TRANSPORT BY THE T2SS MACHINE

The type II secretion apparatus is widespread in Gram-negative bacteria and a wide variety of enzymes and toxins use this pathway. We have already alluded to the species-specificity of this system; i.e. cognate exoproteins from one T2SS are not recognized by another machinery [75]. T2SS substrates are loaded on the nanomachine in the periplasm and translocated across the OM in a folded conformation [122–124]. Moreover, studies in *E. chrysanthemi, K. oxytoca* and *P. aeruginosa* have demonstrated that disulphide bridges are formed within exoproteins before secretion [125–128]. The high specificity demonstrated for T2SSs and their substrates, as well as their specific recognition in the periplasm among all other resident proteins, suggests the existence on folded substrates of a secretion motif that is required for T2SS recognition. Many studies have been carried out on T2SS-dependent exoenzymes in order to define this secretion motif, which is still a biological puzzle [129]. They all converge to the idea of a conformational signal gathering several motifs spread along the primary amino acid sequence of the protein. With the *K. oxytoca* pullulanase PulA, it was shown that two non-adjacent regions were together necessary to promote translocation of PulA-β-lactamase hybrid proteins across the OM [130]. Another study suggested that at least three regions of PulA might contain information that influences its secretion [131]. It was also suggested that *P. aeruginosa* exotoxin A (ToxA) contains two separate secretion signals [132], while alteration of another region also affects secretion efficiency [133]. Finally, the polygalacturonase PehA of *E. carotovora* was found to contain three separate domains involved in T2SS targeting [134,135]. As reported above, the secretion signal may be composed of residues from different locations in the linear polypeptide chain, which are brought together into a conformational patch during protein folding [136]. One alternative to a single structural motif is that successive specific interactions lead to the secretion of exoproteins. These interactions may involve different secretion signals that are not essential individually but are required simultaneously, or sequentially, for optimal secretion. Interestingly, secretion of the *E. chrysanthemi* cellulase Cel5 involves a transitory intramolecular interaction between the cellulase binding domain and

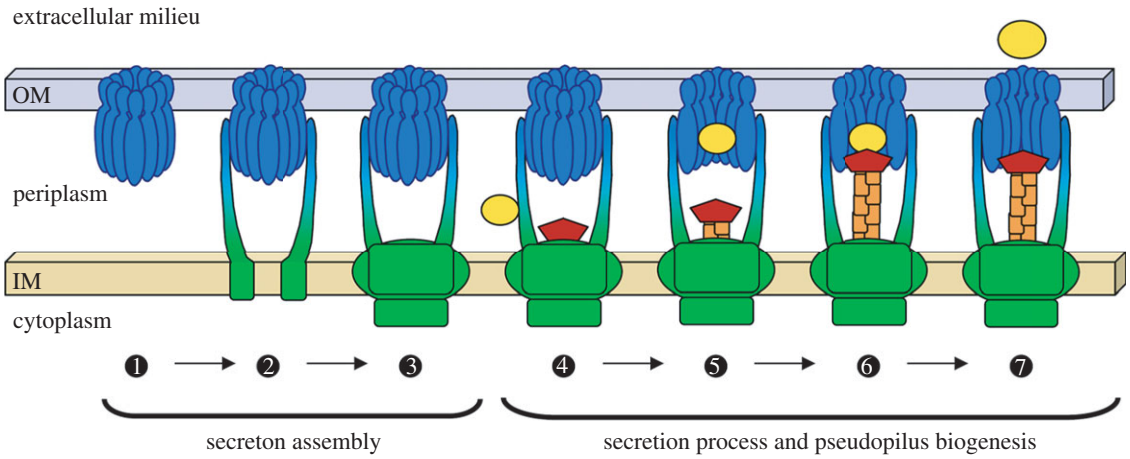

Figure 5. Model of secreton assembly and operation. Schematic of secreton biogenesis as discussed in this review. The first part of the secreton to be assembled is proposed to be the secretin ❶. The next step could be the successive recruitment of the trans-periplasmic protein $GspC_P$ ❷ and of the inner membrane surface ❸. The recognition of the substrate by the T2SS takes place in the periplasm and may involve a peripheral element of the secreton, $GspC_P$ ❹. The substrate is then transferred to the secretin vestibule ❺ in which it could contact the pseudopilus tip complex that is emerging from the inner membrane surface ❻. The exoprotein could then be released in the extracellular medium through the secretin pore ❼. The secretin and periplasmic domain of $GspC_P$ are shown in blue, the components of the inner membrane surface are shown in green, the pseudopilus and the secreted proteins are shown in orange/red and yellow, respectively.

a region close to the active site [137]. In this case, the exoprotein could adopt a secretion-competent conformation prior to secretion and another conformation once released in the extracellular medium [138]. This sequence of events is probably not the case with *P. aeruginosa* and *V. cholerae* T2SSs for which interactions found between substrates and secreton components were detected using purified secreted proteins [41,61,70]. Obviously the question of specific substrate recognition is far from being resolved. One may consider that co-evolution of T2SS machines together with their cognate substrate has resulted in a progressive adaptation to obtain an optimal fit.

Recently, several direct interactions have been identified between secreted substrates and periplasmic domains of secreton components. The previously identified interaction with the secretin [41] was confirmed by two independent studies which both used a highly sensitive technique, surface plasmon resonance or BIAcore [61,70]. By using this technique, Douzi *et al.* [70] have explored the *P. aeruginosa* T2SS periplasmic interaction network and, in addition to the secretin, two other substrate interactants, the transperiplasmic protein $XcpP_C$ and the pseudopilus tip. This set of interactions may suggest that the transperiplasmic element $XcpP_C$ might recruit the substrate and transfer it to the pseudopilus tip which then carries it towards the secretin through which it could be translocated (figure 5).

8. A MODEL FOR SECRETON ASSEMBLY AND MODE OF ACTION

Type II protein secretion occurs in two steps. Secreted proteins are first exported across the IM and then released in the extracellular medium thanks to a sophisticated machine, the secreton. The secreton is composed of at least 12 different proteins embedded in the bacterial envelope and organized in a large multiprotein complex capable of secreting a wide range of folded exoproteins across the OM of Gram-negative bacteria. Based on structural data, protein–protein interactions, and phenotypic observations described in this review, it is possible to propose an innovative model for secreton biogenesis and functioning (figure 5). In this model, secreton biogenesis starts by the insertion of secretin in the OM, thus defining the secretion site [42]. In the second step, the transperiplasmic element $GspC_P$ binds the secretin, therefore allowing docking of IMP. Indeed, cellular localization experiments performed in *V. cholerae* have shown that, in contrast to secretin, $GspC_P$ needs $GspD_Q$ but not $GspM_Z$ which itself needs both $GspC_P$ and $GspD_Q$ for proper localization [42]. We propose that the transitory periplasmic T2SS substrates are first recruited by the peripheral element $GspC_P$ and then transferred to the secretin vestibule. The substrate could then be contacted by the pseudopilus tip, and could be pushed and expelled from the cell through the secretin by a growing pseudopilus. This tentative model is in good agreement with the majority of the data collected so far about the T2SSs, but as any working model, it should be challenged and used to design experimental approaches that will confirm or disprove the views presented in this review. For example: (i) Is there a dedicated location for the T2SS in the cell envelope? If yes, how is the first element of the system, say the secretin, targeted there? (ii) Even if we are getting closer to understand substrate recognition by the machinery, the identity of the secretion signal remains enigmatic. (iii) Does the binding of the substrate to $XcpP_C$ trigger the assembly of the whole system as previously shown for the type I secretion system [139] or does it only trigger pseudopilus elongation? (iv) Does the pseudopilus elongate upon contact of the substrate on $GspC_P$ or later when it is positioned in the secretin vestibule? (v) How is the substrate released from the secretin? Is it through a mechanical movement operated by the pseudopilus or following conformational changes within the secretin or both? (vi) Does the pseudopilus effectively

retract? If yes, what are the molecular mechanism and energy source associated with this event?

Whereas all these questions remained to be addressed, it is remarkable to see the improvement of our understanding of the T2SS over the past few years. This is largely due to the ever increasing performance of structural and biochemical techniques that have generated a lot of new data and come to complement and back up all the original genetic data. Ideally, one would like to see the three-dimensional reconstruction of the whole T2SS machine and even better have this megastructure in motion while transporting the exoprotein. Such level of achievement might not be so far away considering the advances that are currently being made in understanding motion in proteins using molecular dynamics computer simulations [31,140].

We should like to thank M. Tegoni, C. Cambillau, C. Bernard, S. Alphonse, K. T. Forest, L. Franz and D. Dyer for fruitful collaborations, as well as E. Durand, G. Ball, B. Ize, S. Bleves and G. Michel for their contribution to the research undertaken in our laboratory on type II secretion. Research on the bacterial type II secretion system in the laboratory of Romé Voulhoux is supported by the Centre National de la Recherche Scientifique (CNRS) and the '3D-Pilus' young researcher ANR grant (ANR-JC 07-183230). Badreddine Douzi is supported by a BDI-PED PhD grant. Alain Filloux is supported by the Royal Society.

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