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Review

The underlying mechanisms of type II protein secretion

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Abstract

The cell envelope of Gram-negative bacteria is composed of two membranes, which are separated by the peptidoglycan-containing periplasm. Whereas the envelope forms an essential barrier against harmful substances, it is nevertheless a compartment of intense traffic for large proteins such as enzymes and toxins. Numerous studies dealing with the molecular mechanism of protein secretion have revealed that Gram-negative bacteria evolved different strategies to achieve this process. Among them, the type II secretion mechanism is part of a two-step process. Exoproteins following this pathway are synthesized as signal peptide-containing precursors. After cleavage of the signal peptide, the mature exoproteins are released into the periplasm, where they fold. The type II machinery, also known as the secreton, is responsible for the translocation of the periplasmic intermediates across the OM. The type II system is broadly conserved in Gram-negative bacteria and involves a set of 12–16 different proteins named GspC-M, GspAB, GspN, GspO, and GspS. The type II secretion system is highly reminiscent of the type IV piliation assembly system. Based on findings about the subcellular localisation of the Gsp components, protein–protein interactions between Gsps and their multimerisation status, structural data and electron microscopy observation, it could be proposed a working model that strikingly runs both systems in parallel.
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1. Introduction

Bacterial membranes are essential barriers, which preserve the integrity of the organism. However, the cell envelope must be sufficiently permeable to allow the traffic of molecules into and out of the cells. This is essential for the acquisition of nutrients, the uptake of DNA, the liberation of enzymes or toxins, or the assembly of organelles such as pili and flagella on the cell surface. The cell envelope of gram-negative bacteria consists of two membranes delimiting the periplasm. Nutrient molecules are small enough to diffuse through outer membrane porins and are subsequently actively transported across the inner membrane (IM) into the cytoplasm. Extracellular enzymes and toxins are much larger molecules that use dedicated secretory pathways.

The general secretory pathway (GSP) is a mode of translocation, in which the exoproteins cross successively, in two steps, the inner and outer membranes (OM). The first step involves translocation across the cytoplasmic membrane. The transported protein is synthesised as a precursor containing an N-terminal cleavable signal peptide. This precursor is targeted and transported through the IM via a proteinaceous complex constituting the Sec translocon [1]. The signal peptide is then cleaved by the leader peptidase and the mature protein is released into the periplasm. This first series of events is called the general export pathway (GEP). The exoprotein then requires another machinery, an extension of the GEP, to assist its translocation across the OM. This second event is called the terminal branch of the GSP. Several branches have been identified.

This review will focus on a particular transport process, the type II secretion mechanism or main terminal branch (MTB) of the GSP, which involves 12–16 different proteins that constitutes the "secreton". It should be mentioned that it is now demonstrated that the type II secreton can also be supplied with substrates by another IM translocation apparatus, known as the Tat system [2]. This Tat system is dedicated to the transport of folded co-factor containing proteins with a special signal sequence featuring a twin-arginine motif [3]. Hence, the designation "general secretory pathway" is not entirely accurate. The components of

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the type II secreton were first discovered in *Klebsiella oxytoca* [4], and their subsequent identification in *Pseudomonas aeruginosa* demonstrated that they are conserved among most gram-negative bacteria [5]. The type II secreton appeared to be an important determinant of bacterial virulence and was recently identified as such in bacterial pathogens such as *Legionella pneumophila* [6] or *Yersinia entrocolitica* [7]. In *K. oxytoca*, the secreton components are called Pul, whereas they are called Xcp in the case of *P. aeruginosa*. More recently, a second type II cluster, called *hxc*, has been identified in *P. aeruginosa* [8]. The fourth letter for the designations of the homologous components between the different systems is usually identical, e.g. PulE of *K. oxytoca* is homologous to OutE of *Erwinia chrysanthemi*. Unfortunately, however, for historical reasons, the fourth letter of the designations of the pseudomonad Xcp components is deviating; for example, XcpR is homologous to PulE. Gsp is currently the common term used to describe any proteins of the type II secreton. For clarity, we will slightly modify the nomenclature, for example, we will use $GspE_R$, indicating that XcpR belongs to the GspE family.

2. The GSP proteins

The genetic determinant of all known type II systems is a set of 12–16 genes mostly clustered and organised into large operons including *gspC*$_P$-*O*$_A$, *gspAB*, *gspN* and *gspS* (Fig. 1). In case of *P. aeruginosa* and *Pseudomonas alcaligenes*, the genes are organized into two divergently transcribed operons [9]. Mutations in most of these genes abort the secretion process resulting in the accumulation of exoproteins within the periplasm. Thus, *gsp* genes are essential for OM translocation. Surprisingly, the deduced amino acid sequences and initial characterisation of the proteins indicated that only two of the 16 are effectively located in the OM. All but one of the other proteins are anchored in the cytoplasmic membrane, with the remaining protein peripherally associated with the cytoplasmic side of the IM.

2.1. Pseudopilins and their dedicated peptidase

2.1.1. GspO$_A$, a specialised peptidase

P. aeruginosa and *Aeromonas hydrophila gsp* gene clusters have an unusual feature in that *gspO*$_A$, usually the last gene in the operon, is not clustered with the other *gsp* genes (Fig. 1). It is found at another chromosomal location, clustered with genes required for the assembly of type IV pili, and called *pilD* and *tapD*, respectively [10,11]. Type IV pili were originally defined as long cell surface appendages located at the poles and involved in a particular type of motion called twitching motility [12]. PilD and TapD are the prepilin peptidases required for the processing of the N-terminal leader peptide of the pilin subunit before its assembly into pilus. The leader peptide of these subunits consists of a stretch of six to seven residues with an overall positive charge, preceding a hydrophobic domain of approximately 20 residues (Fig. 2) [13]. In some cases the leader peptide could be longer, about 25 residues, these preproteins being referred as type IVb (instead of type IVa for the short leader peptides). The processing site is located immediately before the hydrophobic region, after a highly conserved glycine residue (G – 1). Mature pilin subunits are then helically packed via interactions between their hydrophobic N-terminal domains and are assembled into pilus. A conserved glutamate residue, at position + 5 within the hydrophobic domain (E + 5), may be a key element in the registration mechanism associated with the assembly of the pilin subunits [14]. The new N-terminal residue of pilin, a conserved phenylalanine (F + 1) or a hydrophobic residue such as the methionine of the TcpA pilin of *Vibrio cholerae* [15], is methylated during processing. This second posttranslational

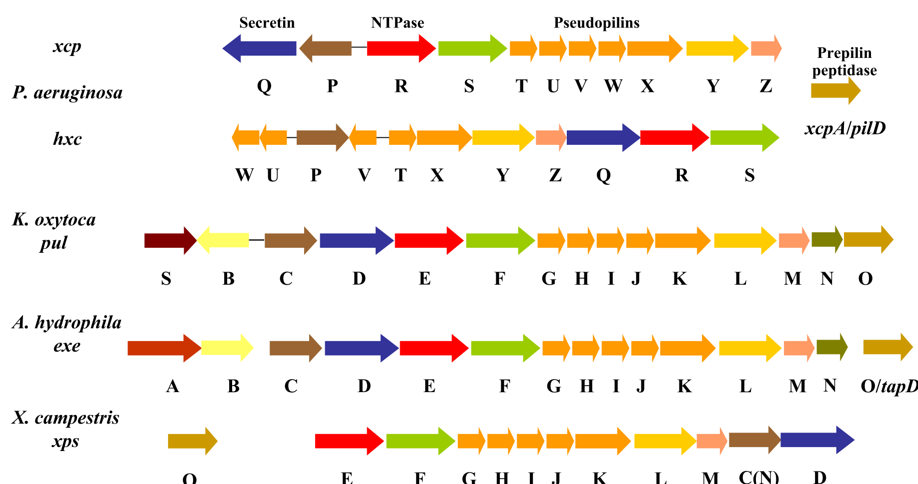

Fig. 1. Gene organisation of type II gene clusters from *P. aeruginosa* (*xcp* and *hxc*), *K. oxytoca* (*pul*), *A. hydrophila* (*exe*) and *X. campestris* (*xps*). Common terms used for a number of Gsp components are indicated. In case of *X. campestris* the *xpsN* gene has tentatively been renamed *xpsC*. Unlinked genes are located at another chromosomal locus.

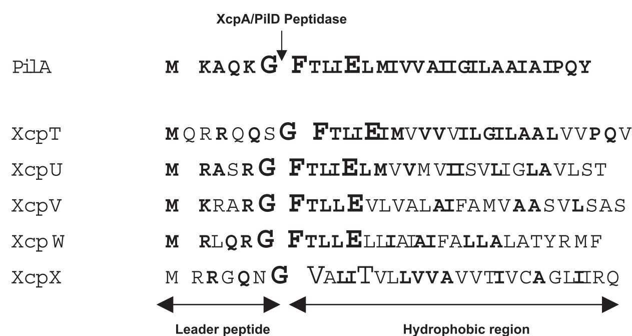

Fig. 2. Sequence alignment of the N-terminal domains of the PilA pilin subunit and pseudopilins of the Xcp-type II secretory pathway from *P. aeruginosa*. XcpT,-U, -V, -W and -X are also more generally named GspG, -H, -I, -J and -K, respectively. The residues in the pseudopilins that are identical to those in the PilA sequence are shown in bold. Here R and K, and also I and L, are treated as "identical" residues. The conserved G − 1, F + 1 and E + 5 residues are shown in uppercase. The position of the leader sequence cleavage site is indicated with an arrow. The leader peptide and the hydrophobic region are shown.

modification is also catalysed by the prepilin peptidase, which is thus a bifunctional enzyme [16]. The G − 1 residue is essential for cleavage of the pilin subunits and assembly into pilus [17]. There is no strict requirement for phenylalanine at position + 1 or for glutamate at position + 5 for prepilin processing. However, although an E + 5V substitution did not affect leader peptide cleavage, it did abolish methylation and piliation. In case of an F + 1S substitution, pilin was assembled into pili despite the complete absence of *N*-methylation. This observation raises questions as to the function of methylation during pilus biogenesis.

Mutations in the prepilin peptidase *pilD* and *tapD* genes affect not only type IV piliation but also abolish the protein secretion process. These proteins, despite the particular location of their genes within the genome, are thus real Gsp components, namely $GspO_A$. The $GspO_A$ proteins are polytopic IM proteins with eight transmembrane segments, as demonstrated for *E. chrysanthemi* OutO [18]. The first cytoplasmic loop of these proteins is large and contains a tetracysteine consensus motif, C-X-X-C...X_{21}...C-X-X-C. However, this motif does not seem to be required for peptidase activity, as shown both in vivo and in vitro with the TcpJ prepilin peptidase from *V. cholerae* [19]. The sole residues required for peptidase activity are aspartate located in the second and third cytoplasmic loops. These studies on TcpJ have led to the classification of prepilin peptidase as being a novel family of bilobed aspartate proteases. In *V. cholerae*, two prepilin peptidases coexist, TcpJ dedicated to type IV pili assembly, and VcpD, which is required for toxin secretion. It should be noted that similar aspartate proteases, such as FlaK in *Methanococcus voltae* [20], are involved in processing archaeal preflagellins.

2.1.2. $GspG_T$, H_U, I_V, J_W and K_X, the so-called pseudopilins

Five of the Gsp proteins, $GspG_T$–K_X, have an N terminus similar to type IV pilins, except for $GspK_X$ that lacks the E + 5 residue (see Section 2.1.3). This sequence similarity has led to name these proteins *pseudopilins*, despite the C-terminal domain of pseudopilins and pilins being rather different. One relevant difference is that the two conserved cysteines at the extreme C terminus of the pilin subunit, which form a disulfide bridge, are not present in Gsp pseudopilins. The cys-bond formed in pilin is important for bacterial adhesion involving type IV pili [21].

The $GspO_A$-dependent cleavage of N-terminal pseudopilin leader peptides has been demonstrated in several organisms [22–25]. As with the pilins, the new N-terminal residue is methylated [26]. Topology studies of the pseudopilin indicate that they are bitopic IM proteins with a single hydrophobic N-terminal segment [18]. The in vivo location of these proteins is more ambiguous, since upon overproduction they are partly found into the OM fraction, particularly the mature form [22]. This suggests that there may be a redistribution of the pseudopilin after its processing. This may involve the relocation of the molecule to the OM, or the assembly of pseudopilins into a macromolecular complex, thereby changing fractionation behaviour. The similarity between pilins and pseudopilins suggests that pseudopilins assemble into a pilus-like structure tentatively called "pseudopilus" [27]. Pseudopilins, like pilins, have been reported to form homomultimers stabilised by chemical cross-linking [28]. In vivo cross-linking experiments with *P. aeruginosa* cells expressing the *gsp* gene cluster from the chromosome have detected mainly pseudopilin dimers [29]. The relative amounts of *P. aeruginosa* $GspG_T$, $GspH_U$, $GspI_V$ and $GspJ_W$ are approximately 16:1:1:4, respectively [26]. A more recent study described the biochemical characterization of XpsG, the $GspG_T$ homolog from *Xanthomonas campestris* [30]. XpsG was found both in the membrane and the soluble cell fractions. In the soluble fraction XpsG is found within a large complex of about 440kDa, which might contain additional pseudopilins, such as XpsH, the $GspH_U$ homolog, and which revealed that a pseudopilus might form inside the cell.

2.1.3. Relationship between pilus and pseudopilus

Recent work has shown that pseudopilins are indeed able to form a pilus-like structure [31]. This was observed upon overexpression of the *K. oxytoca* Pul secreton in the heterologous *E. coli* K12 host. A thicker structure as compared to type IV pili is then visible at the cell surface. Immunogold-labeling showed that this structure was composed of the major pseudopilin $GspG_T$. Similar observations have been done in *P. aeruginosa* (Fig. 3) that clearly showed that overproduction of the sole major pseudopilin $GspG_T$ is sufficient to obtain huge cell surface pseudopili, which form bundles [32]. Bundling may be prevented in some conditions, suggesting that a single filament could represent the native pseudopilus, the filaments being packed together via secondary interactions through the C-terminal end of the pseudopilins [30,32,33]. In case of PulG, a C-terminally

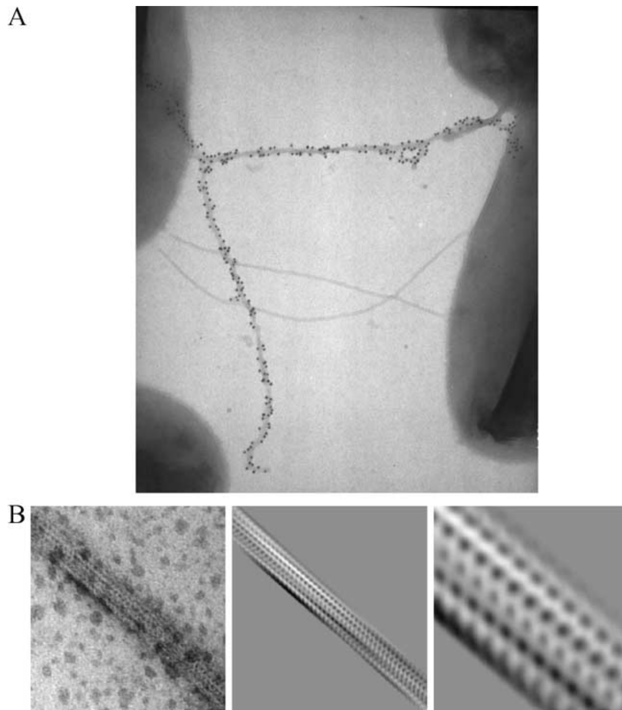

Fig. 3. The $GspG_T$(XcpT)-containing pseudopilus of *P. aeruginosa*. (A) Shown are the results of TEM analysis of the PAO1 strain overproducing the major XcpT pseudopilin after immunogold labelling with antibodies raised against XcpT. The unlabelled filaments are flagella. (B) Image treatment of electron micrographs of a negatively stained portion of the pseudopilus. From left to right: original image; Fourier transformation of the original image and filtered image; enlargement of the filtered image showing the type II pseudopilus substructure.

tagged PulG-His_6 derivative yields non-bundled pili but promotes pullulanase secretion. The PulG-His_6-containing pseudopili could be purified by cobalt affinity chromatography. Protein content analysis revealed that no other species than PulG could be found in the preparation, which thus appeared to be the only pseudopilin within the filament [33]. One may think that the long cell surface pseudopilus is artificial, but it reveals that pseudopilins from the $GspG_T$ family have the ability to pack into helical complex similar to pilus. The fact that occurrence of elongated pseudopilus blocks protein secretion [32] may indicate that permanent occupancy of the secretin channel (see Section 2.2) is incompatible with exoprotein release. It is possible to imagine that the pseudopilus structure involved in type II secretion spans the periplasm without extruding out of the cell, as suggested by the characterization of the intracellular XpsG complex in *X. campestris* [30]. The length of the $GspG_T$-containing pseudopilus could be controlled by the stoichiometry between the five pseudopilins. Upon reaching a threshold for $GspG_T$, pseudopilus elongation is not anymore controllable and it results in the appearance of the extracellular filament.

The four pseudopilins, $GspG_T$–J_W, have the G − 1, F + 1 and E + 5 residues conserved (although some differences are permitted at position F + 1, which can be replaced by hydrophobic residues). Interestingly, in recent years, five genes (*fimT*, *fimU*, *pilV*, *pilW* and *pilE*), the products of which have characteristics in common with Gsp pseudopilins, have been shown to be involved in fimbrial biogenesis in *P. aeruginosa* [34] even though they are not found in the pilus structure itself. These pilin subunits may form a platform within the cytoplasmic membrane for the assembly of the structural PilA subunit, which forms the type IV pilus structure present at the cell surface. The $GspH_U$-J_W pseudopilins may act similarly to promote $GspG_T$ integration into the pseudopilus structure (Fig. 4).

$GspK_X$ is considered to be atypical because it lacks the E + 5 residue and has a higher relative molecular weight (>30 kDa) than classical pilins and pseudopilins (15–20 kDa) [35]. Such atypical subunits have also been found in the type IV piliation system, with PilX from *P. aeruginosa* [36]. It is unclear whether E + 5, which is required for the methylation of the pilin subunit, is required in the case of pseudopilins because the E + 5V substitution in *K. oxytoca* $GspG_T$ did not affect *N*-methylation [37]. The significance of this difference between the two systems is not understood. The E + 5 residue has also been suggested to play a registration role in assembly of the pilus, by participating in the formation of a salt bridge between the negatively charged glutamate of subunit N and the positively charged N terminus of subunit N^{+1} [14]. The absence of E + 5 in the newly incorporated pilin or pseudopilin subunit may disturb incorporation of the subsequent subunit, preventing elongation of the pilus or pseudopilus and initiating disassembly of the structure. This speculation provides clues about the particular role of $GspK_X$ in protein secretion [35]. Interestingly, it has been shown that PulK overexpression abolished formation of the pseudopilus-containing PulG, suggesting that indeed PulK may be involved in pseudopilus length control [33].

2.2. The outer membrane complex

2.2.1. $GspD_Q$, the secretin

$GspD_Q$ proteins are associated with the OM and more generally called *secretins* [38]. Sequence comparisons have shown that in all cases, the C-terminal domain is highly conserved whereas the N-terminal domain is variable [39]. The conserved C-terminal region covers 200–300 residues and contains a very highly conserved block of about 60 amino acids containing invariant glycine and proline residues that have been shown to be functionally important [40]. The $GspD_Q$ proteins are multimers consisting of 12–15 subunits [41–44]. They are extremely stable and in most cases are heat- and detergent-resistant. In case of the *P. aeruginosa* $GspD_Q$, 13 putative transmembrane β-strands were found in the C-terminal domain of the protein [41]. This observation suggests that the C-terminal domain of $GspD_Q$ is required for the insertion of the protein into the OM, whereas the N-terminal domain extends into the periplasm to facilitate interactions with other proteins (see Section 2.5) (Fig. 4).

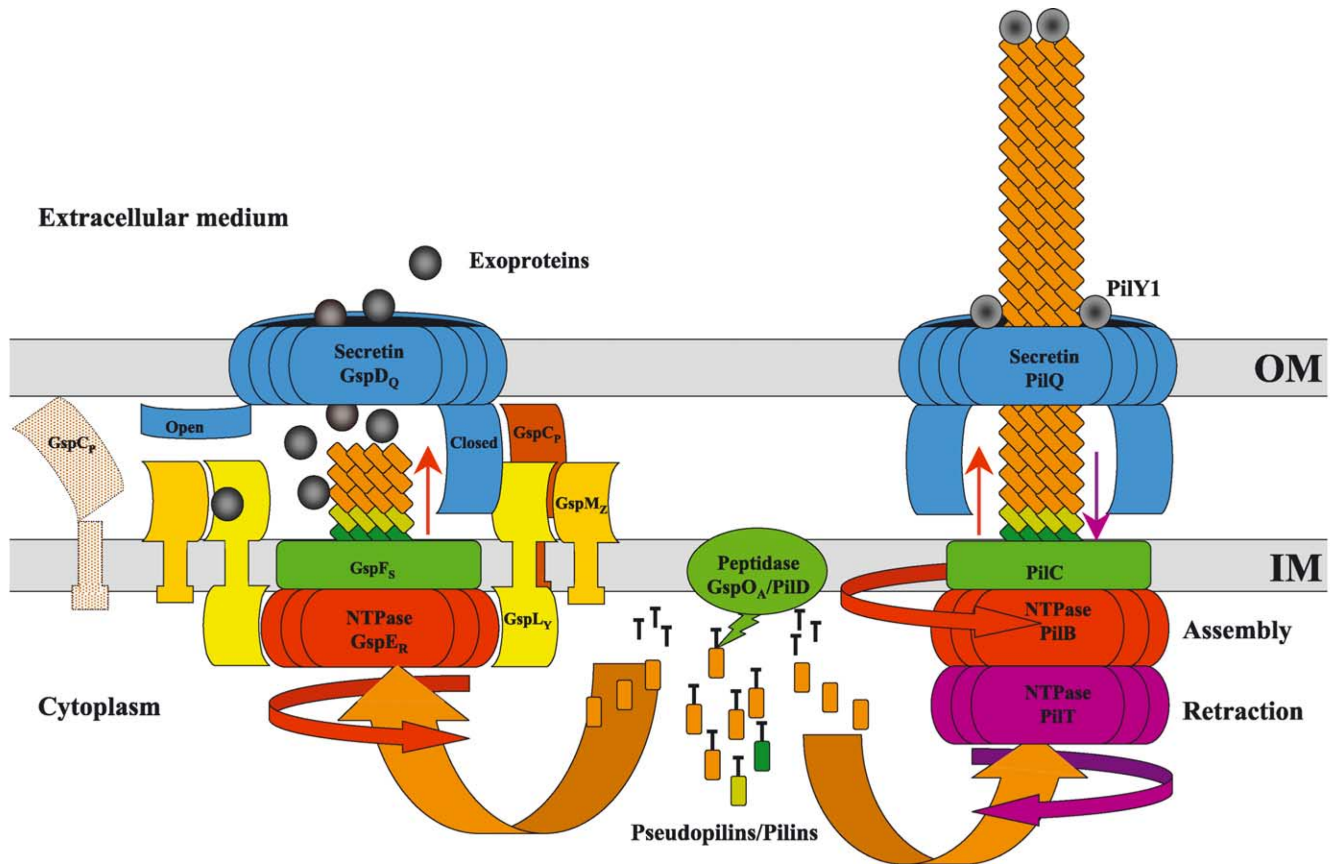

Fig. 4. Model of the Gsp secreton assembly (left), and comparison with a model for type IV pilus biogenesis in *P. aeruginosa* (right). On the left, the Gsp-dependent exoproteins, shown as grey circles, have initially been exported across the IM via the Sec or Tat machinery (not shown). The exoproteins are subsequently recognised by the Gsp machinery and transported across the OM via the secretin, $GspD_Q$. The secretin $GspD_Q$ is shown as a homomultimeric ring forming a channel with a large central opening. $GspM_Z$ is proposed to direct the location of $GspL_Y$ at a specific site into the cell envelope (cell pole in case of *V. cholerae*). $GspL_Y$ is anchoring the $GspE_R$ NTPase (traffic ATPase) to the inner face of the cytoplasmic membrane. The $GspE_R$ NTPase is represented as a homomultimer (hexameric ring). The opening and closure of the $GspE_R$ central ring is linked to cycles of ATP hydrolysis and promotes (as shown by the arrow) the helical assembly of the pseudopilins. Pseudopilins are thus using the central cavity of the $GspE_R$ hexamer to be incorporated into a pseudopilus structure that pushes exoproteins through the secretin channel. The $GspC_P$ component is interacting with the periplasmic N-terminal domain of the $GspD_Q$ secretin (closed). In the absence of such interaction, as shown on the right with the dotted $GspC_P$ form, the central cavity of the $GspD_Q$ secretin is made accessible to exoproteins (open). Preceding assembly into a pseudopilus, the $GspG_T-K_X$ pseudopilins are processed (removal of the leader peptide, shown as a T bar) by the prepilin peptidase, $GspO_A$. The pseudopilus is arbitrarily represented as a succession of different pseudopilins. It should be noted that the major pseudopilin $GspG_T$ is indicated in orange, whereas minor pseudopilins are indicated in green. The atypical pseudopilin $GspK_X$ is represented in dark green and is supposed to arrest pseudopilus elongation. The overexpression of $GspG_T$ may lead to the extrusion of the pseudopilus at the cell surface. The GspA, GspB, GspN and GspS proteins have not been represented. For more details about the Gsp machinery, see text. The homologous components of the type IV piliation system in *P. aeruginosa* (Pil) are shown on the right for comparison. The major pilin, PilA, is represented in orange, whereas minor pilins (PilE, PilV-X and FimT-U) involved in the type IV pilus assembly but that are not found in the extracellular structure are in green. The $GspK_X$ homologue, PilX, is represented in dark green. Type IV pili are retractile appendages. Retraction is promoted by the PilT NTPase in *P. aeruginosa*. The retraction process is represented by the purple arrow, which mimics the movement of the hexameric ring upon ATP hydrolysis. This movement antagonises the movement linked to the PilB NTPase that promotes assembly of the PilA subunit into the pilus. In *P. aeruginosa* the prepilin peptidase $GspO_A$/PilD is one single protein involved both in type II secretion and type IV piliation through its processing activity on pre-pilins and pre-pseudopilins. Finally, the type IV pili-associated adhesins PilY1 is represented as a grey circle.

This was confirmed by the fact that the C terminus of secretins is a protease-resistant domain [45,46].

The multimerisation of $GspD_Q$'s makes it possible to envisage the formation of a pore-like structure in the OM. Biochemical and electron microscopy studies have shown that the *P. aeruginosa* $GspD_Q$ multimer can indeed adopt a ring-shaped structure with a large central cavity of about 95 Å in diameter [41] (Fig. 5). The C-terminal domain of the secretin alone has the property to form these oligomeric rings. Reconstitution in planar lipid bilayers showed that secretins have a pore forming activity with higher single-channel conductance when compared to classical OM porins [45,47,48]. The large size of the channel is consistent with type II secretion-dependent exoproteins being brought across the OM in a folded conformation (see Section 3). For example, the folded elastase from *P. aeruginosa* is 60 Å across in its wider dimension [49]. The presence of such a large pore in the OM could lead to cell death, so the opening

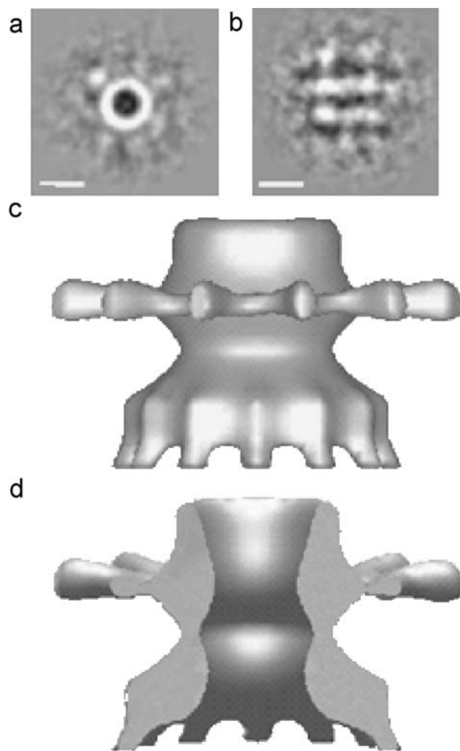

Fig. 5. Cryo-EM analysis of unstained preparations of purified GspD–GspS (PulD–PulS) complex from *K. oxytoca* and preliminary 3D reconstruction. Reprinted with permission from Nouwen et al. [48]. Average end (a) and side (b) views of the PulD–PulS complex. The scale bar in (a) and (b) represents 10 nm. A 3D model of the PulD–PulS complex (c) has been sectioned (d), removing the front of the complex so that the channel and profile of the rings can be clearly seen. The spokes could correspond to GspS.

of this pore is probably controlled. One possibility is that the N terminus of secretins folds back into the cavity formed by the C terminus [46] and/or that interaction with other proteins controls the channel gating (Fig. 4). Other gated OM channels are known, such as the iron–siderophore complex, vitamin B12 and colicin uptake receptors. The mechanism involves a conformational change in the receptor and an energy-transducing process requiring proton-motive-force (pmf) [50]. The mechanism of energy transduction to the OM is unclear but it involves a bitopic cytoplasmic membrane protein called TonB. TonB has a large periplasmic domain, and its energised form interacts with the OM receptor. On substrate binding, TonB induces a conformational change of the receptor, leading to the entry of the substrate into the periplasm. It has been shown that the translocation of proteins across the OM using the type II secreton could be dependent on pmf [51–53]. An analogous system to the TonB mechanism may be involved in protein secretion. In *P. aeruginosa*, *tonB* mutant strains are not secretion-defective (unpublished observation). However, other proteins, particularly bitopic cytoplasmic membrane proteins of the type II secretion machinery, may have a similar function to TonB. $GspC_P$, N or B proteins are good candidates for this function (see Section 2.5).

The $GspD_Q$ or secretin family of proteins is involved not only in type II secretion but also in other membrane translocation systems [27,38,54]. Its members are involved in type III secretion, type IV pilus assembly (PilQ family), DNA uptake (ComE family), assembly of S-layers and filamentous phage assembly and extrusion (pIV family). The size of the central cavity and the numbers of monomers in the final structure may vary from one system to the other [55,56]. It should be noted that one single secretin could be involved in two different transport processes. That is the case for the EpsD secretin from *V. cholerae* that is required for type II secretion and for extrusion of the CTXΦ filamentous phage [57]. Finally, the secretin might in some cases be an OM lipoprotein as shown for XpsD in *X. campestris* type II secretion [44] or BfpB for entheropathogenic *E. coli* bundle-forming pilus [58].

2.2.2. GspS, the secretin-specific chaperone-like protein

In some cases, the insertion of secretin into the OM depends on another protein, GspS [43]. GspS is a small peripheral OM lipoprotein present in the type II secretory systems of *K. oxytoca* and *Erwinia* species. It is essential for the OM insertion of $GspD_Q$ and also protects $GspD_Q$ against proteolytic degradation [59,60]. GspS could also be part of the secretin complex as proposed by Nouwen et al. [48] (Fig. 5). In other bacteria, one cannot exclude the possibility that the *gspS* gene does not belong to the *gsp* gene cluster and may be present at another chromosomal location as proposed with *E. coli* K12 [61]. The protective effect of GspS against proteolytic degradation has been shown using hybrid proteins containing the $GspD_Q$ C terminus, which is thought to be the GspS binding site. The addition of this domain to the pIV secretin or to unrelated proteins such as maltose-binding protein or pectate lyase PelD rendered these proteins dependent on GspS for stability [59,62]. GspS-devoid type II systems may compensate by efficiently partitioning $GspD_Q$ into the OM. In contrast, if kinetic partitioning is too slow, the binding of GspS to $GspD_Q$ may prevent the degradation of the protein prior to membrane insertion and may accelerate insertion as well. In unrelated transport processes involving secretins, a GspS-like function has been for example assigned to PilP, in type IV piliation in *Neisseria gonorrhoeae* [63], and InvH, in type III secretion in *Salmonella typhimurium* [64]. These two proteins are also small OM lipoproteins.

2.3. $GspE_R$, the putative ATP-binding protein

Protein secretion is an active process that requires energy sources. $GspE_R$ proteins have sequences similar to the Walker box A, containing the P-loop $GX_4GK(S/T)$ of an NTP-binding motif, and an atypical, but possible, Walker box B, in which the conserved aspartate residue in the motif Dhhhh*D*E (where h stands for hydrophobic) is replaced by either a glycine or an alanine. Despite the presence of these motifs, it was not possible to demonstrate

the binding of ATP to these $GspE_R$ components that are involved in type II secretion such as PulE or XcpR. However, mutation of the conserved glycine residue within the Walker A box of $GspE_R$ from *P. aeruginosa*, *K. oxytoca*, *E. chrysanthemi* or *V. cholerae* causes the bacteria to become secretion-defective [65–68]. ATP-binding activity could not be demonstrated but autokinase activity has been detected in the case of *V. cholerae* $GspE_R$ [65]. Mutations in the less conserved Walker B box have little or no effect on the secretion process (20–30% reduction in pullulanase secretion for mutations in the *K. oxytoca* $GspE_R$) [68]. The Walker B box is thought to be the determinant for nucleoside recognition, and the weak requirement for this motif may account for the failure to demonstrate ATP-binding activity in $GspE_R$ proteins. Alternatively, binding may be stimulated when $GspE_R$ interacts with other components of the type II secretion machinery or other molecules such as phospholipids. ATP binding and hydrolysis are, for example, stimulated when SecA associates with the preprotein [69].

Within the $GspE_R$ family, there is a highly conserved central region between the Walker A and B boxes consisting of two short aspartate-rich motifs called aspartate box. They are required for the function of $GspE_R$ in the secretion process, as shown by the substantial decrease, 80–90%, in pullulanase secretion, upon replacement of aspartate by asparagine residues in *K. oxytoca* $GspE_R$ [68]. The aspartate residues may be involved in the formation and stabilisation of the nucleotide-binding fold by interacting with Mg^{2+}. A fourth conserved domain could be found downstream the Walker B box, the His box, which includes two histidine residues and whose role in the protein is unknown [70]. Finally, another motif, a tetracysteine motif identical to that described for the $GspO_A$ peptidases (see Section 2.1.1), is present in members of the $GspE_R$ family [71]. It appears to be essential for function, because replacement of any of the cysteine residues by a serine within the *K. oxytoca* $GspE_R$ leads to a large decrease in pullulanase secretion (80%). This similarity between $GspO_A$ and $GspE_R$ proteins may be purely coincidental or may reflect a common function such as co-ordination of a Zn^{2+} ion.

An additional feature of proteins involved in transport processes is their association with the membrane [72]. The deduced amino acid sequence of $GspE_R$ proteins shows that they are mainly hydrophilic and do not possess any hydrophobic domains that could anchor the protein in the membrane. This observation is consistent with the observation that $GspE_R$ proteins are present in the cytoplasm if produced in *E. coli* [65,73–75]. However, these proteins are associated with the cytoplasmic membrane in their original host. This suggests that $GspE_R$ is interacting with other Gsp components to form a functional machinery. It appears that $GspE_R$s are bound to the inner face of the IM thanks to an interaction with $GspL_Y$ (Fig. 4). This is demonstrated by the co-expression of the genes encoding $GspE_R$ and $GspL_Y$ from *V. cholerae* and *P. aeruginosa* in *E. coli*, and by the low level of membrane association of $GspE_R$ in a corresponding *$gspL_Y$* mutant strain [65,73]. It was also shown that $GspE_R$–$GspL_Y$ interaction drives a conformational change of $GspL_Y$, revealing the dynamic of secreton assembly and functioning [67]. $GspL_Y$ is a bitopic cytoplasmic membrane protein with an N_{in}–C_{out} topology [76], and a large cytoplasmic domain, which interacts with $GspE_R$ proteins [67,73] (Fig. 4).

Recently the crystal structure of the *V. cholerae* $GspE_R$ member, EpsE, has been solved [77] (Fig. 6). The protein whose structure was obtained is an N-terminal truncation of EpsE. Several distinct domains could be distinguished. The so-called C1 domain, made up of residues 240–392 and 442–450, contains all four characteristic sequence motifs previously described. A metal ion is found tetrahedrally coordinated in the additional tetracysteine motif, which is located in the Cm subdomain (residues 393–441) that protrudes from the C1 domain. The $GspE_R$ components belong to the large superfamily of "type II/IV secretion NTPases", also called "traffic ATPases", whose classification has previously been reported [78]. Even though classified in the same superfamily, the members that are involved in type IV secretion, T-DNA transfer or bacterial conjugation, whose archetype is VirB11, appeared to be distant homologues of the $GspE_R$ subfamily involved in type II secretion and type IV pili assembly. Typically most VirB11-like ATPases are shorter as compared to the $GspE_R$ members. Interestingly, in contrast to the $GspE_R$ members, several components of the VirB11 subfamily, including VirB11 [79], HP0525 [80–82] or TrwD [70], have been shown to hydrolyse ATP. Moreover, structural data and electron microscopy revealed that components of the VirB11 subfamily, such as TrbB, the component of the conjugative transfer apparatus of the broad-host range plasmid RP4, or HP205, the component of the type IV secretion system from *Helicobacter pylori*, were shown to form homohexamers [81]. In the case of HP205, it is shown that the protein forms a hexameric ring that delimits a central chamber with a diameter of 50 Å [82]. The chamber is open on one side and closed on the other, closure and opening of which is regulated by ATP binding and ADP release [80]. The opening of the HP205 chamber would create a large cylinder, which could allow passage of large proteins.

The exact role of $GspE_R$ in type II secretion is unknown, but the various observations discussed above suggest that it acts as a traffic ATPase for transport across the cytoplasmic membrane. $GspE_R$ proteins involved in type II secretion, including XcpR and OutE, were shown to associate at least as homodimers using a domain of the lambda phage cI repressor as a reporter for dimerisation or the yeast two-hybrid system [67,83]. Moreover, it could be proposed, based on modelling studies, that the type II secretion component EpsE could adopt a hexameric structure, even though it is arranged as a helical filament in the crystals [77]. Such feature was previously described for the T7

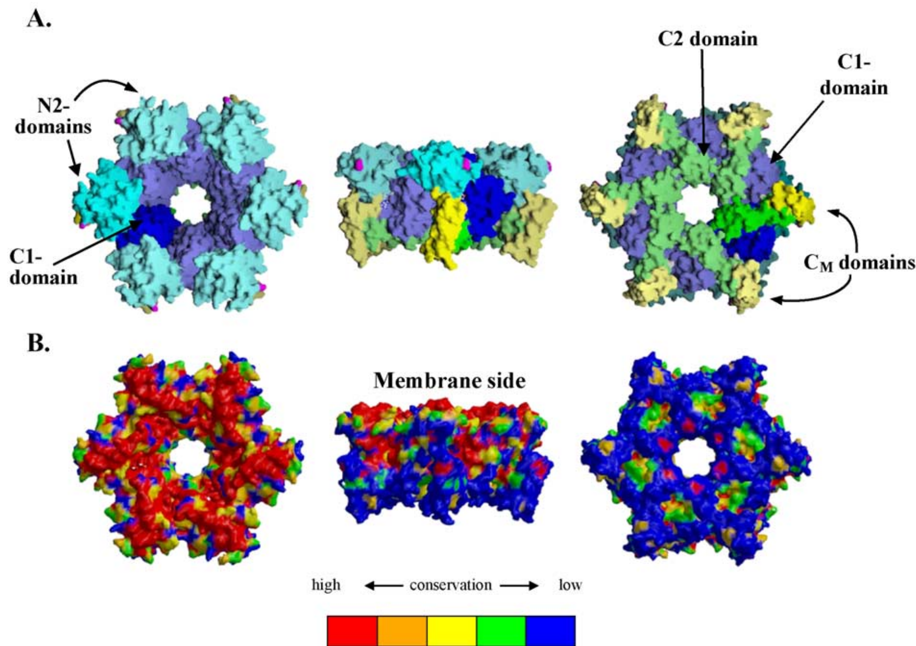

Fig. 6. Hexameric ring model of EpsE from *V. cholerae*. Reprinted with permission from Robien et al. [77]. (A) View from the proposed membrane-facing side (left), side view (middle) and the cytoplasmic face (right) of the hexameric ring model of EpsE. One monomer is shown with the domains colored as follows: N2, cyan; C1, dark blue; CM, yellow; and C2, green. The other five monomers are colored in a lighter shade of the same colors. (B) Same views as in (A), but with the surface colored by sequence conservation.

helicase-primase that forms a hexameric ring upon electron microscopy observation, whereas the isolated helicase domain crystallises as a right-handed helical filament [84]. Considering the features of VirB11 ATPases, it may be suggested that $GspE_R$ components involved in type II secretion and type IV piliation are required for the translocation of pseudopilins and pilins through the cytoplasmic membrane, respectively, before their assembly into ''pseudopilus'' or pilus (Fig. 4). In addition, the effects of thermosensitive (*ts*) mutations within the *P. aeruginosa* $GspG_T$ pseudopilin are suppressed by a secondary mutation within $GspE_R$ [85]. This strongly supports the idea that pseudopilins are substrates for $GspE_R$. Moreover, the *ts* mutations affected the periplasmic domain of the pseudopilin, suggesting that the interaction takes place before translocation, or that $GspE_R$ pushes the pseudopilins through the membrane in a manner similar to the insertion–deinsertion cycles of SecA [86].

2.4. The $GspE_R$, F_S, L_Y, M_Z inner membrane plateform

Comparison between type II protein secretion and type IV piliation has already suggested that homologous components, such as PilD/$GspO_A$, PilB/$GspE_R$, PilA/$GspG_T$–J_W, PilX/$GspK_X$ from *P. aeruginosa* are involved in these processes [27] (Fig. 4). Another Pil protein, PilC, is essential in type IV piliation. It has a Gsp homologue, $GspF_S$. $GspF_S$ is a polytopic integral IM protein with a small periplasmic loop and two larger cytoplasmic domains connected by three transmembrane regions [74]. PilB, C, D and A are clustered in most type IV piliated bacteria, suggesting that PilB and C are simultaneously involved in the translocation/assembly of PilA after processing by PilD [13] (Fig. 4). $GspF_S$ may have a similar function to PilC with respect to pseudopilins, even though $GspL_Y$ is required for the association of $GspE_R$, the PilB homologue, with the membrane. However, recent works proved that $GspF_S$ N-terminus (172 aa) interacts both with $GspE_R$ and $GspL_Y$ from *E. chrysanthemi* [87]. $GspF_S$ may thus strengthen the association of $GspE_R$ with the membrane, and/or be involved in pore formation in the cytoplasmic membrane, allowing pseudopilin translocation (Fig. 4).

No homologues of $GspL_Y$, $GspM_Z$ and $GspC_P$ specifically involved in type IV piliation have yet been identified. These Gsp components are bitopic IM proteins with an N_{in}–C_{out} topology [76]. Interestingly, an Xcp(Gsp)-related system has been found in *Pseudomonas putida* that is required for the secretion of a manganese-oxidizing factor [88]. The gene cluster, named *xcm*, encodes all classical type II secretion components but $GspM_Z$, $GspL_Y$ and $GspC_P$. This observation might indicate that the Xcm system has evolved more recently from the type IV pili assembly machinery, and it is now classified as type IIb secretion system. It is thus distinguishable from the classical type II system, which is characterized by the presence of *gspM*$_Z$, *gspL*$_Y$ and *gspC*$_P$ genes within the *gsp* cluster. A puzzling observation revealed homologues of the *gspC*$_P$, *gspL*$_Y$ and *gspM*$_Z$ genes in the genome sequence of *E. coli* K12, close to a *gspO*$_A$ gene homologue, *pppa*, which encodes a prepilin peptidase [89]. However, this chromosomal region lacks any other *gsp*

homologues. The function of these genes in *E. coli* K12 is unknown. Interestingly, enterotoxigenic *E. coli* strains do contain within this locus a complete *gsp* cluster that is required for secretion of heat stable enterotoxin [90]. Part of this cluster has thus been lost by *E. coli* K12, but it should be noted that this strain possesses another complete type II secretion system, which appeared to contribute to chitinase secretion [61].

Classically, within the type II secreton, $GspM_Z$ was shown to be crucial for the stability of $GspL_Y$ [91]. The stabilisation process is reciprocal because the abundance of $GspM_Z$ in the cell depends on $GspL_Y$, indicating that these two Gsp components interact with each other (Fig. 4). Many other studies have since confirmed this observation [92–94]. Very interestingly, it was shown that the *V. cholerae* type II component $GspM_Z$, EpsM, is located at the cell pole [95]. Moreover, the $GspL_Y$ component, EpsL, is found associated with the cell pole only upon co-expression with EpsM. From these observations, it is tempting to suggest that the cascade of protein–protein interactions is kinetically organised during the assembly of the machinery. The $GspM_Z$ protein may determine the membrane location of the secretion site, recruits the $GspL_Y$ component, which in turn brings $GspE_R$ into association with the membrane.

2.5. GspC and GspN the connecting components?

The function of the $GspC_P$ protein is unknown. Its principal feature is the presence of a PDZ domain in the C-terminal region of the protein [96]. PDZ domains mediate a variety of protein–protein interactions by binding to short sequences (X-T/S-X-V-COO^-), usually at the C termini of target polypeptides, but may also bind homotypically [97]. It is widely thought that PDZ domain-containing proteins mediate the organisation of multi-molecular complexes at sites of membrane specialisation. Some $GspC_P$s have no PDZ domain. This is true of the $GspC_P$s of *P. aeruginosa* and *P. alcaligenes*. However, in both cases, the PDZ region is replaced by a coiled-coil structure, a motif also known to be involved in protein–protein interactions. Hence, $GspC_P$ proteins may form homomultimers or interact with other proteins by one of two mechanisms, one involving a coiled-coil and the other, PDZ domains.

The topology of $GscpC_P$ is similar to that of TonB [76] (see Section 2.2.1). The *P. aeruginosa* $GspC_P$ protein has been proposed to interact with the secretin (Fig. 4), and in case of *K. oxytoca* it has been shown to partly fractionate into the OM [98,99]. In addition, in *P. aeruginosa* and *P. alcaligenes*, the genes encoding $GspC_P$ and $GspD_Q$ are organised in a separate operon (Fig. 1), suggesting their co-ordinated action. These observations led to propose a role for $GspC_P$ in energy transduction and channel gating of the secretin. In support of this hypothesis, it was proposed that $GspC_P$ connects the two sub-secreton complexes, the IM plateform $GspE_R$-F_S-L_Y-M_Z and the OM complex $GspD_Q$-S [92,98,100] (Fig. 4). The suggestion is that a short N-terminal domain of the *P. aeruginosa* $GspC_P$, XcpP, influences the stability of the $GspM_Z$/$GspL_Y$ complex whereas the C-terminal domain negatively controls the opening of the $GspD_Q$ secretin channel [98,100].

At this stage, an interesting parallel can be done with the phage extrusion process [101]. In this case, the pIV secretin interacts with the C-terminal periplasmic domain of the integral cytoplasmic membrane protein, pI [102]. pI contains a nucleotide binding site in its N-terminal cytoplasmic domain, and may be involved in the controlled gating of the pIV pore. The energy transduced for the phage secretion process may therefore come partly from ATP hydrolysis, which has been shown to be required together with the pmf, for filamentous phage assembly [103].

The GspN protein is, like $GspC_P$, a bitopic IM protein. It is absent from many of the known type II systems, and has been identified only in *K. oxytoca*, *Erwinia carotovora*, *X. campestris* and *P. putida*. However, in the case of *K. oxytoca* GspN is not required for type II secretion [92]. In case of the *X. campestris* GspN, an interaction with the secretin was suggested [104]. In addition, it was recently proposed that *X. campestris* GspN participates to the formation of the $GspL_Y$–$GspM_Z$ complex [94,105]. The similar behaviour of the *X. campestris* GspN, XpsN, as compared to the $GspC_P$ family of components appeared really intriguing. Knowing that similarities between various $GspC_P$ are extremely low, it could be proposed that XpsN belongs to this family of type II components. Moreover, the fact that XpsN has a membrane topology similar to $GspC_P$s, that the *xpsN* gene is tandemly organised with the $gspD_Q$ homolog *xpsD* (Fig. 1), and that XpsN has a coiled-coil domain in its C-terminal region, strongly supports this hypothesis.

2.6. GspA and GspB, "energiser", "pilot" or "regulator"

We have seen that there may be small differences in the composition of type II secretory apparatuses. Two additional Gsp proteins have been described in *A. hydrophila*. These two proteins, GspA and GspB, are essential to the protein secretion process [106]. The GspB protein has a sequence and structure similar to that of TonB, whereas GspA is a membrane protein with a consensus ATP binding site. GspA and GspB form a complex within the cytoplasmic membrane and it has been suggested that such a complex transduces energy from ATP hydrolysis, to the protein secretion process. If this is the case, the complex plays a crucial role, and it is not clear why it is not found in most type II systems. In *A. hydrophila*, secretion from the periplasm requires pmf and ATP [52], whereas in *K. oxytoca*, it requires only pmf [51]. This may partly explain the role of GspA and B in *A. hydrophila*.

GspB homologues, but not GspA, are also found in *Erwinia* species (Out) and *K. oxytoca* (Pul). If PulB is not required for secretion [92], OutB is required and may interact with the OutD secretin [107]. A recent report has suggested that the GspA/GspB complex from *A. hydrophila*

(ExeA/ExeB) may help the translocation of the ExeD secretin from the IM to the OM [108]. Interestingly, a consensus sequence for a putative peptidoglycan binding site has been identified in ExeA and may help achieve such function.

GspA and GspB homologues have also been identified in *V. cholerae* and in the *E. coli gsp* gene cluster [109]. Recent work showed that the *E. coli* secreton is functional and allows secretion of a chitinase under particular growth conditions [61]. If the $GspC_P$-O_A secreton is alone sufficient to achieve chitinase secretion, expression of the GspA-B homologues increases the level of secretion [61].

The role of the GspA/B complex remains elusive looking at the panel of putative functions proposed, which include energy transduction, stabilisation or increased expression of the Gsp complex and piloting the secretin to the OM.

3. Exoprotein recognition

Secretion by the type II system is a two-step process. The first step, translocation across the IM, is controlled by the Sec machinery, which recognises signal peptide-bearing exoproteins. Once in the periplasm, the mature polypeptide becomes the substrate for the Gsp machinery, which should discriminate between exoproteins and periplasmic or OM proteins. This process may be based on the recognition of a secretion motif by some of the Gsp proteins.

3.1. Species-specific recognition of exoproteins

The Gsp secretion apparatus is widespread in gram-negative bacteria and a wide variety of enzymes and toxins use this pathway. However, Gsp-dependent exoproteins, such as *K. oxytoca* pullulanase, are not recognised by the Gsp machinery of *P. aeruginosa*, demonstrating that the process is specific [110]. These two bacteria are not closely related and heterologous secretion has been described in closely related organisms. For example, the *Burkholderia glumae* lipase and the *A. hydrophila* aerolysin are secreted by *P. aeruginosa* and *Vibrio*, respectively [111,112]. In addition, the *P. alcaligenes* lipase [113] is secreted by *P. aeruginosa*, whereas *P. aeruginosa* elastase is secreted by *P. alcaligenes* [9]. In contrast, even though the cellulases, Cel5 (ex EGZ) and CelV, from *E. chrysanthemi* and *E. carotovora*, respectively, are very similar (40% identity), these two proteins are recognised exclusively by their own secretion machinery. Thus, the principle on which the exoprotein recognition process is based is unclear but it may rely on the presence of a secretion motif within the secreted protein. *P. aeruginosa* secretes enzymes as diverse as lipase, elastase, alkaline phosphatase, phospholipases and the ADP-ribosylating exotoxin A, all of which use the same secretion machinery. These proteins should, therefore, have a common secretion motif. However, sequence analyses have not identified a linear motif of residues and led researchers in the field to suggest that there is a nonlinear motif, a conformational signal constructed by folding.

3.2. Translocation of folded proteins

Two major observations suggest that Gsp-dependent exoproteins are translocated across the OM in a folded conformation. First, the studies with *E. chrysanthemi* cellulases and pectate lyases and *K. oxytoca* pullulanase have demonstrated that disulfide bridges are formed in exoproteins during secretion [114,115]. The cysteine bond is formed in the periplasm, catalysed by disulfide bond isomerases (Dsb), particularly by DsbA [116,117]. Second, studies with *V. cholerae* and *A. hydrophila* have shown that the B pentamer of the cholera toxin is formed in the periplasm before its secretion [118], and that proaerolysin is secreted as a dimer [119]. Thus, the secretion motif may be formed and presented to the secretory apparatus on the folding of the exoprotein. It is clear that it is not the disulfide bond itself that is recognised by the machinery. CelV, secreted in a Gsp-dependent manner by *E. carotovora*, contains no cysteines [120], and an engineered cysteine-free pullulanase is efficiently secreted [121]. Secretion of the cysteine-free pullulanase is still DsbA-dependent, raising questions about the role of DsbA. Two secreton components, $GspK_X$ and GspS, possess disulfide bonds. The formation of these bonds has been shown to be crucial for $GspK_X$ activity and GspS stability in *K. oxytoca* [122]. However, no GspS is found in *P. aeruginosa* and $GspK_X$ contains only one single cysteine. In this case DsbA is still required for stability of secreted lipase [123]. The results of studies on pullulanase conflict with the work of Bortoli-German et al. [115] who showed that site-directed mutagenesis of either of the cysteines in Cel5 cellulase prevented secretion. The cysteine bond may thus act as a clip, stabilising and fixing the protein in its folded conformation. The requirement for the disulfide bond therefore depends on the stability of the structure adopted by the reduced exoprotein.

The folding of exoprotein within the periplasm seems, in some cases, to involve not only general catalysts such as DsbA, but also specifically dedicated chaperones. This is the case for the propeptide of *P. aeruginosa* elastase [124], and for the Lif proteins, which are required for folding and activation of *Pseudomonas* lipases [125,126]. Other studies proposed that PlcR is required for full hemolytic activity of *P. aeruginosa* phospholipase C, suggesting that it acts as well as a helper for secretion [127].

3.3. The secretion motif

Gsp-dependent exoproteins acquire a highly ordered structure after reaching the periplasm and before OM translocation. In the absence of a linear motif, the secretion signal for the various exoproteins is therefore probably a patch signal involving distal regions brought into close

proximity by the folding of the protein. If this is true, the motif should be very sensitive to any changes in the structure of the exoproteins. This is consistent with the observation made by Py et al. [128] who showed that slight modifications in the *E. chrysanthemi* Cel5 protein result in non-secreted derivatives. In *A. hydrophila*, a single substitution of the W227 residue yields a protein that is not secreted [129]. In contrast, linker insertion experiments within *K. oxytoca* pullulanase resulted in the construction of 23 derivatives, all of which were efficiently secreted [130]. Some of these derivatives were inactive, indicating changes in the enzyme structure, thereby challenging the existence of the conformational motif. However, it is possible that the motif is formed at an intermediate state of folding rather than in the fully active native protein. The fold to a specific secretion-competent conformation prior to secretion may thus be different from the folding state of the secreted species. An interesting model, in which the secretion of Cel5 involves a transient intramolecular interaction between the cellulose-binding domain (CBD) and a region close to the active site, has been proposed [131]. Once secreted, the protein may then vary its fold to allow the CBD to interact with the cellulose substrate.

Two regions in the *K. oxytoca* pullulanase, A and B, which are well separated from each other in the primary protein sequence, together direct the secretion of a PulA–Bla hybrid [132]. These two domains are therefore probably part of a structural motif, which is still formed and recognised within such a hybrid. The polygalacturonase PehA is secreted in a type II-dependent manner by *E. carotovora*. In this case, three separate regions between residues 84–135, 190–242 and 342–369, called A, B and C, respectively, were found to be needed for targeting [133]. It was proposed that region B serves a conformational function, most probably in a proper positioning of regions A and C with respect to each other. The notion of two targeting domains positioned by a third one is reminiscent to what was proposed for exotoxin A [134]. Exotoxin A structure is made of three distinct domains, domain I (host cell binding), domain II (translocation domain) and domain III (catalytic domain). On the one hand, studies identified a single stretch of 60 amino acids (60–120, domain I), which were sufficient to direct Bla secretion [135], whereas other studies have shown that a truncated protein retaining the first 30 and last 305 residues (domain III), but not residues 60–120, is still efficiently secreted [136]. On the other hand, alterations in domain II generates increased changes in the molecular dimension of exotoxin A coinciding with a decrease of its secretion efficiency [134]. Interestingly, these changes did not affect the overall structure of domains I and III, but prevented their proper positioning with respect to each other.

The 3D structures of several type II-dependently secreted proteins are now available. No reliable program allows for the visualisation of clear conserved structural motifs between these proteins. One suggestion is that these proteins have a high β-sheet content [137]. Interestingly, the exotoxin A 60–120 region appeared to be rich in anti-parallel β-sheets [135]. However, a number of type II-dependent proteins, such as the *P. aeruginosa* lipase, have only a moderate β-sheet content, whereas some resident periplasmic proteins, such as LolA [138], mostly contain β-sheets. Since the nature of a secretion motif appears extremely difficult to identify, it may also be considered that some physico-chemical properties of the exoproteins, such as overall surface charge, might be crucial for type II-dependent secretion.

One alternative to a unique structural motif is that successive interactions lead to the secretion of exoproteins. These interactions may involve different secretion signals that are not essential individually but are required simultaneously for optimal secretion. Finally, the secretion motif may not be recognised by a common component of the secretion machinery but by a specific intermediate, such as the previously described dedicated chaperone, notably the elastase propeptide and the lipase foldase (Lif), which will in turn be recognised by a Gsp component of the secretion machinery.

3.4. Recognition by $GspD_Q$

One of the Gsp component involved in the cascade of recognition events required to direct the exoprotein is the $GspD_Q$ secretin. The pectate lyase PelB of *E. chrysanthemi* binds to the N terminus of $GspD_Q$ [139]. The N terminus is the most variable region of the protein, consistent with the notion of specificity for recognition of the exoprotein. Even though contradictory results have been obtained with *K. oxytoca* $GspD_Q$ [140], this observation is consistent with the fact that of all the Gsp components of *E. chrysanthemi* required for pectate lyase secretion, only two cannot be replaced by Gsp homologues from *E. carotovora*, namely $GspC_P$ and $GspD_Q$ [141]. This suggests that these two proteins are intimately involved in the species-specific recognition of the exoprotein by the Gsp system. Another possibility, however, is that these proteins are not incorporated into the heterologous system, because they fail to make specific interactions with the other Gsp components that are required for the assembly of the secretion machinery. This second hypothesis is supported by the work of de Groot et al. [9] who showed that *P. aeruginosa* and *P. alcaligenes* $GspC_P$ or $GspD_Q$ proteins cannot be exchanged even though the two species secrete each other exoproteins. Because $GspC_P$ and $GspD_Q$ can be exchanged simultaneously one may suspect a specific interaction between these two components (see Section 2.5).

4. Conclusions

Based on the observations described in this chapter, it is possible to formulate ideas about several of the main

features of the type II machinery. Secretion occurs in two steps, as shown by Poquet et al. [142], and the Gsp components of the secretory apparatus are involved in the translocation of the transient periplasmic form across the OM. However, it is important to say that the periplasmic form might correspond to an extremely short period or may not exist, both steps of membrane translocation being then tightly connected. It was shown for example that elastase fused to *E. coli* colicin A is efficiently secreted by *P. aeruginosa* suggesting that there is no time for ColA to insert in the membrane from the periplasmic side and exert its toxic effect [143]. The assembly of the secretion machinery and the recognition of the secreted proteins can be summarised in several points:

(1) One can imagine the existence of a trans-periplasmic structure, formed of helically packed pseudopilins, which was named pseudopilus. Assembly would require a peptidase, an ATP-binding protein and possibly several IM proteins to achieve the translocation of pseudopilins across the IM (Fig. 4). In light of the $GspE_R$ structure, it can be proposed that pseudopilin translocation through the cytoplasmic membrane is initiated via its insertion in the $GspE_R$ hexameric ring cavity. Cycles of ATP hydrolysis by $GspE_R$ alternate an open and closed state of the hexameric ring allowing to screw pseudopilins through the membrane via another channel that can be formed by the polytopic $GspF_S$ component (Fig. 4). However, the initial insertion and translocation process of the pseudopilins through the IM via $GspE_R$ remains to be demonstrated since it might still be proposed that pseudopilins are first inserted into the membrane via the more classical Sec pathway. Furthermore, the assembly of the "pseudopilus" may be a key element, helping to push the exoprotein through the secretin for its final release. Type IV pili are retractile organelles, and by comparison pseudopilus retraction might correlate cycles of pushing off (Fig. 4). Alternatively, the pseudopilus may act like a cork, contributing to block the secretin channel, with secretion fully effective only if the structure is retracted. In case of type IV pili, it was indeed shown that the exit pore for the pilus filament is the secretin channel [144]. The pseudopilus may also serve as a guide for routing exoproteins to the secretin channel. The role of the different pseudopilins, the exact composition of the pseudopilus, its physiological length and its ability to retract, such as the type IV pili, might provide crucial information for understanding the way the type II machinery may function. The pseudopilus has probably a key role in the process and it requires most Gsp components for its assembly [31–33]. However, it should be noted that type IV pili retraction involved a second traffic ATPase, PilT, and no identified similar component has been found to be required in the case of the type II secretion system (Fig. 4).

(2) The final channel, allowing exoproteins to reach the extracellular medium, is thus made of the so-called secretin ($GspD_Q$), which multimerises (12–15 subunits) and may form a large hole (95 Å) within the OM. The release of the exoproteins could only occur if the secretin cavity is not occupied by the pseudopilus (Fig. 4).

(3) The contribution of $GspC_P$ might be coordinated to assembly of the pseudopilus. Indeed, $GspC_P$ interacts with the inner platform $GspL_YM_ZE_R$ via its N terminus, and with the secretin $GspD_Q$ via its C terminus [100] (Fig. 4). ATPase activity of $GspE_R$ on the one hand may promote pseudopilus assembly and on the other hand may induce conformational changes in the $GspL_YM_ZP_C$ complex. Such changes may result in looser interaction of $GspC_P$ with the $GspD_Q$ secretin (Fig. 4), giving access to exoprotein release via pseudopilus pushing and extrusion. The molecular characterization of the $GspC_P$–$GspD_Q$ interaction will be helpful to understand the dynamic of the secretin channel and more generally of the secreton. In this respect, availability of structural data on these two components will be required.

(4) Despite the conservation of Gsp systems, most Gsp components are not exchangeable, suggesting that there is specificity within the machinery itself. The probability of producing a functional hybrid machinery depends on the phylogenetic distance between the organisms involved. As described above, only $GspC_P$ and $GspD_Q$ are not exchangeable. More generally, the $GspO_A$ peptidase may be functionally moved from one species to another [145,146], probably because it has an enzymatic rather than a structural function. This idea is supported by the observation that $GspE_R$ hybrids may be functional if the variable N terminus is in a homologous context, whereas the ATP binding site-containing C terminus may be exchanged between species [65,147]. Apart from that, the most permissive Gsp component is $GspG_T$, which can be exchanged, more or less efficiently, in several cases [28,109,141,148].

(5) The secreted proteins must have features (secretion motifs) that mediate specific recognition by Gsp components, such as the secretin $GspD_Q$. The exoproteins are folded in the periplasm and the secretion motif is probably conformational rather than linear. However, this issue remains only poorly experimentally supported and additional and concordant data will be required before definite conclusions could be reached. As they are folded, the exoproteins need the large hole formed by the secretin for their translocation across the OM. This translocation of folded exoproteins contrasts with the "dogmatic" unfolded competent state for translocation across the bacterial cytoplasmic membrane via the Sec machinery. However, a novel Sec-independent translocation pathway has been shown to translocate metalloenzymes into the periplasm in a stably folded

conformation [149]. Interestingly, recent work indicates that the Tat system might in some cases be used as the first step of the GSP instead of the Sec system [2].

Finally, it is clear that the components of the type II secretion machinery have now been well established. However, we do not yet understand the precise organisation of the machinery and the mechanisms controlling its secretion function. The function of the "pseudopilus" is probably one of the key feature of the type II secretion apparatus. At that stage it is worth noting that the type IV pili-associated adhesins, PilC from *N. gonorrhoeae* [150] or PilY1 from *P. aeruginosa* [36], do have a Sec-dependent signal peptide that targets them first to the periplasm. Their final exposition at the cell surface is thus dependent on type IV pilus assembly, which will play the role of a pseudopilus, whereas PilC or PilY1 play the role of the secreted proteins.

Looking at the multiple interactions between the Gsp components, and looking also at the fact that most of them are included in the complex as homomultimers, the secreton appears to be an extremely large structure of the bacterial cell envelope. The structural characterization of the individual components and the integration within the 3D-image reconstitution of the whole secreton by using electron microscopy technologies will be the next steps forward to gain understanding on the mechanism of this secretion system. It should also be noted that the proper incorporation of the secreton might involve the interaction with additional constituents of the envelope like it was suggested with lipopolysaccharides [151].

Gsp-like components are also involved in the transport and assembly of various macromolecules through the membranes of gram-negative bacteria. Therefore, specific mechanisms may be required to adapt regions of the cell envelope, including the peptidoglycan layer, to such transport processes. It is possible that these sites are not spread out evenly over the envelope but are rather confined to particular areas, such as the pole of the cell. Several indications revealed that the type II secreton might be polarly located as seen with the use of $GspM_Z$–GFP fusion [95] or the polar pseudopilus localisation [31]. This distribution would be reminiscent of that for type IV pili.

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