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Review

Type II secretion system: A magic beanstalk or a protein escalator☆

Mangayarkarasi Nivaskumar [a,b,c], Olivera Francetic [a,b,*]

[a] Institut Pasteur, Molecular Genetics Unit, 25 rue du Dr. Roux, Paris CEDEX 15, France
[b] CNRS ERL3526, 25 rue du Dr. Roux, Paris CEDEX 15, France
[c] University Paris VII, 25 rue du Dr. Roux, Paris CEDEX 15, France

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ABSTRACT

Type II protein secretion systems (T2SS) are molecular machines that promote specific transport of folded periplasmic proteins in Gram-negative bacteria, across a dedicated channel in the outer membrane. Secreted substrates, released to the milieu or displayed on the cell surface, contribute to bacterial adaptation to a range of habitats, from deep-sea waters to animal and plant tissues. The past decade has seen remarkable progress in structural, biochemical and functional analysis of T2SS and related systems, bringing new mechanistic insights into these dynamic complexes. This review focuses on recent advances in the field, and discusses open questions regarding the secretion mechanism. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

1. Introduction

Since its discovery in the 1980s in the genus *Klebsiella* [1], the type 2 secretion system (T2SS) has been identified and studied in many environmental or pathogenic strains including *Pseudomonas* [2], *Aeromonas*, *Erwinia* [3,4], *Xanthomonas* [5,6], *Vibrio* [7] and enterotoxigenic *Escherichia coli* (ETEC) [8]. Genetic and biochemical studies of these models have provided precious insights into the T2SS composition and molecular organization. To date, genome studies indicate the presence of T2SSs in hundreds of bacterial species, belonging to all classes of Proteobacteria, but also to other major groups like Chlamydia, Spirochetes or Cyanobacteria.

Structural analysis has been essential for dissecting the secretion mechanism, which involves a series of dynamic protein–protein interactions, as discussed in detail in several excellent reviews [8–10]. The readers are referred to other recent reviews that summarize specific aspects such as secretin channels [9], surface display of exoprotein substrates [10], assembly of T2SS [11] and pseudopilus biogenesis [12]. Here we will provide an update on the more recent findings in the field and discuss mechanistic and functional aspects of type II secretion process.

2. T2SS substrates: biogenesis, diversity and biological functions

Gram-negative bacteria have developed several strategies to energize protein secretion across their complex two-membrane envelope. Successful strategies are typically used not only for protein transport but also for assembly of surface organelles. The T2SSs are highly similar to type 4 pilus assembly systems (T4PS) [12–14] as well as to archaeal pili and flagella [15], suggesting their early common origins. These ubiquitous prokaryotic systems generate mechanical energy through ATP hydrolysis in the cytoplasm to promote assembly of filamentous structures anchored in the inner membrane (IM). In T2SS these filaments are localized in the periplasm and have been coined pseudopili, in contrast to the surface-exposed pili in T4PS that perform a range of functions in adhesion, signaling and motility. Pseudopilus components and assembly machinery are essential for exoprotein transport across the outer membrane (OM) channel called the secretin. Together they form a large and dynamic trans-envelope complex composed of up to 15 different, typically oligomeric, components.

Although T2SSs have been mostly studied in human, animal and plant pathogens [16,17], from the bacterial perspective the main function of T2SS is nutrient acquisition. The majority of exoproteins identified so far are hydrolytic enzymes that degrade biopolymers — carbohydrates, lipids, proteins or nucleic acids. In addition, T2SSs promote secretion of toxins, adhesins, slime proteins or cytochromes and function in respiration, motility or biofilm formation, as discussed below.

Together with the type 6 secretion systems (reviewed in this issue [18]) the T2SSs promote secretion of proteins that have attained their

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* Corresponding author at: Institut Pasteur, Molecular Genetics Unit, 25 rue du Dr. Roux, 75724 Paris CEDEX 15, France. Tel.: +33 1 40 61 36 81; fax: +33 1 45 68 89 60.
E-mail address: ofrancet@pasteur.fr (O. Francetic).

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native three-dimensional structure. The distinguishing feature of T2SS substrates is the presence of an N-terminal signal sequence that targets them to the periplasm via Sec or Tat protein export systems. The Sec substrates are exported as linear polypeptides to adopt their native state in the periplasmic airlock aided by chaperones [19], Dsb oxido-reductases [20] and calcium [21–23]. T2SS is the only known way out for the Tat substrates, which fold in the cytoplasm prior to export [24]. Both exoprotein classes converge in the periplasm to form tightly folded intermediates, sometimes associated with cofactors (Cu, Mb, Fe–S clusters) or with other proteins. In *Vibrio cholerae*, one of the first identified T2SS substrates, the cholera toxin (CT) is a hetero-hexamer, wherein the toxin subunit A is associated with B subunit pentamer, which serves as carrier during secretion and cellular uptake [25]. Not surprisingly, due to their high stability, the T2SS substrates are found in many bacterial species that are adapted to extreme conditions of pressure, temperature and salt [26–29].

Substrate repertoire, very limited in some species and vast in others, appears to be a result of ongoing adaptive evolution. In *Aeromonas* and *Vibrio* spp. this evolution led to secretion of over 20 different exoproteins [30] and to dependence on T2SS for viability and OM integrity [31,32]. Free-living *Yersinia* and *Vibrio* use T2SS to degrade chitin and related compounds, highly abundant in aquatic environments [30,33,34]. Interestingly, GbpA, a chitin binding protein secreted by *V. cholerae*, promotes attachment to chitin to colonize zooplankton, but also to mucin in intestinal epithelia during the colonization of mammalian host [35]. Freshwater *Legionella pneumophila*, which lives either free or associated with protozoa, secretes, besides chitinases, at least 20 enzymes including lipase, cholesterol acyltransferase, aminopeptidase, RNase [36,37] and slime proteins involved in motility [38]. An obligate intracellular pathogen *Chlamydia* depends on T2SS for growth on glycogen reserves of its host [39]. Plant pathogens *Dickeya dadantii*, *Pectobacterium carotovorum* (formerly *Erwinia chrysanthemi* and *Erwinia carotovora*) or *Xanthomonas campestris* secrete a variety of plant cell wall degrading enzymes.

The majority of exoprotein enzymes are released to the surrounding milieu to generate small nutrient forms available for uptake. The *Klebsiella oxytoca* pullulanase, a lipoprotein that degrades branched maltotriose polymers had been a rare example of an exoprotein that remains surface-associated upon secretion [40]. Recently, other surface lipoproteins have been identified including SslE implicated in colonization and biofilm formation in enteropathogenic *E. coli* [41] or invasin DraD secreted by uropathogenic *E. coli*, associated with surface fimbriae [42]. The Tat-dependent lipoprotein UxpB from *Pseudomonas putida* is surface associated, but also released specifically to the medium under low oxygen conditions [43]. Similarly, phosphate starvation in *Caulobacter crescentus* induces T2SS-dependent release of the lipoprotein ElpS, an alkaline phosphatase, presumably by promoting secretion of a specific starvation-induced protease [44]. Protein cell association/release might be regulated by quorum sensing and environmental cues as a function of planktonic or community lifestyles and growth conditions. PnlH, a substrate of the Stt T2SS of *D. dadantii* uses a non-cleaved Tat signal sequence as a cell surface anchor [45]. In the absence of T2SS, the same signal targets PnlH to the OM via periplasmic chaperones [46].

Perhaps the most remarkable T2SS substrates of the lipoprotein class are the multiheme c-type cytochromes displayed on the surface of *Geobacter* or *Shewanella* spp. The exquisite alignment of dozens of heme clusters of these proteins on the bacterial surface allows them to relay electrons from the respiratory chain to insoluble Fe(III) and Mn(IV) oxides, uranium or DMSO as terminal acceptors [47–51]. Protein "nanowires" containing T4P in these species transport electrons over longer distances, further enhancing the contact area and transfer efficiency [52]. Besides providing a major primary source of bioavailable metals, these systems have remarkable potential for applications in biotechnology and bioremediation.

3. Modular architecture of T2S and CMF systems

Between 12 and 15 genes designated *gsp* (for General Secretory Pathway) [53] encode T2S machineries. The core genes are typically organized in one large operon *gspCDEFGHIJKLMNO* (Fig. 1A). In some species, including *Aeromonas hydrophyla* depicted here, *gspO* is found in a different locus with T4PS-encoding genes. Several additional genes code for factors involved in the correct localization and assembly of the OM channel-forming protein GspD, also called the secretin (reviewed in [9]). These include *gspS* and the recently identified *yghG* [54] or *aspS* [55], encoding "pilotin" proteins that target GspD to the OM. In *Vibrio*, *Aeromonas* and *E. coli* an additional operon *gspAB* encodes a peptidoglycan-binding complex that facilitates GspD multimerization [56], while in some species only the *gspB* component is present.

The T2SSs are highly similar in composition and structure to systems that build archaeal pili and flagella [57,58] and bacterial T4P that include class a, class b and competence pili [12,14,59]. The common feature of all these systems is the ability to promote assembly of helical filaments composed of plasma membrane-embedded pilin subunits. Here we will use the abbreviation CMF to designate this superfamily of *c*ytoplasmic *m*embrane-anchored *f*ibers. Comparative analysis of CMF members highlights their modular organization, which might provide clues on T2SS assembly, functions of different components and on the molecular mechanism of protein transport.

The original CMF module is found in all systems and consists of four basic protein elements. These include the pilin subunits (one major and up to four minor ones), a hexameric assembly-ATPase in the cytoplasm and two polytopic membrane proteins: the prepilin peptidase involved in pilin maturation and a membrane platform protein with two large cytoplasmic domains. In bacteria, four additional low-abundance (minor) pilin subunits are required to prime fiber assembly. Together, these eight elements seem to be sufficient for fiber assembly in monoderm bacteria, as exemplified by the competence T4P in *Streptococcus pneumoniae* [60] (Fig. 1B). In T4P of *Pseudomonas aeruginosa* these basic elements are organized in two operons: *pilABCD* (encoding the major pilin PilA, ATPase PilB, membrane platform protein PilC and prepilin peptidase PilD) and *fimU-pilVWX* encoding core minor pilins (Fig. 1C).

In diderm bacteria, the presence of the second, outer membrane barrier is linked to the acquisition of a new module in CMF systems to ensure fiber/substrate surface exposure. The channel forming protein secretin GspD in T2SS (PilQ in the *P. aeruginosa* T4P) is the central component of this module. It is typically associated with GspC (in T2SS) and PilP (in T4PS) [61–63]. In T2SS adjacent *gspCD* genes are always co-transcribed, sometimes in the opposite orientation relative to other *gsp* genes. Systematic exchanges of components between different T2SSs provided evidence for their species-specific interactions [64–66]. In *P. aeruginosa* the second $XphA^{GspC}$–$XqhA^{GspD}$ pair forms a functional module that can associate with the Xcp T2SS machinery and replace the endogenous pair $XcpP^{GspC}$–$XspQ^{GspD}$ [67]. In *P. aeruginosa*, the secretin PilQ and its partner PilP are encoded by the surface exposure operon *pilMNOPQ* (Fig. 1C).

The last two proteins of this module are GspL and GspM, belonging to the so-called IM assembly platform, a complex also containing GspE and GspF [68]. So far these proteins have not been found in the *Streptococcus* competence T4P (Fig. 1B). Their role could be to coordinate fiber assembly and its specific function. The cytoplasmic domain of GspL binds to the extended N-domain of the assembly ATPase GspE in a species-specific fashion [69,70], while its periplasmic domain interacts with GspM [71–73]. The two GspL domains correspond to PilM and PilN in *P. aeruginosa* T4P, while GspM has its homologue in PilO [14,74].

Some *gsp* genes like *gspN*, *gspB* or *gspH* are not absolutely essential for secretion [75] and are lacking in some systems. Their absence might be compensated by the overexpression of other components [71,76]. For example, the Stt system of *D. dadantii* does not have a homologue of the minor pseudopilin gene *gspH* [45].

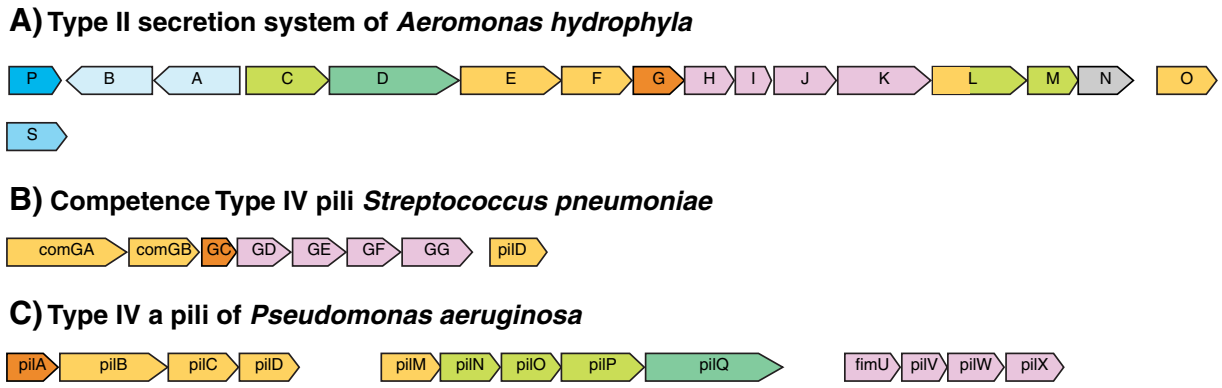

Fig. 1. Genetic organization and composition of type II secretion systems (A) in comparison with type IV pilus assembly systems in Gram-positive (B) and in Gram-negative bacteria (C). Common color code is used for genes belonging to the corresponding modules. Components of the same module are depicted with common colors: the CMF module gene encoding the major pilin subunit (in dark orange); the assembly ATPase, the membrane platform protein, genes *pilM* and *gspLcyto* encoding the cytoplasmic domains/factors and the prepilin peptidase (in pale orange); the minor pilin genes encoding the switch module (in pink). Genes encoding the connecting module in Gram-negative bacteria are depicted in light green and the secretin gene is in dark green. In blue are genes encoding the secretin targeting/biogenesis factors. The *gspN* gene encoding the IM protein of unknown function is shown in gray. GenBank Accession numbers: for *A. hydrophila* X66504.1, X81473.1 and YP_858310.1; for *S. pneumoniae* AP200 NC_014494.1 and for *P. aeruginosa* NC_002516.2.

4. The CMF assembly module

The high degree of similarity between T2SS and T4PS has been recognized early on, leading to models of their common basic mechanism [13,77,78]. T2SS has been proposed to assemble a filamentous structure termed "pseudopilus" to push exoproteins through the secretin channel via T4P-like extension and retraction dynamics acting as a piston [76,79–81]. An observation that T2SS assemble pili on the bacterial surface in plate culture and overexpression condition has provided a very useful handle on this fiber [75]. Biochemical, structural and functional analyses of this extended pseudopilus in the *K. oxytoca* Pul system [75,76,82] and Xcp T2SS of *P. aeruginosa* [83,84] have aimed at defining the mechanistic link between fiber assembly and protein secretion. Extracellular T2SS pili, also called hyper-pseudopili, are composed only of the major subunit GspG, while minor pseudopilins GspH, I, J and K have not been detected in these fibers, possibly due to their low abundance, degradation or retention in the periplasm [76,84]. Fiber analysis in *X. campestris* suggests that the native periplasmic pseudopilus is a complex of at least 400 kDa containing $GspG^{XpsG}$ and $GspH^{XpsH}$ [85].

All (pseudo)pilins are type II membrane proteins [86], which use SRP and Sec systems for membrane targeting and insertion [87,88]. Their correct N-in C-out orientation in the IM requires a positively charged N-terminal signal anchor, 6–8 residues long in T2SS pseudopilins. Their extraction from the membrane and assembly into helical fibers requires removal of this anchor by the prepilin peptidase GspO. This intra-membrane aspartate protease of the presenilin family [89] cleaves pilins after a conserved Gly residue on the cytoplasmic face of the IM. The crystal structure of its archaeal homologue FlaK has provided insights into this 8 TM-segment enzyme and its active site with a conserved GXDG motif [90]. In bacteria, GspO has an additional domain formed by the two N-terminal TM segments and a cytoplasmic loop containing Zn^{2+}-binding tetra-cysteine motifs [91]. This region catalyzes the transfer of a methyl group to the first residue of mature pilin, typically a Phe [77,92]. The role of this modification, non-essential for pilin processing, is still unclear [93–96].

Similar to T4 pilins, major pseudopilins are elongated lollipop-shaped proteins [86]. Their S-shaped α-helical stem is typically 53-residues long and membrane-embedded through its hydrophobic N-terminal segment, highly conserved among pilins. The periplasmic end of the stem (α-C) is followed by an α–β loop, extending into the globular beta-sheet domain [86]. Structural analysis of GspG from several species revealed a conserved C-terminal loop in this β-sheet stabilized by a Ca^{2+} ion [97,98]. In *V. cholerae* $EpsG^{GspG}$ Ala substitutions of Ca^{2+} binding residues severely impair protein secretion [97]. In $PulG^{GspG}$ of *K. oxytoca*, the equivalent residues are essential for pseudopilin folding or stability [99]. GspG, like its homologues, builds helical fibers, which are restricted to the periplasmic compartment under physiological expression levels in liquid culture [82,83]. Cryo-EM analysis of $PulG^{GspG}$ fibers combined with X-ray crystallography have provided the first insights into their organization and structure [82]. Based on the predictions generated using flexible structural modeling, the detailed and experimentally validated structure of T2SS pili revealed right-handed helix protomer organization in the fiber [99], highly similar to that of gonococcal T4P [99,100]. In both fiber types, pilins form inter-protomer contacts along the 1-start as well as 3- and 4-start helix. In PulG pili two salt bridges at the 1-start helix interface are essential for pseudopilus assembly and highly conserved in all major pseudopilins [99]. Importantly, these contacts are also essential for protein secretion under physiological conditions, showing a direct correlation between these two processes.

In addition to the proton motive force, crucial for function [101,102], it is now established that GspE ATP binding and hydrolysis provide energy for pseudopilus assembly and protein secretion [103]. The AAA+ hexameric ATPase motor GspE is structurally related to the T4P assembly ATPase PilB and the retraction ATPase PilT. Structural studies in *V. cholerae* and *X. campestris* revealed a bi-lobed monomer wherein ATP binds at the hinge between the N-terminal and C-terminal domains [104,105]. The recently obtained crystal forms of EpsE hexamers show 20-fold higher ATPase activity compared to monomers [106]. ATP hydrolysis requires contacts between N and C domains of adjacent monomers. These contacts involve specific Arg residues at the interface that have been identified by site-directed mutagenesis based on the *V. cholerae* $EpsE^{GspE}$ hexamer model [103]. Large displacements of N and C domains occur between the ATP and ADP bound states, as seen in many members of this ATPase family, presumably creating the power stroke of this remarkable motor. Cryo-EM and crystallographic studies of the T4P retraction ATPase PilT suggest that conformational changes induced by ATP binding and hydrolysis in one active site affect the overall conformation of the hexamer, possibly coordinating the ready, active and release ATPase conformations [107,108]. In *Vibrio* and other T2SSs GspE has an extended N-terminal domain forming a stable complex with the cytoplasmic domain of GspL, characterized by X-ray crystallography [70]. The species-specific GspE–GspL contact might be involved in energy transfer to membrane assembly complex, as discussed below. The close genetic link between GspE and GspF suggests that these proteins function as a complex. The essential CMF component GspF is fully embedded in the IM via the three TM segments [109,110]. The two cytoplasmic domains of GspF probably share the same 6-helix bundle structure, which has been revealed by X-ray

analysis of the N-terminal domain Cyto1 [111]. PilC, the GspF homologue involved in T4P biogenesis in *Thermus thermophilus* showed the same cytoplasmic domain structure and biochemical studies suggested it forms a tetramer [112]. Yeast two-hybrid and pull-down studies suggest that GspF Cyto1 interacts with GspE and GspL, forming the so-called T2SS assembly platform that also includes GspM [68]. GspF requires GspL and GspE for full stability [113]. Although the role of GspF in CMF assembly is crucial in T2SS and in *P. aeruginosa* T4P [114], its homologue is not essential for assembly of in meningococcal T4P [115]. Clearly, a lot remains to be learned about its molecular function.

5. Minor pseudopilins: the switch module?

Four minor pilin subunits (shown in pink in Fig. 2) form a separate module in CMF systems [115–118]. Like GspG, minor pseudopilins GspH, GspI, GspJ and GspK are processed and N-methylated by the prepilin peptidase GspO [116]. Although all these features allow their incorporation into pili, so far these proteins could not be detected in pilus fractions [76,84]. In T2SSs X-ray crystallography brought structural insights into their periplasmic domains [119–122]. Particularly remarkable, the structure of the GspJ–GspI–GspK periplasmic domain trimer shows a quasi-helical symmetry and staggered subunit arrangement [123]. GspI occupies a central place in the trimer, consistent with the strong requirement for GspI during PulG^{GspG} and XcpT^{GspG} pilus assembly [75,84,124] and with biochemical studies showing independent binding of GspI^{XcpV} to GspJ^{XcpW} and GspK^{XcpX} [125]. GspK at the summit caps this complex via its α-helical domain. Molecular dynamics (MD) and interaction studies of the full-length GspJ–GspI–GspK complex suggest that sequential binding events lead to initiation of pseudopilus assembly [124]. In MD simulations, the GspI–GspJ dimer tilts in the membrane to facilitate binding of GspK and promote partial extraction of GspK from the membrane [124]. These events, independent of other T2SS factors are essential for the efficient initiation of pseudopilus assembly and protein secretion. They might act as a switch to transduce a signal across the IM to activate the assembly ATPase GspE. In the simplest model, membrane extraction of GspK could exert a pulling force on the GspE N-domain via GspL. This would promote GspE binding to cardiolipin to stimulate the initial rounds of ATP hydrolysis [124,126] that are required for assembly of GspG. GspH, which binds this initiating tip complex in vitro via its globular domain [125], could ensure a transition between initiation and ATPase-catalyzed elongation, required for maximal efficiency of protein secretion under native conditions [124]. Consistent with the estimated low levels of minor pseudopilin subunits under physiological conditions [84], one initiation event would allow continued pseudopilus elongation catalyzed by GspE. A theoretical model of length control suggests that proteolysis is an important factor in maintaining a steady pilin pool and fiber length [127]. Interestingly, the tip pseudopilin GspK^{XcpX} has been reported to increase protease sensitivity of the major pilin GspG^{XcpT} in *P. aeruginosa* [84].

Although minor pseudopilins have been implicated in secretin channel opening, their presence in Gram-positive T4P systems lacking the secretin [60], argues against this model. Another major role proposed for minor pseudopilins, predicted by the piston model of type 2 secretion, is their specific binding to exoprotein substrates. Indeed, recent studies using surface plasmon resonance show that the globular domains of the minor pseudopilins form a stable quaternary complex that interacts specifically with the exoprotein substrate of the Xcp T2SS of *P. aeruginosa* [81].

6. The secretin channel

The T2SS component GspD forms a dodecameric, megadalton-size complex in the OM. It is a member of the secretin class of multidomain proteins that function as OM channels in many Gram-negative secretion machineries [9] (Fig. 2). Domains N3 and C in T2SS secretins

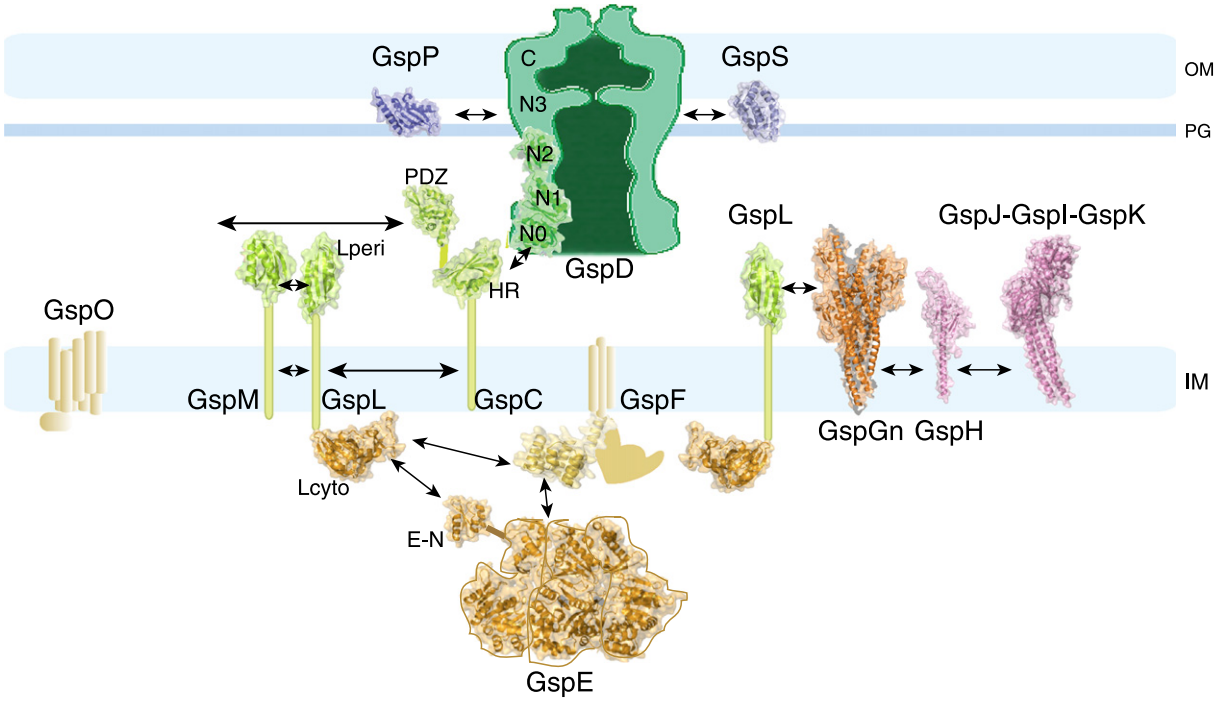

Fig. 2. The localization and interactions of T2SS components in the Gram-negative bacterial envelope. Components/domains of known structure are shown in cartoon representation with a transparent surface envelope, using the same color code as in Fig. 1A. Proteins and their parts of unknown structure (TM segments, linkers, etc.) are represented as cylinders and lines. Proteins are labeled with standard Gsp single-letter code and individual domains are indicated in some cases. The pilotins GspSβ or AspS are designated as GspP. Documented interactions between different T2SS components are represented by arrows, except for GspJ, GspI and GspK, which are shown as a complex. Protein Data Bank entries were used as templates GspC-HR (2LNV), GspC PDZ (2I4S), GspC–GspD complex (3OSS), GspD (3EZJ), GspE (1P9R and 2BH1), GspF (2VMA), GspH (2KNQ), GspJ-I-K (3CI0), GspL (1W9 and 2W7V), GspM (1UV7), GspS (3SOL) and AspS (4FTF) to generate protein images using PyMol software. GspG polymer and GspJ-I-K trimer models with TM segments were described in [99] and [124].

are required to form the membrane embedded core of the channel, which is trypsin-resistant in PulD^{GspD} of *K. oxytoca* [128,129]. In several T2SS secretins these domains have the capacity to form closed channel multimers in vivo and in vitro [130] and are separated by a constriction into a large periplasmic vestibule and an external chamber (Fig. 2) [128,129,131]. The N-terminal domains N0, N1 and N2 further extend into the periplasm and are well resolved in the cryo-EM map of *V. cholerae* EpsD^{GspD} [131], but are highly flexible in *K. oxytoca* PulD^{GspD} [132]. X-ray crystallography revealed the periplasmic domains N0, N1 and N2 to be connected by flexible linkers [133]. Biochemical and functional studies on *D. dadantii* OutD support the idea that these domains undergo large conformational changes and that secretin subunits are organized as a hexamer of dimers [61,134]. Recent structural analysis of XcpQ^{GspD} in *P. aeruginosa* suggested a similar model [135]. On the other hand, EM studies and the recent crystal structure of the superhelix formed by the N0 domain of ETEC GspD suggest rather the 12-fold symmetry of the secretin dodecamer [136]. In vitro studies of the *K. oxytoca* PulD^{GspD} indicate that assembly is initiated by monomer addition [137]. It is plausible that dodecamers with high and low symmetries represent particular conformations that secretins cycle through. Further structural, biophysical and functional studies will be required to understand the conformational switch involved in GspD multimerization and membrane insertion.

When produced in the bacterial cytoplasm, PulD^{GspD} multimers insert into the IM to form lethal holes, a property used to select mutants defective in multimer formation [138]. Such mutations map in multiple PulD^{GspD} domains, suggesting a large multimerization interface, perhaps rationalizing the exceptional resistance of these channels to heat and detergent denaturation. Although the secretin channel is thought to be generally closed in its resting state [128,131], small constitutive or transient openings cause proton leakage upon its insertion in the inner membrane, inducing the phage shock response and PspA production to repair this damage [139]. Mutations that increase the size of these gaps were mapped in the C domain of the filamentous phage secretin pIV, defining two loop regions, Gate 1 and Gate 2 [140]. A recent study of PulD^{GspD} indicates the presence of a constitutively open small pore with size similar to that of porins, allowing passage of small molecules (~600 Da) [141]. The passage of pili (6 nm in diameter), or exoprotein substrates, clearly requires a much larger channel, suggesting dramatic conformational changes to promote a wider gate opening. How this happens without causing the leakage of periplasmic contents remains a major question.

7. The secretin targeting and OM assembly module

Several factors have evolved to control the efficiency of secretin insertion in the OM. Small OM lipoproteins of the GspS family referred to as 'pilotins' [142] allow efficient targeting to the OM via the lipoprotein sorting pathway [143]. Recent studies have revealed the structural basis of interaction between PulS^{GspS} and the C-terminal S domain of the secretin PulD^{GspD}, which is intrinsically disordered [144]. In the absence of the pilotin, the S-domain is a target of periplasmic proteases, providing a quality control mechanism that ensures degradation of incorrectly localized secretins. The PulS^{GspS} is composed of four alpha helices that form a binding groove for the S-domain peptide [145]. Very similar results were obtained for the *D. dadantii* OutS pilotin, showing that the disordered C-terminal secretin peptide adopts alpha-helical structure when bound to the pilotin [146,147].

In *D. dadantii* a second factor OutB^{GspB} is required for stability of OutD^{GspD}, probably through a direct interaction [148]. OutB is a homologue of ExeB and GspB IM proteins, encoded by the *gspAB* operon in *Aeromonas*, *Vibrio* and *E. coli.* Its homologue ExeB together with the ExeA ATPase in *A. hydrophila*, are required for assembly ExeD^{GspD} secretin in the OM [149]. The large ExeA-ExeB IM complex could interact with the secretin via ExeB [150,151]. In *Vibrio vulnificus* a gene fusion event resulted in a single EpsAB hybrid protein [152] whose structure suggests similarities with peptidoglycan remodeling enzymes. Interestingly, *gspAB* deletion does not affect protein secretion in *Vibrio*, indicating functional redundancy [56]. While this group of bacteria lacks GspS homologues, recent studies have identified its functional analogues, providing a nice example of convergent evolution. In *E. coli* a gene upstream of the T2SS cluster encodes YghG, a small OM lipoprotein with significant beta-strand content and a binding groove for the C-terminal GspD peptide. While this new pilotin has been named AspS in *Vibrio* [55] and GspSβ in ETEC [54], we propose the designation GspP as an appropriate single letter code for pilotins of this new family (Fig. 2).

Interestingly, in *P. aeruginosa*, which assembles the secretin XcpQ^{GspD} and the lipo-secretin HxcQ^{GspD} [153], a similar presumed targeting factor has been identified, encoded by the PA3611 gene [55]. It remains to be determined whether this factor has a targeting or anti-proteolytic function in *Pseudomonas*. Precise molecular function of GspS/GspP pilotins is still unclear and it remains possible that they act as OM receptors for the secretin, stabilizing its OM intermediate during biogenesis. The targeting function might also require general periplasmic chaperons, like DegP, SurA or Skp [154].

8. The connecting module

In T4P of *P. aeruginosa* 4 proteins, PilM, PilN, PilO and PilP link the IM pilus assembly complex with the secretin channel PilQ. Recent studies suggest that none of these proteins is essential for assembly per se, but rather for the fiber exposure on the bacterial surface [114]. Their structural homologues in T2SS are GspL, GspM and GspC. The cytoplasmic domain of GspL and PilM share an actin-like fold [155], which is also found in the cytoplasmic protein FliH of archaella [156]. Unlike PilM and FliH, their homologue, the cytoplasmic GspL domain (GspL$_{cyto}$) does not bind ATP. The co-crystal structure of a complex between GspL$_{cyto}$ and GspE N-domain shows an extensive interaction surface providing an explanation for the species-specificity of this protein pair [70]. The role of GspL might be not only to provide the physical link between the ATPase and the membrane, but also to regulate its activity and allow coupling to pilus assembly. Studies in *X. campestris* show that only the ATP-bound form of GspE^{XpsE} interacts with GspL^{XpsL} [157]. The last 11 residues of the cytoplasmic domain of GspL are crucial for cardiolipin binding, which stimulates GspE ATPase activity [126]. Importantly, EpsL^{GspL} could be cross-linked to the major pseudopilin EpsG^{GspG}, providing a molecular basis for the role of ATPase in pseudopilus assembly [158].

GspM is a bitopic IM protein that interacts with GspL [68,71–73,159]. The two periplasmic domains share a ferredoxin fold and stabilize each other, forming heterodimers and ensuring the correct cellular localization [70,155,160]. While GspL is essential for both fiber assembly and secretion, the absence of GspM still allows pilus assembly, suggesting that overproduction of other components partially compensates for its absence [12,84]. The C-terminal part of GspM has been implicated in an essential function unrelated to its interaction with GspL, but the molecular basis of this role remains to be identified [72,126,155]. Recent studies suggest that GspL and GpsM interact through their TM segments with GspC and form dynamic homo and heterodimers [161]. Recent structural studies revealed more details on the complex of periplasmic domains of the GspL and GspM homologues PilN and PilO in *T. thermophilus* and show that major pilins bind these domains during pilus elongation [154]. GspC is an IM-anchored protein, with two periplasmic domains, the homology region (HR) near the membrane and the C-terminal PDZ domain that might be involved in substrate specificity [162]. The structure of the HR domain revealed high similarity with the IM lipoprotein PilP of T4PS [62,163,164]. The TM segment of OutC^{GspC} mediates essential dimerization in *D. dadantii* [165], while the HR domain interacts with N0 domain of GspD [166], as shown by the recent structural and surface plasmon resonance studies [62,81,164]. This contact, essential for function, provides a trans-envelope connection between the assembly platform in the IM and the

OM channel. The tight link between GspC and GspD is probably the basis for the species-specificity identified in systematic exchange experiments of Out T2SS components of *D. dadantii* and *P. carotovorum* [64]. Studies in *D. dadantii* T2SS suggest however that this link is flexible and involves multiple dynamic interfaces, and also that the stoichiometry of the C–D complex might vary [61,134].

Fluorescent protein fusions have provided evidence that T2SS assembles at discrete sites in the bacterial envelope, visible as foci in live cells [160]. In *E. coli* the secretin GspD^{PulD} forms such foci at the cell periphery even in absence of other T2SS factors [167]. The secretin auto-assembly led to the model wherein GspC–GspD pair provides the key connection that drives assembly of IM T2SS components [160]. In *V. cholerae* formation of GspC^{EpsC} foci in the IM depends on the assembly of GspD^{EpsD} in the OM [160]. Although the secretin and its link with assembly platform are essential for surface exposure of pili and for protein secretion, fibers can still form in the periplasm in absence of secretin, indicating CMF assembly functions as an independent module both in T2SS and T4PS [76,115,124,168].

A role of GspC and the periplasmic flexible domains of GspD in substrate binding and selection has been proposed based on the domain exchange studies in the Out T2SS of *D. dadantii* and *P. carotovorum* [162]. Further support for the roles of both GspC and GspD in substrate binding comes from in vitro studies showing that GspG^{XcpP} binds specifically to the Xcp system substrate LasB [81]. The C-terminal PDZ/coiled-coil motif of GspC has been implicated in these protein–protein interactions [166]. However, this motif is required for secretion of only a subset of substrates in the *D. dadantii* Out T2SS [162]. In vitro, cholera toxin binds to the periplasmic secretin vestibule in the absence of GspC [169].

9. Substrate recognition and protein transport mechanism

The folded nature of T2SS substrates suggests that a structural motif on exoprotein surface determines specific interactions leading to secretion. Despite a large number of studies and structural information available for dozens of substrates, the nature of this signal has remained elusive. T2SS substrates are for the most part large proteins rich in beta-strand and loop motifs, without obvious common sequence or structural features [170]. Exoproteins might make specific yet transient contacts with one or more interacting partners among the T2SS components during different steps of the secretion process. Furthermore, each exoprotein might use a different sequence determinant for specific binding to their partners in T2SS. In many exoproteins, mutations that impair secretion also reduce substrate stability, which further complicates interpretation of the results [171].

Several regions of primary exoprotein sequence are typically implicated in secretion, involving loop or beta-strand regions localized near the N-terminus of the substrate, but also one or more other regions that are clearly distant on the 3D structure of the protein. Examples include the B-subunits of CT and LT [172], exotoxin A of *P. aeruginosa* [173], PulA of *K. oxytoca* [174] and the polygalacturonase PehA [171]. Although many studies suggest multiple short signals and raise the possibility of functional redundancy, single residue substitutions can block secretion. This is the case of aerolysin of *Aeromonas salmonicida* [175] and the soluble colonization factor TcpF, secreted by the toxin-coregulated pilus in *V. cholerae*, in which a mutation of conserved motif YXS abolished secretion [176].

Which substrate-binding T2SS components determine specificity and how is still unclear. Early exchange experiments between T2SS components of *D. dadantii* and *P. carotovorum*, suggested a role of OutC^{GspC} and OutD^{GspD} as specificity "gatekeepers" [64]. While these species were originally classified in the same genus (*Erwinia*) it is now clear that they are more distant and this divergence might preclude formation of a functional GspC–GspD complex [62,65,66]. Other experiments implicate particular regions of these proteins in substrate recognition [162]. Surface plasmon resonance analysis of *P. aeruginosa* T2SSs shows specific exoprotein binding to GspC and to the minor pseudopilin complex [81], while EM analysis shows cholera toxin binding to EpsD^{GspD} [131]. In vivo structure–function analysis is required to understand the functional significance of this binding. Furthermore, complementation studies between T2SS components need to be revisited, as the role of some T2SS components in specificity might have been overlooked due to overexpression [64,71]. Similar questions are relevant for the bi-functional CMF systems of *Vibrio* [176], *Dichelobacter nodosus* [177] or *Francisella novicida* [178], which promote T4P assembly and protein secretion. In several T2SS major pseudopilins are not interchangeable, even when overproduced, as in *Klebsiella* and *Xanthomonas*, or the two T2SSs of *P. aeruginosa* [71,76,179]. Interestingly, a few point mutations are sufficient to confer to HxcT^{GspG} the ability to promote surface piliation and to secrete the Xcp T2SS substrate LapA [179].

At least two models for how pseudopilus drives protein secretion have been proposed: the piston mechanism [79] and rotary ratchet/Archimedes' screw [13,78,180]. While the piston model has been extensively discussed in the literature, the screw model deserves serious consideration, in view of the structural and evolutionary relation of GspE and other macromolecular transport motors with ATP synthase [181]. In this model, pseudopilus function in T2SS would be comparable to a spiral escalator connecting the IM to the bacterial cell surface. GspG would build steps of this helical treadmill powered by the cytoplasmic, "basement" motor composed of the GspE–GspF complex. The minor pseudopilin module would provide the switch to turn the treadmill on. Once activated, it would function permanently, as long as the highly abundant GspG building blocks and energy are available. One of the many major challenges is to determine how exoproteins specifically enter into this pathway and whether this specificity is determined at several levels.

In physiological and liquid culture conditions pseudopilus fibers are not surface exposed, suggesting that pseudopilus length is limited, possibly through action of periplasmic proteases. The C-terminal GspG loop stabilized by Ca^{2+} might be the weak point of the structure and loss of Ca^{2+} might expose this loop to proteolysis, playing a regulatory role, similar to the one proposed for PilY in T4P [182]. Although this might seem energetically expensive, the benefit of nutrient availability and uptake provided by T2SS would likely out-weigh the cost. Once assembled, the same machinery would be used for secretion of many exoproteins via the continuous assembly of GspG, which could also be recycled via proteolysis. Similarly, recent studies of type VI secretion systems show an essential role of proteolysis in its dynamic activity [183].

The limiting step in protein secretion is probably the entry of substrates into the secretin vestibule, which, together with GspC is likely to restrain protein access to the base of the machinery (Fig. 3). As suggested recently, different substrates might accommodate differently to this flexible passageway, precluding nonspecific leakage of solutes and proteins [136]. Given the interaction of the secretin N0 domain with the HR domain of GspC, high flexibility is required to provide substrate access to the secretin vestibule. Similar considerations led to a model of radially aligned PilM, PilN and PilO in *P. aeruginosa* T4P that could provide a conduit for pilins to the assembly site [63]. Although exoproteins are often depicted as floating in the periplasm, the same entryway at the IM base is predicted at least in the case of IM-anchored lipoproteins.

10. Concluding remarks

The past decade has witnessed remarkable progress in T2SS analysis providing structural information for nearly all soluble domains of its components and of exoprotein substrates. Further understanding of these dynamic complexes will require complete structural information on the missing TM segments that mediate dynamic and functional connections between these domains. To understand the workings of this machine, detailed descriptions of functional sub-complexes and their allosteric changes will be required. Presumably driven by the

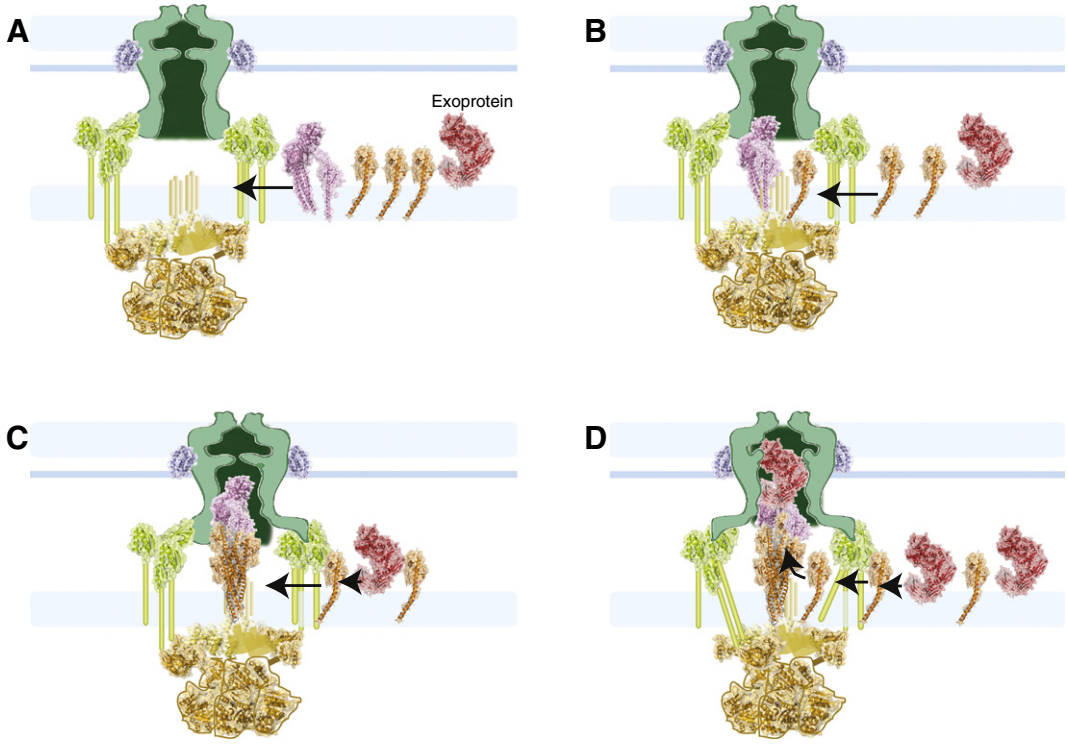

Fig. 3. Model of type 2 secretion, illustrating the unresolved questions. A. Assembly of T2SS components in the OM and IM delineates a compartment, the access to which is limited. B. Minor pseudopilins GspJ-I-K form a complex that recruits GspH and GspG. Targeting of GspG to GspL might provide access to the assembly platform complex with GspF (depicted here as a dimer) and GspE ATPase. C. Assembly of GspG is initiated. Pseudopilus elongation could drive conformational changes of the GspD N domains and GspC to provide access to the exoprotein (in red). D. The growing pseudopilus opens the channel wide and exoprotein binding to the fiber or mere entry into the channel allows its unidirectional transport along the growing pseudopilus.

ATPase motor, modulated by the secreted cargo, housekeeping proteases and chaperones, these conformational transitions need to be described in molecular detail and their individual functions and dynamics require in vivo studies.

The wealth of biochemical and structural information on T2SS and related systems provides a solid basis for further functional and interaction studies. Based on structural predictions and quantitative analysis of different phenotypes, gaining mechanistic insight into these systems will require converging data using different complementary approaches. Among these, interaction studies, molecular modeling, MD in model membranes, mass spectrometry, high-resolution cryo-EM and solid state NMR are well suited to study membrane protein complexes. Single molecule analysis and biophysical methods will also be required to gain insight into their highly dynamic function. Available sequence data generated through life-size natural selection need to be exploited to identify sequence conservation and coevolution of substrates and T2SS components.

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