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Bacterial protein secretion systems: Game of types

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Abstract

Protein trafficking across the bacterial envelope is a process that contributes to the organisation and integrity of the cell. It is the foundation for establishing contact and exchange between the environment and the cytosol. It helps cells to communicate with one another, whether they establish symbiotic or competitive behaviours. It is instrumental for pathogenesis and for bacteria to subvert the host immune response. Understanding the formation of envelope conduits and the manifold strategies employed for moving macromolecules across these channels is a fascinating playground. The diversity of the nanomachines involved in this process logically resulted in an attempt to classify them, which is where the protein secretion system types emerged. As our knowledge grew, so did the number of types, and their rightful nomenclature started to be questioned. While this may seem a semantic or philosophical issue, it also reflects scientific rigour when it comes to assimilating findings into textbooks and science history. Here I give an overview on bacterial protein secretion systems, their history, their nomenclature and why it can be misleading for newcomers in the field. Note that I do not try to suggest a new nomenclature. Instead, I explore the reasons why naming could have escaped our control and I try to reiterate basic concepts that underlie protein trafficking cross membranes.

INTRODUCTION

All cells are surrounded by one or more membranes which protect them from harmful environmental substances [1]. The cell membrane/s form a hydrophobic barrier made of lipids/phospholipids that prevents the free transit of hydrophilic molecules between internal and external compartments. Yet communications with the environment is of prime importance to capture nutriments and release waste. The cell has in place membrane proteins which form channels that allow passive or active diffusion of molecules. The inserted channels can result from the multimerization/oligomerisation of a single protein such as TatA [2], although it is likely that parts of TatB and TatC also form the channel, or the hetero-oligomerisation of several subunits such as the translocon SecYEG [3], both pores being involved in protein translocation. Alternatively, a single protein that has several transmembrane segments can fold in the membrane so that it builds a channel structure, such as LacY [4], a permease that allows the passage of lactose.

Based on the structure of their cell envelopes, bacteria have been grouped into two categories, i.e. Gram-negative and Gram-positive. This empirical classification arises from a staining method using crystal violet and various steps of coloration-decolouration [5]. It subsequently appeared, that the bacteria not retaining the stain had a cell envelope made of two hydrophobic membranes concealing a thin layer of peptidoglycan (PG) within the periplasm. Instead, those staining positive had a single membrane and a thick layer of PG trapping the stain. The cell envelope of Gram-negative bacteria was further characterized, and the cytoplasmic membrane or inner membrane is made of a symmetric bilayer of phospholipids, whereas the outer membrane is asymmetrical with the outer layer being made of lipopolysaccharides and the inner layer of phospholipids [6]. In Gram-negative bacteria communication with the external environment requires inner membrane proteins, such as LacY, but additionally channels in the outer membrane [7]. Lactose uptake across the outer membrane of *Escherichia coli* is mediated by the porins LamB or OmpF before transport across the inner membrane by LacY [8]. Whereas the membrane

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Abbreviations: CDI, contact-dependent inhibition; FHA, filamentous haemagglutinin; GSP, general secretion pathway; POTRA, polypeptide transport-associated; Tat, twin arginine translocation; T4P, type IV pilus; TPS, two-partner secretion; T0SS, type zero secretion system; T1SS, type I secretion system; T2SS, type II secretion system; T3SS, type III secretion system; T4SS, type IV secretion system; T5SS, type V secretion system; T6SS, type VI secretion system; T7SS, type VII secretion system; T8SS, type VIII secretion system; T9SS, type IX secretion system; T10SS, type X secretion system; T11SS, type XI secretion system.
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spanning region of LacY is made of hydrophobic alpha-helices, the membrane integrated portion of outer membrane proteins comprises amphipathic β-strands.

It is obvious from the description of the cell envelope that some proteins must be targeted to various extra cytoplasmic compartments. It was discovered that specific transport systems, primarily the Sec system [9], is required to transport proteins across the cytoplasmic membrane. The exported proteins that use the Sec pathway carry a cleavable N-terminal targeting signal [10], known as a signal peptide, and traverse through the inner membrane channel via the pore formed by the SecYEG translocon [9]. This is a narrow pore and only unfolded proteins can pass, so cytoplasmic chaperones, such as SecB [11], are required to maintain Sec substrates in an export-competent state. Once in the periplasm, proteins can fold such as MalE, a maltose binding protein, or be inserted in the outer membrane such as the LamB porin. It has also been shown that some exported proteins must fold in the cytosol before being transported, and in that case will use another membrane translocation system called Tat [2]. The Tat transport channel is much larger than SecYEG and receives proteins that have a particular signal peptide containing a recognisable twin arginine motif [12].

In Gram-positive bacteria, the transport of proteins by the Sec or Tat systems could result in extracellular localisation, whereas in Gram-negative bacteria it does not because these pathways deliver proteins only to the periplasm. Secretion in Gram-negative bacteria only refers to those proteins that pass beyond the outer membrane. From a semantic point of view this observation is key and must be accepted before one can go into the definition of protein secretion systems, which we will see in the next section progressively became classified by types.

THE DEFINITION OF A SECRETION SYSTEM AND THE EMERGENCE OF TYPES

Transport across the bacterial cell envelope

If one considers that a bacterial protein secretion system releases proteins into the extracellular milieu then in Gram-positive bacteria Sec and Tat could be sufficient to achieve this goal. In Gram-negative bacteria that is not the case and this is the main reason why Sec [9] and Tat [12] are not generally referred to as secretion systems but as export machines or accessory to 'secretion types'. Instead, secretion types were identified as systems that allow the transport of proteins across the cell envelope, and specifically the outer membrane, of Gram-negative bacteria [13].

Historically protein secretion systems were discovered in bacteria such as *Escherichia coli* [14], *Neisseria gonorrhoea* [15], *Klebsiella pneumoniae* [16], *Yersinia enterocolitica* [17] and *Pseudomonas aeruginosa* [18]. Among the early studies to describe protein secretion systems was the discovery of accessory proteins that allow the release of *E. coli* haemolysin [19]. Interestingly, secretion of the *K. pneumoniae* pullulanase also required accessory proteins but those were different from the *E. coli* haemolysin system [20]. There was also a fascinating report about the secretion of *N. gonorrhoea* IgA protease, which did not need any accessory proteins for transport including release from the cell surface, which happens upon autocleavage [15]. Finally, Yop proteins which accumulated as aggregates in the extracellular medium of *Y. enterocolitica* cultures were proposed to be secreted by yet another mechanism [17]. In other words, these studies led to the implication that individual Gram-negative bacteria evolved specific and distinct secretion mechanisms. Furthermore, some systems appeared to be an extension of the Sec pathway, such as for IgA protease or pullulanase secretion [21], whereas other exoproteins such as haemolysin or Yops did not display a canonical signal peptide and were proposed to cross the whole cell envelope in a single step.

It is difficult to imagine that every single Gram-negative bacterial species would put in place a unique mechanism for secretion across the outer membrane, and indeed it was not long before it became apparent that despite the existence of many different systems, they are conserved among Gram-negative bacteria. The first hint about this conservation was the finding that two of the accessory proteins involved in pullulanase secretion, namely PulL and PulM [22], have homologues in *P. aeruginosa*, XcpY and XcpZ, respectively [23]. In *P. aeruginosa*, the Xcp system is involved in the secretion of many enzymes including elastase, exotoxin A, lipase, phospholipase and alkaline phosphatase [24], which differs from the Pul system that is entirely dedicated to the secretion of the single pullulanase enzyme. PulL/M and XcpY/Z are integral cytoplasmic membrane proteins, but their absence abrogated secretion into the extracellular medium, and instead resulted in the accumulation of the secretion substrates in the periplasm. These substrates have a signal peptide which explained their Sec-dependent transport into the periplasm. It then become obvious that PulL/M or XcpY/Z are part of a larger cell envelope macromolecular complex, including outer membrane proteins, i.e. PulD/XcpQ [25], which is responsible for collecting the proteins in the periplasm and transporting them across the outer membrane. This was the start of the protein secretion types - the Pul/Xcp system became the type II secretion system (T2SS), while the haemolysin transport system was called T1SS, the IgA protease (initially called an auto-transporter) would be part of the T5SS, and the Yops require the T3SS for secretion. Importantly individual bacterial species can encode simultaneously multiple secretion types, for example *P. aeruginosa* uses the T1SS for secretion of the alkaline protease (AprA) [26] and the T3SS for secretion of several anti-eukaryotic effectors such as ExoSTYU [27]. Furthermore, bacteria can have several examples of each system, like in *P. aeruginosa* with the T1SS called Apr [28] for AprA secretion and the T1SS called Has for the release of the haem-binding protein HasA [29].

The protein secretion types in Gram-negative bacteria

As outlined in the previous section, protein secretion systems are large macromolecular complexes which can span the whole cell envelope and allow transit of proteins all the way to the extracellular medium. The canonical T1SS, which is exemplified by the haemolysin system, involves three components. HlyB is a multi-spanning inner membrane protein that carries an ATP binding and hydrolysis domain located on its cytosolic side. The T1SS substrates do not carry Sec-dependent signal peptides, but instead have a C-terminal non-cleavable targeting signal [30] which direct them to HlyB. HlyB drives the energy-dependent transport across the inner membrane [31] and triggers the recruitment and assembly of the two other accessory proteins [32]. HlyD, initially called a 'membrane fusion protein' interacts with HlyB as well as with the outer membrane protein TolC, so that a continuous channel is generated spanning from the cytosol to the extracellular environment, completely bypassing the periplasm. TolC has a remarkable 3D structure, being a trimer that forms an outer membrane-embedded β-barrel with an alpha-helical periplasmic conduit [33].

The T2SS is far more complex. For example, the *P. aeruginosa* Xcp system involves a dozen accessory proteins [34]. In addition to the XcpYZ proteins [35], the inner membrane platform includes a further protein, XcpS [36]. The membrane platform is connected to a peripheral ATPase called XcpR which interacts with XcpY [37]. In contrast to the T1SS membrane ATPase, the XcpR ATPase associated with the Xcp inner membrane platform is not directly involved in exoprotein translocation across the cell envelope but instead supports the assembly of a periplasmic proteinaceous structure which is called the pseudopilus [38]. The pseudopilus is made of pseudopilins including the major pseudopilin XcpT [39] and four minor pseudopilins XcpUVWX capping the pseudopilus [40, 41]. The pseudopilins are inserted in the inner membrane in a Sec-dependent manner, then slide sideways towards the Xcp inner membrane platform [42]. The prepilin peptidase, XcpA, cleaves a short N terminal leader sequence in the pseudopilin [43], which will aid the XcpR-dependent and ATP-driven extraction from the membrane and subsequent assembly into a periplasmic pseudopilus. The inner membrane platform is connected to the outer membrane channel XcpQ by the inner membrane protein XcpP, which interacts both with XcpZ and XcpQ, and whose multimerization likely encases the periplasmic pseudopilus [44, 45]. The XcpQ protein belongs to the secretin family which have remarkably large dodecameric structures and form gated channels [46]. The dimensions of the channel would be adequate to accommodate pre-folded exoproteins, which may gain access to the inside of the XcpP cage and be pushed into the secretin by the piston-like movement of the pseudopilus, ultimately being released into the extracellular medium [47]. The nomenclature XcpP-Z might sound odd but originates from the initial discovery of the *xcpYZ* genes which appeared to be the last two genes of a larger operon [23]. The nomenclature has not been entirely unified for the T2SS but mostly *Pseudomonas* species will have the *xcp* names [48] while for any other bacterial species it will be named after the *gsp* nomenclature with the correlation of *xcpPQRSTUVWYZ* to *gspCDEFGHIJKLM*.

In *Y. enterocolitica* the Yop proteins found aggregated in the culture supernatants were artefactual, and their ultimate destination was in fact meant to be the cytosol of eukaryotic target cells [49], where they may, like YopE or YopT, alter the dynamic of the cytoskeleton by interfering with Rho GTPases [50]. The system that allows the injection of these subversive effectors directly into the eukaryotic cytosol is the T3SS which assembles into a needle-like structure at the surface of the bacterium [51]. The T3SS is now known as the injectisome [52] and it involves ca. 25–30 accessory proteins named Ysc in *Yersinia* or variously Inv/Prg/Spa in *Salmonella*. An attempt to unify the nomenclature under a generic Sct has been made but has yet to be widely adopted [53]. In essence the T3SS can be described as a series of rings which are like the C ring (cytoplasmic) and MS ring (inner membrane) described for the basal body of the flagellar assembly apparatus [54]. Positioned within the ring at the cytosolic side is the so-called export apparatus, which in case of the flagellum is required for the assembly of the hook and flagellin subunits whereas in the T3SS it is required for the assembly of the so-called needle. As such, the T3SS is evolutionarily related to the flagellar export apparatus. The assembly process [55] is proposed to be energized by an ATPase which is called InvC for *Salmonella*, YscN for *Yersinia* or SctN in the universal nomenclature. However, the energy requirement for driving T3SS-dependent secretion is unclear and the proton motive force (pmf) might also be required [56]. The needle is made by the polymerisation of a single subunit, SctF, that protrudes at the bacterial surface by passage through an outer membrane protein of the secretin family, SctC, also known as YscC or InvG [57] in *Yersinia* and *Salmonella*, respectively. The purification and visualisation of the entire T3SS was first described for the *Salmonella* system, and electron microscopy revealed it to have a syringe-like shape [51]. The T3SS effectors to be transported transit through the needle in a defined order, starting with three distinct translocon proteins (SctA, B and E) which are not released into the target cells but instead SctEB (also known as PopBD in *Pseudomonas*, YopBD in *Yersinia* and IpaBC in *Shigella*) insert into the plasma membrane to form a pore [58]. Once the translocon is in place the genuine effectors are sequentially transported through the needle and the translocon into the cytosol of target cells to exert their toxic effects. The translocon itself is not considered a toxic effector but it has been proposed to subvert the host response once inserted in the plasma membrane [59]. An additional T3SS protein that is key in the assembly process is the ruler protein which determines the length of the needle, and which is YscP in *Yersinia* [60]. In case of the T3SS, the effectors are transported in a Sec-independent manner. They are kept unfolded before transport by direct interaction with chaperones and are targeted directly to the T3SS by an N-terminal non-cleavable signal [61].

The T1SS, T2SS and T3SS are thus extremely different in terms of composition and mechanism. Instead, the T3SS is very similar to the flagellar assembly system [54]. These parallels between a secretion system and another machine with a distinct function

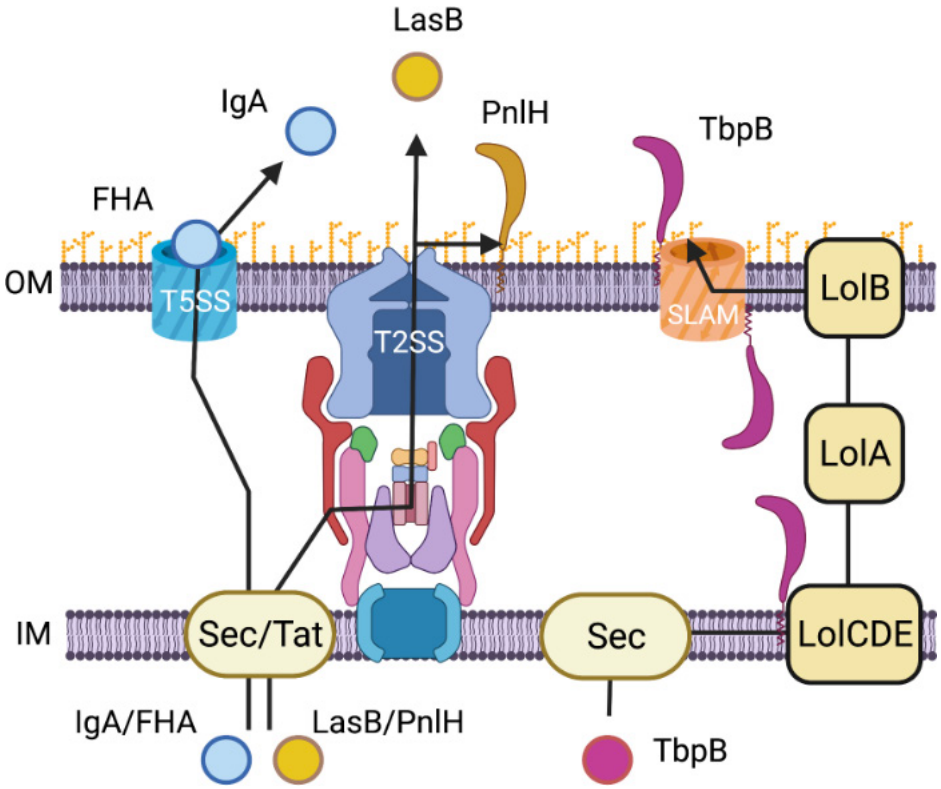

Fig. 1. Sec/Tat-dependent transport of exoproteins. The translocation of proteins across the inner membrane (IM) of Gram-negative bacteria involves the Sec and eventually Tat machinery. Several secretion systems use this as a first step towards the extracellular medium and subsequent translocation across the outer membrane (OM). Some proteins will remain attached to the surface as is the case for T5SS-dependent FHA (*Bordetella pertussis*) or T2SS-dependent PnlH (*Dickeya dadantii*). Others will be released free in the extracellular environment such as the T5SS-dependent IgA (*Neisseria gonorrhoea*) or T2SS-dependent LasB (*Pseudomonas aeruginosa*). The Slam outer membrane transporter can be quoted as belonging to the T11SS and is responsible for the cell surface exposition of lipoproteins, such as TbpB (*Neisseria gonorrhoea*), which will be moved from the IM to the OM in a Lol-dependent manner. It is to be noted that in case of the T5SS it is unlikely to have a first step involving the Tat system since the beta barrel needs to be unfolded in the periplasm for recognition of the individual beta strands by the Bam machinery.

is not unique. The T2SS for example has high similarities with the machine that assembles type IV pili (T4P) at the cell surface, which are involved in twitching motility [62]. The T4P are assembled from pilin subunits, and the machine that polymerises the pili and exposes them at the surface is nearly identical to the T2SS [62]. Indeed, remarkably both systems share a component in *P. aeruginosa* which is the prepilin peptidase XcpA/PilD responsible for cleaving both pilins and pseudopilins [43, 63]. The gene encoding XcpA is part of the *pil* gene operon and not found in the *xcp* cluster [64]. While the T1SS does not share similarities with any nanomachine assembling cell surface appendages, it is related to pumps that are involved in the efflux of small molecules including most known antibiotics [65]. At this stage it is thus tempting to hypothesize that secretion systems have not been created *de novo*, but instead have evolved from exploiting various existing complexes forming trans-envelope channels and adapted their structure and function to the release of exoproteins. This may underlie why there are so many distinct secretion types which achieve a similar goal.

The multimodal T5SS

Intriguingly, the idea of the necessity of having accessory components to form a secretion system was challenged right from the beginning with the mechanism involved in IgA protease secretion [15] (Fig. 1). This single protein carries all the information needed to make its way across the Gram-negative cell envelope. It has an N-terminal signal peptide that allows Sec-dependent translocation. Once released in the periplasm, the C terminus of the protein can adopt the fold of outer membrane proteins and thus creates a channel through which the N terminus can pass to access the surface. Once exposed the protein uses its own protease activity to trigger detachment from the domain embedded in the outer membrane, and thus fully achieve the secretion process. For this reason, it was termed autotransporter [66]. However, it should be noted that at each membrane translocation step the autotransporter requires an accessory machine, Sec for transport across the cytoplasmic membrane, and the β-barrel assembly machinery (Bam) for insertion into the outer membrane [7, 67].

Considering a mosaic protein such as the IgA protease suggests that in other cases the two domains of the proteins, which have entirely different functions, could be encoded by two distinct genes. In this case that would be a gene for the outer membrane channel and one for the exoprotein to be transported through the channel [68]. It is no surprise that many examples of this kind have been found, and one of the very first such systems to be characterized was the filamentous haemagglutinin (FHA) from *Bordetella pertussis* [69]. In this case the protein that forms the outer membrane channel is encoded by the *fhaC* gene and it is found in tandem with the FHA-encoding gene on the chromosome. This system has also been called a two-partner secretion (TPS) system, with the generic name for the pore being TpsB and for the passenger TpsA [67]. Finally, to provide a better fit with the secretion type nomenclature the autotransporter and TPS systems were subsequently designated T5aSS and T5bSS.

An ambiguous T4SS

The *Bordetella pertussis* toxin belongs to the AB toxin family which also includes cholera toxin [70]. The A catalytic subunit (S1) is bound by a pentameric ring of B subunits (S2/S3/S4×2/S5) whose role is to bind onto receptors of target eukaryotic cells. Both A and B subunits are synthesized with a signal peptide and are exported into the periplasm in a Sec-dependent manner, where they assemble into the complete toxin. The AB5 assembly is then secreted into the extracellular milieu, internalised into eukaryotic cells upon B-dependent binding and undergoes retrograde transport through the endoplasmic reticulum [71]. Once released in the cytosol the toxin ADP-ribosylates G proteins to subvert cell communication. The secretion of the toxin is dependent on a set of eight genes which are found next to the toxin genes. These genes were called *ptlA-I* [72] and are highly like the *virB* genes encoded on the Ti plasmid involved in the transfer of *Agrobacterium tumefaciens* T-DNA into plant cells [73].

The structure of this secretion system, now called the T4SS, has been characterized in detail, notably using the VirB system as a canonical example [74]. The organisation and structure of the VirB complex describes a nanomachine that spans the cell envelope, including components located in the cytoplasmic membrane (VirB3 (PtlB), VirB6 (PtlD) and VirB8 (PtlE), components bridging inner and outer membrane (VirB10 (PtlG)), proteins predicted to be periplasmic like VirB9 (PtlF), or localized in the outer membrane, the VirB7 (PtlI) lipoprotein. In the case of the VirB system the identification of what could be the outer membrane channel has long been debated until the cryo-EM structure of the complex showed that it is in fact formed from the association of the C terminus of VirB10 together with VirB7 and VirB9 [75]. Notably the C terminus of VirB10 includes an amphipathic alpha-helix which when bundling upon VirB10 oligomerisation forms an alpha barrel in the outer membrane while the VirB7 and VirB9 wrap around the protruding periplasmic part of VirB10. This way of inserting a protein in the outer membrane is quite unique since it was long considered that integral outer membrane proteins are mainly beta-barrels [76]. As in the case of the T2SS and T4P, the VirB system assembles a pilus, which will subsequently contribute to the T-DNA transport, and/or create contact with the target cell [77]. This T-pilus is mainly formed by polymerisation of the VirB2 (PtlA) subunit but also includes VirB5 at the distal end [78]. Finally, the assembly/transport process is energized by a set of three ATPases, VirB11 (PtlH), VirB4 (PtlC) and VirD4, whose respective roles are not entirely elucidated. The substrates are likely recognized by the coupling ATPase VirD4 [79] and those could be the T-DNA-bound relaxase VirD2 or effectors and virulence proteins such as *A. tumefaciens* VirE2, VirE3, VirF or VirD5 [80]. Instead, the VirB11 and VirB4 ATPases may be linked with the assembly of the T-pilus [77].

The T4SS has now been described in many other species but in all cases, it was proposed that the secreted proteins were not released in the milieu, like with the pertussis toxin, but directly injected into target cells such as for the CagA protein of *Helicobacter pylori* [81] or the many T4SS effectors from *Legionella pneumophila* [82]. These T4SS effectors have no Sec-dependent signal peptide but instead carry a non-cleavable unstructured C-terminal motif [83] to help targeting to the secretion machine and the coupling protein VirD4 [84]. The T4SS like the T3SS is a potent anti-eukaryotic weapon but has also brought several ambiguities to the concept of protein secretion system. Among these are the transport of DNA attached to proteins, a non-conventional outer membrane channel, and either a one-step (CagA) or two-step process (pertussis toxin) depending on the organism.

The T6SS bacterial killing machine

Another important observation about the T4SS is that it has similarities with bacterial conjugation systems that assemble an F-pilus for transferring DNA from one bacterial cell to another [85]. In that case the system involves far more components than the one described for VirB, which is more like the Dot/Icm T4SS system described in *L. pneumophila* [82]. Consequently, subtypes have been allocated with VirB belonging to Type IVA and Dot/Icm to Type IVB. The distinction between secretion types would primarily be that the secretion systems have totally different protein composition and therefore different mechanisms, which might not be the case for the Type IVA and type IVB. Yet some of the secretion system types can share a few homologous proteins, like the secretin in case of T2SS and T3SS [25], or the ATPase in the T2SS and the T4SS (VirB11) [86]. Interestingly, *dotU* and *icmF* homologues were found in *Rhizobium* species that were part of a gene cluster in which all other genes had no similarities with T4SS genes [87]. It appeared that this gene cluster encoded a novel type of secretion system which was called T6SS when it was later described in *V. cholerae* and *P. aeruginosa* [88, 89].

The T6SS mechanism came as a rather unique discovery since it requires puncturing of the bacterial cell envelope in a similar way that bacteriophages puncture prey cells to inject their DNA [90, 91]. The T6SS is made of three main parts. The membrane complex (TssLMJ) connects inner and outer membranes and has no homologues in the phage system [92]. The rest of the machine

is, however, very reminiscent of contractile phage tails. A hetero-multimeric ring-shaped complex called baseplate (TssAEFGK) is positioned onto the membrane complex [93–96]. This baseplate is the site of assembly for a contractile sheath (TssBC) [97, 98] which extends in the cytosol and is a twelve-fold helical tube wrapping around a pile of hexameric Hcp rings [88]. The baseplate is surrounding a so-called puncturing device, which is a conical shape trimeric VgrG [91] and the flat side of the cone interacts with the first Hcp ring [88]. The conical side is facing the membrane complex and can be sharpened by the positioning of a PAAR protein on top [99]. Once the assembling TssBC sheath spans the cytoplasm entirely and contacts the membrane opposite to the baseplate position, contraction is triggered [93, 96, 100]. This is accompanied by a conformational change in the shape of the baseplate [101] and the outwards movement of the VgrG puncturing device and the Hcp stacks through the membrane complex, which force their way out through the TssJ multimer in the outer membrane [102].

The proteins secreted by the T6SS are associated either with the puncturing device (VgrG/PAAR) [103–106] or the Hcp ring [107, 108]. One of the first reported T6SS effectors is VgrG1 from *V. cholerae* and this VgrG carries a C-terminal extension with actin cross-linking activity [91]. It was shown that it is injected in eukaryotic cells to interfere with the organisation of the cytoskeleton. As such it was assumed that this was yet another secretion system to inject subversive effectors into host cells. However, a few years later it was reported from studies on *P. aeruginosa*, that the main function of the T6SS is as a nanomachine that injects toxins into bacterial competitors to kill them [109]. This was a remarkable new concept involving contact-dependent assault between bacteria [110, 111]. Yet one could argue that contact-dependent killing was already described in the case of *E. coli*, and the contact-dependent inhibition (CDI) system initially reported by Aoki and collaborators in 2005 [112]. In this case the system is in fact a T5SS in which CdiB is the outer membrane transporter and CdiA the passenger [113]. The CdiA passenger has the remarkable peculiarity of having a short C terminus which carries the toxic domain, this domain being released into prey cells upon CdiA-dependent contact. One main difference between CDI and T6SS is in the target range, CDI requires a receptor on prey cells (e.g. BamA) and therefore competes only with related species [114], whereas the T6SS is non-specific and injects a cocktail of diverse effectors into all type of prey cells, including bacterial, fungal or mammalian cells [115, 116].

THE CHALLENGES OF CLASSIFYING BY SECRETION TYPES

Is the T7SS a Gram-negative protein secretion system?

The organisation of the cell envelope of Gram-negative bacteria, and the presence of an outer membrane, is very important for the definition of a secretion type, otherwise the Sec and Tat systems could also be allocated type numbers. However, this definition also has limitations if one considers that the distinction between Gram-positive and Gram-negative bacteria is based on staining which may not rigorously reflect the exact structure of the cell envelope. Mycobacteria appear weakly Gram-positive, but the cell wall is not simply made of a cytoplasmic membrane and a thick layer of peptidoglycan and in some ways resembles the organisation seen in Gram-negative bacteria [117]. Indeed, the peptidoglycan layer of Mycobacteria is connected by arabinogalactans to an additional layer, termed the mycomembrane, that is comparable to an outer membrane [118]. This mycomembrane is made of an inner layer which is essentially mycolic acids while the outer side is enriched in lipids including phenolic glycolipids. Remarkably, porins inserted in this mycomembrane have been identified such as *Mycobacterium smegmatis* MspA which allow the transport of small hydrophilic molecules [119]. It is thus obvious that secretion of mycobacterial effectors and toxins, e.g. ESAT-6 (EsxA) [120], might then require a system allowing transport across the mycomembrane, and that would merit being assigned a type number [121].

The genes in the vicinity of the gene encoding ESAT-6 [122], were proposed to represent such secretion system which was named ESX, and later coined the T7SS [123]. Genomic and biochemical studies suggested a minimal organisation of the T7SS involving the inner membrane components EccB, EccC, EccD, EccE and MycP [124–126]. In case of the *M. tuberculosis* ESX-5 system, the cryo-EM structure of the purified complex displays a trimer of dimers for each subunit except for EccD which is present in a total of 12 copies [127]. Each dimer is then associated with a copy of the bitopic membrane protein MycP, which in all results in a supramolecular assembly of 2.3 MDa. EccD is found at the periphery of the putative channel, or secretion pore, formed by the six copies of EccC. At the cytosolic side of the complex, the EccC stalks might contribute as a gate for the channel. EccC has a AAA +ATPase activity which could trigger conformational changes important for the secretion process and movement of substrates through the secretion pore. EccC is also the protein, which recognises T7SS substrates [128], while another ATPase, EccA, a cytoplasmic ATPase found in some T7SS, might help dissociate the substrates from their cytosolic chaperones prior to translocation [129].

Proteins secreted by the Mycobacterial T7SS display a secretion signal made of two conserved motifs, YXXXD/E and WXG [126]. When genes encoding WXG-containing proteins clustered with an *essC* gene (*eccC* homologue) were described in classical Gram-positive bacteria such as *Staphylococcus aureus* [130, 131], the T7SS nomenclature was then adopted for a secretion system that does not involve transport across a bipartite cell envelope. The firmicute system has been called T7bSS since apart from *essC* and one or more WXG-substrate encoding genes, other genes in the cluster share no detectable homology with the *ecc* genes. Instead, a supramolecular complex would be formed essentially from four membrane proteins EssA, EssB, EssC (EccC homologue) and EsaA [126]. In this case transport across the T7bSS would result in secretion, just as is the case for proteins

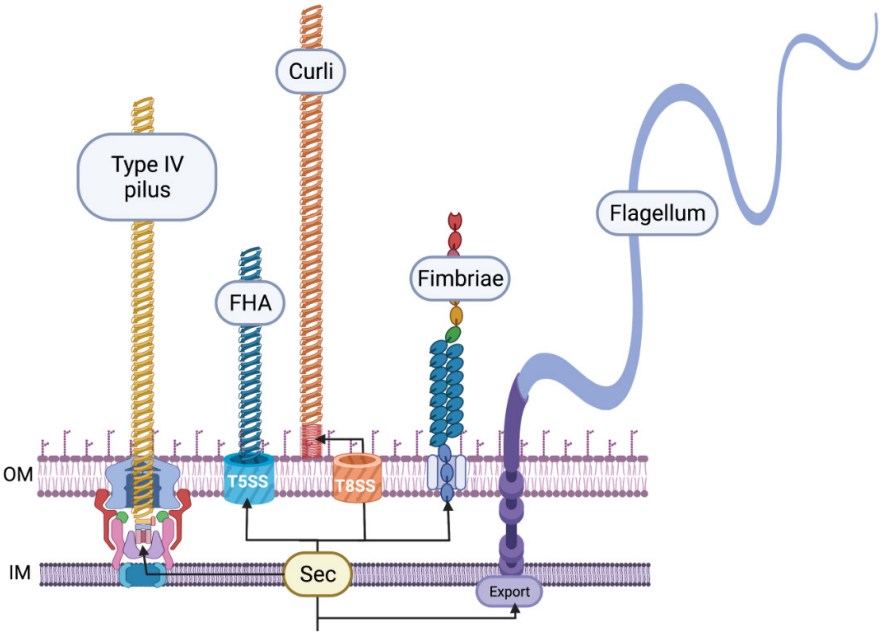

Fig. 2. Cell surface appendages in Gram-negative bacteria. Cell surface appendages at the bacterial cell surface may result from the polymerisation of single subunit into a filamentous structure or exposition of a filamentous protein. The assembly of curli polymers (e.g. *E. coli*) has been associated with the T8SS while the exposition of the filamentous protein FHA is T5SS-dependent. The polymerisation of a type IV pilus requires a machine with high similarity with the T2SS while assembly of the flagellum requires an export/secretory apparatus which is alike the T3SS. The transport, exposition and assembly of fimbrial subunits (e.g. *E. coli*) has not been assigned a secretion type, nor the usher resembles any outer membrane components in classified secretion systems.

transported by the Sec or Tat pathways in firmicutes. Herein lies the controversy about whether the T7SS is a genuine secretion system. The mycomembrane conduit for any of the ESX systems has yet to be identified and is not obviously encoded at any of the *esx* gene clusters. Furthermore, all the available structures of ESX indicate that it solely transports proteins across the cytoplasmic membrane, and therefore could be considered as the first step of translocation as described for Sec or Tat in the case of the T2SS [132]. However, since the T7SS nomenclature is now firmly established in the literature, it is not advisable to change the name to avoid any further unnecessary confusion.

Are cell surface anchored proteins secreted?

To continue with semantic issues a question that arises is whether proteins that remain attached to the surface of the bacterium can be considered as secreted. In the early days of secretion system analysis, the IgA protease has represented the archetype of what is now known as the T5SS [15]. This enzyme is ultimately released from the bacterial surface by autocleavage but now it is known that most proteins transported by the T5SS remain surface anchored and act as adhesins, such as FHA from *B. pertussis* [133].

One of the reasons that this concept of release of the protein from the cell surface is important is when considering extracellular appendages. Indeed, the machines involved in the assembly of T4P or flagella, although highly like the T2SS [134] and T3SS [135], respectively, are not necessarily coined as such. However, every single pilin or flagellin subunit is transported to the surface of the bacterium and yet never released, but instead assembled into a polymeric cell surface appendage (Fig. 2). The chaperone-usher pathway for the biogenesis of Type I pili [136, 137] is another case in point. Here the fimbrial subunits are transported into the periplasm in a Sec-dependent manner and then require a specific outer membrane component, called the usher, for surface display. Fimbrial subunits are protected from degradation in the periplasm when bound to their cognate chaperone, and the complex is targeted to the usher. The fimbrial subunit dissociates from the chaperone when it engages with the usher and the liberated subunit tightly interacts with the previously engaged subunit. This interaction involves a mechanism known as donor strand exchange, generating a stable polymeric structure which will remain anchored at the surface. The fact that the subunit is never released on its own but kept in a polymer while emerging from the usher might be the reason it has never been assigned a secretion type. Another class of extracellular filaments or fibres produced by proteobacteria are the so-called curli [138]. As for the chaperone-usher pathway curli assembly is also best described in *E. coli*. The CsgA or CsgB subunits are transported into the periplasm in a Sec-dependent manner before being delivered to the outer membrane channel called CsgG, while chaperones prevent premature polymerisation in the periplasm. The mechanism overall resembles the chaperone-usher concept although the CsgG structure is distinct from that of the usher [139]. Interestingly, this system has later been given a type number and called

the T8SS [13] (Fig. 2). This ambiguity might come from the fact that the curli subunits are first released free as unfolded proteins in the medium before aggregating back at the surface of the cell while forming the curli fibres.

There will be always conflicting views on what is exposed or secreted and what merits being assigned a secretion type. The T9SS for example has been assigned to a system almost exclusively found in Bacteroidetes species, including *Porphyromonas gingivalis*, and which mainly transport proteins that are surface attached [140]. The system, initially called PorSS [141], comprises two Por proteins (PorLM) localized in the cytoplasmic membrane and 12 components associated with the outer membrane, while the proteins transported to the surface have distinct function in virulence, attachment, iron uptake or gliding motility. If cell surface exposure is a genuine criterion for assigning a secretion system, then the recently described Slam system [142] could be a valid candidate to be considered. Slam is a 14-beta strands beta-barrel outer membrane protein which was discovered in *Neisseria* as being involved in exposing outer membrane lipoproteins at the cell surface, notably TbpB and LbpB which are used to bind transferrin and lactoferrin, respectively. Importantly it was well described that targeting and anchoring of lipoproteins at the inner face of the outer membrane involves the so-called Lol system [143], but no mechanism was described for flipping a lipoprotein at the surface. In this respect Slam could be seen as a T5SS specifically dedicated to lipoprotein 'secretion' [144], if surface anchoring is coined secretion. However, Slam does not have similarity with the T5SS transporter TpsB, which in fact belongs to the so-called Omp85 family including BamA the protein responsible for the insertion of beta-barrels in the outer membrane [7, 145–147]. Instead, it was shown that Slam belongs to a family of proteins carrying a DUF560 domain [148]. Importantly, some of these are proposed to be involved in the release of soluble proteins into the medium, such as the haem binding protein from *Haemophilus haemolyticus* [149]. As such it was proposed that systems involving DUF560 proteins be called type XI secretion system (T11SS) [148].

It could be here slightly ironic that the first substrate identified for a T2SS was pullulanase, which effectively is a lipoprotein using a T2SS for display at the cell surface, although it can be released with the fatty acyls still attached [150]. There are now more examples featuring this specificity like the T2SS-dependent transport of the *Dickeya dadantii* PnlH lipoprotein [151].

HOW FAR CAN WE STRETCH THE DEFINITION OF A SECRETION SYSTEM?

Where cell lysis may clash with the concept of secretion

Colicins are toxins released by *E. coli* and able to kill related species upon specific uptake [152]. The uptake steps in the process were studied long before protein secretion systems had been characterised. To be released from cells colicins require a so-called 'release' or 'lysis' protein which is a lipoprotein anchored at the inner face of the outer membrane. In the absence of the lysis protein colicins will accumulate in the cytoplasm which suggests that despite its final localization in the outer membrane the lysis protein helps colicins to cross the inner membrane. Intriguingly, the key role of the release protein is to trigger activation of an outer membrane phospholipase, OmpLA [153], which will impact the integrity of the outer membrane. When this happens most of the colicin producing population will lyse. As such the release of the colicins might be seen as a totally non-specific mechanism, although supported by the role of specific accessory proteins.

A protein secretion system that has been previously disregarded for the crime of non-specific release due to potential cell lysis has since been coined the T10SS [154]. *Serratia marcescens* produces a chitinase ChiC, whose secretion requires two cytoplasmic membrane proteins, ChiY and ChiW, a periplasmic endopeptidase, ChiX [155], and an outer membrane lipoprotein ChiZ that interacts with ChiY. These four proteins have similarities with a so-called lambda phage lysis cassette, and ChiW is a holin homologue that allows transport of ChiX to the periplasm. Once ChiX is in the periplasm it degrades peptidoglycan, which may trigger cell envelope permeability. However, it was shown that the periplasmic-accumulated chitinase is released in the absence of cell lysis and therefore represents a *bona fide* secretion system. Interestingly, it was also proposed that combinations of holins and endolysins are involved in the release of extracellular DNA from *P. aeruginos*a [156].

Outer membrane vesicles as a last resort secretion option

Whenever proteins are secreted by non-characterized or poorly understood mechanisms a frequently used suggestion is that it is mediated by budding and shedding of vesicles from the outer membrane [157, 158]. This apparently non-proteinaceous mechanism has been tentatively called type 0 or T0SS [159]. Are we pushing things a bit too far here? Obviously, it all depends on how we view it. For example, it was proposed that *P. gingivalis* accumulates cell surface proteins in the outer membrane using the T9SS, proteins which are then released upon formation of membrane vesicles [160]. These vesicles are then able to fuse with target cells in which the virulence factors are delivered. In the case of the T10SS there are no components in the outer membrane which have been described for facilitating the release of the periplasmic chitinase [154]. Of course, these might yet be discovered. Alternatively, the endopeptidase ChiX which degrades the peptidoglycan and challenges the integrity of the cell wall may trigger the production of vesicles that would package the periplasmic contents, including the chitinase, resulting in its secretion. Vesicles could then be the terminal branch of the T9SS/T10SS, just like the T2SS is the terminal branch of the so-called General Secretion Pathway (GSP) [21] with Sec and Tat being the proximal branches. The limits of the vesicle concept reside in the fact that there might be a lack of specificity and whatever is in the membrane or periplasm is released [161]. One can then choose the cargo to

pinpoint as the secreted element but that might be heavily biassed. The other limitation would be that a secretion system might be proteinaceous and be represented by at least one component which would be the secretion machine. If vesicles are spontaneously produced upon any cell envelope stress [162] they might not fit this definition. In other words, without a clear molecular basis to the process, vesicle release might be more akin to partial lysis rather than specific secretion. One could argue that it is the blockade of a proteinaceous machine, like the Tol/Pal system [163], which would trigger vesicle formation, and again it will remain a semantic issue.

This conceptual problem is reminiscent of nanotube formation [164]. The nanotubes appear to be mainly lipidic, looking like pili but are mostly membrane extension, and no associated molecular machine has been clearly identified. It could involve some elements of the flagellar basal body [165] but might as well result from cell envelope stress and possibly be a post-mortem event as proposed in *Bacillus subtilis* [166].

Protein or macromolecule secretion systems?

Among the protein secretion systems so far described it has become obvious that some may not just transport proteins but also other macromolecules, such as DNA for the T4SS [167]. In this case the DNA is not targeted as a protein-free complex but instead is piloted by other proteins such as the VirD2 relaxase [168] which may itself be targeted to the T4SS. In that case the DNA becomes a VirD2-hitchicker. Now there is no usurpation to the T4SS nomenclature since in many other instances the system transports exclusively proteinaceous virulence factors.

It is also tempting to look at complex nanomachines spanning the cell envelope of Gram-negative bacteria and involve in the transport of biopolymers as a secretion type. The description of Bcs complex involved in cellulose production and secretion in *E. coli* is such an example [169]. It has a large outer membrane channel, BcsC, which connects with an inner membrane conduit BcsAB. The synthesis/elongation of cellulose correlates the movement of the polysaccharide through this cell envelope channel. Naturally, it would make concepts far more too complex/subtle if we were to broaden even further the concept of secretion systems and forget about proteins, in which case the vesicles could even be seen as a means to secrete lipids.

How many subtypes in a type?

There is already a plethora of protein secretion types, and there is emerging an even more complex classification of subtypes. This might be a good thing in helping to rectify misannotations and to accommodate some unrecognized major differences although sometimes it looks more like splitting hairs.

The T5SS for example now has up to six subtypes [170]. The T5aSS is clear since it reflects the classical autotransporter concept. It has also been called T5eSS when the domains are inverted that is when the beta-barrel region encompasses the N terminus and the passenger the C terminus, like the *E. coli* intimin. For the b, c and d subtypes it is all about the structure of the outer membrane channel. A T5bSS, which has the β-barrel and cargo domains encoded as separate polypeptides uses a separate 16-beta strand barrel, whereas the barrel of classical autotransporters has 12 beta strands. One recent addition is also the so called T5fSS which has a 8-stranded β-barrel. The T5cSS differs because in this case the barrel of an autotransporter is formed by the trimerization of the protein, just like the outer membrane protein TolC fold [33]. However, the T5dSS [171] is somehow more subtle since it is also an autotransporter (i.e. a single polypeptide) but otherwise resembles the T5bSS, forming a beta barrel with 16 strands and harbouring a periplasmic POTRA domain [172]. Although many interesting conclusions can be made from an evolutionary perspective, these distinctions are often far too subtle for the secretion neophyte.

To date the T2SS has not been split in subtypes, but there would be grounds to do so. For example, it could be based on the insertion mechanism of the secretin in the outer membrane which may require or may not be dependent on a lipoprotein called pilotin [173]. Does this change the mechanism of the secretion? Most likely not. Making a distinction based on the fate of exoproteins might also be a possibility. For example, cell surface anchored lipoproteins versus fully secreted substrates. This is less appropriate though since again there would be no difference in the machine itself. The T3SS could also be divided into subtypes, e.g. whether there is a protruding naked needle or if it is present within a shaft such as the EspA protein described in pathogenic *E. coli* [174]. The T1SS could be split into those that have their own specific outer membrane conduit, e.g. *P. aeruginosa* AprDEF [28], or those exploiting existing outer membrane proteins, e.g. TolC, such as the *E. coli* HlyBD [175] and CvaAB [176]. The T6SS could also be sub-divided based on differences in the TssA component which is involved in T6SS dynamics and speed of firing, and which has very distinctive features in sequence and structure [93, 95, 100].

The T4SS has also been subclassified, but here the distinction between the components involved is obvious [167]. The type IVA exemplified by the VirB system comprises 12 proteins. Most of these are found in the type IVB, e.g. *L. pneumophila* Dot/Icm, which however also requires further components and a total of more than 25 distinct proteins to assemble the secretion machine. However, whether the overall architecture is distinct to a point that the mechanism is drastically different between the two subtypes remains to be shown. The T7SS should also rightly be split in subtypes since the two systems described so far share only one component. Or maybe these are different types, and that might well be the case since the mycobacterial and firmicutes

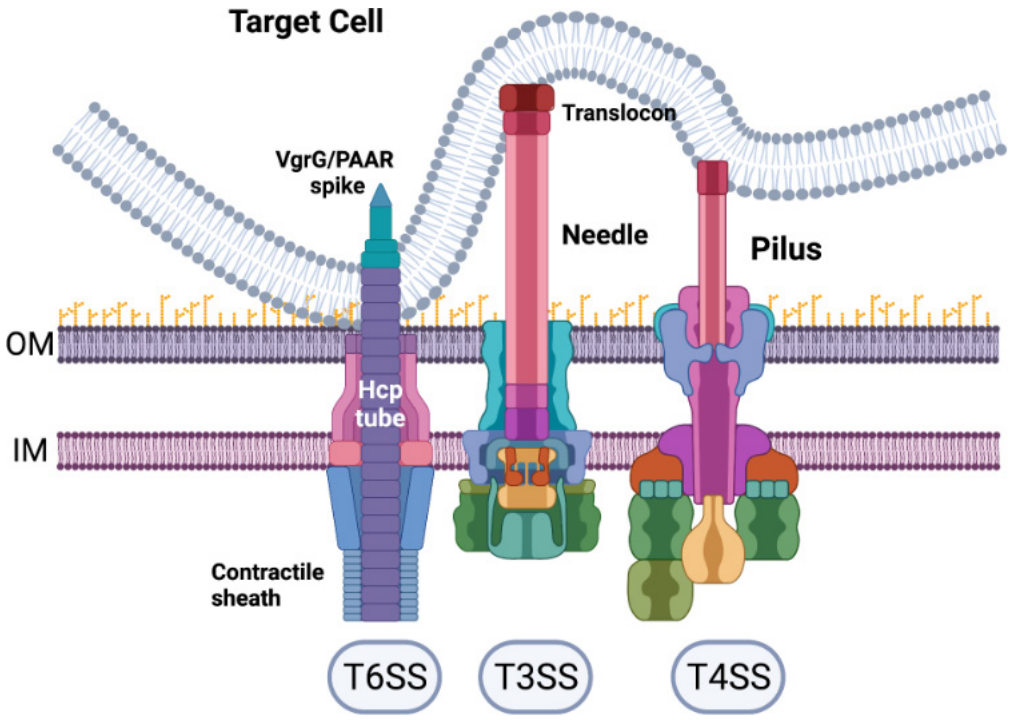

Fig. 3. Secretion and injection machines. Some of the classified secretion systems can be responsible for the direct injection of proteins/effectors/toxins into target cells. That is the case of the T6SS in which the cytosolic contractile sheath propels from close range a spike or puncturing device or the T3SS, the needle of which insert a translocon into the target cell membrane through which subsequent injection of toxic effectors will be driven. In the T4SS the contact with the target cell requires the assembly of the pilus whereas the route followed by transported proteins is still under debate.

cell envelopes are different. One should recall that the T3SS and the T2SS both have the secretin component in common [25] while the T4SS and the T6SS share DotU/IcmF [177] but that is where the similarity stops.

THE SECRETED PROTEINS DESIGN THE FUNCTION OF THE SECRETION SYSTEM

Overall, one can say that the main question is not so much about types but about what a secretion type is used for. The straight answer to this is that, irrespective of the type, what is important is the secreted proteins and their biological roles. The T3SS was the first example of a mechanism essential for bacterial pathogenesis and injecting toxic effectors into host cells. I am not necessarily aware of a T3SS that does not do this, but there are now so many T3SS clusters found in non-pathogenic bacteria that one may rightfully think that it possibly also has other functions. For example, in *Ramlibacter tataouinensis* it has been suggested that the T3SS might secrete a chitinase [178]. The cytotoxic impact of secretion systems has been further supported when the T4SS was discovered and shown to display similar abilities to the T3SS in delivering effectors in host cells. Overall, these T3SS/T4SS effectors can often be very alike in structure and function [179] and manipulate/subvert many signalling networks within eukaryotic cells. Even the T6SS can be cytotoxic [180] (Fig. 3). Would that mean that cytotoxicity is a prerogative of an injecting secretion machine? Certainly not. For example, cholera toxin [181] and exotoxin A [182] are released into the extracellular environment in a T2SS-dependent manner before being recaptured by target cells. The *P. aeruginosa* TesG effector is released by the T1SS and when internalized in host cells suppresses the immune response [183, 184]. The concept of the T3SS being the most effective cytotoxic weapon can also be challenged. There are *P. aeruginosa* strains that have lost all their T3SS equipment but are even more toxic than those which have a fully functional T3SS [185]. Indeed, these strains have acquired a T5bSS ExlA/ExlB, in which ExlA appears to be an extremely potent exolysin.

There is surely no commitment of a system towards a specific function. The T6SS was discovered as an anti-host weapon [91] but is now far better known as an antimicrobial machine delivering toxins into competitors to kill them [111]. This has awakened interest in looking at the other systems and T1SS, T4SS, T5SS, T7SS all appear to be professional bacterial killers engaging in combat in which direct contact is needed for injecting the toxins into competitors [186]. This may also include the T1SS-dependent secretion of colicin V [176], which unlike most other colicins is released in a specific manner and not through lysis.

It is important to recognize that in the early days of protein secretion system discovery the focus was about releasing enzymes to degrade extracellular and complex substrates for nutrient acquisition or capturing rare resources such as with haem-binding

proteins [187]. The T2SS and T1SS are extremely prolific at doing this, but again the function comes from the exoproteins and not from the system. The T6SS after being coined an anti-eukaryotic weapon, then an anti-prokaryotic weapon, now expands its role to the acquisition of common goods [188–192]. So, there is no universal definition as for the role of protein secretion systems and it is likely that they are all multifunctional.

The multifunctions of protein secretion systems can even take us back to the distinction separating them from systems involved in assembling extracellular appendages. The T2SS and T4P assembly machines are practically one unique system that diverged to fulfil entirely different roles [62, 134]. The T2SS machine stopped protruding the filament out of the cell while using it to push exoproteins outside. Or had the T4P machine already the ability to perform this function as could be proposed for the Sec-dependent PilY1 protein which is bound to but not part of the pilus [193]? This is even more true for TcpF which is secreted by the T4P machine of *V. cholerae* [194]. This very same idea could hold true with a flagellar apparatus that would transport not only flagellar subunits but also secrete proteins such as is the case for FlaC of *Campylobacter jejuni* [195].

CONCLUDING REMARKS

There have been already several attempts to resolve the semantic issues I described above [13] and by no means do I want to try to offer any unifying nomenclature. The most important is for newcomers in the field to navigate through the concepts and understand that secretion biology is so rich and the evolution trajectories of organisms so clever that one cannot hold everything into a neat and polished golden box. There will always be exceptions, there would always be things that were not known at a given time which once discovered challenge all previously accepted concepts. That is the way Science moves forward but nevertheless one should make sure we can teach these discoveries properly and avoid any misunderstanding that could mislead the field. We must accept that cells are using available tools and mix and match molecules during evolution and develop new tools and new functions. What was one can sometimes become two or more.

One example to be taken as a type of secreted proteins are the AB5 toxins. The cholera toxin or even the heat labile toxin use the T2SS [196]. This could have been functionally more suited for direct injection by the T3SS and avoid all the complexity of retrograde transport into target cells. Yet those toxins must be assembled into a pentameric structure that would not fit into the channel of the T3SS but will fit the secretin of the T2SS. However, the pertussis toxin uses the T4SS [197]. Although it is not clear whether such system could fit an assembled toxin, *B. pertussis* has no T2SS, so the toxin required another mechanism for being secreted. This is the sign of remarkable adaptability, since T4SS substrates usually come directly from the cytosol whereas here the pertussis toxin is assembled into the periplasm. This secretion mosaic is thus a mix and match game and a fine example of evolutive trajectories. In this genomic era, mining for gene clusters and genetic organisations encoding any known secretion components, analysing any protein domains known to be involved in secretion process, would deliver on new types and new function, I have little doubt about that.

There is also a plethora of moonlighting proteins which are cytoplasmic enzymes involved in housekeeping metabolic process which are reported to reach the surface and likely adopt new functions [198]. How would they get there? A rather striking example are the nanowires of *Shewanella oneidensis* or *Geobacter sulfurreducens* that are made by a series of piled cytochromes, from the cytoplasmic membrane to the cell surface [199]. At the surface one can then visualize a nanowire that looks like a pilus, but which results from the polymerisation of cytochromes, e.g. OmcS and OmcZ, that would then allow long distance extracellular respiration [200].

I started this review by putting emphasis on how we shall be clear about the cell envelope organisation to define a type. Would there be a genuine T7SS in proteobacteria? Finding candidate WXG proteins produced by these bacteria will suggest it is going to be the case. Now I fear that being strict on definition would again mislead the field. Phylogenetic analysis has recently shown that there is a branch of firmicutes, so Gram-positive bacteria, that have kept a cell envelope made of two membranes, inner and outer [201]. In other words, diderm instead of monoderm. That is for example the ironically named clade of Negativicutes, such as *Veillonella parvula*, for which a class of T5SS autotransporter is now described [202]. This is all taking us back to where we started and the concept of cell envelope organisation to define a type. Shall we now ignore the brilliant concept that Gram developed although we know that it does no longer stand as a tool for classification? Surely not, but it's anybody's type now and it will be hard to rule the seven kingdoms and more since we already reached eleven types and counting. One, two, three, four five, once I caught a fish alive; six, seven, eight, nine, ten, then I let it go again….

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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