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Abstract

The evolution of protein secretion systems of Bacteria, and related nanomachines, remains enigmatic. Secretion is important for biotic and abiotic interactions, and secretion systems evolved by co-option of machinery for motility, conjugation, injection, or adhesion. Some secretion systems emerged many times, whereas others are unique. Their evolution occurred by successive rounds of gene accretion, deletion, and horizontal transfer, resulting in machines that can be very different from the original ones. The frequency of co-option depends on the complexity of the systems, their differences to the ancestral machines, the availability of genetic material to tinker with, and possibly on the mechanisms of effector recognition. Understanding the evolution of secretion systems illuminates their functional diversification and could drive the discovery of novel systems.

Keywords: Molecular evolution; horizontal gene transfer; exaptation; secretion; functional innovation.

Glossary

Co-option/Exaptation: use of an existing biological function for a novel adaptive purpose.

Diversifying selection: process where natural selection favors the rapid diversification of a gene; frequent in proteins targeted by immune defenses in bacterial pathogens.

Effector protein: a secreted protein that has an effect on another cell, *e.g.* a virulence factor that subverts the function of an eukaryotic cell.

Functional innovation: change in a function to provide an adaptive response.

Monoderms/Diderms: Bacteria lacking/having an outer membrane. Diderms tend to be gram negative and monoderms gram positive, but several exceptions are known.

Neo-functionalization: a process where a gene acquires a new function.

Protein secretion systems: systems allowing the transfer of proteins across the outer membrane of diderm Bacteria. Systems with homologous components may exist in monoderms, and are sometimes also called protein secretion systems.

Sub-functionalization: a process where a gene with several functions specializes in a subset of them.

Tinkering: recruitment of a component into a new biological system (pathway, protein complex, regulon).

Main text

The mechanisms and the plausibility of the evolution of complex functions have been one of the most intriguing and contentious points in evolutionary biology [1]. The increasing mechanistic understanding of some of these functions opens the possibility of integrating molecular and evolutionary biology to detail their evolution. Microbes have an increasingly important role in these studies because they can be easily manipulated, evolved, and analyzed in the laboratory. In nature, their large effective population sizes and their ability to exchange genes horizontally render selection processes efficient [2-4]. Furthermore, Bacteria and Archaea emerged in the planet billions of years before Eukaryotes, and were responsible for many structural, biochemical and genetic innovations.

The emergence of novel functions requires the modification of existing genes, the acquisition of genes from other genomes or the establishment of novel associations between gene products. This tinkering of genetic structures by evolutionary processes [5] results from mutations, deletions or accretions of genetic material. These may eventually recombine. The resulting variants — in terms of biochemistry, cell localization, genetic regulation — are submitted to natural selection and eventually purged or amplified in the population. In addition, horizontal gene transfer in Bacteria and Archaea has the potential to spread novel functions to distinct species, as currently observed in the evolution of antibiotic-resistant Bacteria [6, 7]. Genes newly arriving at a genome by transfer are sometimes under weak or no selection, many are probably never expressed, providing genetic material that may become the substrate for further functional innovation. For example, genomes contain many mobile genetic elements that can evolve novel functions for the host advantage [8]. In this process, a gene or system that originally evolved to respond to a given adaptive need is co-opted (or exapted) to provide a function that tackles a different need [9]. This article details how the tinkering of existing cellular machineries was at the origin of most, if not all, protein secretion systems of Bacteria.

Protein secretion systems and related nanomachines

Bacteria and Archaea use secreted proteins (effectors and auxiliary proteins) to protect themselves, manipulate their environment, and interact with other individuals [10, 11]. Protein secretion systems are defined as machines that transfer proteins across the outer

membrane in diderm Bacteria [12], and are called “TXSS” for type “X” secretion system, where X is a number from 1 to 9 (for broad reviews see [13, 14]). Protein secretion systems transfer proteins directly from the cytoplasm (T1SS, T3SS, T4SS, T6SS, T7SS) or from the periplasm (T2SS, T5SS, T8SS, T9SS). Some systems deliver proteins into other cells using specialized pili (T3SS, T4SS, T6SS), whereas the others deliver them in the extracellular space. The complexity of protein secretion systems is variable. They all need to assemble at the cell envelope and transport at least one protein across the outer membrane. But in some cases, they also transport proteins across the cytoplasmic membrane, deliver proteins directly into other cells, and have complex mechanisms to recognize effectors. As a consequence, some systems are very simple and made of one single protein (T5SS autotransporters), whereas others have more than a dozen different components. While we adhere here to the convention that secretion systems only concern diderms [12], it is important to note that some systems, *e.g.* T4SS, have homologous machineries in monoderms (bacteria lacking an outer membrane). Such systems have usually specific adaptations to the differences in the cell envelope and may also secrete proteins (*e.g.* pilins).

The comparison of the key components of protein secretion systems, and related machineries, shows an extensive network of homology between components (Figure 1). Some of these components have homologs across several secretion systems. This is most remarkably the case of the large family of AAA+ ATPases present in T1SS, T2SS, T4SS, and T6SS. Members of this family also perform other types of cellular functions, such as chromosome segregation, and they are probably very ancient [15, 16]. Yet, the majority of components has recognizable homologs in only a few other nanomachines. These evolutionary associations result in numerous structural and sequence similarities. For example, the T2SS is evolutionarily related to the super-family of type IV filaments (TFF) [17, 18], the T3SS is homologous to the secretion system of the bacterial flagellum [19, 20], the T4SS is closely related to the conjugative pilus [21, 22], and a key part of the T6SS is strikingly similar to bacteriophage tails [23, 24]. The study of the evolutionary processes underlying these similarities has the power to illuminate many aspects of the structure, assembly, genetics, and taxonomic distribution of secretion systems. It may also provide means of finding novel systems and ways to manipulate secretion [25]. In the following sections, we describe the evolution of four complex protein secretion systems to illustrate the avenues/paths underlying their natural history and thereby reveal some common evolutionary principles.

The T4SS evolved novel functions by minor tweaks

Many biological systems have multiple functions that can evolve in a few steps. An interesting example is provided by the T4SS, a system that originated for DNA exchange by conjugation and gave rise to a protein secretion system that is used by many pathogenic bacteria to secrete virulence factors into Eukaryotic cells [26] (Figure 2b). In conjugation, a nucleoprotein complex composed of a single-stranded DNA molecule and the relaxase is transferred into another cell by the mating pair formation (MPF) apparatus (reviewed in [26-28], see Figure 2b). The MPF of diderms contains a T4SS whose phylogeny can be divided into eight major clades with some different accessory components [22]. Phylogenetic analyses indicate that the T4SS was originally involved in conjugation in diderm Bacteria and only later was transferred to monoderms (Bacteria and Archaea) where two sub-types adapted to the absence of an outer membrane. Two AAA+ ATPases of the T4SS — VirB4 and the coupling protein (T4CP) — arose from an event of gene duplication preceding the emergence of the modern systems. The T4CP specialized in mediating the interaction between the relaxase and the T4SS, whereas VirB4 became tightly involved in the function and/or the assembly of T4SS. Other homologous ATPases are found in some sub-types of T4SS, *e.g.* the VirB11 ATPase that was recruited from a TFF [29]. There are alternative mechanisms of DNA transfer by conjugation that are driven by AAA+ ATPases. The protein TraB transports double stranded DNA in Actinobacteria forming mycelia [30] and the protein TdtA has a key role in a recently discovered transformation-dependent conjugation process (transjugation) [31]. Hence, one family of ATPases has diversified early in natural history to provide a very large panel of functions, many of which implicate interaction with membrane components and transport of molecules across the cell envelope.

Conjugation is intrinsically a mechanism of protein secretion because the relaxase is recognized by the T4CP and then secreted by the T4SS with the covalently linked DNA [32, 33]. This facilitated its co-option into a machine that is specialized in the secretion of proteins. This process was followed by the evolution of the ability to secrete a broad range of effectors (Box 1). Recently, a T4SS was shown to secrete toxins into other bacteria broadening the type of hosts into which T4SS can deliver proteins [34]. T4SS thus evolved to transfer mobile genetic elements or protein effectors to both Eukaryotes and Bacteria, and phylogenetic analyses show many independent co-options of T4SS into specialized protein secretion systems [22].

The experimentally verified ones have all occurred in just two of the eight T4SS clades (named T and I). We don't know the reasons for this apparent concentration of co-option events in two clades. One possibility is that some conjugative pili are better at interacting with different cell types, as suggested by the observation that broad host range conjugative systems tend to be of type T [35]. Alternatively, the evolution of the ability to recognize different effectors may be simpler in certain types of conjugative systems. While conjugative pilus typically recognize one or a few different relaxases, the T4SS of *Legionella* spp. can deliver numerous different proteins because its T4CP binds adaptors that recruit distinct subsets of effectors [36, 37]. The ability to recognize different effectors, and how these effectors arise, remains one of the least understood aspects of the evolution of protein secretion systems (Box 1).

The functional promiscuity of the T4SS, *i.e.* its ability to intrinsically secrete proteins, implicates that the evolution of a protein secretion system by neo-functionalization of an adequate conjugation pilus can take place in a small number of steps: acquisition of a T4CP-interacting domain by the effector (or horizontal transfer of the effector) and loss of the relaxase. Accordingly, T4SS with intermediate properties have been observed, including some systems that are able to secrete the relaxase without attached DNA [38], and systems that are able of both DNA conjugation and secretion of virulence factors [39]. In contrast, the evolution of a T4SS of Campylobacterales into a competence pilus for DNA uptake, first discovered in *Helicobacter pylori*, required many more changes and seems to have taken place only once [40]. In summary, the evolution of T4SS illustrates how a few changes can dramatically affect the function and role of a molecular system.

Initial specialization of flagellar secretion led to the injectisome

The bacterial flagellum includes a secretion system — the flagellar T3SS (F-T3SS) — to export its filament to the outside of the cell. Flagella are complex machines containing the F-T3SS, the motor, the hook and the filament (Figure 2a). While the key function of the flagellum is to allow motility, it can provide many other functions including adhesion, biofilm formation, and interaction with immune systems [41]. Several bacteria also use the flagellum to secrete toxins and other proteins to the extracellular milieu [42-44], suggesting that this trait evolved multiple times in the F-T3SS. *Buchnera* spp. are endo-mutualistic bacteria with very small genomes containing a few flagellar genes. These genes are insufficient to produce a complete flagellum. Instead, they code for a flagellar basal body devoid of most extracellular structures

169 at the cell surface that could function as a secretion system [45]. The extracellular components
170 of the flagellum are not required for secretion and flagellar motility is useless in this obligatory
171 intracellular bacteria, suggesting that *Buchnera* co-opted the F-T3SS for protein secretion. This
172 reduction of the flagellar structure is reminiscent of the mechanism of ejection of the
173 flagellum hook and filament observed in diverse γ-Proteobacteria bacteria to cut the costs on
174 cell motility under nutrient depletion [46]. Hence, simple gene losses could have driven the
175 specialization of the F-T3SS into systems that cannot provide motility to the cell, but are able
176 to secrete proteins in the extracellular space.
177 One particular case of flagellar reduction led to the subsequent evolution of the non-flagellar
178 T3SS or injectisome (NF-T3SS, here shortened to T3SS). The T3SS has many genes homologous
179 to the flagellum, even if it has fewer components (flagella are encoded by ~50 genes, T3SS by
180 less than half) [20, 47, 48] (Figure 2a). The analysis of the components of the T3SS, and of their
181 phylogeny, shows that most core components of the T3SS were directly derived from the
182 ancestral flagellum, including the filament and the F-T3SS. This process was accompanied by
183 the loss of many flagellar genes, which may have led to intermediate systems involved in
184 protein transport to the periplasm or to the extracellular space [19]. A few systems of
185 unknown function in *Myxococcales* spp. may be representative of such intermediate systems.
186 Some crucial gene gains then led to the T3SS, a machine that secretes a plethora of effectors
187 directly into eukaryotic cells.
188 The only recognizably ubiquitous component of the T3SS that lacks a homolog in the flagellum
189 is the pore-forming secretin, which was shown by phylogenetic inference to have been
190 acquired by T3SS at least three times from different systems (see Box 2 and Figure 3). Other
191 key components found in the T3SS and lacking in the flagellum are the devices puncturing the
192 eukaryotic cells (translocon and tip of the needle, Figure 2a). They provide functions relevant
193 for the T3SS, but not for the F-T3SS. The relations of homology between these two
194 components across sub-types of T3SS cannot always be ascertained, because they evolve very
195 fast and the analogous components often lack significant sequence similarity. For the same
196 reason, it's not known if they were co-opted from other cellular machineries. The rapid
197 evolution of these components may result from diversifying selection, since they are in direct
198 contact with the host and are targeted by immune responses [49]. Following the initial crucial
199 steps of gene losses, the gains of secretins and needles led to the modern T3SS. Specific T3SS
200 sub-types are specialized in puncturing either animal, plant or fungal cells [50] (Figure 2a).

Intriguingly, and contrary to T4SS and T6SS, we have no knowledge of reports showing the use of T3SS to secrete effectors into other bacterial cells (see Outstanding Questions). T3SS have spread by horizontal gene transfer to many diderm Bacteria. This is a mechanism that affects much less frequently the evolution of flagellar systems [51, 52]. As a result, some Bacteria (*e.g. Burkholderia*) encode multiple T3SS to interact with multiple types of eukaryotic hosts [53]. In summary, the natural history of T3SS shows that nanomachines enduring processes of functional simplification can subsequently acquire novel complex functions.

TFF evolved different functions from a common set of components

The T2SS uses a complex machinery to secrete proteins from the cell periplasm to the extracellular space. It initially evolved from a type IVa pilus (T4aP) presumably involved in twitching motility. Together with other systems of Archaea and Bacteria (both monoderm and diderm), T2SS and T4aP are part of the super-family of type IV filaments (TFF) [18, 54]. TFFs typically include five to six core components: AAA+ ATPase(s), major and minor pilins, cytoplasmic membrane platform(s), a prepilin peptidase and sometimes a secretin (Figure 4). The phylogeny of the ATPase family places the root of the tree between the bacterial and the archaeal groups of TFF, suggesting that the system pre-dated the last common ancestor of all cellular life forms [18]. Most other key components have recognizable homologs across TFF, suggesting that they were already present in the ancestor of all TFF. Unfortunately, they rarely have homologs in non-TFF systems, implicating that only the ATPases can be used to trace the very initial stages of TFF evolution. The ancestral system diversified by a succession of mutations, gene deletions, duplications, fissions and fusions that led to TFFs with very different functions. They include adhesion (many TFFs), protein secretion (T2SS), twitching motility (T4aP and T4bP), flagellar motility (Archaellum, unrelated with the F-T3SS) and DNA uptake (Com, T4aP) [40, 55-58] (Figure 4). For example, T2SS have evolved pseudo-pili producing shorter structures than the pili of the ancestral T4aP from which they were derived. These specialized components drive the transport of the effectors across the outer membrane [59].

The phylogeny of some TFFs suggests complex evolutionary scenarios. Soon after the divergence of the ancestral TFF into an archaeal and a bacterial branch, the ATPase of the bacterial systems was duplicated into a pair of proteins specialized in pilus extension (PilB) and retraction (PilT). This event was thought to have endowed the T4aP with the ability to

provide twitching motility by rounds of pilus extension and forceful contraction. Interestingly, the single homologous ATPase of the tight adherence (Tad) pilus systems can extend and contract the pilus [29], and PilT mutants of T4aP can weakly retract the pilus [60]. Hence, the PilB/PilT duplication seems to have allowed a specialization of two ancestral functions (sub-functionalization) where PilT evolved to provide the high-force retraction required for twitching motility. In some TFF there was yet another amplification the ATPase family giving rise to PilU (Figure 1). Its function is to coordinate the activity of retraction with PilT [61 , 62]. When the T2SS evolved from a T4aP, the retraction function was unnecessary and the corresponding specialized ATPase was lost.

The majority of TFFs specialized in the secretion of protein effectors (T2SS-like) are monophyletic and present across Proteobacteria [63]. But several recently uncovered T2SS-like systems may have evolved in different ways. The phylogenies of the components of the Chlamydiales T2SS-like systems show that they were recruited from diverse TFFs [18]. The T2SS-like systems from Bacteroidetes, *e.g.* those of *Cytophaga*, constitute completely independent co-options of (different) T4aP [18]. This suggests that a specialized protein secretion system may require relatively few steps to evolve from the T4aP, possibly because the latter already secretes a specific family of proteins (pilins). Once the different T2SS-like systems evolved into specialized secretion systems, they became able to secrete many different types of proteins [64, 65] (Box 1). This versatility may have contributed to the evolutionary success of T2SS.

The spread of TFFs among Bacteria and Archaea was promoted by horizontal transfers. For example, the Tad pilus originated from an archaeal pilus that was transferred to diderm Bacteria, where it recruited a secretin, and was then transferred to other phyla [18] (see Box 2). There is a close interplay between horizontal transfer and the genetic organization of systems because transfer is facilitated when the systems are encoded in a single locus. Such systems, which among TFF include most T2SS and Tad, are more frequently transferred horizontally than those that tend to be encoded in multiple loci [18]. This is presumably because one single event of transfer is enough to provide a novel function to the recipient when all the system is encoded in a single locus [66]. Horizontal transfer spreads these novel functions to other cells where they are eventually fixed when they provide a competitive advantage. Importantly, by transferring systems in new genetic backgrounds this process may

drive further innovation to allow the accommodation of the function to its novel cell. In this way, a relatively small set of components can diverge to produce very diverse functions.

Co-option of phages led to the radical invention of T6SS

The T6SS delivers effectors directly into eukaryotic or bacterial cells and is implicated in inter-specific competition, virulence, resource scavenging, and genetic exchanges [67-69]. It is composed of a baseplate-like platform bound to the membrane, which anchors a contractile sheathed tube decorated by a puncturing device that allows toxins to penetrate the target cells (Figure 2c). The structure of many of these components resemble bacteriophage proteins and the overall T6SS resembles an inverted phage tail [23, 24, 70-72], which suggests that a large part of the T6SS derived from a phage. Unfortunately, sequence similarities between phage and T6SS homologs are very low and preclude the study of the initial processes of co-option using standard phylogenetic approaches. The similarities between T6SS and phages extend to the assembly/disassembly dynamics of the respective structures. In particular, T6SS sheaths are contractile in order to project the tube towards target cells, in a similar fashion that phage tails contract to deliver DNA into target cells [73]. This activity is dependent on the ClpV ATPase [73], which is an AAA+ ATPase homologous to those of T2SS and T4SS, but lacking close homologs in phages (see Figure 1). The T6SS also has two components homologous to the type IVb pilus (T4bP) of the TFF super-family, which led to its initial misnaming as a T4bP [74]. This suggests that the co-option of a phage tail was subsequently integrated with components from other cellular machines to produce extant T6SS.

Some genomes encode many different T6SS. For example, *Burkholderia thailandensis* have five different systems, one of which is specialized in the interaction with the host and another with competing Bacteria [75]. As some structural components of the T6SS are also effectors (Box 1), different systems may be associated with different effectors. In spite of the multiplicity of T6SS in certain genomes, the four known major variants of T6SS, called T6SS^{i} to T6SS^{iv}, tend to be present in different types of Bacteria. Variants of the most widespread T6SS (T6SS^{i}) were initially found in *Francisella* (T6SS^{ii}) and in Bacteroidetes (T6SS^{iii}) [76-78]. In spite of having specific components, these systems include most of the T6SS^{i} core components and are presumed to have derived from the same ancestor. More remarkably, the T6SS^{iv} resembles the other variants in terms of the structure of the puncturing device, but seems to lack components not associated to phages, such as the ATPase or the trans-envelope complex

296 [79]. The phylogenetic analyses of sheath protein sequences suggest that it could have
297 emerged from phages independently from other T6SS (Figure 2c) [79]. These results suggest
298 that even such complex co-option events may occur several times in parallel. This might seem
299 surprising, but one should take into consideration that the accumulation of machineries in the
300 genome that can be used for such complex evolutionary processes may increase the frequency
301 at which they occur. Half of the bacteria are lysogens and many of these encode several
302 prophages [80], and these may provide abundant genetic material for evolutionary tinkering
303 leading to the evolution of puncturing devices.
304

305 ## Concluding Remarks and Perspectives

306 The evolution of secretion systems illuminates some general properties of the processes of
307 co-option. The evolution of T4SS exemplifies how a small number of changes suffices to evolve
308 a protein secretion system when there is functional promiscuity in the original system (which
309 is inherently capable of secreting a protein attached to the DNA). The simplicity of this process
310 explains why it evolved many times independently in natural history [22]. Analogously, the
311 evolution of the T2SS was facilitated by the fact that most TFF secrete to the extracellular
312 space the proteins composing their filaments. In both cases, the evolutionary success of the
313 innovations may be due to the ability of the derived secretion systems to secrete a large panel
314 of effectors. Unfortunately, the evolution of mechanisms of effector recognition remain
315 poorly known (Box 1) and this hampers our ability to fully understand these processes.
316

317 The evolution of the two other systems was much more complex. The loss of genes of the F-
318 T3SS results in systems that may be involved in protein transport. This initial sub-
319 functionalization was followed by the accretion of novel components that led to the T3SS.
320 Some steps of this process occurred multiple times: the loss of the motility component of
321 flagella, the subsequent acquisition of secretins (Box 2), and possibly the acquisition of
322 translocons [19]. Since all known T3SS are monophyletic, the complete path from the
323 flagellum to the T3SS occurred only once. This suggests that the frequency of the processes of
324 co-option depends on the complexity of the evolutionary path. Similarly, the T6SS endured a
325 complex evolutionary process that one might think unique in natural history. The T6SS^{iV}, which
326 may have emerged independently from the other T6SS, seems to lack some of the functions
327 of the other systems [79], and could result from the initial steps of phage tail co-option that

were extended in the case of the other T6SS. If correct, both T3SS and T6SS evolved by an initial process of sub-functionalization (co-opting basal flagellar basal bodies in one case and phage tails in the other) that produced intermediary structures. These were later integrated with other cellular components to produce a complex novel secretion system. One could regard such processes as rare, but the frequency of co-option events is certainly also shaped by the availability of machineries that can be tinkered by natural selection for functional innovation. Both flagellar loci and prophages are frequent in bacterial genomes.

The existence of analogous functions across secretion systems and other cellular machineries, sometimes performed by homologous proteins (Figure 1), may facilitate the combination of components of different origins into novel systems. For example, the T4SS of type I, also called T4SSb, includes a T4bP that is essential for conjugative transfer of plasmid R64 in liquid media and for adherence to host cells by the T4SS of *Legionella* [81]. A combination of a T4SS and a TFF secretin is used by some *Haemophilus* spp. to export DNA to the environment [82]. Finally, the T1SS is a combination of two systems that pre-existed autonomously: an outer membrane porin and an ABC transporter [83]. The frequency with which components were combined to produce novel systems during natural history suggests that novel systems may be uncovered by looking at atypical combinations of known components. Furthermore, it opens the possibility that humans could engineer secretion systems with novel features by recruiting components of diverse membrane-associated machines.

This review focused on the most-studied secretion systems, but many relations of homology between protein secretion systems and other machines have been reported. It was recently proposed that some core components of the flagellum and T3SS are a functional unit capable of forming nanotubes between cells for the transport of nutrients [84, 85]. The ESX inner membrane transport system in monoderms has components homologous to the T4SS and to the T7SS of Mycobacteria [86, 87], the autotransporters (T5SS) are structurally homologous to the porins of the novel FAP system that exports amyloid subunits in *Pseudomonas* [88], and there are intriguing homologies between components of several secretion systems and a system putatively involved in the transport of proteins between mother and forespore cells in Firmicutes [89]. Finally, a TonB-dependent transporter (TBDT) of *Myxococcus xanthus*, a widespread family of systems usually involved in protein and nutrient import, was recently shown to be involved in the two-step protein secretion of a protein [90]. Further work is needed to understand the evolution of some other secretion systems. Notably, the T9SS is

present in many bacteria of the Fibrobacteres-Chlorobi-Bacteroidetes super-phylum where it is involved in both gliding motility and protein secretion [91, 92], but its evolutionary associations with other cellular components are unknown. The T7SS is present in Mycobacteria and is regarded as a *bona fide* secretion system because these bacteria have an outer membrane (that differs in composition from those of Proteobacteria) [87]. The T7SS is partly homologous to systems involved in protein transport across the cytoplasmic membrane of Firmicutes [86, 87]. Interestingly, these two systems contain AAA+ ATPases like those of T4SS and T2SS, suggesting ancient evolutionary associations that need to be unravelled.

Based on past experience, there are probably protein secretion systems yet to be discovered, especially in the numerous clades that are being uncovered by environmental metagenomics. The evolutionary principles described in this article suggest that many of these novel systems will include components homologous to those of known membrane-associated machines. This informed hypothesis can be leveraged to identify the novel systems. Such endeavour can now also rely on abundant genomic data to identify components homologous to those of membrane-associated machineries, metagenomics data to investigate conditions and environments where they are expressed, structural and microbial cell biology to understand their function, and evolutionary biology to integrate this information. Altogether, these elements will likely accelerate the pace of discovery of novel protein secretion systems (see Outstanding Questions). In turn, these may illuminate some yet obscure aspects of the very ancient history of complex machineries at the origin of known secretion systems, such as the flagellum or the conjugative system.

Box 1: Where do all effectors come from?

At a given moment in evolution, protein secretion systems acquired the key ability to recognize novel effectors. This is particularly remarkable for systems able to secrete many different effectors, such as T2SS, T3SS, T4SS, T6SS, or T9SS, many of which evolved from machines secreting only a few different proteins (pilins, flagellins, relaxases, etc). Systems capable of secreting many different effectors may be more economical than systems secreting a single one, because the production of one single machine is sufficient to secrete a large panel of proteins. Furthermore, some sets of effectors must interact to be efficient, which means they must be secreted at the same time. For example, T3SS effectors require co-secretion of specific protein translocases to traverse the membrane of eukaryotic cells [93]. Selection for the ability to secrete many different effectors in a single cell may have been a driver of the evolution of the complex machineries of T2SS, T3SS, T4SS, and T6SS [48]. On the other hand, systems with fewer components, but usually associated with one or a few effectors like the T1SS and the T5SS, can be horizontally transferred with their effectors across bacteria.

Secretion systems discriminate effectors using mechanisms that provide some clues on how effector recognition evolved. Some T6SS effectors are structural components of the system [94], and some T4SS effectors have domains that are structurally similar to the recognition domain of the relaxase [95]. In both cases, genetic fusions of proteins with domains recognized by the secretion system can result in novel effectors. Such gene fusions are frequent in secreted proteins, as revealed by the combinatorial variation of domains of secreted polymorphic toxins [96].

The evolution of other mechanisms of effector recognition by secretion systems is unclear, since the mechanisms of effector recognition are themselves poorly understood. Interestingly, some effectors can evolve to be recognized by multiple systems. For example, a toxin was recently shown to be secreted by both T2SS and T3SS in *Vibrio* [97]. It is unclear if this involved adaptation of the secretion system to the effector or the other way around. This raises the complementary question of how effectors evolve to be recognized by the secretion systems. Some effectors might evolve by co-option, just like their cognate secretion systems. For example, a large fraction of the repertoire of *Legionella* T4SS effectors may have been co-opted from proteins of Eukaryotes [36], and *Burkholderia* deploy a T3SS anti-fungal protein that may have been co-opted from a prophage tail-like protein [98]. Proteins that evolved to become secreted by a specialized system can subsequently endure processes of gene

414 duplication and transfer to diversify into novel functions. Actually, genes encoding secreted
415 proteins are very often on mobile genetic elements, such as plasmids [10] and temperate
416 phages [99].
417

BOX 2. Sharing and recruiting components

The transfer of a system to another bacterium may require the accretion of novel functions to fit in the novel genetic background. When the pertinent functions are encoded in the new host genome, they may be initially shared with the other systems. An interesting example is provided by the T1SS, a system composed of an ABC transporter, a porin, and a fusion protein connecting the two. Several T1SS of *E. coli* share the porin function encoded by a single gene (*tolC*), which is also involved in the transport of small molecules [100]. Hence, a single gene, distant from the other genes of the T1SS, provides multiple functions to the cell. Similar gene sharing is observed in TFF, where some prepilin peptidases contribute to the assembly of both T4P and T2SS [101]. However, a component that is shared by several systems accumulates functional, structural and regulatory constraints [102]. This may lead to subsequent gene duplication and sub-functionalization events that will eventually result in the presence of multiple homologous genes in the genome (one per system).

The secretin has been recruited by many systems independently. At the initial stages of this process there was probably a single gene, which implicates that the protein may have been shared between systems. The secretin was co-opted by the ancestor of the Tad where it allowed the pilus to cross the outer membrane after its transfer from Archaea to diderm Bacteria. Even more strikingly, the co-option of the secretin from the TFF took place three times independently during the evolution of the T3SS, one of which from a Tad pilus that had obtained it previously from a T4aP (Figure 3). The secretin was also co-opted from the T2SS by some filamentous phages that use this porin to secrete virions from living cells (Figure 3). The grafting of secretins into secretion systems requires a remarkable structural flexibility, especially if this takes place while the same protein remains a component of another machinery. Secretins of the T2SS assemble independently of the rest of the system, and the substitution of a single amino-acid in some T4P secretins was shown to make them capable of self-assembly [103]. Yet this structure is not stable in the absence of the inner-membrane components with which its N-terminus domains interact [104, 105]. It is tempting to speculate that the ability of secretins to assemble autonomously from the rest of the system for a short period of time has facilitated the initial recruitment of the secretin by many different systems.

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Figure legends

Figure 1 – Pairwise HMM profile alignments between all the proteins of the TXSS-related systems. The HMM profiles were obtained from TXSScan [18, 63]. The color of nodes represents systems in which the proteins were found. To establish relationships of homology between the components of the different systems, *i.e.* to draw edges between nodes, we made pairwise alignments of their HMM protein profiles using HHSearch v3.0.3 (p-value threshold of 0.001). Groups of proteins that gathered more than two components from at least two systems are displayed. The function attributed to each group is written in black in its background. Given the current difficulty in precisely delineating the functions of the TFF of Archaea, they were grouped under "Archaeal-T4P".

Figure 2 – The evolution of protein secretion systems delivering effectors directly into other cells. (a) The diversification of the T3SS from the bacterial flagellum involved initially the loss of flagellum-specific genes and the motility function. The subsequent multiple acquisitions of pore-forming secretins, translocons, and a few other genes led to the extant T3SS. On the right, the rooted cladogram represents the history of the T3SS [19]. The *Myxococcales* system is not a genuine T3SS since, to the best of our knowledge, it lacks an outer membrane porin. (b) Conjugative apparatuses, involved in ssDNA conjugation, were co-opted multiple times independently into T4SS secreting proteins into other cells. On the right, the rooted cladogram represents the evolution of T4SS [22]. MPF stands for mating pair formation and includes the T4SS. (c) The T6SS resulted from the co-option of contractile tail phage genes (and their integration with other genes). This resulted in a contractile structure able to puncture eukaryotic or bacterial cells and deliver effectors, often toxins. On the right is presented a non-rooted cladogram of the history of T6SS [79]. MAC stands for metamorphosis- inducing structures. Afps stands for insecticidal anti-feeding prophages. The drawings of the systems are based on [48]. OM stands for outer-membrane, IM for inner (cytoplasmic) membrane; the periplasm is shown in brown between the IM and OM; "Entero" stands for enterobacteria.

Figure 3 – Phylogeny of secretin proteins. The tree was built using the secretin domain of the protein sequences from [18], with the addition of T3SS and phage sequences from [19]. We aligned the sequences using MAFFT v7.273 (einsi algorithm) [106], selected informative sites in the multiple alignment using Noisy v1.5.12 [107] (default parameters), and inferred the maximum-likelihood tree from these alignments with IQ-TREE v1.6.7.2 [108] (using the best evolutionary model, options -MF, BIC criterion, -allnni, -ntop 1000, -nm 10000). Node supports displayed at nodes were estimated using the option -bb 1000 for ultrafast bootstraps [109]. The tree is consistent with the results of several previous studies. The root was positioned between T4bP and the remaining clades, as suggested elsewhere [18, 19, 103]. If correct, secretins were first components of the TFF superfamily and subsequently co-opted by phages and T3SS (three times independently from Tad for Rhizobiales, from T2SS for Chlamydiae, and from T4aP for other proteobacterial T3SS). They were also recruited by the Tad upon the transfer of the ancestor of this system from Archaea. The color of circles at the tip of the tree corresponds to different bacterial phyla. The colors of groups and drawings on the right depict the different systems where secretins have been identified.

Figure 4 & KEY FIGURE – Diversification of the type IV filament (TFF) superfamily around a common set of components. The TFF superfamily diversified into many different systems using a few homologous core components (in the middle) and integrating some new ones (in circles, described in the bottom legend). One should note that some of the acquired components may have been already present in the ancestral system but evolved so fast that one can't trace homology. The different machines were able to diversify into functions as different as secretion of toxins, DNA uptake, motility, adhesion to surface. Components colored in the same way correspond to homologs. The drawings of the systems are based on [110].

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745

Highlights

- Recent discoveries in molecular, structural and cell biology revealed interactions between components and unexpected functions for protein secretion systems. They illuminate the evolutionary history of these machines.
- Many protein secretion systems evolved from evolutionary tinkering of other pre-existing cellular machines by co-option of components or entire functional modules.
- The extent of changes involved in these evolutionary processes depends on the complexity of the systems, functional differences to the original co-opted system, abundance of genetic elements to be tinkered with, and, possibly, the evolution of the mechanisms for interacting with effectors and discriminate them.
- Some protein families, like the secretin, were recruited multiple times to different protein secretion systems and may have endured periods where the same gene was shared by several systems.

Outstanding questions

- Are there novel systems yet to be discovered?

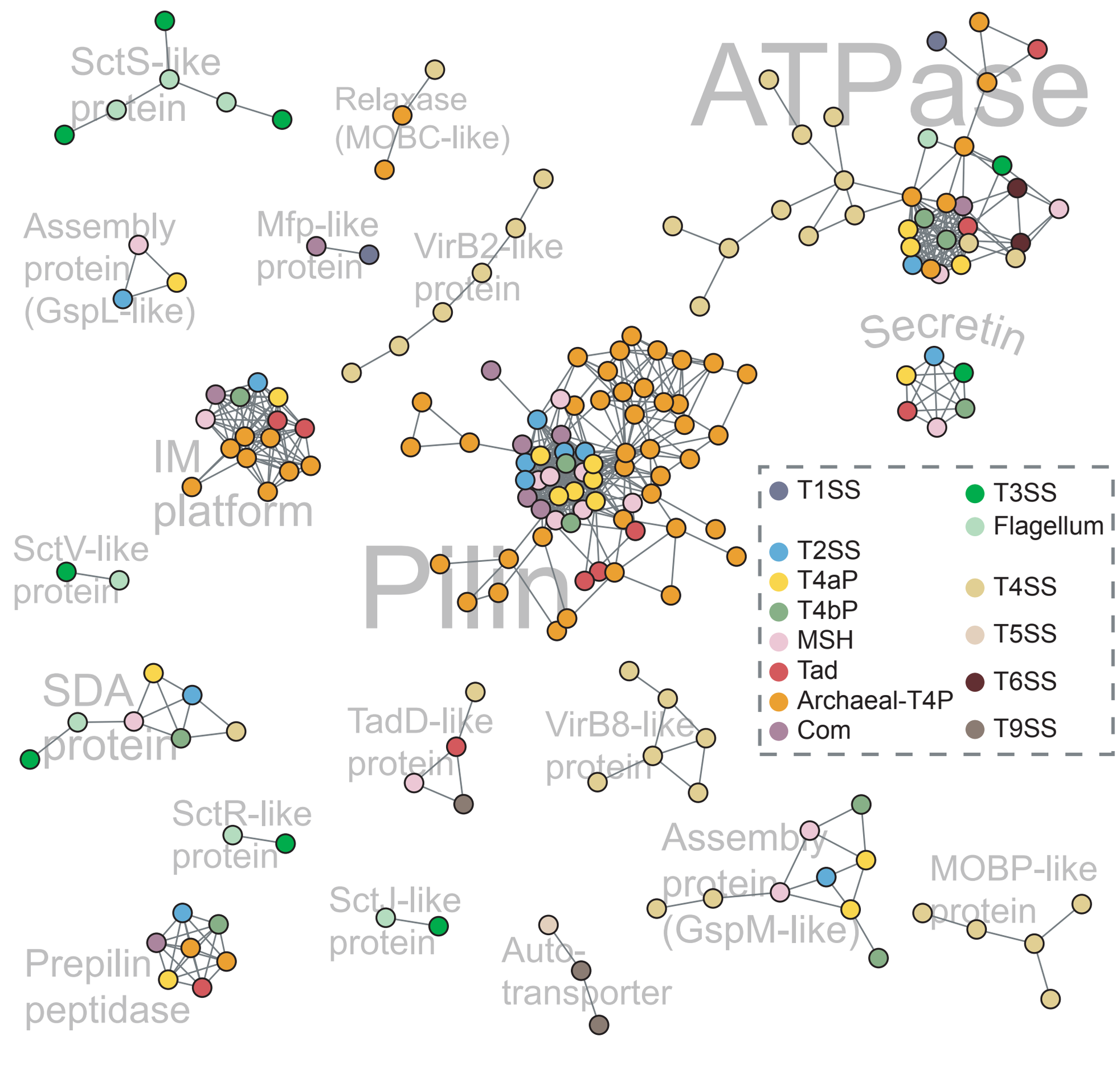
- Are systems that deliver proteins inside other cells exclusively present in diderm Bacteria? If so, why?

- Are there specific structural traits facilitating the integration of components in a secretion system?

- Can we combine components from known systems to design novel ones with specific functions?

- How did the recognition of effectors at the onset of secretion systems evolve?

- Can T3SS be used to secrete proteins directly into bacterial cells?


SctS-like protein
Relaxase (MOBC-like)
ATPase
Assembly protein (GspL-like)
Mfp-like protein
VirB2-like protein
Secretin
IM platform
Pilin
SctV-like protein
T1SS
T2SS
T4aP
T4bP
MSH
Tad
Archaeal-T4P
Com
T3SS
Flagellum
T4SS
T5SS
T6SS
T9SS
SDA protein
TadD-like protein
VirB8-like protein
SctR-like protein
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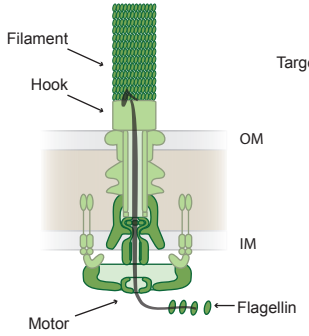
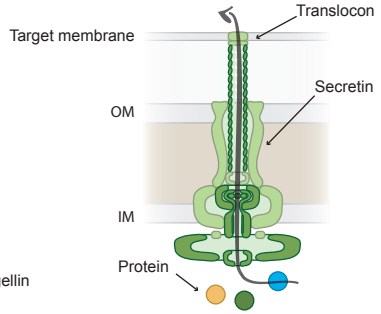
SctJ-like protein
Prepilin peptidase
Auto-transporter

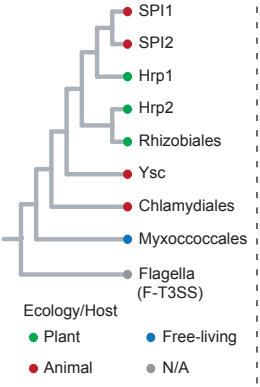
a- From the flagellum to the T3SS



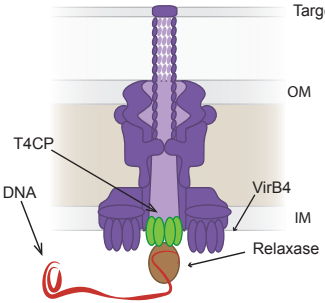
Flagellum



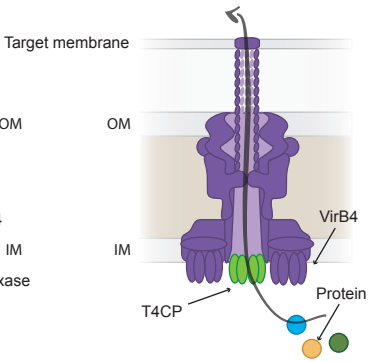
T3SS



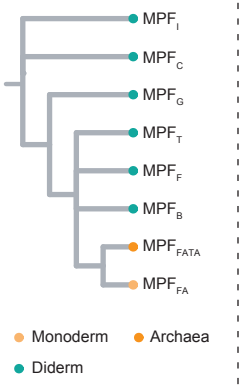
b- The T4SS: from conjugation to protein secretion



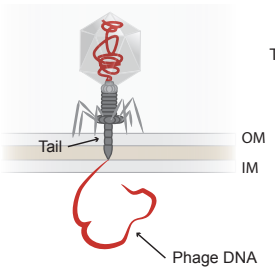
Conjugative apparatus



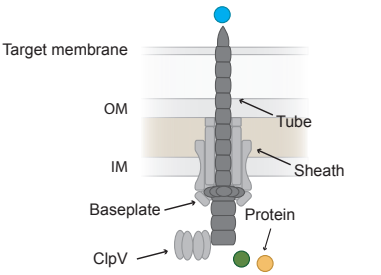
T4SS



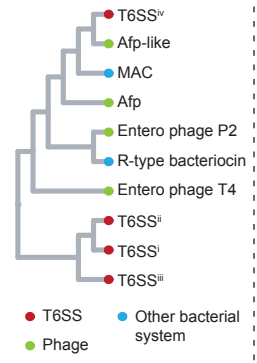
c- From a phage to the T6SS

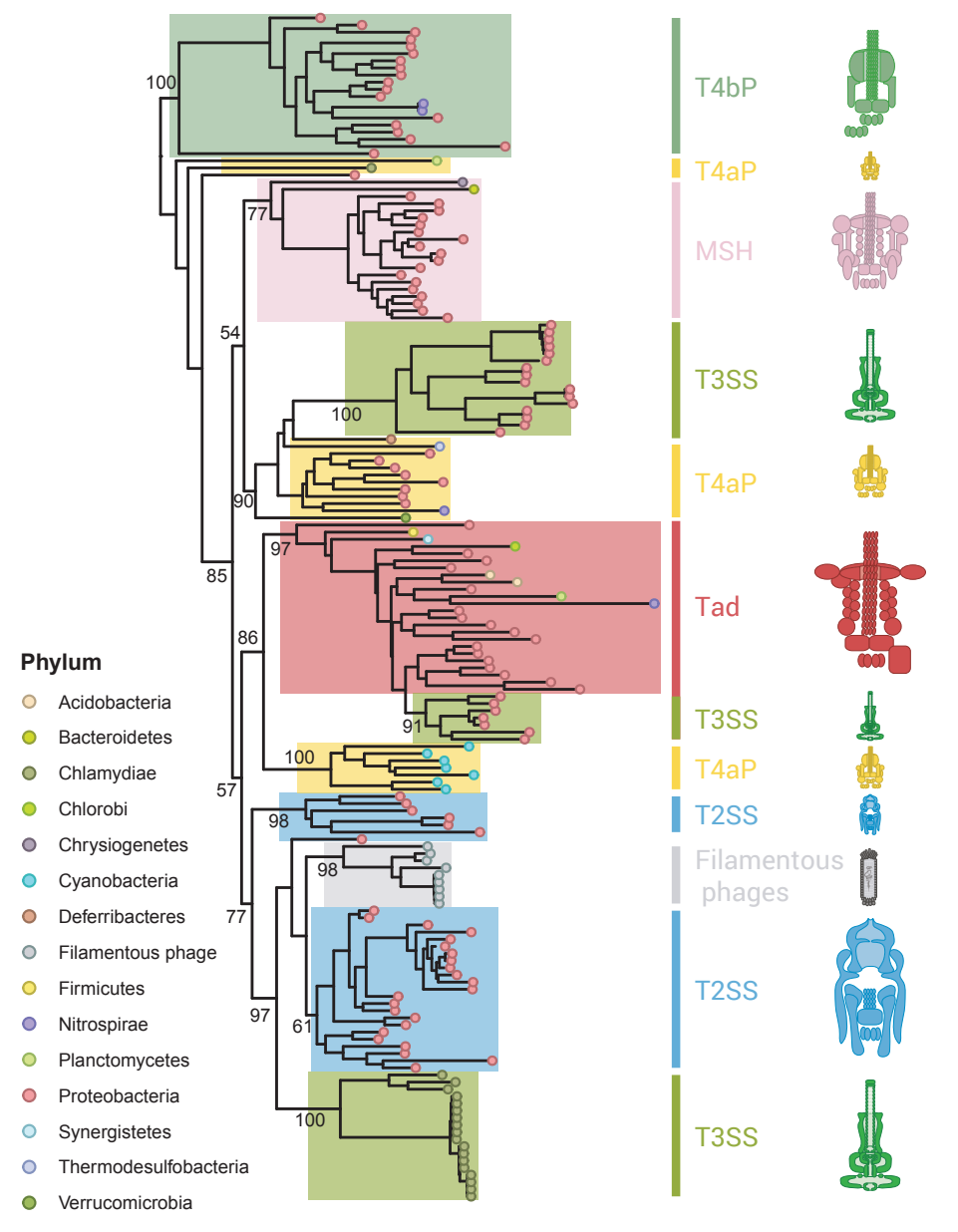


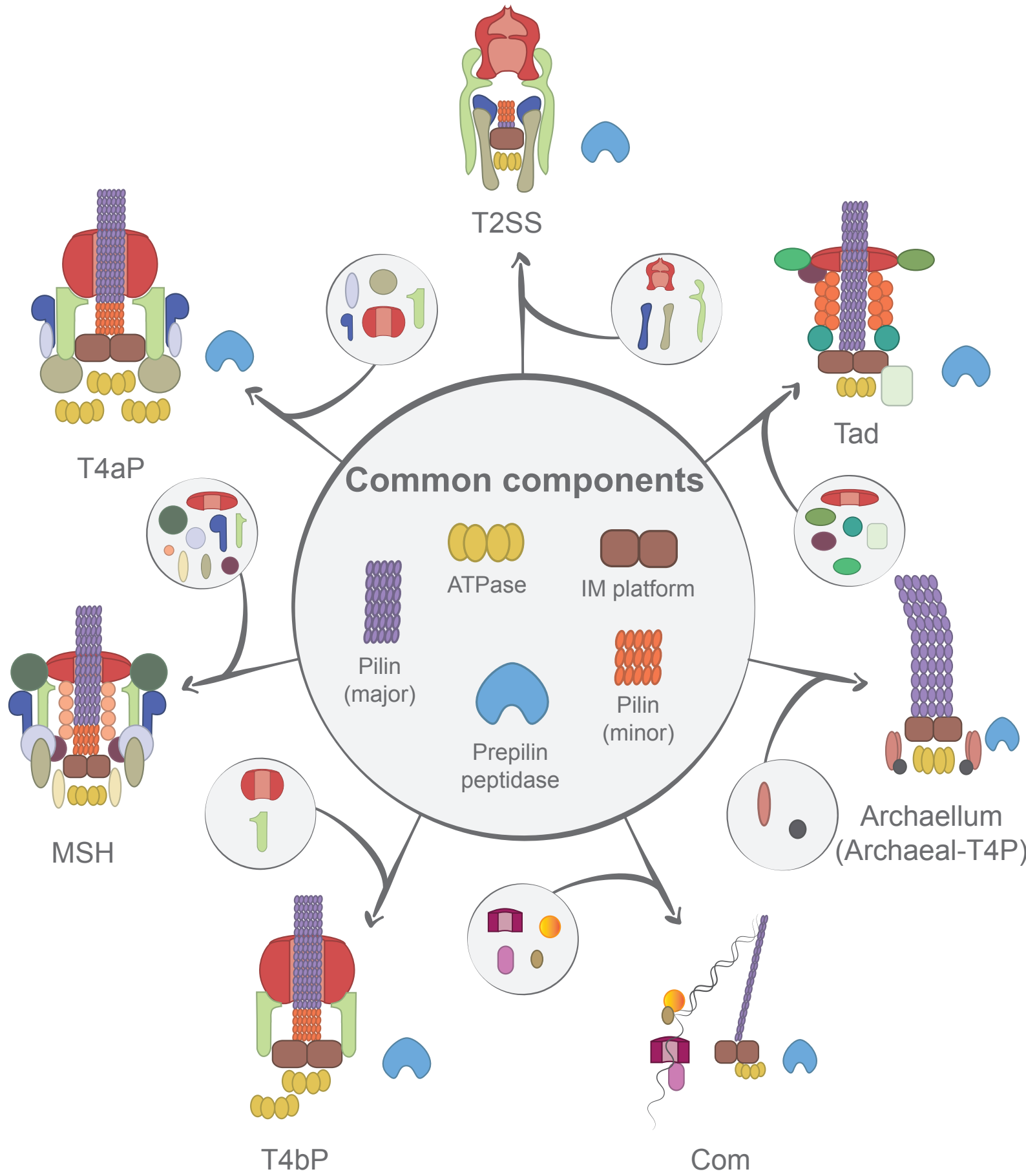
Phage



T6SS




100
77
54
100
90
85
97
86
91
100
57
98
98
77
97
61
100
T4bP
T4aP
MSH
T3SS
T4aP
Tad
T3SS
T4aP
T2SS
Filamentous phages
T2SS
T3SS
Phylum
Acidobacteria
Bacteroidetes
Chlamydiae
Chlorobi
Chrysiogenetes
Cyanobacteria
Deferribacteres
Filamentous phage
Firmicutes
Nitrospirae
Planctomycetes
Proteobacteria
Synergistetes
Thermodesulfobacteria
Verrucomicrobia


T2SS
T4aP
Tad
Common components
ATPase
IM platform
Pilin
(major)
Pilin
(minor)
Prepilin
peptidase
MSH
Archaellum
(Archaeal-T4P)
T4bP
Com