Protein Injection Machines in Bacteria

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Abstract

Many bacteria have evolved specialized nanomachines with the remarkable ability to inject multiple bacterially encoded effector proteins into eukaryotic or prokaryotic cells. Known as type III, type IV, and type VI secretion systems, these machines play a central role in the pathogenic or symbiotic interactions between multiple bacteria and their eukaryotic hosts, or in the establishment of bacterial communities in a diversity of environments. Here we focus on recent progress elucidating the structure and assembly pathways of these machines. As many of the interactions shaped by these machines are of medical importance, they provide an opportunity to develop novel therapeutic approaches to combat important human diseases.

Introduction

One of the most exciting recent developments in the field of bacterial pathogenesis is the discovery that many bacterial pathogens have the capacity to transfer bacterially encoded proteins to eukaryotic target cells. These bacterial proteins collectively known as "effectors" are endowed with the ability to modulate a myriad of eukaryotic host cell functions (Galán, 2009; Grant et al., 2006; Jennings et al., 2017; Khan et al., 2016). It is therefore not surprising that the discovery of these machines originated from the study of bacterial pathogens that have evolved very close interactions with their eukaryotic hosts (Galán and Curtiss III, 1989; Michiels and Cornelis, 1991; Rosqvist et al., 1991). In fact, it is now known that these systems operate not just in the context of pathogenic relationships but also in the context of symbiotic interactions between bacteria and their respective eukaryotic hosts, including animals, plants, insects and a variety of protists. The remarkable feat of injecting proteins into eukaryotic cells is accomplished by

extraordinary multi-protein machines endowed with the capacity to specifically select their cargos and move them through the physical barriers imposed by the multiple membranes that separate the bacterial cytoplasm from their final destination within target cells.

Evolution seems to have built at least two structurally and ancestrally different machines, collectively known as type III and type IV protein secretion systems, that are capable of transferring bacterial proteins into eukaryotic cells (Christie, 2016; Galán et al., 2014). A third and unrelated type of machine, the type VI secretion system, has evolved a different but no less remarkable activity, which is to inject proteins into other bacteria (Russell et al., 2014). However, these machines also appear in some circumstances to be able to deliver bacterial effector proteins into target eukaryotic cells (Pukatzki et al., 2007). As expected from evolution, these protein injection machines have evolved from distant ancestors that, while related, perform rather different functions. Thus, these systems have evolved from machines involved in motility (type III), DNA transfer (type IV), or bacteriophage infection (type VI) (Abby and Rocha, 2012; Guglielmini et al., 2013 ; Leiman et al., 2009). The study of the multiple functions of the plethora of effector proteins delivered by type III or type IV secretions systems indicate that they have <u>cell modulating</u> functions rather than <u>cell destructive</u> activities (Galán, 2009), which are most often associated with bacterial exotoxins (Alouf, 2000; Galán, 2009). Importantly, the complex modulation of cellular functions mediated by type III or type IV machines most often requires the activity of several effector proteins that must work in concert with one another. Therefore, the driving evolutionary force that led to the emergence of these very complex machines most likely stem from the need to deliver <u>multiple</u> bacterial proteins to the <u>same</u> cell, a complicated feat unattainable through the simpler mechanisms utilized by bacterial exotoxins. Due to their central importance in bacterial pathogenesis and their widespread presence in many important pathogens, these protein injection machines are quickly emerging as prime targets for the development of novel therapeutic and prevention strategies (Anantharajah et al., 2016; Gu et al., 2015). In addition, these machines are also becoming extremely useful tools to understand

fundamental issues in biology just as protein transport across membranes, organelle assembly, and signal transduction. Although these protein injection machines are conserved across different bacterial species, the biology in which they are involved and the effectors that they deliver are highly customized for the bacteria that encode them. Due to space constrains, in this review we will only provide a general overview of the more salient structural and functional features of type III, type IV and type VI protein injection machines. For more detail information readers are referred to recent reviews on the individual systems or on the biology of the effectors that they deliver (Buttner and He, 2009; Byndloss et al., 2017; Chandran and Waksman, 2015; Deng et al., 2017; Galán, 2009; Galán et al., 2014; Jennings et al., 2017; Journet and Cascales, 2016; Kapitein and Mogk, 2013 ; Notti and Stebbins, 2016; Russell et al., 2014 ; Zechner et al., 2012).

Type III secretion systems

Evolution and function

Type III protein secretion systems (T3SSs) are encoded by a broad range of gram-negative bacteria with pathogenic or symbiotic relationships with a great variety of hosts including vertebrates, plants, insects, and nematodes (Hu et al., 2017b). Among these bacteria are pathogens of great Public Health significance including *Salmonella enterica*, *Shigella* spp., *Yersinia pestis, Vibrio cholera*, *Bordetella pertussis,* and *Pseudomonas aeruginosa*. Although the origin of T3SS has been the subject of a lively debate, it is now accepted that T3SSs most likely evolved as an exaptation from the flagellar apparatus (Abby and Rocha, 2012; Hu et al., 2017b; Pallen et al., 2005). Indeed, to export its extracellular components during assembly, the flagellum relies on a protein secretion mechanism that shares many features with T3SSs (Diepold and Armitage, 2015). Recent phylogenetic analysis have provided evidence that the last common ancestor of the non-flagellar T3SSs was most likely not competent for protein delivery into

eukaryotic cells (Abby and Rocha, 2012). In fact, vestiges of this original structure may be apparent in *Myxococcales* spp. Subsequent acquisition of additional components not shared by the flagellar apparatus resulted in T3SS machines competent for protein injection into eukaryotic cells. Interestingly, this last step may have independently occurred more than once during T3SS evolution (Abby and Rocha, 2012; Hu et al., 2017b).

Although the components of different T3SSs are highly conserved, the composite of effectors that they deliver are not (Hu et al., 2017b). Consequently, T3SSs are involved in a variety of activities that share in common the need for a close interaction between the pathogen or symbiont and its host cell. Examples include the modulation of actin cytoskeleton dynamics to invade cells, the manipulation of vesicle trafficking processes to establish an intracellular niche, the stimulation of transcriptional responses to stimulate inflammation, and the interference with host innate immune responses (Dean, 2011; Galán, 2009; Jennings et al., 2017; Macho, 2016) (Fig. 1A). The manipulation of these various cellular processes is achieved by the delivery of multiple effectors with a vast array of biochemical activities including GEFs and GAPs for Rho and Rab family GTPases, kinases, phosphatases, ubiquitin ligases, highly specific proteases, and a variety of enzymes that can introduce specific covalent modifications to specific host cell targets. A common theme in many of these effectors is that they exert their activity by mimicking host proteins (Stebbins and Galan, 2001). This mimicry is often the product of convergent evolution since many of these effectors do not share primary amino acid sequence or even structural homology with their eukaryotic counterparts. Mimicry as a strategy to exert their functions allows the modulation of vital cellular process while preserving cellular homeostasis. In fact, the preservation of cellular homeostasis is often central to the activities of some T3SSs, which often deliver effector proteins whose sole purpose is to counter potentially harmful agonistic activities of other effector proteins (Fu and Galan, 1999; Sun et al., 2016). These Yin and Yang relationships between effectors underscore the difference between "effectors" and "toxins". While effectors delivered by protein injection machines most often modulate cellular activities, a function

that often requires the simultaneous activities of various effectors, toxins act on their own to simply disrupt cellular processes in a manner that most often leads to the demise of the intoxicated cell.

Architecture and Assembly

The central element of T3SSs is the injectisome, a multiprotein structure that mediates the selection of effectors in the bacterial cytoplasm, and their subsequent delivery to target eukaryotic cell (Fig. 1). A combination of various experimental approaches has provided a detailed view of the structure of the entire injectisomes (Hu et al., 2017a; Loquet et al., 2012; Schraidt and Marlovits, 2011; Worrall et al., 2016), which is composed of several substructures that are associated with specific tasks during the delivery process (Fig. 1).

The needle complex. The best characterized of the substructures of the injectisome is the 3.5 Md needle complex (NC), which mediates the passage of the effectors through the bacterial envelope (Kubori et al., 1998) (Schraidt et al., 2010) (Worrall et al., 2016) (Fig. 1B). The NC is composed of a multi-ring base ~25 nm in diameter and a needle-like structure that protrudes several nanometers from the bacterial surface and is anchored to the base by the inner rod. The base is composed of just three proteins, SctD and SctJ, which make up the inner rings, and SctC, a member of the secretin family of proteins, which makes up the outer ring and neck [note, for simplicity we will use a previously proposed universal nomenclature (Hueck, 1998)]. SctD and SctJ are arranged in two concentric rings with 24-fold symmetry. These rings connect through a periplasmic neck region to an outer ring that exhibits 15-fold symmetry. Therefore the resulting structure has an overall 3-fold rotational symmetry and a symmetry mismatch between the outer and inner rings. The helical needle filament, which is formed by a single self-polymerizing protein (SctF), is ~6 nm in width and encloses an inner channel ~2.5 nm in diameter through which unfolded proteins travel on their way to their final destination within the target eukaryotic cells. The needle is capped at its distal end by the tip substructure, which exhibits significant variation

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across different T3SSs (Mueller et al., 2008; Wang et al., 2006; Wang et al., 2008). While in some T3SSs the tip complexes are formed by a homopentamer of a single protein, in others, the needle is capped by a long polymeric helical structure made of a single protein that extends the reach of the injectisome. At its cytoplasmic proximal end, the needle is anchored to the base through the inner rod, a multimeric structure made of a single protein, SctI (Marlovits et al., 2006). Finally, in the center of the NC inner rings seats the export apparatus, which is composed of several inner membrane proteins (SctV, SctR, SctS, SctT, and SctU) that serve as conduit for the T3SS substrates to traverse the inner membrane. Aside for the crystal structures of some soluble domains, there is little structural information on the the export apparatus components. The cytoplasmic domain of SctV organizes in a nonameric ring, which cryo electron tomographic studies have located immediately beneath the center of the NC (Abrusci et al., 2013; Hu et al., 2017a). The cytoplasmic domain of SctU, on the other hand, is an autoprotease that adopts different configuration before and after self-cleavage (Zarivach et al., 2008).

The sorting platform. The NC is linked on its cytoplasmic side to the sorting platform, a very large, multi-protein complex that serves as a hub to engage, sort, and prepare the substrates for their initiation into the secretion pathway (Lara-Tejero et al., 2011) (Fig. 1C and 1D). The sorting platform is composed of three scaffolding proteins, SctK, SctQ, SctL, and an ATPase (SctN), which is linked to the export apparatus through another component, SctO. These proteins form of a six-pod structure 23 nm in height and 36 nm in width (Hu et al., 2017a; Hu et al., 2015) (Fig. 1C and 1D). The pods, which are largely formed by SctQ, are joined to the cytoplasmic ring of the NC trough a linker protein, SctK, and are capped on their cytoplasmic-facing side by a six-spoke wheel-like structure with a central nave-like hub 12 nm in diameter formed by SctL. The ATPase SctN lays on top of the nave of the sorting platform scaffold and is linked to the cytoplasmic nonameric ring of the export apparatus component SctV by a small helical linker formed by SctO.

The entire structure encloses a chamber-like space where most likely substrates are engaged and unfolded prior to their targeting to the export apparatus.

Assembly of the type III secretion injectisome. Assembly of the entire injectisome occurs in a highly organized step-wise manner (Diepold and Wagner, 2014) (Fig. 1E). The assembly process is initiated by the formation of a complex of a subset of the inner membrane protein components of the export apparatus (SctR, SctS, SctT and SctU), which in turn scaffolds the assembly of the inner rings of the NC. The secretin protein SctC can form the outer rings independently with the help of a dedicated chaperone or "pilotin", but in the absence of the inner rings, the stability of this structure is severely compromised (Crago and Koronakis, 1998; Daefler and Russel, 1998). These observations indicate that the outer ring must "dock" with the inner rings shortly after its assembly. Once the base of the NC is assembled, it becomes competent for secretion by scaffolding the assembly of the sorting platform (Zhang et al., 2017). The resulting structure is competent for the secretion of only a very limited number of substrates, the needle and inner rod subunits (SctF and SctI), and the regulatory protein SctP. The needle is assembled by addition of the building subunit at the distal end of the growing filament after its transport through the central channel of the nascent filament. It is not known how the inner rod is assembled but there is evidence indicating that its assembly occurs independently from the needle. Once the injectisome is fully assembled, it switches specificity in that it can no longer engage the early substrates (i. e. those necessary for the completion of its assembly) becoming competent for the secretion of the effectors and the proteins necessary for their delivery through the eukaryotic cell plasma membrane (i. e. the "protein translocases", see below). The process leading to substrate specificity switching is poorly understood but at least two models have been proposed [reviewed in (Diepold and Wagner, 2014)]. Briefly, one of the models proposes that the conclusion of the assembly of the inner rod, which requires the regulatory protein SctP, results in the firm anchoring of the needle substructure to the base and a significant conformational change in the export

apparatus component SctU, leading to substrate switching. In this model the role of the regulatory protein SctP is largely indirect since in its absence, the inner rod cannot assemble and consequently needle anchoring cannot take place (Lefebre and Galan, 2014). An alternative model proposes that the secreted "extended" SctP acts as a molecular ruler within the secretion channel, "measuring" the length of the needle by binding through its N-terminus the growing tip while simultaneously interacting with SctU through its C-terminus to trigger substrate switching and the termination of needle assembly (Journet et al., 2003). More experiments will be required to define which of the mechanisms, or combination of mechanisms, control needle length.

Mechanisms of secretion

One of the unique aspects of T3SSs is that at least in some bacteria they are programmed to be active only after contacting eukaryotic cells, a process that presumably prevents the unproductive secretion of effectors. The mechanisms by which the T3SS machine senses cell contact and transduces the activating signal to the secretion machine are poorly understood. However, there is evidence indicating that the needle tip structure may be involved in sensing the host cells and generating the activating signal, which is subsequently transduced through the needle filament to the secretion machine components in the bacterial cytoplasm (Barta et al., 2012; Blocker et al., 2008; Deane et al., 2006). How the secreted proteins are subsequently targeted to the secretion machine, unfolded, and initiated in the secretion channel is also incompletely understood. In addition, secretion is hierarchical in that certain substrates clearly have priority over others for early secretion (Lara-Tejero et al., 2011). Type III secreted proteins are guided to the secretion machine by a secretion signal located within the first ~20 N-terminal amino acids (Cornelis, 2003). This secretion signal is poorly conserved at the primary amino acid sequence level although it exhibits some specific features such as being inherently unstructured and enriched for serine, threonine, isoleucine and proline. In addition, substrate targeting requires a family of customized

chaperones that bind a ~100 amino acid domain located immediately downstream of the amino terminal secretion signal. Binding of the chaperone keeps this domain in a partially unfolded state that retains secondary structure, a configuration that has been proposed to serve as an additional targeting signal, determine the place of the bound substrate in the secretion hierarchy, and prime the substrates for secretion (Lara-Tejero et al., 2011; Stebbins and Galan, 2003). How substrates are recognized and initiated in the secretion process is not understood but there is evidence indicating a critical role for the sorting platform-associated ATPase SctN, which has been shown to bind and dissociate the chaperone effector complexes, resulting in the unfolding of the secreted protein (Akeda and Galan, 2005). It has been proposed that the unfolded substrates would then be threaded first through the cytoplasmic nonameric ring formed by one of the export apparatus components (SctV), and subsequently through the inner membrane protein channel within the needle complex (Dietsche et al., 2016). The initiated substrates would then progress through the central secretion channel of the needle complex by mechanisms that are not understood but that most likely involve the proton motive force (Lee and Rietsch, 2015). Finally, effector proteins need to traverse the eukaryotic plasma membrane, a process that is strictly dependent on the protein translocases, which themselves are secreted by the T3SS (Blocker et al., 1999). The translocases are poorly conserved at the primary amino acid sequence level, which may be in keeping with the fact that different T3SSs target rather different cells and deliver different effectors. The deployment of the protein translocases (usually two) on the target eukaryotic cell membrane is strictly dependent on the presence of the tip complex, which is thought to orchestrate their membrane insertion and subsequent assembly of the protein channel. However, how the effectors move through this protein channel to gain access to the target cell is not known.

Type IV secretion systems

Evolution and function

Type IV secretions systems (T4SSs) are versatile nano-machines capable of transporting proteins, DNA, or nucleo-protein complexes that are encoded by Gram-negative and Gram-positive bacteria (Christie, 2016; Zechner et al., 2012). They are ancestrally related to conjugation systems, and are involved in horizontal gene transfer and virulence. Phylogenetic analyses suggest that ssDNA conjugation arose first in diderm bacteria, possibly Proteobacteria, and then spread to other bacterial phyla, including bacterial monoderms and Archaea (Guglielmini et al., 2013). Gram-negative bacterial T4SSs are minimally composed of 12 components, termed VirB1-11 and VirD4 (Chandran and Waksman, 2015). Three ATPases (VirB4, VirB11, and VirD4) power the system although some T4SSs lack VirB11. The ATPase VirD4 is otherwise known as the coupling protein as it is believed to form the platform onto which T4SS substrates dock before their engagement by the secretion machine. Most T4SSs form a distinct appendage or "pilus", whose function is still not entirely elucidated, possibly forming a conduit for transport and/or a device for attachment to the target bacterial cell. The T4SS pili (which should not be confused with the type IV pilus associated with Type 2 secretion) is made up of the major VirB2 and minor VirB5 subunits. All other VirB proteins form a large complex embedded in both the inner and outer membranes. T4SSs have been classified based on their complexity representing simpler (type A) and more complex (type B) systems (Christie and Vogel, 2000)], based on their function [DNA conjugation, effector protein delivery, or DNA uptake (Fronzes et al., 2009a)], or based on their phylogeny (Guglielmini et al., 2013). Here for convenience and brevity, we consider T4SSs that mediate DNA transfer and those that mediate effector protein translocation

DNA transfer. The most widespread role for T4SSs is the transfer of DNA from a donor to a recipient bacterial cell by conjugation (Fig. 2A). This fundamental process in bacterial evolution was discovered in the mid 1940s by Lederberg and Tatum (Lederberg and Tatum, 1953). Most commonly, conjugation mediates transfer of plasmids and other mobile, extrachromosomic

genetic elements but it can also mediate the transfer of chromosomal DNA. In addition, plasmids containing arrays of antibiotics resistance genes can be propagated through bacterial populations by this mechanism, thereby contributing to the spread of antibiotic resistance. Simplified versions of T4SSs can also mediate DNA uptake adding another mechanism by which these systems contribute to genome evolution.

Bacterial virulence. A number of bacterial pathogens use T4SSs for pathogenicity including *Legionella* spp., *Bartonella* spp., *Brucella* spp. *Coxiella* spp. and *Helicobacter pylori* (Fig. 2A). *L. pneumophila,* a cause of acute pneumonia in humans, utilizes a T4SS (termed Dot/Icm) to build an intracellular niche permissive for replication by delivering over 300 effector proteins with the capacity to modulate a myriad of host cell functions (Berger and Isberg, 1993; Brand et al., 1994; Hubber and Roy, 2010). Similarly, *Coxiella burnetti,* the cause of Q fever in humans, utilizes a related T4SS to survive and replicate within host cells (Kohler and Roy, 2015). In the case of *Helicobacter pylori*, the causative agent of stomach ulcers, the T4SS contributes to the most severe forms of the disease by delivering a single effector protein, CagA, which is capable of interfering with major signaling pathways in the host (Covacci et al., 1999; Odenbreit et al., 2000; Segal et al., 1999). In contrast, the plant pathogen *Agrobacterium tumefaciens* utilizes its T4SS to inject a fragment of its Ti plasmid DNA into plant cells to cause crown galls (Christie, 2016). Of note, the *Agrobacterium* Ti plasmid with its associated T4SS has been re-engineered to serve as the most widespread technological platform to create genetically modified plants. More recently, a role for T4SSs in inter bacterial species killing has been reported (Souza et al., 2015).

Architecture

The architecture of the conjugation-associated T4SS consists of a 1 Mda outer-membrane core complex mounted onto an inner membrane complex via a connecting stalk (Low et al., 2014) (Fig. 2B). The outer membrane core complex is composed of 14 copies each of VirB7, VirB9 and

VirB10, which form the so called I- and O-layers (Fronzes et al., 2009b; Rivera-Calzada et al., 2013). The crystal structure of the O-layer defines a cap formed by 14 VirB10 proteins, which by contributing a helical hairpin, come together to form a channel in the outer membrane. In addition to lining the interior of the core complex, VirB10 inserts a transmembrane segment in the inner membrane (Chandran et al., 2009; Rivera-Calzada et al., 2013), thus placing it in a position where it can mediate the conformational changes induced by the cytoplasmic and inner membrane ATPases to gate the T4SS outer membrane channel (Cascales and Christie, 2004). The 2.6 Mda inner membrane complex is composed of two arches on its periplasmic side, a large membrane-embedded platform, and two barrel-like leg structures protruding into the cytoplasm (Low et al., 2014). This complex is made up of 12 copies each of VirB3, VirB4, VirB5, VirB6 and VirB8. The two barrel-like legs are composed of 6 copies each of VirB4, one of the three T4SS ATPases that power the system. No channel is apparent in the structure suggesting that T4SS substrates may pass through the inner membrane ATPases before reaching the periplasm where they may be engaged by the core complex to cross the outer membrane. Recently, the VirD4 ATPase, which is involved in substrate recruitment, was shown to locate between the VirB4 barrels on either side of the inner membrane complex (Redzej et al., 2017). The location of VirB11 is still unknown and the composition and structure of the stalk remains unclear but it might act as a pre-pilus onto which the pilus itself can be built.

The pilus is made up of the inner membrane protein VirB2 bound to a phosphatidylglycerol phospholipid (Costa et al., 2016). The phospholipid headgroups are directed towards the lumen of the pilus, neutralising basic pilin residues that line the lumen thus creating a slightly electronegative environment that facilitates ssDNA transport. VirB5 is believed to be at the tip of the pilus where it might serve as an adhesion molecule that facilitates contact with target cells prior to substrate injection.

A recent cryo-electron tomography study in *Legionella pneumophila* has provided a low-resolution glimpse of a T4SS associated with effector protein transfer (Fig. 2B). This study showed

a structure that is largely consistent with the one observed in the conjugation-associated T4SSs (Ghosal et al., 2017). Like conjugative T4SSs, the *Legionella* T4SS consists of a 14-fold symmetrical outer membrane core complex connecting to a two-barreled inner membrane complex via a stalk. However, the *Legionella* T4SS is larger, made of many more components than most conjugative T4SSs. Combined with the recent structure of the outer membrane core complex from *H. pylori* Cag T4SS also showing a 14-fold symmetry, these studies indicate that the architecture of the different types of T4SSs is likely to be conserved (Frick-Cheng et al., 2016).

Mechanism of secretion

The VirD4 protein plays a crucial role in the recruitment of T4SS substrates to the secretion machine, working either on its own (as seen in conjugative T4SSs) (Ilangovan et al., 2015) or in complex with other proteins (as seen in the *Legionella* spp. T4SS) (Kwak et al., 2017). In most instances, the substrate is bound to other proteins such as chaperones (as in the case of *Legionella*) or substrate-processing machineries (as in the case conjugative T4SSs). In *Legionella*, IcmS and IcmW serve as chaperones for at least a subset of effectors. However, their role in protein secretion is poorly understood and it is unlikely to be similar to the role of T3SS chaperones since IcmS/IcmW do not appear to unfold the effectors prior to transport. Rather, these chaperones appear to form an extended platform that target the effectors to the VirD4 recruitment complex (Kwak et al., 2017).

Processing of DNA substrates by conjugative T4SSs requires a dedicated machinery known as "the relaxosome" (Ilangovan et al., 2015 ; Zechner et al., 2012), which consists of the super-coiled plasmid DNA, a large protein called "the relaxase", and several accessory proteins (Fig. 2C). This complex assembles around a discrete plasmid sequence known as the OriT (for "origin of transfer"), which contains the processing site (termed "nic") for the relaxase. This enzyme is sometimes bifunctional containing trans-esterase and helicase activities. The trans-

esterase activity nicks a single strand (the T strand) of the plasmid DNA at the *nic* site and covalently links its tyrosine catalytic residue to the resulting free 5'-phosphate, resulting in a covalently bound relaxase/single strand DNA (ssDNA) complex, which serves as substrate for its transport by the T4SS. The helicase activity, on the other hand, serves to unwind the DNA prior to its transfer. Some mechanistic details have recently emerged indicating that the OriT is bound by two relaxase molecules, one at each side of the *nic* site. One of the relaxase molecules binds on the 5' side of the *nic* site, nicking the *nic* site and covalently linking it to the T strand. The other binds on the 3' side of the *nic* site acting as a helicase to unwind the DNA in the 5'-3' direction (Ilangovan et al., 2017).

Conjugative T4SSs can function both as pilus biogenesis and ssDNA transfer machines. As the pilus is presumably required for making contact with recipient cells and possibly as a conduit for transport, it is likely that its assembly occurs first (Fig. 2C). However, the mechanistic details of pilus biogenesis are unknown. Once the pilus is formed, the T4SS is thought to switch modes to function as a DNA-transfer device (Zechner et al., 2012; Ilangovan et al., 2015; Ilangovan et al., 2017). First, the relaxosome docks onto the T4SS via VirD4 to form a pre-initiation complex. At this point, the relaxosome contains just one molecule of the relaxase bound to the *nic* site and is poised to initiate DNA transfer. However, DNA transfer does not occur and the "pre-initiation complex" lays dormant until contact with a recipient cell is made. This contact triggers activation of the T4SS ATPases and the relaxosome resulting in the formation of a ssDNA "bubble" around the *nic* site. At this stage, nicking occurs and transport of the resulting covalent relaxase-ssDNA complex is initiated. Simultaneously, a second relaxase molecule is loaded onto the ssDNA bubble on the 3' side of the *nic* site, unwinding the DNA and feeding the T strand to the T4SS transport machine. While the T-strand is being transferred to the recipient cell, the complementary strand remaining in the donor cell undergoes replication. Although the mechanisms by which the virulence-associated T4SS inject effector proteins are not known, they are likely to differ substantially from the mechanism described above in that pilus biogenesis might

not be required and docking of effectors onto the VirD4-platform might be mediated by specific chaperones. Future research will need to address the mechanistic reasons why a duplication of each of the T4SS ATPases is required for function.

Type VI secretion systems

Evolution and function

The type VI secretion systems (T6SSs) are widespread in Proteobacteria and Bacteroidetes. Like the T4SSs, the T6SSs can target both eukaryotic and prokaryotic cells and, as such, they are involved in a number of biological processes including bacteria-to-bacteria warfare as well as pathogenesis towards humans, animals and plants (Kapitein and Mogk, 2013 ; Russell et al., 2014) (Fig. 3A). The T6SS machine is made of 13 components assembled into a large complex that resembles an "inverted" bacteriophage tail. Similar to tailed phages such as T4, T6SSs include a "baseplate" connecting to a trans-membrane complex and an extended tube-like structure (the tail) (Fig. 3B and 3C). However, unlike bacteriophages which infect cells from the outside, the T6SS is largely located within the bacterial cytoplasm. The cytoplasmic tube is composed of Hcp hexamers surrounded by a contractile sheath assembled from two proteins, TssB and TssC. The Hcp tube is capped on the membrane-proximal side by two components, VgrG and PAAR, which form a "spike" at the tip of the tube. Both the spike and the Hcp tube are injected into target cells, thus the VgrG, PAAR, and Hcp proteins are both structural components as well as effectors of the T6SSs (Brackmann et al., 2017).

Approximately 1/3 of all sequenced Gram-negative bacteria encode T6SS clusters playing multiple roles in modulating inter-bacterial as well as host-pathogen interactions (Basler et al., 2013). T6SSs can serve as effective weapons against Gram-negative bacteria both of the same or different species (Hood et al., 2010 ; Journet and Cascales, 2016; Kapitein and Mogk, 2013 ;

Russell et al., 2014). To protect against the potentially lethal effect of effectors, bacteria that encode T6SSs most often produce a set of corresponding "anti-effectors" (immunity proteins) that confer "immunity" to effectors of their own or from other bacteria (Journet and Cascales, 2016). Although the contribution of T6SSs to shaping bacterial communities is well established, their direct role in bacterial pathogenesis is less clear. T6SSs can play an important role in the ability of many pathogenic bacteria to colonize their hosts (Durand et al., 2014; Hachani et al., 2016). However, in most cases their contribution to colonization is exerted through their ability to help the pathogen compete against the resident microbiota rather than by the action of effectors on host cells. The biochemical activities of antibacterial T6SS effectors vary significantly and include nucleases, NAD(P)+ glycohydrolases, or amidases (Journet and Cascales, 2016), which have the capacity to inflict damage within the target cells.

Architecture

The structural components T6SSs are homologous to the contractile tails of phages, which consist of a spike-capped inner tube surrounded by a contractile sheath and a baseplate complex at the base of the sheath (Fig. 3B and 3C). The sheath can adopt extended or contracted states and it is the rapid transition between these states what propels the inner tube and its payload of effectors into targeted cells. The T6SS machine consists of 13 subunits and is anchored to the cell envelope by a 1.6 Mda membrane-spanning complex composed of 10 copies each of TssJ, TssL, and TssM (Durand et al., 2015). This membrane-spanning complex serves not only as a channel for the passage of the inner tube during contraction, but also as a docking platform for the entire cytosolic machinery including the tube, the phase plate, and the sheath. The contractile structure is composed of the inner tube made of stacks of Hcp hexamers, surrounded by a sheath-like structure consisting of TssB and TssC. At the base of the sheath and connected to the trans-membrane TssJ/TssL/TssM complex lays the baseplate composed of TssE, TssF, TssG, TssK, and VgrG. The baseplate serves as a platform for inner tube and sheath polymerization. The

recently determined structure of the T4 phage baseplate (Taylor et al., 2016) fits remarkably well in the *in situ* cryoelectron tomogram of the T6SS from *Amoebophilus asiaticus* (Böck et al., 2017). These observations emphasize the close evolutionary relationship between T6SSs and phage injection devices. Another critical component of the T6SS machine is TssA, which appears to have a different location in different systems. For example, in a T6SS from *E. coli*, TssA interacts with the TssJ/TssL/TssM trans-membrane complex, the baseplate, and the Hcp/sheath complex. This observation suggests that TssA may coordinate the polymerization of the sheath and the inner tube and maintain the distal extremity of the sheath attached to the inner tube during contraction (Zoued et al., 2016). In contrast, in a *P. aeruginosa* T6SS TssA is thought to be an integral component of the baseplate (Planamente et al., 2016), although it is not clear whether these differences reflect fundamental differences between these systems.

The inner tube is primarily composed of a stack of Hcp hexamers forming a 1,000 nm tubular structure capped at its membrane-proximal end by a trimer of VgrG, a structure equivalent to the bacteriophage T4 cell-puncturing device (Leiman et al., 2009; Mougous et al., 2006). T6SSs have additional components with homology to phage spikes components, such as the so-called PAAR (Proline, Alanine, Alanine, Arginine) proteins, which are located at the tip of the inner tube cap. The Hcp/VgrG/PAAR tube is surrounded by a sheath structure composed of TssB and TssC (also known as VipA and VipB) that can adopt an extended and contracted configuration and plays a fundamental role in the injection process. Although Hcp is required for the assembly of the extended sheath, the contracted sheath can still form in the absence of other T6SS components (Brackmann et al., 2017). Thus, the contracted state represents the "relaxed" state of the sheath, while the extended state represents its "tense" state, requiring stabilization presumably through interaction with the inner tube. The structures of both states revealed that the sheath subunits form a six-star helix stabilized by a core domain assembled from four beta strands donated by one TssB and two TssC molecules (Kudryashev et al., 2015; Wang et al.,

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2017). These structures also show that transitioning from the extended to the contracted state leads to profound rearrangements affecting interactions between subunits.

Mechanism of secretion

T6SSs assemble in a sequential manner starting with the trans-membrane complex, followed by the baseplate, and the polymerization of the inner tube and sheath. Effectors can be delivered by apparently different mechanisms. For example, some effectors are fused to the VgrG protein or have a PAAR domain thus becoming an integral part of the machine and are therefore injected through contraction of the sheath that propels the inner tube components into the target cells. Other effectors bind to components of the machine indirectly or are located within the Hcp tube. In these cases it is unclear how they are loaded onto the system or how they are ultimately delivered into target cells (Durand et al., 2014; Shneider et al., 2013). The injection process is presumably initiated by conformational rearrangement of the baseplate components leading to the contraction of the sheath by releasing the constraints that stabilize the extended state of the sheath. These conformational changes lead to the opening of the baseplate allowing the passage of the inner tube and its payload. The trigger for such rearrangement is unknown although it is presumed to be the proximity of a target cell. However, the triggering event must be highly selective. For example, *P. aeruginosa* fires its T6SS in response to a T6SS-mediated attack by other bacteria (Basler et al., 2013), or the presence of a neighboring bacteria with an active conjugation system (Ho et al., 2013). Therefore, the close interactions with surface structures appear to be an essential component of the sensing and triggering mechanisms that lead to the T6SS response. Once injection has occurred, the sheath is disassembled through a mechanism implicating a protein with amino acid sequence similarity to the ClpV family of ATP-dependent unfoldases. This protein recognizes the exposed N-terminus of TssC, resulting in the rapid disassembly of the sheath and the release of the subunits that can be later recycled for the building of a new T6SS structure. Although much progress has been made, it is clear that much

more research is needed to clarify the mechanisms of sensing, activation, and effector delivery by these secretion machines.

Concluding remarks

Ever since their discovery, protein injection machines have captured the imagination of researchers in a variety of fields. Research during the last few years has begun to provide a close-up view of these amazing machines and have revealed multiple activities in which they are involved. Their central role in pathogenesis or in the shaping of microbial communities important for human health has sparked interest in targeting them for medical purposes. Efforts are under way to develop inhibitors of these machines as anti-infectants (Cabezón et al., 2017; Charro and Mota, 2015), to harness their activities as antigen delivery systems for vaccine development (Russmann et al., 1998) or, perhaps in the future, to deploy them to shape the composition of the resident microbiota for medical purposes. Although progress in the understanding of the structure and function of these machines has been remarkable, much remains to be learned. The complete atomic structures of these machines are still not available and the mechanistic understanding of critical aspects of their function such as assembly and activation, signal transduction, substrate engagement and substrate delivery are in their infancy. It is expected that answering these critical questions will require the leveraging of multiple imaging and biochemical approaches capable of interrogating these machines not just in a test tube but also in live bacteria. "Catching them in the act" will undoubtedly bring amazing surprises...stay tuned.

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Figure legends:

Figure 1. Type III secretion systems

(A) Phenotypes associated with T3SSs in pathogenic or symbiotic bacteria. **(B)** Structure of the T3SS needle complex. Surface views of the 3D reconstruction of the cryo EM map of the *S. Typhimurium* needle complex and the docking of the atomic structures of the different needle complex components onto the 3D cryo-EM map are shown. The different substructures are noted. OR1: outer ring 1; OR2: outer ring 2; IR1: inner ring 1; IR2: inner ring 2. (**C** and **D**) *in situ* cryo-ET structure (**C**) and molecular model **(D)** of the entire T3SS injectisome [adapted from (Hu et al., 2017a)]. A central section of a global average cryo ET structure of the intact T3SS injectisome is shown (**C**) as well as the molecular model with the fitting of the available atomic structure of the different components (**D**). (**E**) Model for the assembly of the type III secretion needle complex and associated structures (adapted from (Galán et al., 2014). The export apparatus inner membrane protein components are sequentially assembled into a complex that nucleates the assembly of the inner membrane rings of the NC. The outer ring of the NC assembles independently and docks to the inner rings through its long periplasmic neck. The sorting platform components are then recruited leading to the assembly of a functional T3SS that is only competent for the secretion of inner rod and needle filament proteins. Once assembly is finished, the machine becomes competent for the secretion of translocases and effector proteins [components are designated according to a universal nomenclature (Hueck, 1998)]. OM: outer membrane; PG: peptidoglycan; IM: inner membrane.

Figure 2. Type IV secretion systems

(**A**) T4SSs are involved in conjugation, DNA release and uptake, and in pathogenicity of eukaryotic hosts (Adapted from Grohmann et al. (2017)). (**B**) EM structure of the T4SS. A side view of the structure of a conjugative T4SS is shown featuring the outer membrane core complex

(Core/OMC), the stalk, and the inner membrane complex (IMC) (top left, adapted from Low et al. 2014). The corresponding schematic diagram of the same structure with the location of the various VirB proteins is shown (top right). A cryo-electron tomogram of the structure of the *Legionella* Icm/Dot T4SS is also shown (bottom left) with the schematic diagram of the system (bottom right) (reproduced with permission from Ghosal et al., 2017). (**C**) Secretion mechanism of the relaxase-ssDNA complex by conjugative T4SSs as exemplified by the F plasmid. The T4SS is shown as in panel B with the addition of the VirD4 coupling protein. The relaxosome is composed of a relaxase molecule and several accessory components. Secretion starts with the formation of a pre-initiation T4SS-Relaxosome complex (step I), which lies dormant until stimulated by the contact of the pilus with a recipient cell. Upon stimulation (shown with a lightning bolt), the T4SS ATPases are activated, which in turn activate the relaxosome resulting in the formation of a single-strand DNA bubble (step II). A second relaxase molecule can then load onto the ssDNA bubble through its helicase domains (step III). Concomitantly, the first relaxase molecule cleaves OriT at *nic* and attaches covalently to the resulting 5' phosphate of the T-strand (step IV). Finally, the ssDNA-bound relaxase is transported, while the second relaxase molecule unwinds the DNA and pumps the resulting ssDNA through the system (presumably also in coordination with T4SS ATPases). While the T-strand is being transferred to the recipient cell, the complementary strand remaining in the donor cell undergoes replication via PolIII. The relaxase must be present in the recipient cell to catalyze the end-joining recircularization of the DNA once a copy of the entire plasmid has been transferred.

Figure 3. Type VI secretion systems

(**A**) T6SSs are involved in pathogenicity during host infection (left) and in killing of competitor bacteria (right). Upon puncturing of the eukaryotic host membrane, *V. cholera* VgrG1 reaches the host cytosol and mediates actin crosslinking via its C-terminal ACD domain, thereby inhibiting actin polymerisation (left). *P. aeruginosa* delivers the effector proteins Tse1 and Tse3 in a T6SS-

dependent manner into the periplasm of target cells (right). The amidase Tse1 and the muramidase Tse3 degrade the cell wall of target cells, causing cell lysis. Specific immunity proteins (Tsi1/3) prevent self-killing. Panels are adapted from Kapitein and Mogk (20130. (**B**) Structures of the T6SS extended and contracted sheath/tube complexes. The top left panel shows the extended sheath wrapping around a modeled Hcp tube (grey), the crystal structure of the VgrG (green), the PAAR complex (orange), and the EM structure of the membrane-embedded core complex (pale yellow). The baseplate is shown as a semi-transparent cone surrounding VgrG/PAAR. The top right panel shows the structure of the contracted sheath. The top panels were created by Marek Basler and coworkers. The bottom panels show the cryo-electron tomographic structure of the extended and contracted states of the T6SS from *Amoebophilus Asiaticus* [used with permission from (Böck et al., 2017)]. (**C**) Secretion by contractile nanomachines [adapted from (Brackmann et al., 2017)]. The two left panels show a schematic diagram of the extended (upper left panel) and contracted (lower left panel) state of the contractile structure by T6SS delivering protein effectors (left), phage T4 delivering DNA (middle) and R-type pyocins generating holes in the cell envelope (right). In the right panel, a schematic diagram shows the organization of the base plate: TssK attaches the baseplate to the membrane complex, TssF and TssG form a wedge and a core bundle of the baseplate, and TssE is a sheath initiator. VgrG and PAAR-repeat-proteins form the spike and spike tip complex and Hcp forms the tube. ClpV disassembles the contracted T6SS-sheath. Respective phage gene products (gp) are in brackets.