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concept

The type VI secretion toolkit

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Bacterial secretion systems are macromolecular complexes that release virulence factors into the medium or translocate them into the target host cell. These systems are widespread in bacteria allowing them to infect eukaryotic cells and survive or replicate within them. A new secretion system, the type VI secretion system (T6SS), was recently described and characterized in several pathogens. Genomic data suggest that T6SS exist in most bacteria that come into close contact with eukaryotic cells, including plant and animal pathogens. Many research groups are now investigating the underlying mechanisms and the way in which the effector proteins translocated through this machine subvert host defences. This review provides an overview of our current knowledge about type VI secretion, focusing on gene regulation, components of the secretion machine, substrate secretion and the cellular consequences for the host cell.

Keywords: type VI secretion; Hcp; Vgr; IcmF; bacterial pathogenesis

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Introduction

Pathogenic bacteria secrete toxin proteins into the external medium, or directly into the eukaryotic target cell, by means of dedicated macromolecular complexes known as secretion systems. Seven secretion systems—types I to VII (T1SS to T7SS)—have been identified, and they each require the function of between 1 and more than 20 proteins. Although they now have pathogenic purposes, these secretion systems evolved from bacterial organelles involved in specific cell functions other than virulence. T1SS are similar to ATP-binding cassette (ABC) transporters, whereas T2SS, T3SS and T4SS are structurally and evolutionarily related to type IV pili, flagella and conjugation machines, respectively. This review does not discuss these various systems in detail, but focuses primarily on T6SS.

T6SS have only recently been identified and described, although data obtained in the mid-1990s already suggested their existence. Type VI secretion (T6S) clusters contain 12 to more than 20 genes, but the composition and organization vary between species. The minimal toolkit—or core components—is described below. Most bacterial species have one or two copies of T6S clusters, but a few species, including *Burkholderia* species, have four to six clusters, suggesting redundancy for virulence or specificities for particular niches or hosts.

Identification of T6S clusters: from type IVB to type VI

Historically, T6SS were identified as T4bSS-related machines because two subunits of T4bSS—IcmF and IcmH (or DotU)—are encoded on conserved gene clusters, initially known as IcmF-associated homologous proteins (IAHP; Das & Chaudhuri, 2003). *In silico* analyses have shown that T6S clusters are found in approximately 100 sequenced genomes, including a wide range of Gram-negative bacteria. However, most T6SS were identified by screening procedures for loss of virulence towards the targeted host cell, for survival in a model of infection, for genes transcriptionally induced during infection, or by the identification of proteins secreted into the culture supernatant. Many T6S clusters have now been identified using *in silico* analyses (Das & Chaudhuri, 2003; Barker & Klose, 2007; Bingle *et al*, 2008; see supplementary Table 1 online). A recent study on the T6SS identified so far showed the existence of five distinct phylogenetic groups (Bingle *et al*, 2008).

Gene organization: themes and variations

T6S clusters are usually found within a pathogenicity island—for example, *Pseudomonas aeruginosa* HSI (Hcp-secretion island), EAEC (enteroaggregative *Escherichia coli*) *pheU*, *Salmonella typhimurium* SCI (*Salmonella* centrisome island), *Francisella tularensis* FPI (*Francisella* pathogenicity island)—or on chromosomes that show a bias towards virulence or survival in the host. In most cases, the G+C content of T6S clusters is not very different to the rest of the chromosome, suggesting that, together with extensive sequence rearrangements and shuffling (Fig 1), these clusters were not recently acquired by horizontal transfer. However, acquisition by lateral transfer might have occurred in some cases because many T6S clusters are genetically linked to transfer RNA (tRNA), ribosomal RNA (rRNA) or rearrangement hot spot (rhs) elements. Rhs elements are reservoirs of sequence repetition that mediate chromosomal rearrangement or acquisition of new genetic information (Hill, 1999). T6SS exist as multiple copies in many micro-organisms, although, whether these clusters arise from duplication or distinct genetic transfer is not clear. However, the observation that the three *P. aeruginosa* T6SS have distinct evolutionary histories suggests that they have been acquired by horizontal transfer (Bingle *et al*, 2008).

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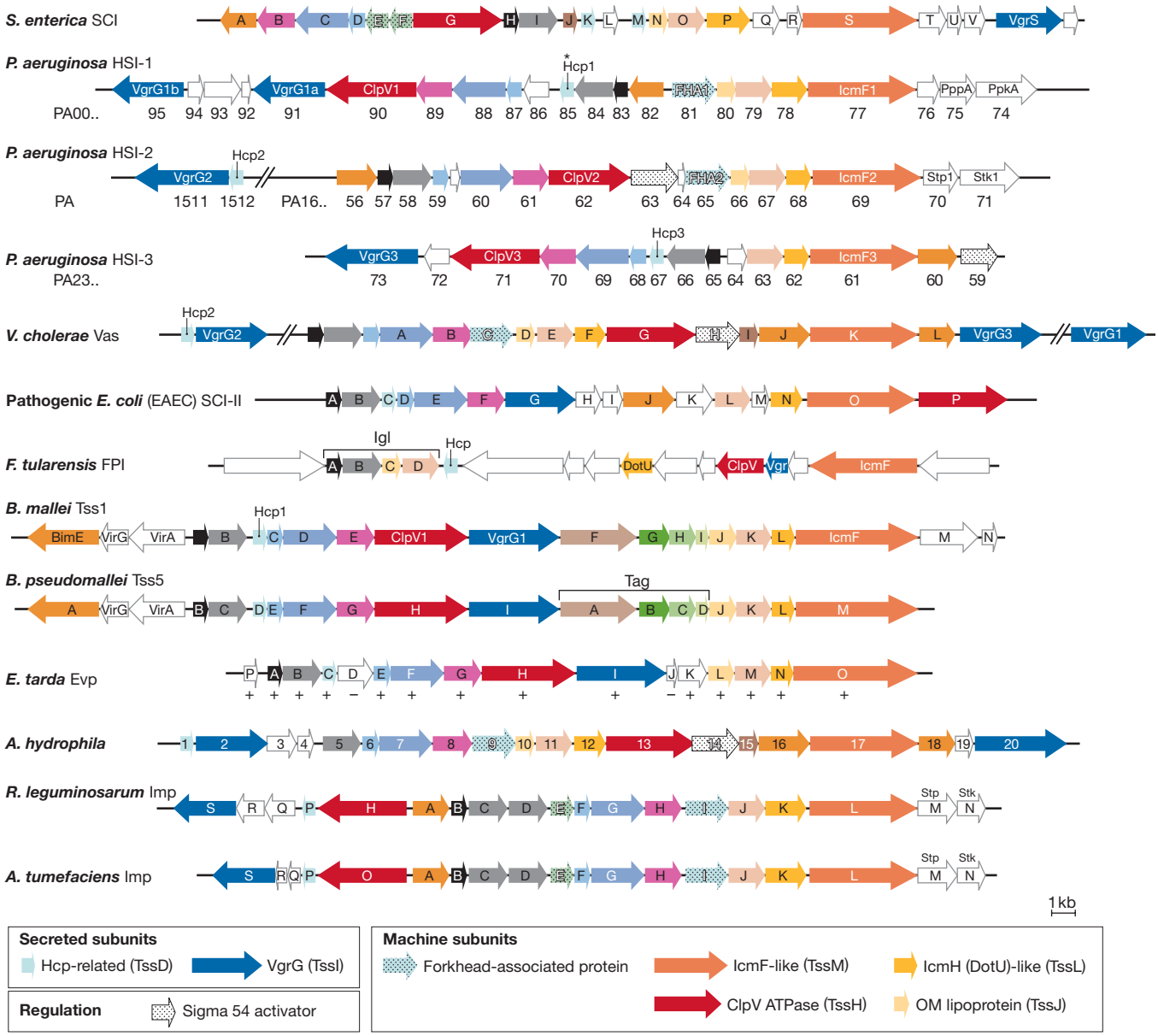

Fig 1 | Gene organization of type VI secretion clusters. Homologues and related genes are indicated with the same colour, whereas genes with no homologues in other type VI secretion systems are coloured white. Characteristic features of the gene products are indicated in supplementary Table 2 online. Genes necessary for type VI secretion determined by the systematic deletion approach on the *Edwardsiella tarda* Evp cluster are indicated by (+). FHA, forkhead-associated protein; Stk, Ser/Thr kinase; Stp, Ser/Thr phosphatase.

On the basis of the organization of the identified T6S clusters (Fig 1), it is clear that T6S machines involve various combinations of proteins. Some of these proteins are conserved in all T6SS and might therefore correspond to the 'core components' of the machine. These proteins include the T4SS IcmF- and IcmH-like proteins, a putative lipoprotein, the ClpV AAA$^+$ ATPase, and the Hcp (haemolysin co-regulated protein) and VgrG (valine–glycine repeats) proteins. These last two proteins were found in the culture supernatant of most bacteria carrying T6SS gene clusters. However, the genes encoding these proteins are associated with several genes encoding the T6S machine itself or secreted subunits (Fig 1; supplementary Table 2). In many cases, regulatory elements such as the sigma 54 alternative activator of RNA polymerase or two-component systems are also encoded within the cluster.

The structure of the operon has been shown for the EAEC *aai* and *Edwardsiella tarda evp* gene clusters (Dudley *et al*, 2006; Zheng & Leung, 2007) suggesting that T6S clusters are likely to belong to a single transcribed unit, controlled by a single promoter.

The recent identification of a significant number of T6SS has led to problems relating to gene nomenclature. The lack of a systematic nomenclature has resulted in several names being assigned to the same gene, or unrelated genes designated by the same alphabetic

character. For example, the IcmF-like proteins are known as VasK, EvpO or ImpL. A unified nomenclature has recently been proposed, making it easier to deal with this complexity (Shalom *et al*, 2007; supplementary Table 2). As these changes seem difficult to integrate now that gene names have been assigned, one alternative would be to use the current name followed by the alphabetical character designated by Shalom *et al*, in subscript. Using this system, the IcmF-like proteins would then be known as $VasK_M$, $EvpO_M$ or $ImpL_M$.

Regulation of T6S clusters: a complicated story

The regulatory mechanisms of T6S clusters involve transcriptional activators of the AraC family or sigma 54.

The AggR activator of the AraC family controls the expression of the EAEC *aai* cluster (Dudley *et al*, 2006). Regulation of the *Burkholderia mallei* T6S cluster involves both the VirAG two-component system and an AraC-like protein (Schell *et al*, 2007). The *evp* cluster of *E. tarda* is positively regulated by the EsrA/EsrB two-component system, through EsrC, which is a transcriptional activator of the AraC family. This regulatory mechanism is identical to that of T3S, suggesting a functional relationship between the T3S and T6S clusters (Rao *et al*, 2004; Zheng *et al*, 2005). Opposite patterns of regulation for the T3S and T6S clusters have been shown in *Salmonella enterica* and *P. aeruginosa*. In *S. enterica*, T6S transcripts are only detected in the late phase of infection (Parsons & Heffron, 2005) because transcription is negatively controlled by SsrB, a two-component regulator that activates the SPI-2 T3SS during the early phase of infection and promotes intracellular replication. Later in infection, *ssrB* is downregulated, switching off T3S genes but activating T6S genes (Parsons & Heffron, 2005). In *P. aeruginosa*, the HSI-1 cluster is positively regulated by the chronic infection-specific activator LadS and negatively regulated by RetS, an activator of the acute phase of infection (Mougous *et al*, 2006), whereas the T3SS has an opposite requirement for LadS/RetS regulation. In *Burkholderia cenocepacia*, T6S genes are negatively regulated by a RetS homologue, AtsR (Aubert *et al*, 2008).

The T6S clusters of *Vibrio cholerae* and *Aeromonas hydrophila* are positively regulated by sigma 54 (encoded by the *rpoN* gene) through VasH—a sigma 54 activator encoded within the T6S cluster (Pukatzki *et al*, 2006; Suarez *et al*, 2008). Interestingly, a mutation conferring virulence attenuation towards *Dictyostelium discoideum* occurs in the *rpoN* locus (Pukatzki *et al*, 2006). *P. aeruginosa* HSI-2 and HSI-3 clusters encode sigma 54 activators, but sigma 54-dependent regulation of these clusters has not yet been shown. However, HSI-2 has been shown to be regulated by the LasR/RhlR quorum sensing system (Schuster *et al*, 2003).

Upregulation of most T6S clusters is dependent on contact with, or intracellular growth inside, the host cell during the infection process (supplementary Table 1 online). In *F. tularensis*, this is consistent with the demonstration that T6SS is positively regulated by MglA and MglB, two transcriptional regulators that are activated during intracellular growth (de Bruin *et al*, 2007). These complex regulatory mechanisms suggest that an optimal timing of T6S gene expression is necessary to ensure correct function. This has been confirmed by a subtle post-transcriptional regulatory mechanism dissected in *P. aeruginosa* (Mougous *et al*, 2007; see below).

T6S subunits

One of the crucial issues in current research on the T6SS is the distinction between genes encoding machine subunits and secreted factors. Homologues of Hcp and VgrG have been detected in the culture supernatants of most bacteria with T6SS and in the fluids of infected eukaryotic cells, suggesting that these proteins are exported. However, as discussed below, mutations of the *hcp* or *vgrG* genes in *Vibrio* and *E. tarda* prevents secretion of both Hcp and VgrG, suggesting that these proteins are machine components or targeting elements. The other genes of the cluster might encode subunits involved in transport through the cell envelope or chaperone proteins, and are therefore probably required for substrate export (supplementary Table 2 online).

Machine subunits. By definition, mutations in subunits involved in machine assembly or substrate transport abolish substrate secretion. Most studies aimed at defining machine subunits have targeted the conserved IcmF-like and ClpV ATPase proteins.

ClpV ATPases are a subfamily of the ClpB family, which comprises hexameric enzymes involved in protein quality control. ClpBs use ATP to unfold protein substrates to be degraded. T6S ClpV members might unfold substrates to be secreted as suggested for the T4SS VirB11 and the T3SS InvC ATPases (Christie *et al*, 2005; Akeda & Galán, 2005). However, the *S. enterica* ClpV protein forms oligomeric complexes with ATP hydrolysis activity but fails to unfold aggregated proteins (Schlieker *et al*, 2005). It has been suggested that these complexes provide the force required for the assembly of the secretion machine or for the translocation of exported proteins.

In contrast to other secretion systems, sequence analysis softwares predict a cytoplasmic location for most of the subunits (supplementary Table 2 online). Two genes encode putative inner membrane proteins with one (IcmH) or three (IcmF) transmembrane domains. In many cases, it is predicted that IcmH have a carboxy-terminal extension that resembles the peptidoglycan-binding motif found in the OmpA or Pal proteins, suggesting that it anchors the T6SS to the bacterial cell wall. In addition, one conserved gene encodes a probable outer membrane lipoprotein.

A recent study on the *E. tarda* Evp machine—the most comprehensive attempt so far to identify the components of the T6S machine—showed that 13 out of the 16 genes of the *evp* cluster are required for the efficient export of EvpC (Hcp-like), EvpI (VgrG) and EvpP (Zheng & Leung, 2007; see Fig 1).

Assembly of the secretion machine. Little is known about the protein–protein interactions between secretion machine subunits. In the *Legionella pneumophila* T4bSS, the IcmF protein is required for IcmH stability and the IcmF-H complex stabilizes the Dot/Icm apparatus (Sexton *et al*, 2004). In *E. tarda*, the IcmF-like protein EvpO interacts with the IcmH-like protein EvpN, the EvpL outer membrane lipoprotein and the cytoplasmic EvpA protein in a yeast two-hybrid assay (Zheng & Leung, 2007). This, together with the requirement of all non-secreted Evp proteins for the secretion of EvpC, I and P, strongly suggests that these proteins form a transport apparatus and that these act synergistically in substrate translocation. Indeed, a functional ClpV fused to the green fluorescent protein (GFP) localizes to discrete foci in *P. aeruginosa*. The correct spatial localization of this protein requires IcmF, Hcp, and a forkhead-associated (FHA) protein (Mougous *et al*, 2006). Assembly is probably controlled by environmental factors, as ClpV recruitment depends on FHA phosphorylation. This modification is controlled by the antagonist activities of two proteins encoded within the HSI-1 cluster, PpkA (a serine/threonine (S/T) kinase) and PppA (a S/T phosphatase). PpkA phosphorylates FHA, thereby promoting the clustering of ClpV,

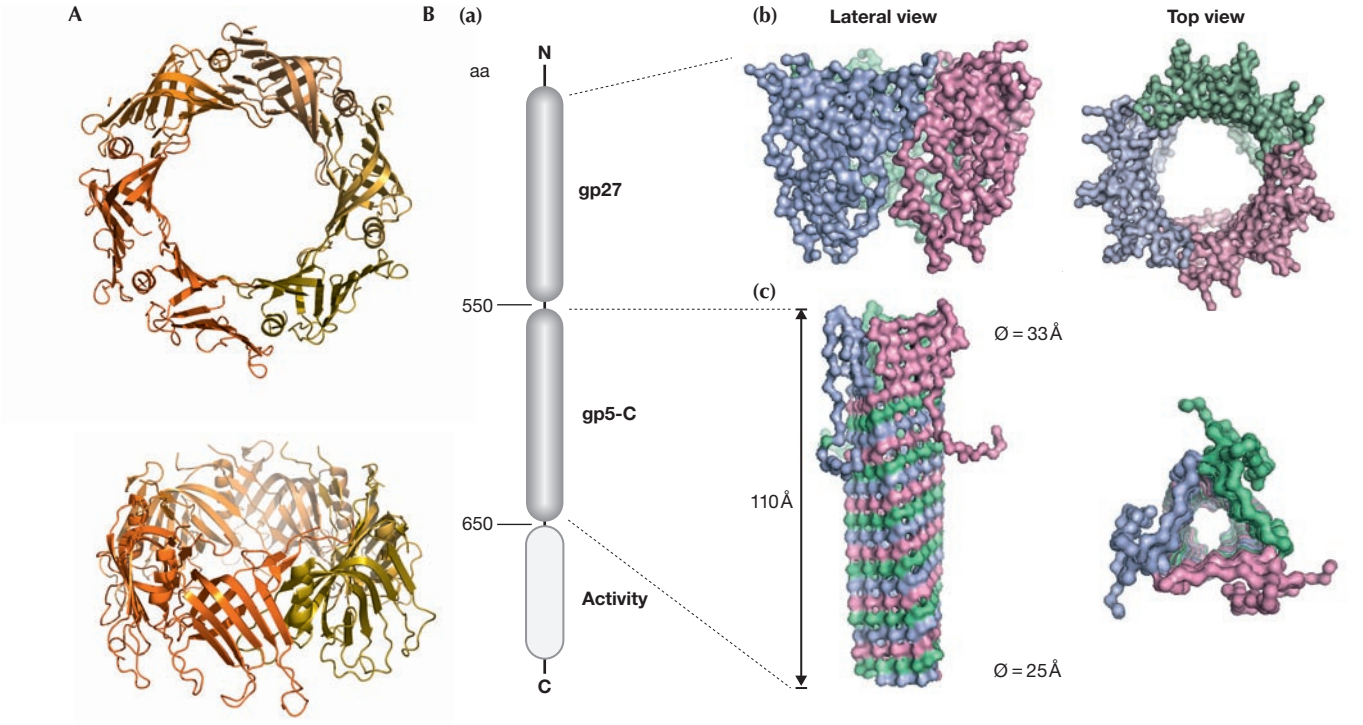

Fig 2 | Structural information on type VI secretion subunits. **(A)** Crystal structure of the Hcp protein. Top and edge-on ribbon views of the Hcp protein assembled as a hexameric ring-shaped structure delimiting a 40 Å cavity. **(B)** Domain organization of VgrG. The modular structure of the VgrG protein family is shown (**a**). The carboxy-terminal domain, which probably represents the 'true effector', carries an activity (see text for details). VgrG proteins assemble as trimers and show structural homology with the hub proteins of the bacteriophage T4 base-plate. The amino-terminal domain has homology with the bacteriophage T4 gp27 protein (lateral and top view of the gp27 trimer shown in panel (**b**)). The central domain shares homology with the bacteriophage T4 gp5 C-terminal domain. This triangular prism forms a 110 Å-long puncturing needle-like structure (**c**). Models have been drawn using Pymol (http://pymol.sourceforge.net/) after Mougous *et al*, 2006 (**A**) and Kanamaru *et al*, 2002 (**B**).

correct assembly of the machine and subsequent substrate translocation. In the absence of PpkA, Hcp accumulates in the periplasm, suggesting that FHA phosphorylation controls passage through the outer membrane. PppA inhibits Hcp secretion by dephosphorylating FHA and dissociating ClpV-GFP (Mougous *et al,* 2007). Therefore, PpkA and PppA act as a molecular switch to control T6SS assembly and activity on detection of an environmental signal.

Secreted proteins: a dual function. By definition, effector proteins are secreted through a dedicated secretion system and share similarity to eukaryotic proteins with enzymatic, regulatory or signalling activities. The Hcp and VgrG proteins are found in the culture supernatants of most bacteria carrying a T6SS. Their transport is T6SS-dependent and, in many cases, the C-terminal domain of VgrG resembles known virulence factors or eukaryotic domains. These characteristics identify Hcp and VgrG as putative substrates; however, Hcp and VgrG display mutual dependence for secretion in *V. cholerae*, *E. tarda* and EAEC (Pukatzki *et al*, 2007; Zheng & Leung, 2007; Dudley *et al*, 2006). This suggests that Hcp and VgrG are not only secreted proteins but also might act as machine components. This 'dual function' is consistent with several pieces of experimental evidence.

The C-terminal domains of VgrG proteins are similar to various bacterial and eukaryotic enzymes, suggesting that they probably interfere with signalling pathways or cellular function of the host. The C-terminal domain of the *V. cholerae* VgrG1 protein resembles the actin cross-linking domain (ACD) of the RtxA toxin. Indeed, expression of this domain in eukaryotic cells causes actin cross-linking (Sheahan *et al*, 2004), and VgrG1-dependent actin cross-linking of macrophages has been reported during infection (Pukatzki *et al*, 2007). Other VgrG C-terminal extensions carry tropomyosin-like, pertactin-like or YadA-like activities or share similarities with peptidoglycan- or fibronectin-binding sequences. BLAST analyses using the *A. hydrophila* VgrG C-terminal extension show that it shares homologies with the eukaryotic lysosomal cathepsin D protein, suggesting a control of host-cell apoptosis. Further investigation of VgrG protein function could provide exciting new insights into the way in which host-cell functions are mimicked or hijacked by T6S effectors. Hcp is not only found in culture supernantants, but also in the cytosol and the membrane of human epithelial cells on infection with *A. hydrophila* (Suarez *et al*, 2008). The ectopic production of *A. hydrophila* Hcp in HeLa cells increases the rate of apoptosis mediated by caspase 3 activation (Suarez *et al*, 2008). These findings are consistent with Hcp and VgrGs being secreted T6S substrates.

Electron microscopy and single-particle analyses have shown that *P. aeruginosa* Hcp_{HSI-1} forms oligomeric rings with a large internal diameter. The Hcp crystal structure showed that Hcp is a ring-shaped hexamer delimiting a 40 Å diameter interior (Fig 2A; Mougous *et al,* 2006). Hcp might therefore assemble as a channel or pilus with an internal diameter that is sufficient to accommodate a protein in transit.

The amino-terminal and central domains of VgrG proteins resemble the T4 bacteriophage gp27 protein and the C-terminal domain of gp5, respectively (Pukatzki *et al*, 2007). These two proteins associate with a stoichiometry of 3:3 to form a structure that resembles a torch, which constitutes the tail spike complex of the bacteriophage baseplate that is inserted into the bacterial outer membrane during phage infection (Kanamaru *et al*, 2002). The gp27 trimer forms a cylinder with an internal diameter of 30 Å, whereas the three gp5 C-terminal domains are structurally arranged to form a slightly twisted β-helical prism. The width of the prism narrows from 33 Å at the base to 25 Å at the tip, resulting in a needle-like structure (Kanamaru *et al*, 2002). The *V. cholerae* VgrG proteins form trimers (Pukatzki *et al*, 2007), suggesting that the VgrG trimeric structure is organized in a similar way to the $(gp27)_3$–$(gp5)_3$ complex (Fig 2B). The VgrG complex might therefore puncture the host-cell membrane and act as a syringe for substrate injection, providing further support for the hypothesis that VgrG is part of the transport apparatus. This membrane insertion would deliver the C-terminal catalytic domain into the host cytosol.

Other putative secreted proteins. Candidates for secretion include the *Rhizobium leguminosarum* RbsB, and the *B. mallei* TssB and TssM proteins (Bladergroen *et al*, 2003; Schell *et al*, 2007). TssB is necessary for T6SS activity, but is not required for Hcp secretion. TssM is an exciting candidate because it carries a eukaryotic ubiquitin-specific proteinase domain that might interfere with the ubiquitin proteasome degradation complex in host cells (Schell *et al*, 2007).

Mechanism of substrate recognition and transport. As the presence of the substrate promotes assembly of the cytoplasmic ClpV ATPase, one might suggest that recognition and transport are initiated at the cytoplasmic face of the inner membrane. However, it remains unclear whether transport is a one-step or a two-step process. With the exception of the *R. leguminosarum* RbsB protein, the T6S substrates identified so far lack a canonical hydrophobic (Sec) or arginine-rich (Tat) N-terminal signal sequence. Hcps purified from culture supernatants are intact, indicating an absence of cleavage during transport (Williams *et al*, 1996; Wu *et al*, 2008). These findings are consistent with a one-step model. However, Mougous *et al* showed that Hcp accumulates in the periplasm in the absence of PpkA (Mougous *et al*, 2007). This could indicate that substrate transit through the T6S machine is a two-step mechanism in which both steps—inner and outer membrane translocations—are mediated by components of the T6SS (Fig 3).

Phenotypic consequences of substrate translocation

In many pathogens, the presence of T6S clusters in distinct isolates correlates with the ability to induce host diseases or disorders. Many virulence defects are associated with mutations affecting T6S subunits or the regulatory network controlling their production. Processes facilitated by T6SS include adherence, cytotoxicity, host-cell invasion, intracellular growth within macrophages or survival and persistence within the host (supplementary Table 3 online).

The T6SS of *R. leguminosarum* prevents pea nodulation and nitrogen fixation (Bladergroen *et al*, 2003). In *Pectobacterium atrosepticum*, overproduction of Hcp increases overall virulence, which results in a severe rotting of potato stems and tubers (Mattinen *et al*, 2007).

The overproduction of a ClpV dominant-negative mutant in wild-type *S. typhimurium* or *Yersinia pseudotuberculosis* strains decreases their ability to invade epithelial HEp-2 cells (Schlieker *et al*, 2005).

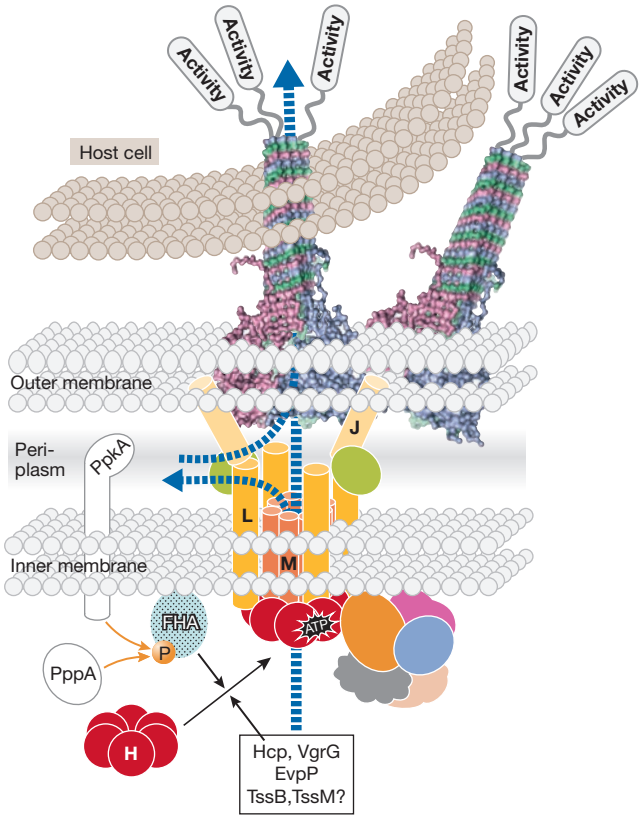

Fig 3 | A model for type VI secretion system assembly and function. A putative model that integrates the current data is proposed. An inner membrane channel formed by the IcmF-like and IcmH-like proteins interacts at the cytoplasmic side of the IM with a complex composed of the probable cytosolic type VI secretion (T6S) subunits and the ClpV AAA^+ ATPase. Recruitment of the ClpV multimer is induced by the regulation of forkhead-associated (FHA) phosphorylation through the activities of PpkA and PppA, and by the presence of the Hcp protein. A multimer of the putative lipoprotein in association with periplasmic subunits is shown at the outer membrane. Putative routes for substrate translocation are depicted through the cell envelope and the host-cell membrane (blue arrow) including a 'one-step' mechanism through a unique channel, and a 'two-step' mechanism, in which both steps are catalysed by T6S subunits with transient accumulation in the periplasm (P). This hypothetical model shows a trimeric VgrG inserted into the OM through the amino-terminal domain and puncturing the host cell through the needle-like structure formed by the central domains, releasing the activity domain into the host cytosol (for eukaryotic-like activities) or in the medium (for binding or adhesion activities). J, L, M and H (TssJ, TssL, TssM and TssH respectively) represent the T6S core components, following the nomenclature of Shalom *et al*, 2007.

Similar effects on invasion were observed when the *S. enterica* SCI pathogenicity island was deleted; however, no defect was observed in a BALB/c mouse model of infection (Folkesson *et al*, 2002). Further studies indicate that the *S. enterica* T6SS limits intracellular growth in macrophages at late stages of infection, resulting in a decrease in mortality rates in a mouse model of infection (Parsons & Heffron, 2005). The T6SS therefore limits the bacterial toxic effect and favours persistence within the host.

In EAEC, mutations in the *aai* T6S cluster lead to significant changes in the morphology of HEp-2 cells with no effect on survival in BALB/c mice (Dudley *et al*, 2006).

E. tarda evp mutants show lower rates of replication than wild type in gourami phagocytes, such that infections are not fatal (Rao *et al*, 2004). In *A. hydrophila*, the T6S cluster is required to inhibit the phagocytic activity of macrophages. T6S mutants are less cytotoxic towards macrophages and human epithelial HeLa cells than wild type, and are less virulent in a mouse model of septicaemia infection (Suarez *et al*, 2008).

F. tularensis IglA-D and VgrG are essential for the regulation of phagosome biogenesis and disruption, escape to the cytosol and intracellular replication (de Bruin *et al*, 2007; Barker & Klose, 2007).

The T6SS of *V. cholerae* O37 and *B. cenocepacia* are responsible for the ability of these bacteria to resist predation by the social amoebae *D. discoideum* (Pukatzki *et al*, 2006; Aubert *et al*, 2008) and to affect the morphology of mammalian macrophages. The cytotoxicity of *V. cholerae* towards macrophages has been associated with the actin cross-linking activity of VgrG1 (Pukatzki *et al*, 2007). The *Burkholderia* T6S clusters are required for survival in relevant models of infection (Aubert *et al*, 2008; Schell *et al*, 2007).

In *P. aeruginosa*, mutations in the HSI-1 cluster decrease or abolish virulence in the rat model of chronic pulmonary infection (Potvin *et al*, 2003). As noted above, this cluster is induced in the chronic phase of infection and, similar to the *S. enterica* SCI T6SS, might favour persistence within the host.

Finally, other phenotypes, such as defects in biofilm formation in *Vibrio parahaemolyticus* have been assigned to T6SS mutants (Enos-Berlage *et al*, 2005). Further evidence for a role of secreted Hcp in biofilm development has been provided by the maximal levels of production of Hcp during the late stages of biofilm development in *P. aeruginosa* (Southey-Pillig *et al*, 2005).

Conclusions and future directions

It is now clear that many pathogenic bacteria known to manipulate host-cell physiologies are equipped with T6SS. This recently discovered secretion apparatus delivers macromolecules designed to subvert host-cell defences. T6SS facilitate bacterial survival and propagation in the harsh environment of the eukaryotic host, which is rendered more habitable by the exported proteins. This process seems to involve the subversion of crucial host-cell functions such as signalling cascades, inflammatory responses, intracellular transport, cytoskeleton dynamics or key regulatory or metabolic pathways. Several putative effectors have been identified. Interestingly, Hcp and VgrG are both secreted and part of the secretion machine. Finally, several subunits share homologies with subunits of the bacteriophage T4 tail spike, suggesting that the T6S might have evolved from the bacteriophage base-plate or that the two systems share a common ancestor. The relationship between the T6SS and the bacteriophage base-plate is particularly interesting given that all secretion systems described so far have been shown to have evolved from bacterial organelles.

Research on the T6SS is progressing rapidly, with each week bringing new findings about regulation, substrate function and secretion, and raising new questions. Progress over the next few months and years can undoubtedly only confirm the fascinating nature of bacterial secretion systems.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org)

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