Type Six Secretion: A Beginner's Guide

Running Title: Type Six Secretion

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Summary

The type VI secretion system (T6SS) is a newly described apparatus for protein transport across the cell envelope of Gram-negative bacteria. Components that have been partially characterised include an IcmF homologue, the ATPase ClpV, a regulatory FHA domain protein and the secreted VgrG and Hcp proteins. The T6SS is clearly a key virulence factor for some important pathogenic bacteria and T6SS-dependent translocation of a potential effector protein into eukaryotic cells has been described for one system (*Vibrio cholerae*). However, T6SSs are widespread in nature and not confined to known pathogens. In accordance with the general rule that the expression of protein secretion systems is tightly regulated, expression of T6SS is controlled at both transcriptional and post-transcriptional levels.

Introduction

When it comes to understanding type VI secretion (T6S), we are all largely beginners, given that this mechanism earned its place alongside the previous five categories of Gram-negative protein secretion as recently as 2006. All six types of secretion system transport proteins from the cell's interior across the cell envelope to the external milieu. Before examining type VI secretion, it is worth pausing to outline what we know about systems I-V (reviewed in [1]). Type II and type V secretion systems generally require the presence of an N-terminal signal peptide in order to utilise the Sec pathway for translocation from cytoplasm to periplasm. In contrast type I, type III and usually also type IV systems, can secrete a protein without any such signal. While type I and type V systems are relatively simple, consisting of only two or three proteins, secretion systems of types II - IV are large multi-protein complexes that span the entire cell envelope. Type III and type IV systems have been particularly implicated in interactions between bacterial and eukaryotic cells and in the translocation of bacterial effector molecules directly into the eukaryotic cytosol. These two secretion systems are commonly encoded in large clusters of contiguous genes, which, when they occur in pathogens, are often considered pathogenicity islands (PAIs). The following review will briefly set the discovery of the T6SS in a historical context and then describe the current state of knowledge about these important protein transporters.

First hints of the existence of type VI secretion

Despite being named only in 2006, hints of the existence of T6SS go back over a decade. As long ago as 1996, the haemolysin co-regulated protein, Hcp, from *Vibrio cholerae*, was shown to be secreted without cleavage of a signal peptide, unlike other known secreted proteins of *V. cholerae* [2]. However, no link was found between the protein and virulence or colonization in a murine model of cholera. The following year, a chromosomal locus (later

termed the *imp* locus and now linked to T6S) that negatively affected pea nodulation was discovered in *Rhizobium leguminosarum* [3]. In 1998, Wang *et al.* identified an infection-induced gene encoding a serine-threonine protein kinase (STPK) in *Pseudomonas aeruginosa* that contributed to virulence in neutropenic mice [4]. In the same year, the gene cluster *icmGCDJBF* on the chromosome of *Legionella pneumophila* was shown to be required for the killing of human macrophages [5]. In 2000, Das and co-workers from Calcutta, India, discovered a homologue of *icmF* in *Vibrio cholerae* that was induced in a rabbit ileal loop model of infection [6].

Soon, the conserved gene clusters that were later recognised as T6SS clusters began to be recognised. In 2002, Pallen *et al.* carried out a survey of bacterial proteins that contained FHA domains, known to bind reversibly to the targets of STPKs. [7,8]. They noted that several species of pathogenic or symbiotic Gram-negative bacteria possessed homologous gene clusters including a gene encoding an FHA domain. Some of these clusters also encoded a STPK and a serine-threonine protein phosphatase (STPP), thought to be involved with the FHA domain in phosphorylation-dependent signalling. That same year, a Swedish group described a novel genomic island (SCI) in *Salmonella enterica*, related to other *icmF* gene clusters, and showed that deletion of this island reduced the ability of the bacteria to enter eukaryotic cells [9].

In 2003, Das *et al.* provided the first detailed *in silico* analysis of type VI secretion gene loci [10], which they termed IAHP (IcmF-associated homologous protein) clusters. Although these clusters were characterised by their encoding a homologue of the IcmF protein, associated with type IV secretion in *Legionella pneumophilia*, the majority of proteins encoded did not seem to be homologous to type IV (or any other known) secretion system components. A role for the IAHP proteins in secretion was suggested and this was subsequently confirmed by a study showing that the *imp* cluster from *R. leguminosarum* had a

role in temperature-dependent protein secretion [11]. In 2004 and 2005, work from Leung's group in Singapore showed that the Evp proteins from the fish pathogen, *Edwardisella tarda* were also implicated in pathogenesis and suggested that they might be components of a novel secretion mechanism [12,13]. By the end of 2005, a *Salmonella* homologue of IcmF, SciS, had been shown to control intracellular bacterial levels and attenuate virulence [14] and a new class of ClpB-like proteins (the ClpV family) had been recognized and linked to bacteria-host cell interactions [15]. By 2006, the stage was set for the dramatic appearance of type VI secretion.

Type VI secretion in full view

Type VI secretion moved centre-stage with the publication of two papers from the Mekalanos group in 2006. The first paper described extracellular export of Hcp and three related VgrG proteins from *V. cholerae*, twinned with evidence that the associated genes were required for contact-dependent cytotoxicity in amoebae and macrophages [16]. In view of the facts that all secreted proteins lacked signal sequences and that sequencing the genome of this strain did not reveal a recognisable type III or type IV secretion system, the authors coined the term "VAS" (virulence-associated secretion) for the genes and proposed that they encoded a prototypical "type VI secretion system". The second paper documented the existence of a similar protein secretion apparatus encoded by the HIS-I virulence locus of *Pseudomonas aeruginosa* [17]. Assembly of the HSI-I T6SS, subject to a reciprocal regulation by RetS (also regulates T3S) and LadS, was shown to allow export of a ring-forming protein, Hcp1, that evoked an immune response in cystic fibrosis patients [17,18].

Shortly after the appearance of these two seminal papers, a publication from the Nataro group described two T6SSs in enteroaggregative Escherichia coli (EAEC), both encoded within a PAI [19]. Also in 2006, the presence of T6SSs in the emerging human

pathogen *Aeromonas hydrophila* and many pathogenic *E. coli* strains was noted [20,21]. More recently, type VI secretion has been shown to play a major role in the virulence of *Burkholderia mallei* [22]. In the past year, more extensive studies on the T6SS from *Edwardsiella tarda* have identified two new proteins that are secreted and thirteen proteins that are required for secretion (including the secreted Hcp homologue) [23].

Further investigations of the prototypic *V. cholerae* T6SS have shown that one of the secreted VgrG proteins (VgrG-1) can covalently cross-link actin *in vitro*, and this activity has been exploited to prove that it can be translocated into macrophages by a T6SS-dependent mechanism [24]. However, additional studies suggested that all three VgrG proteins form a complex that might act as both a membrane-penetrating structure, delivering the effector domain of VgrG-1 to the host cytoplasm and also as a conduit for protein translocation. Thus it remains unclear whether VgrG-1 acts primarily as a translocator or an effector under physiological conditions, or whether both functions are equally important [24].

In *Francisella tularensis*, the *Francisella* pathogenicity island (FPI) encodes at least seven homologues of genes found in other T6SSs (Bingle & Pallen, unpublished & [25]), but in terms of sequence similarity and gene complement, the FPI sits as an outgroup, at a distance from other known T6SSs (Figure 1). Although clearly implicated in virulence, it remains unclear how far the function of the FPI protein complex overlaps with that of other more typical T6SSs.

Regulation of type VI secretion systems

Gene expression in many T6SSs can be subject to control by global regulators that regulate other virulence genes, including T3SS genes, and several T6SSs are known to be induced *in vivo* [12-14,16,17,19,22,26]. Some of the first T6SS clusters to be identified contained homologues of a σ^{54} dependent transcriptional activator [10]. Disruption of this

gene, or of the *rpoN* gene, in *V. cholerae* resulted in attenuated virulence and reduced expression of effectors known to be secreted by the T6SS [16]. However, our observations indicate that this regulator is present in only a minority of T6SS (Bingle & Pallen, unpublished), so RpoN-dependent regulation cannot be a universal feature of type VI secretion. In line with predictions from Pallen *et al.*, a threonine-phosphylation signaling cascade has recently been shown to regulate type VI secretion in *P. aeruginosa* [7,27,28]. Although such post-translational regulation of protein secretion by threonine phosphorylation is unprecedented in bacteria, recent studies on the PknD / YscD protein pair in *Chlamydia* suggest that it is likely, as previously predicted [7], that a similar mechanism regulates some type III secretion systems [29]. Again, the absence of STPKs and STPPs from many T6SS and most T3SS gene clusters suggests that this mode of regulation is not universal.

The naming of parts

There is a highly conserved set of "core" T6SS component proteins (Table 1): for example, out of a total of 16 proteins encoded by the *E. tarda* cluster, all but three are found in at least four out of six other clusters examined [23]. T6SS gene clusters were originally identified by their common possession of an *icmF* homologue. In the *L. pneumophilia* type IV secretion system, IcmF has an accessory function and is not an essential part of the core apparatus [30,31]; however the conservation of IcmF among T6SSs suggests a more central role in secretion. T6SS clusters typically also include a homologue of DotU (IcmH), another accessory protein from the *L. pneumophilia* T4SS [30,31]. In type III and type IV secretion, protein secretion is energized by one or more ATPases [32-34]. The best candidate for a similar role in type VI secretion is ClpV [15]. Some ClpV proteins are known to form hexameric structures with ATPase activity [15] and loss of ClpV1 in *P. aeruginosa* , or even just loss of its ATPase motif, is enough to abrogate secretion [17]. ClpV is homologous to

ClpB, a member of the Clp/Hsp100 family, which acts with DnaK/Hsp70 to disassemble, translocate and refold aggregated proteins [35]. ClpV proteins apparently lack dissaggregation activity, but retain a translocation function. Genes encoding domains of unknown function DUF877 and DUF770 always occur in T6SS gene clusters as a tandem pair, suggesting that the associated proteins interact and play some important function [36,37]. However, the DUF877 protein from the *B. mallei T6SS* is not required for Hcp1 protein secretion, although it is essential for virulence [22]. This may be because the DUF877-DUF770 complex is a also substrate for secretion, or is some kind of chaperone [36].

Identification of the proteins secreted by a particular T6SS may be facilitated by the fact that they are commonly encoded by genes that cluster with the genes for the T6SS apparatus [12,17,19] - important exceptions include the Hcp protein secreted by the VAS cluster [16]. However, distinguishing T6SS effectors from structural proteins may not be easy - as noted, it has been suggested that VgrG proteins, although secreted, can also act as components of the translocation apparatus [24]. Similar considerations apply to Hcp, which is arguably more likely to be part of the secretion system than to be an end substrate.

The view from the genomic high ground

In preparing this review, we performed a survey of T6SS gene clusters in over 400 bacterial genome sequences and performed a phylogenetic analysis on representative clusters (see supplementary information and Figure 1). These investigations revealed the presence of T6SS gene clusters in more than a quarter of genome-sequenced bacteria. Although largely confined to the Proteobacteria, T6SSs occur in at least two other phyla— the Planctomycetes and the Acidobacteria. However, phylogenetic analysis suggests that these outliers might be the result of horizontal gene transfer from the Proteobacteria. There are four major groups of

T6SS, which we have named A-D, with the *Francisella* FPI complex forming a fifth divergent group (Figure 1 and [12]).

In several dozen cases, a single genome encodes more than one T6SS. Although this phenomenon has already been recognized in several species [10,19,22,26], we were intrigued to find as many as four T6SS gene clusters in the genomes of *Yersinia pestis* and *Photorhabus luminescens*. In some cases, it is clear that multiple T6SS clusters have not arisen by duplications within a given lineage— for example the three T6SS clusters in the genome of *P. aeruginosa* PAO1 have distinct evolutionary histories and are likely to have been acquired by horizontal gene transfer (Figure 1). These three clusters do not seem to be coregulated, suggesting that they perform different roles for the bacterial cell [17].

A key lesson from the field of bacterial pathogenomics is that systems involved in host-pathogen interactions are often best understood in an eco-evo context, that takes into account the organism's evolution and ecology [38]. Thus, from an evolutionary viewpoint, not all T6SS gene clusters should be assumed to be fully functional—for example, several apparently degenerate T6SS clusters occur in the genome of *Photorhabus asymbiotica.* In addition, as expected on the eco-evo view, our survey reveals the presence of T6SS genes in many bacterial species that are not normally thought of as pathogens or symbionts—for example, in soil bacteria such as *Pseudomonas putida* and *Myxococcus xanthus* (Figure 1). To accommodate this finding, we have to recognize T6SSs as more than just virulence factors and the future focus of research into type VI secretion will have to extend from pathology to ecology.

Future prospects

Although a latecomer to the scene of bacterial protein secretion, it seems likely that within a few years research into type VI secretion will rival that on better characterised

systems in terms of excitement, scope and ability to deliver telling insights into molecular mechanisms and cellular interactions. Many questions remain unanswered—for example what is the signal that targets substrates for secretion or mediates interactions between substrates and the secretion apparatus [39]? The components that span the inner and outer membranes and periplasm remain unknown and the details of the structure, function and localisation of most T6SS proteins remain mysterious. Finally, there are strong practical reasons for investigating type VI secretion, given that these secretion systems are promising targets for the development of new approaches to diagnosis, vaccine development and antimicrobial drug design [40,41].

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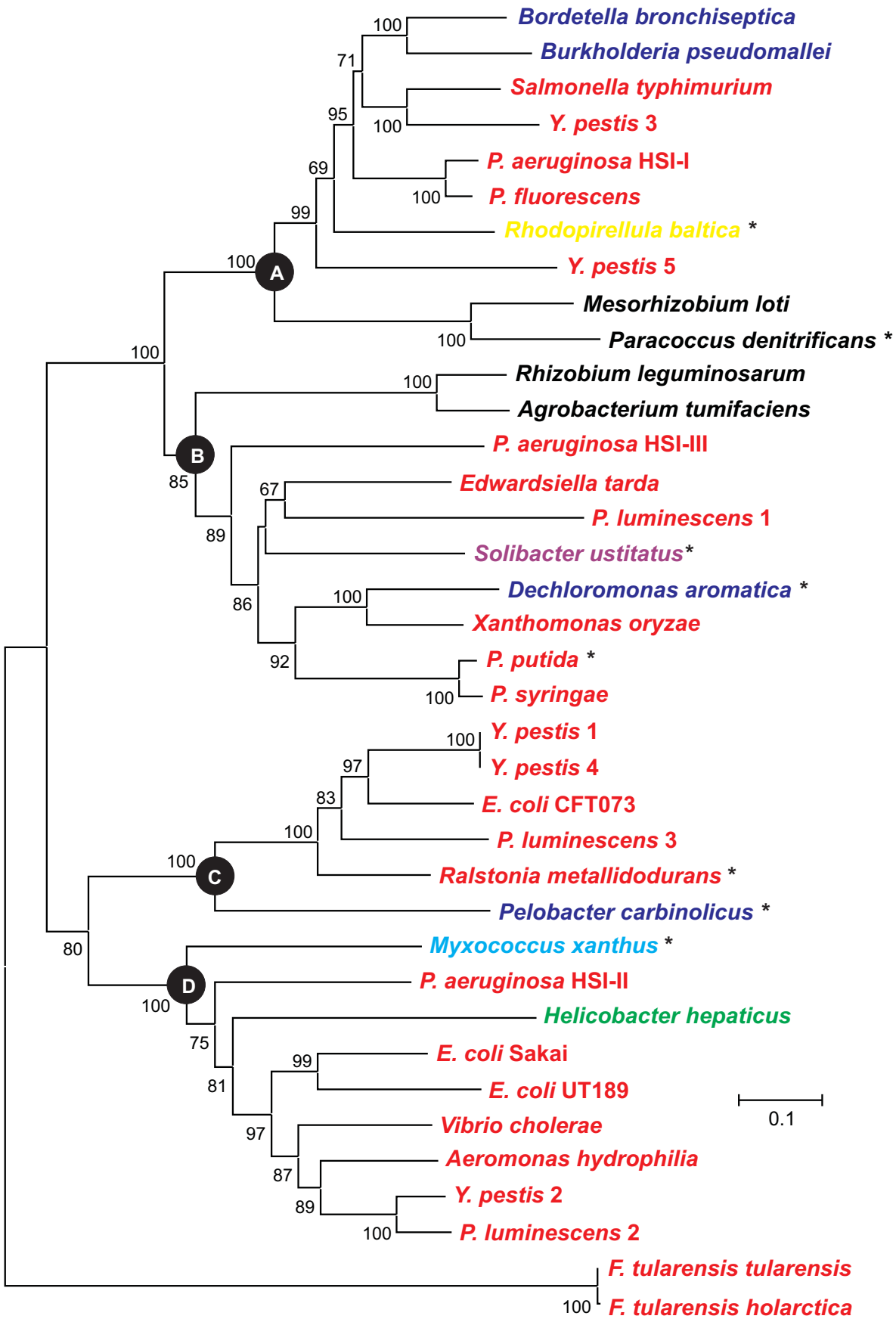
Figure 1. Evolutionary relationships of Type 6 Secretion Systems. The distance tree (Neighbour-Joining) shown here was calculated from an alignment of concatenated DUF770 and DUF877 protein sequences from all classes of bacteria possessing T6SS. Bootstrap values (% from 1000 replicates) over 50 % are indicated at the nodes. The same four major groups (labelled A-D at their basal nodes) were obtained in a maximum parsimony analysis of the same data: bootstrap support values A, 99 %; B, 94 %; C, 98 % ; D, 100 %. Bacterial taxons are indicated by font colour: Alphaproteobacteria, black; Betaproteobacteria, blue; Gammaproteobacteria, red; Deltaproteobacteria, turquoise; Epsilonproteobacteria, light green; Acidobacteria, purple, Planctomycetales, yellow. Asterisk indicates species that are not considered to be pathogens or symbionts. The scale bar indicates 0.1 substitutions per site. See supplementary information for further information.


Bordetella bronchiseptica
Burkholderia pseudomallei
Salmonella typhimurium
Y. pestis 3
P. aeruginosa HSI-I
P. fluorescens
Rhodopirellula baltica *
Y. pestis 5
Mesorhizobium loti
Paracoccus denitrificans *
Rhizobium leguminosarum
Agrobacterium tumifaciens
P. aeruginosa HSI-III
Edwardsiella tarda
P. luminescens 1
Solibacter ustitatus*
Dechloromonas aromatica *
Xanthomonas oryzae
P. putida *
P. syringae
Y. pestis 1
Y. pestis 4
E. coli CFT073
P. luminescens 3
Ralstonia metallidodurans *
Pelobacter carbinolicus *
Myxococcus xanthus *
P. aeruginosa HSI-II
Helicobacter hepaticus
E. coli Sakai
E. coli UT189
Vibrio cholerae
Aeromonas hydrophilia
Y. pestis 2
P. luminescens 2
F. tularensis tularensis
F. tularensis holarctica
A
B
C
D
0.1

Conserved domain(s)	**T6SS Example**	**Subcellular location**	**Proposed function**
ImpA N-terminal related / COG3515	VC_A0119	Cytoplasm	?
IcmF-related / DUF1215 / COG3523	VC_A0120	Membrane	?
DUF879 / COG3519	VC_A0110	?	?
DUF877 / COG3517	VC_A0108	Cytoplasm? [36]	Interacts with DUF770 [36]
DUF876 / COG3522	VC_A0114	?	?
DUF770 / COG3516	VC_A0107	Cytoplasm [9,36]	Interacts with DUF877 [36]
DUF1305 / COG3520	VC_A0111	?	?
ClpV	VC_A0116	?	Protein translocation
FHA domain / COG3456	VC_A0112	Inner Membrane	Signal transduction
COG3521	VC_A0113	?	Lipoprotein
DotU (IcmH)-related / COG3455	VC_A0115	?	?
Pfam04965 / COG3518	VC_A0109	?	T4 phage Gene 25-like lysozyme
Hcp / DUF796 / COG3157	VC_A0017	Extracellular [12,16,17,22,42]	Secreted (effector / translocator?)
VgrG / DUF586 / COG3501	VC_A0018 / VC_A0123 / VC1416	Extracellular [16]	Secreted (effector / translocator?)

Table 1. T6SS Core Proteins. Conserved domains refer to Pfam (DUF = Domain of Unknown Function) [43] and / or COG (Cluster of Orthologous Groups) [44] families where possible. Examples are from the genome of *V. cholerae* N16961. Subcellular location for each protein

is indicated according to experimental data (where reference is given) or as predicted by PSORTb v2.0 [45]) –see supplementary information for details.

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Use of a protozoan model of infection, together with transposon mutagenesis of a non-O1/non-O139 strain of *V. cholerae* leads to a description of the prototypic T6SS, including details of the secretion of Hcp and VgrG proteins and the involvement of Hcp in secretion of VgrG proteins. This T6SS is shown to mediate virulence of *V. cholerae* towards both *Dictyostelium* amoebae and a mammalian macrophage cell line.

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This paper demonstrates a strong link between one of the three T6SSs of *P. aeruginosa* PAO1 and the pathogenicity of this bacterium towards cystic fibrosis patients. The pattern of transcriptional control by global virulence regulators uncovered by microarray studies suggests a role during chronic infection. Structural studies of *P. aeruginosa* Hcp1 detailed here indicate that it can self-associate, forming a hexameric ring with a wide lumen consistent with a role in forming a channel to translocate secreted proteins.

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This paper describes an Hcp-secreting T6SS of *B. mallei*, the causative agent of Glanders. Expression of the T6SS genes (along with many other virulence genes) is strongly upregulated by the VirAG two-component system and is essential for virulence in a hamster model of infection. This system has been shown to secrete a homologue of Hcp that is recognized in the horse, human and mouse antibody response to infection. The authors describe a search of available bacterial genomes sequences and their discovery of five additional distinct T6SS clusters that are found in *Burkholderia* species.

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This important paper includes details of a systematic mutagenesis of all genes in a T6SS cluster and the effects that these mutations have on type VI secretion. This has allowed the definition of a core set of T6SS proteins required for secretion and also to identify two novel secreted proteins (EvpI and EvpP). The authors have also begun to catalogue the protein-protein interactions involved in assembly of the secretion system.

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Bioinformatic analysis allowed the prediction of a trimeric complex, homologous to the tail spike of phage T4, between the three secreted VgrG proteins and this prediction is supported by immunoprecipitation data. The authors experimentally demonstrate an effector role for one of these proteins (the VgrG-1 protein is translocated into macrophage cells where it has an actin crosslinking function), allowing them to suggest multiple functions for the VgrG complex. VgrG proteins may have a variable C-terminal domain; this domain of *V. cholerae* VgrG-1 is homologous to the actin crosslinking domain of the RtxA toxin [46] and VgrG homologues from other pathogenic bacteria possess domains that resemble known virulence proteins.

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