

Type VI secretion: a beginner's guide

Lewis EH Bingle, Christopher M Bailey and Mark J Pallen

Type VI secretion is a newly described mechanism 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. Type VI secretion is clearly a key virulence factor for some important pathogenic bacteria and has been implicated in the translocation of a potential effector protein into eukaryotic cells by at least one organism (*Vibrio cholerae*). However, type VI secretion systems (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 type VI secretion is controlled at both transcriptional and post-transcriptional levels.

Addresses

Centre for Systems Biology, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

Corresponding author: Bingle, Lewis EH (l.e.bingle@bham.ac.uk), Bailey, Christopher M (c.bailey@bham.ac.uk) and Pallen, Mark J (m.pallen@bham.ac.uk)

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Introduction

When it comes to 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 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 secretion 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. By 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 in particular have been 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. 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 secretion systems.

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 colonisation 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 *et al.* from Calcutta, India, discovered a homologue of *icmF* in *V. cholerae* that was induced in a rabbit ileal loop model of infection [6].

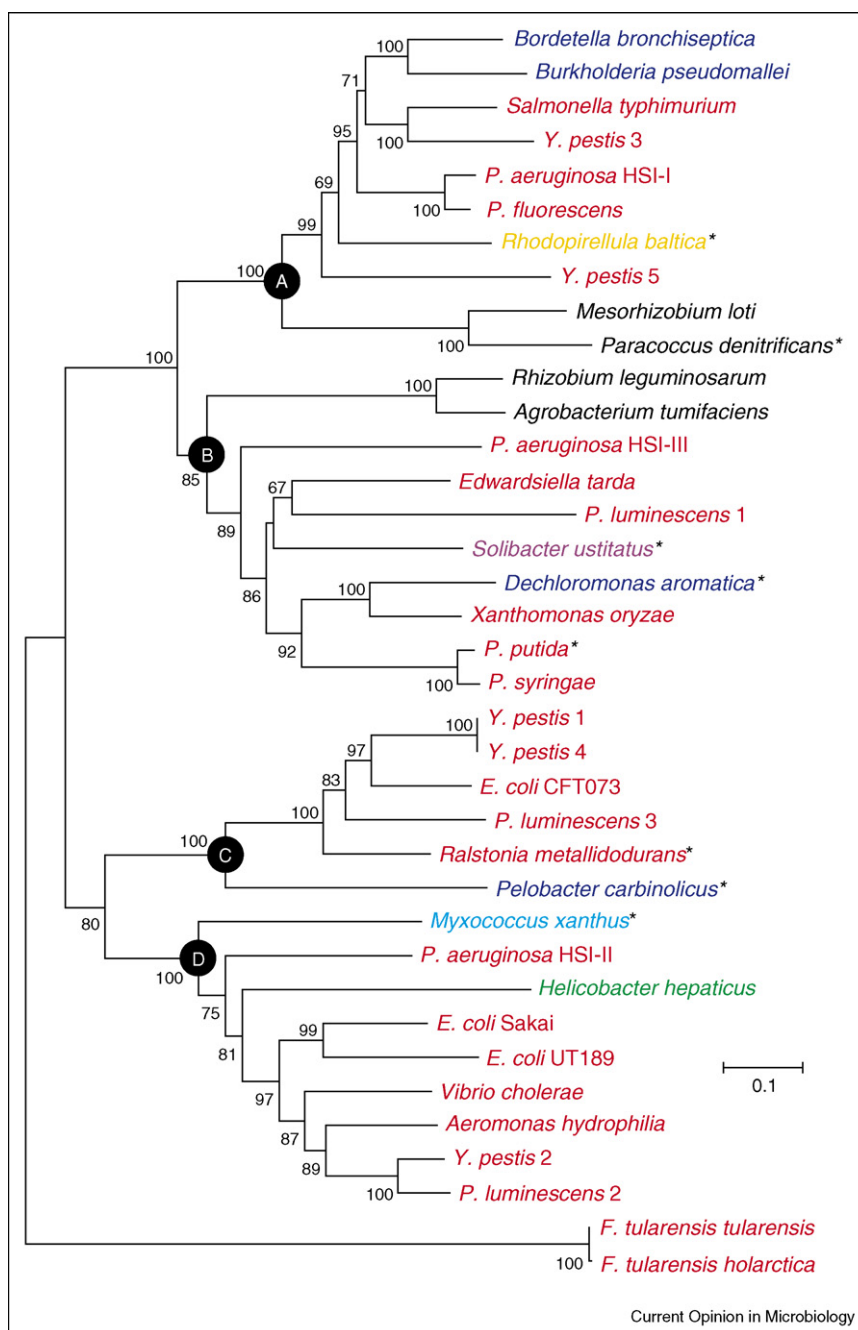
Soon, the conserved gene clusters that were later recognised as T6SS loci began to attract attention. In 2002, Pallen *et al.* carried out a survey of bacterial proteins that contained FHA domains, known to bind reversibly to the targets of STPKs [7,8]. They noted that several species of pathogenic or symbiotic Gram-negative bacteria possessed homologous gene clusters centred on 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 *L. pneumophila*, 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

Figure 1



Evolutionary relationships of type VI 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 taxa 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.

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 *Edwardsiella 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 recognised 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 gene cluster was required for contact-dependent cytotoxicity in amoebae and macrophages [16^{••}]. In view of the facts that all secreted proteins lacked signal sequences and that the genome of this strain did not encode a (non-flagellar) type III or a 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 HSI-I virulence locus of *P. 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 *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 2 new proteins that are secreted and 13 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, or 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 and 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.

Table 1

T6SS core proteins

Conserved domain(s)	T6SS example	Subcellular location	Proposed function
ImpA N-terminal related/COG3515	VC_A0119	Cytoplasm	Unknown
IcmF-related/DUF1215/COG3523	VC_A0120	Membrane	Unknown
DUF879/COG3519	VC_A0110	Unknown	Unknown
DUF877/COG3517	VC_A0108	Cytoplasm? [36 [•]]	Interacts with DUF770 [36 [•]]
DUF876/COG3522	VC_A0114	Unknown	Unknown
DUF770/COG3516	VC_A0107	Cytoplasm [36 [•]]	Interacts with DUF877 [36 [•]]
DUF1305/COG3520	VC_A0111	Unknown	Unknown
ClpV	VC_A0116	Unknown	Protein translocation
FHA domain/COG3456	VC_A0112	Unknown	Signal transduction
COG3521	VC_A0113	Unknown	Lipoprotein
DotU (IcmH)-related/COG3455	VC_A0115	Unknown	Unknown
Pfam04965/COG3518	VC_A0109	Unknown	T4 phage Gene 25-like lysozyme
Hcp/DUF796/COG3157	VC_A0017	Extracellular [12,16 ^{••} ,17 ^{••} ,22 ^{••} ,24 ^{••} ,42]	Secreted (effector/translocator?)
VgrG/DUF586/COG3501	VC_A0018/VC_A0123/VC1416	Extracellular [16 ^{••} ,24 ^{••}]	Secreted (effector/translocator?)

Conserved domains refer to Pfam (DUF: domain of unknown function) [43] and/or COGs (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.

Regulation of type VI secretion systems

Gene expression in many T6SSs can be subject to control by global regulators of 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 and 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-phosphorylation signalling 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

Some T6SS component proteins are well conserved (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. pneumophila* type IV secretion system (T4SS), 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. pneumophila* T4SS [30,31]. In type III and type IV secretion, secretion systems are associated with 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 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 disaggregation 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 in fact a substrate for secretion, or is a chaperone for some but not all type-VI secreted proteins [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, though 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 two conserved protein sequences (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 recognised 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 *Photobacterium 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 co-regulated, 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 *Photobacterium symbiotica*. 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 recognise 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mib.2008.01.006.

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