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Secretion Systems Used by Bacteria to Subvert Host Functions

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Abstract

In this article we examine the use of secretion systems by bacteria to subvert host functions. Bacteria have evolved multiple systems to interact with and overcome their eukaryotic host and other prokaryotes. Secretion systems are required for the release of several effectors through the bacterial membrane(s) into the extracellular space or directly into the cytoplasm of the host. We review the secretion systems of Gram-positive and Gram-negative bacteria and describe briefly the structural composition of the seven secretion systems that have been associated with increased virulence through subversion of host functions. Some of the effects of such systems on eukaryotic host processes have been studied extensively. We also describe the best-characterized effectors of each secretion system to give an overview of the molecular mechanisms employed by bacteria to hide from the immune system and convert eukaryotic cells into optimal ecological niches for their replication.

Introduction

Bacteria are the dominant form of life on earth. Around 50% of our cells are bacterial, and they have evolved to adapt to a multitude of ecological niches including multicellular eukaryotes (Sender *et al.*, 2016). Bacterial pathogenicity is greatly enhanced by their ability to evade and weaken the host defences, and to outcompete other microorganisms growing in the same milieu. To achieve that, bacteria can express and secrete several different macromolecules and toxins, called effectors, into the extracellular medium and in some cases directly into the cytoplasm of their target. Sometimes the macromolecules released will trigger a response from the host defences, in which case they are called virulence factors or elicitors (Jimenez *et al.*, 2016).

The machinery required to release such factors and the macromolecules themselves are usually encoded in pathogenicity islands in the bacterial genome or in plasmids that have been acquired through horizontal gene transfer, since the secretion machines are well conserved across species (Abby *et al.*, 2016).

Bacteria are contained in one or two membranes separated by a peptidoglycan layer. The passage of the effectors through these barriers cannot occur without expenditure of energy and is carried out by one of nine secretion systems (types I to IX). These complex multiprotein assemblies evolved from existing ones such as the bacterial phage tail (type VI) from the machinery required for shuffling macromolecules through organelles (type I), for piliation (type II), flagellar or gliding motion (types III and IX), or conjugation (type IV). Each secretion system has its own unique mechanism; some release the effector in one stage, others require an additional secretion step carried out by another membrane protein. Some of them are encoded in operons; each species might express none, one or more of the systems and when present they are normally associated with increased virulence and pathogenicity. Very few secretion systems (types I and III) are expressed in commensal organisms and mutations that lead to loss of function in one of their components results in phenotypically avirulent population. For this reason the presence of secretion systems on the surface makes them a likely target of host defence mechanisms to discriminate highly pathogenic from commensal species (Vance *et al.*, 2009).

Gram-positive and Gram-negative bacteria share some of the components of type IV and type VII secretion systems, but each has evolved their own unique systems to export proteins through one or two membranes (Schneewind and Missiakas, 2012).

The type VIII secretion system is encoded by two *csg* operons (Hammar *et al.*, 1995) and is involved in the secretion of curli fibres composed of the protein CsgA through a pore formed by CsgG monomers (Goyal *et al.*, 2014). Curli are involved in biofilm formation and surface attachment (Olsén *et al.*, 1989; Pawar *et al.*, 2005) and will not be discussed further in this review.

In this review we provide a brief overview of the eight bacterial secretion systems involved in the subversion of the host system and their mechanisms of action, with a focus on some of the best-characterized secreted effectors and how they function. The review is divided in three main sections: (i) Mechanism of protein translocation by secretion systems; (ii) mechanism of protein secretion by Gram-positive bacteria; and (iii) secreted virulence factors that interact with the host cell.

Mechanism of protein translocation by bacterial secretion systems

Bacteria communicate with their host and other organisms by secreting macromolecules into the external environment (Waters and Bassler, 2005). Bacterial secretion systems that span one or two membranes modulate such function through direct or indirect host interaction.

In Gram-negative bacteria, where the effectors have to be secreted through two membranes, this could be done in one step through the type I, III, IV, VI and VII, or in two steps by secreting first the effector in the periplasm through a common Sec/TAT pathway and finally secreting it into the extracellular space through the type II, type V or type IX secretion system (see *Secretion of effectors to the periplasm*). Gram-positive bacteria only have the outer

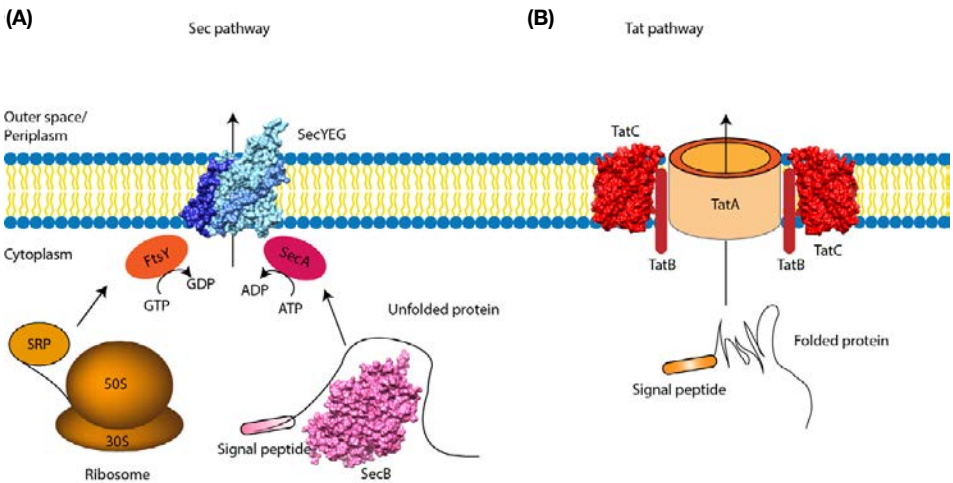


Figure 1.1 Sec and Tat pathway of protein secretion. (A) Diagram of the Sec secretion pathways. SecY in light blue, SecG in dark blue and SecE in cornflower blue (PDB: 5AWW). The nascent peptide binds to either SRP or SecB (PDB: 1OZB) and is secreted by SecYEG in an unfolded state with the assistance of FtsY or SecA respectively. (B) Diagram of the TAT secretion system. Folded proteins with an appropriate signal peptide are secreted through a pore formed by TatA, B and C (PDB: 4HTS).

portion of the two-step mechanism as they only have one membrane. Mycobacteria are an exception, since that have a membrane surrounding the cytoplasm and a unique mycomembrane, and thus require a one-step secretion system (Brennan and Nikaido, 1995). We will describe the first Sec/TAT systems required for the two-step mechanism used by the type II, V and IX secretion systems, which will then be described. We will continue this section by describing the rest of secretion systems (type I, III, IV, VI and VII), which perform the secretion of effectors in only one step.

Secretion of effectors to the periplasm: the two-step mechanism requires the Sec translocase complex or the TAT system

The effectors that require secretion through the type II and V systems are first exported to the periplasm through the inner membrane. The Sec translocase is used if effectors are secreted in an unfolded conformation, but if they require cytoplasmic-assisted folding the twin-arginine-dependent translocation (TAT) system is used (Voulhoux, 2001).

The Sec translocase complex consists of a pore made of three membrane proteins, SecYEG, and a membrane associated ATPase (SecA) that provides the energy for translocation. Once the nascent protein emerges from the ribosome and the signal peptide becomes available for binding, two possible pathways of Sec-dependent secretion are possible (Fig. 1.1):

- 1 If the N-terminal signal sequence is hydrophobic it slows down translation and allows the signal recognition particle (SRP) to bind to it (Pechmann *et al.*, 2014). SRP then associates to its receptor FtsY, located on the membrane and transfers the nascent polypeptide chain to the SecYEG channel (Gilmore *et al.*, 1982).

- For non-hydrophobic signal sequences, the trigger factor (TF) chaperone binds and transfers the nascent protein to SecB, another chaperone that keeps it into an unfolded state and subsequently to SecA, an ATPase that powers protein transport across SecYEG (Randall *et al.*, 1997).

The twin-arginine-dependent translocation (TAT) apparatus can transport across the plasma membrane fully folded proteins that contain the specific signal sequence SRRXFLK (Weiner *et al.*, 1998). It is composed of three essential transmembrane proteins: two single-pass proteins (TatA and TatB) and a protein with six transmembrane helices (TatC). The mechanism by which the protein is transported fully folded is not yet clear. It is thought that TatA might form oligomers that destabilize transiently the plasma membrane and allow the protein to pass through its pore (Westermann *et al.*, 2006) (Fig. 1.1).

Once in the periplasm the effector protein can be secreted through the type II, V or IX secretion systems.

Type II secretion systems are homologous to type IV pili

The type II secretion system (T2SS) is widely expressed in pathogenic and non-pathogenic bacteria such as *Klebsiella pneumoniae* (encoded by *pul* genes) (d'Enfert *et al.*, 1987), *Vibrio cholerae* (*eps*) (Sandkvist *et al.*, 1997), *Pseudomonas aeruginosa* (*xcp* and *hxc*) (Bally *et al.*, 1992; Nampiaparampil and Shmerling, 2004), *Yersinia enterocolitica* (*yts*) (Iwobi *et al.*, 2003), *Erwinia chrysanthemi* (*out*) (He *et al.*, 1991) and in *E. coli* where it is encoded in an operon consisting of 12–15 general secretion pathway proteins (*gsp*) (Francetic and Pugsley, 1996). Some proteins are not conserved across all species that express the T2SS; the core genes essential for secretion are GspC–O. GspA and GspB are accessory single-pass membrane proteins that, when present, assemble into a large heteromultimeric complexes capable of rearranging the peptidoglycan layer for better assembly of the core complex (Strozen *et al.*, 2011). Mutations in any of the core genes abolish secretion. Fig. 1.2 shows the arrangement of the operon in *V. cholerae* and *E. coli* K12 coloured on the basis of the subcomplex composition and their location in the membrane. The nomenclature of the proteins across species is not well conserved, for clarity in this review we will be using only the *gsp* nomenclature used in *E. coli*, other reviews have a comprehensive list of all the different nomenclatures used in other species (Douzi *et al.*, 2012; Korotkov *et al.*, 2012).

The complex is divided into four subcomplexes: the pseudopilus (in red the major pseudopilin GspG and in yellow the minor pseudopilins GspH, I, J K), the inner-membrane platform (in green, GspC, E, F, L, M), the outer membrane complex (in blue, GspD) and the secretion ATPase (in orange, GspE).

The secretion ATPase GspE, which is anchored to the membrane through GspL (Sandkvist *et al.*, 1995), provides the energy through ATP hydrolysis for GspF to assemble the pseudopilus, a filament composed of major and minor pseudopilins. The pseudopilus is thought to act as a piston to push the proteins through the outer membrane secretin GspD channel, a dodecameric bell-shaped structure with a hollow centre and a gate at the periplasmic edge (Fig. 1.2) (Reichow *et al.*, 2010). Secretins are well conserved components of the type III (Koster *et al.*, 1997) and type IV (Collins *et al.*, 2001) secretion systems, and in type II secretion systems they interact with GspC, a transperiplasmic protein that is the least conserved of the core proteins (Korotkov *et al.*, 2011). The outer membrane complex aids in the formation of the inner-membrane complex (Lybarger *et al.*, 2009). The substrates

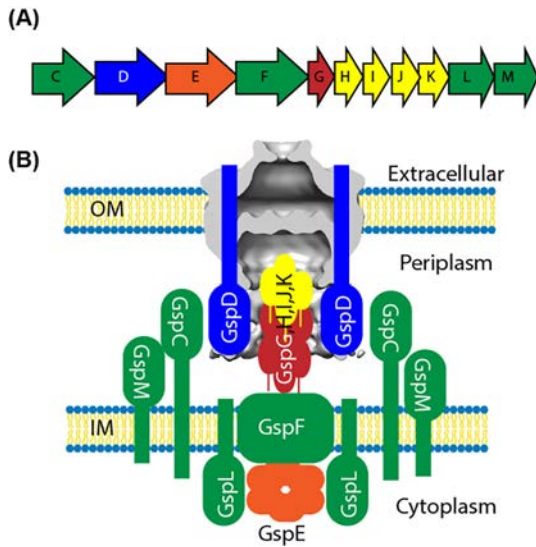


Figure 1.2 The type II secretion system structural organization. (A) Operon composition of the type II secretion system. In blue is the secretin component, in red and yellow the major and minor pilins, in green the inner-membrane components and in orange the ATPase. (B) Structure of the secretin of type II secretion system (EMDB 1763) and a diagram of protein–protein interaction of all the other components. The colouring scheme is the same as in (A).

that are transported across the type II secretion system are usually folded in the periplasm, they are exclusively targeted for excretion to the type II secretion system and they contain a specific three-dimensional signal that has not yet been identified (Filloux, 2010).

Several proteins are released by the type II secretion systems, most notably the cholera toxin and other enzymes such as phosphatases, lipases, proteases that function mostly extracellularly and are required for the bacterial motility and its virulence. None of these proteins is involved in evasion of host defences (Parsot, 2009; Sandkvist, 2001).

Type V secretion systems and the autotransport mechanism

Type V secretion systems were identified in several pathogenic bacteria such as *Neisseria gonorrhoeae* (Pohlner *et al.*, 1986), *Neisseria meningitidis* (van Ulsen *et al.*, 2003), *Pseudomonas aeruginosa* (Salacha *et al.*, 2010) and *Bordetella pertussis* (Leininger *et al.*, 1991). Several variants of this system exist, broadly classified into five types based on their composition and insertion mechanism into the membrane (Leo *et al.*, 2012). They all share the common feature of being a Sec-dependent transport system that requires neither ATP nor a proton gradient. The only energy available for insertion into the membrane and transport is the free-energy of protein folding (Junker *et al.*, 2009).

The secretion process involves the following steps (Fig. 1.3):

- 1 All proteins involved in secretion are expressed with an N-terminal signal that targets them for transport to the periplasm through the Sec pathway (Brandon *et al.*, 2003; Sijbrandi *et al.*, 2003). The proteins are unfolded at the time of transport and are likely kept in a translocation-competent state by chaperones (Adams *et al.*, 2005; Ruiz-Perez *et al.*, 2009).

- 2 The β -barrel or translocator domain of one of the proteins inserts in the outer membrane (OM) independently or through the Bam (β -barrel assembly) complex (Rossiter *et al.*, 2011).
- 3 The secreted domain or protein, termed passenger, binds to at least one periplasmic polypeptide transport-associated (POTRA) domain on BamA or the translocase protein. It then translocates through the pore of the β -barrel and is either secreted directly, cleaved by a serine protease domain and secreted, or it is secreted to the extracellular space while remaining anchored to the translocator domain (e.g. adhesins) (Leo *et al.*, 2012).

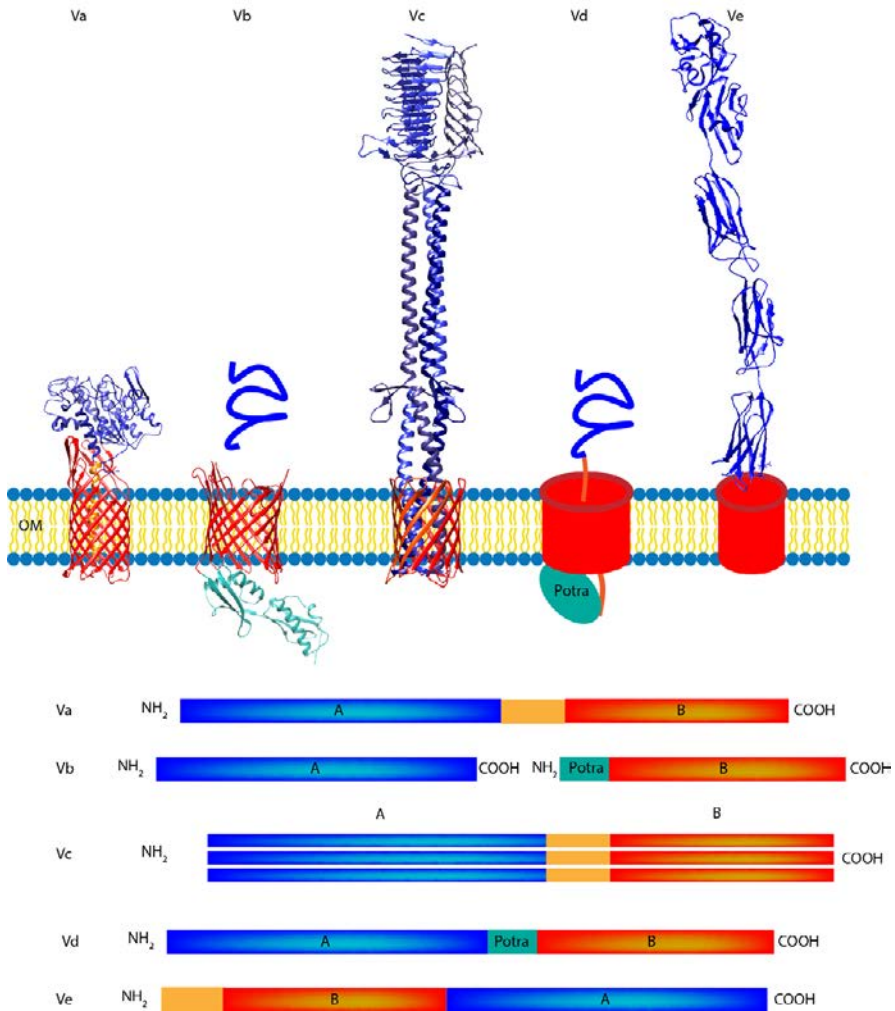


Figure 1.3 Summary of type V secretion mechanisms. There are five known mechanisms for secretion of type V (Va–e). The difference between the mechanisms involves the direction of insertion and the presence of one or more polypeptides. Several structures have been solved by crystallography: 3KVN for the type Va, 4QKY for type Vb, 2XQH and 2GR7 for type Vc, and 1CWV.

Table 1.1 Summary of type V secretion systems classes

Class	Alternative name	Translocator/ passenger	Bam-assisted	POTRA location	Direction of secretion
Va	Autotransporter	1 protein	Yes	BamA	N to C
Vb	Two-partner	2 proteins (TpsA and TpsB)	No	TpsB	N to C
Vc	Trimeric autotransporter	3 copies of 1 protein	Yes	BamA	N to C
Vd	Fused two-partner	1 proteins	No	Translocase	N to C
Ve	Inverted autotransport	1 protein	Yes	None	C to N

More than one class of type V secretion systems can co-exist in one organism. Their characteristics are summarized in Table 1.1.

Several proteins are secreted through the type V secretion system, most are involved in pathogenicity through adhesion (AIDA-I, Pertactin, *shdA*), biofilm formation (Ag43, BcpA), serum resistance (*BrkA*), cytotoxicity (*EspP*) and cell communication (van Ulsen *et al.*, 2014). Type V effectors will be discussed further in the following section.

Type IX secretion system is also a gliding motility apparatus

In *Porphyromonas gingivalis*, a periodontopathic pathogen that lacks type I to VIII secretion systems, a new mechanism for protein secretion and gliding motility was recently discovered (Sato *et al.*, 2010a). The type IX secretion system (T9SS) is composed of 11 proteins (*porK-X* and *sov*) scattered along the genome. *PorK*, L, M and N form a large complex of 1.4MDa resistant to SDS PAGE electrophoresis (Sato *et al.*, 2010b) and smaller subcomplexes of *porK* and *porN* assemble into multimeric rings of 32–26 subunits in the outer membrane (Gorasia *et al.*, 2016). *PorL* and *porM* are inner-membrane proteins that provide the energy required for secretion (Hammar *et al.*, 1995).

Proteins are targeted for secretion mainly by the presence of an N-terminal signal that targets them for translocation across the IM through the Sec system (Pavloff *et al.*, 1995), and C-terminal signal sequence (Glew *et al.*, 2012) that is cleaved by the peptidase *PorU* (Gorasia *et al.*, 2015). The secretion can then take place across the OM through a pore likely made of *porK* and *porN* (Hammar *et al.*, 1995). The secreted effectors will be discussed in section 3.

With the exception of the type II, V and IX secretion systems, all other systems require no periplasmic intermediary step and the secretion occurs in one step.

The type I secretion system is a bacterial efflux system

The type I secretion system (T1SS) consists of three proteins that are essential to its function: an ABC (ATP-binding cassette) transporter fused to an inner membrane-spanning domain, a membrane fusion protein (MFP) in the periplasm and an outer membrane factor (OMF) homologous to TolC receptors (Fig. 1.4).

The ABC transporter most likely works as a dimer (Dawson and Locher, 2006) and contains a nucleotide binding domain (NBD) that shuttles between a cytoplasm-facing to periplasm-facing conformations upon nucleotide binding (Fig. 1.4; Yang and Rees, 2015).

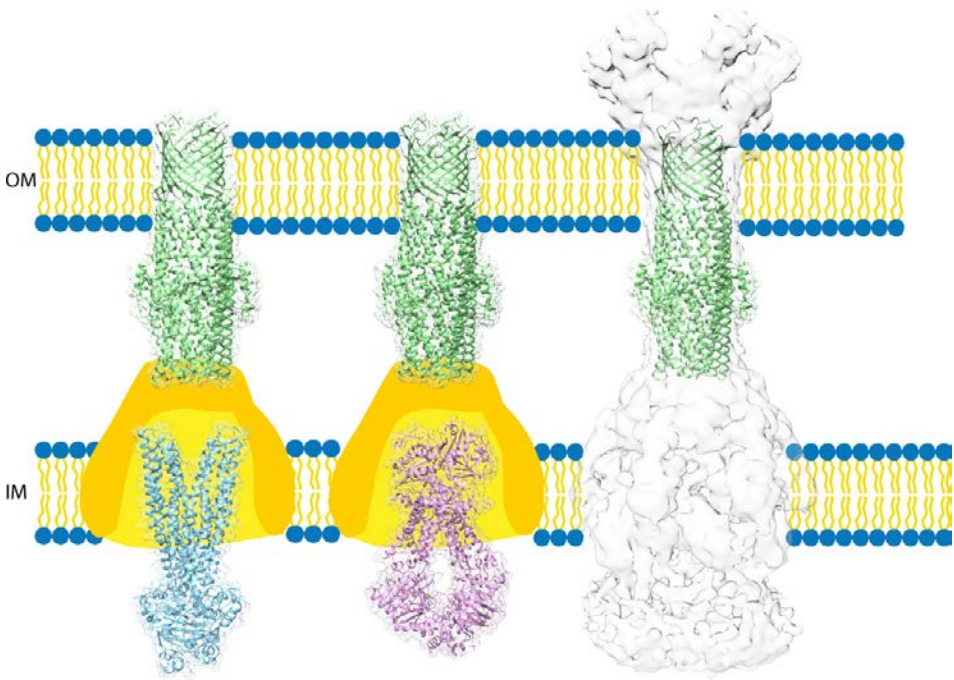


Figure 1.4 Type I secretion system proposed structure. In green is the TolC receptor (1EK9), in orange is the membrane fusion protein (MFP) whose structure is unknown and in blue (2ONJ) is the closed state of the ABC (ATP-binding cassette) transporter and in magenta is the open state (2ONK) modelled on a multi drug transporter from *S. aureus*. The structure at high resolution of a related drug transporter shows the tolC in an open conformation depicting a potential mechanism of secretion (EMD 3330).

The OMF consists of a short cytoplasmic domain, a membrane-spanning domain and a periplasmic region that anchors it to the ABC transporter. It is associated to the ABC transporter even in the absence of the secreted substrate (Uhlén *et al.*, 2000a) and it is thought to form hexamers as its analogue AcrA, a member of the resistance-nodulation-division (RND) family of multidrug efflux pumps (Du *et al.*, 2014; Jeong *et al.*, 2016).

The TolC protein trimerizes and forms a long tube made up of a large β -barrel with a very long but narrow periplasmic domain of several α -helices (Koronakis *et al.*, 2000). The α -helices form a narrow channel in the unbound tolC (Koronakis *et al.*, 2000) but open up upon binding to the OMF and the ABC transporter (Du *et al.*, 2014; Jeong *et al.*, 2016). The assembly of the complex is initiated by the substrate binding to the ABC transporter (Balakrishnan *et al.*, 2001). All the substrates transported across T1SS are unfolded during transport and contain a glycine rich motif (GGxGxDxxx) at their C-terminus that targets them for secretion (Nicaud *et al.*, 1986). The glycines form calcium-binding domains that promote the protein folding once outside the cell at a calcium concentration above its K_d (Gangola and Rosen, 1987).

The substrates secreted by T1SS vary in size between 19 kDa, e.g. the haemophore HasA (Létoffé *et al.*, 1994), to 900 kDa, e.g. LapA, surface layer protein (Hinsa *et al.*, 2003), and

most notably include pore-forming haemolysin A (Uhlén *et al.*, 2000b), toxins, lipases, adenylate cyclases and proteases, all proteins associated with increased virulence. T1SS effectors will be further discussed in *Virulence factors in host cell interaction*.

Type III secretion systems inject the effectors directly into the cytoplasm of the host

Type III secretion systems (T3SS) have been often associated with pathogenicity in bacteria, though they can also be involved in symbiosis (Dale *et al.*, 2002). They have been identified mostly through genome sequencing in *Salmonella* (Ochman and Groisman, 1996), *Shigella*, *Vibrio* (Park *et al.*, 2004), *Yersinia* (Cornelis, 2002), *Pseudomonas* species (Yahr *et al.*, 1996), some *E. coli* strains (Ren *et al.*, 2004), and many more species (Pallen *et al.*, 2005). There are eight subclasses of T3SS identified, each with a different target (animals, plants or other pathogens) and effect on the host. More than one T3SS can be encoded in the genome of the same species, though usually of different subclasses. Examples are the SPI-1 and SPI-2 systems found in *Salmonella*, required respectively for entry into the host, and survival and replication (SPI-2) at the intracellular stage of infection (Diepold and Armitage, 2015). T3SS is derived from the flagellar export system in bacteria and the two systems share a strong homology (Abby and Rocha, 2012). While the flagellar genes are encoded in the genome of the bacterium and co-evolved with it, the T3SS is encoded in virulence plasmids or pathogenicity islands and thus is more well-conserved across species as a result of horizontal gene transfer (Gophna *et al.*, 2003). The expression of the genes on the pathogenic islands is not constitutive but is induced by contact with the host (Pettersson *et al.*, 1996) since the complex formation requires a large energy expenditure to express and assemble more than 20 proteins that form a needle spanning the two membranes (Fig. 1.5). In this review we will be using the Sct (secretion and cellular translocation) nomenclature except for proteins that are exclusively expressed in certain species (Hueck, 1998).

The secretion complex can be divided into several substructures, described below.

The basal body

Stacks of concentric rings, referred to as the basal body, connect the inner and outer membrane leaflets. The outer membrane (OM) ring is a member of the secretin family analogous to that of T2SS (Koster *et al.*, 1997; Reichow *et al.*, 2010). It is composed of 12–15 copies of the SctC protein giving the overall basal body structure a 12- and a 3-fold symmetry respectively (Hodgkinson *et al.*, 2009; Schraidt and Marlovits, 2011). The inner-membrane (IM) ring is composed of two concentric circles of different diameters, the inner ring is made up of 24 molecules of the lipoprotein SctJ and the outer ring of 24 molecules of SctD, both single-pass membrane-spanning proteins (Hodgkinson *et al.*, 2009).

Export apparatus

The export apparatus is essential to secretion and is composed of five membrane-spanning proteins: SctR, SctS and SctT being mostly periplasmic (Berger *et al.*, 2010), and SctU and SctV with mostly cytoplasmic domains (Abrusci *et al.*, 2013; Zarivach *et al.*, 2008). The export apparatus is thought to recognize and bind to the substrate and proceeds to its secretion. It is visible in the EM structure as a central density at the height of the IM (Fig. 1.5) (Hodgkinson *et al.*, 2009).

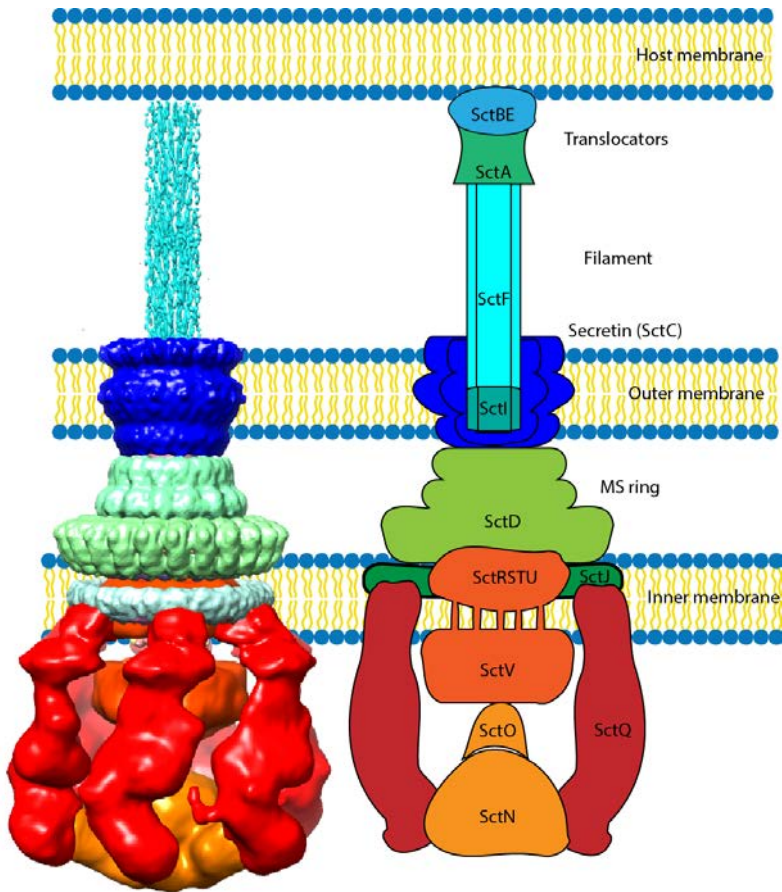


Figure 1.5 Type III secretion system architecture. Left. The EM structure of type III secretion system obtained from the superimposition of tomographic, single particle and helical reconstruction cryoEM studies (EMD 2667 and EMD 1875, EMD-5352 respectively). Right. A summary of the different components of the secretion system coloured according to their position in the bacterial membrane.

Cytosolic components

The cytosolic components make up a very dynamic complex that stabilizes upon contact with the host (Nans *et al.*, 2015). This complex, homologous to V/F-type ATPases (Pallen *et al.*, 2006), is composed of five proteins: sctN, sctO, sctQ, sctL and sctK. sctN, the hexameric ATPase, upon contact with the substrate–chaperone complex, disassembles it and unfolds the substrate through ATP hydrolysis to prepare it for secretion (Akedo and Galán, 2005). The other proteins anchor the ATPase to the membrane and assist in the secretion process (Lara-Tejero *et al.*, 2011).

The needle

The needle is a helical oligomer of SctF (Loquet *et al.*, 2012) that probably self assembles (Fujii *et al.*, 2012) and whose length is controlled by SctP (Kubori *et al.*, 2000) and SctI

(Lefebvre and Galán, 2014). It projects out of the bacterial envelope towards the target's membrane (Kubori, 1998). It is hollow inside and the unfolded proteins are secreted through the lumen (Radics *et al.*, 2014). The tip of the needle contains a pentameric hydrophilic protein (SctA-IpaD) that provides a scaffold for two translocator proteins (SctB-IpaB and E-IpaC) to insert into the host membrane, thus connecting the two cytosols (Cheung *et al.*, 2015).

The T3SS complex is assembled independently of any stimuli, but substrate export is activated upon contact with the host cell (Ménard *et al.*, 1994). Following a change in pH of the cytosol, a 'plug' protein complex (SctW) is released from the cytosolic component and the translocator proteins can be successfully exported to the tip of the needle, leading to the formation of a pore on the target membrane (Yu *et al.*, 2010).

Type IV is a secretion and conjugation apparatus that spans two membranes and the membrane of the host

The type IV secretion systems are found in both Gram-negative and Gram-positive bacteria such as *Helicobacter pylori* (Hofreuter *et al.*, 2001; Kersulyte *et al.*, 2003; Odenbreit, 2000), *Brucella* (O'Callaghan, 1999), *Bartonella* (Schmiederer and Anderson, 2000), and *Streptococcus* species (Zhang *et al.*, 2012). They can be broadly classified into three subfamilies based either on their function, the coding region of the genomic DNA or the plasmid that encodes them. Type IV secretion systems possible functions are conjugation (Lawley *et al.*, 2003), DNA uptake and release (Hamilton *et al.*, 2005), and effector secretion.

Based on the coding region of their DNA, they are divided into type IVa, b and c (Fig. 1.6). Type IVa is composed of proteins homologous to the *virB* and *VirD* operons in *Agrobacterium tumefaciens* (Ghai and Das, 1989; Ward *et al.*, 1988). Type IVb has no sequence similarity but is functionally and structurally related to the type IVa proteins (Ghosal *et al.*, 2016) and is composed of 25 subunits encoded in *Legionella pneumophila* by the *Dot/Icm* (defective in organelle 45 trafficking/intracellular multiplication) operon (Segal and Shuman, 1997). Finally, the type IVc system, mainly found in Gram-positive bacteria, is a complex composed of 5 subunits derived from the type IVa system (Zhang *et al.*, 2012) (Fig. 1.6). These classifications are not rigid since the same secretion systems can have more than one function and proteins can be exchanged between the different systems.

The secretion complex of type IVa can be divided into several substructures:

Type 4 coupling protein (T4CP)

The type IV coupling protein (T4CP) *VirD4* is an ATPase that mediates the interaction between cytoplasmic proteins (chaperones, substrates or accessory proteins) and the inner-membrane complex (IMC) (Atmakuri *et al.*, 2004). *VirD4* and its homologues *DotL* and *TrwB*, are integral membrane proteins that form hexamers and have a large cytoplasmic domain (Gomis-Rüth, 2001). They interact with the IMC through the transmembrane portion of the *VirB10* protein (Gomis-Rüth, 2001).

Inner-membrane complex (IMC)

The bipartite inner-membrane complex is made of *VirB3*, *VirB4*, *VirB5*, *VirB6*, *VirB8*, and *VirB11*, together forming the stalk and the arch in the structure of the whole system (Low *et al.*, 2014). *VirB8* is the nucleating protein around which the other proteins assemble (Kumar *et al.*, 2000). *VirB3* interacts with *VirB4* and *VirB5* (Shamaei-Tousi *et al.*, 2004), and has shares some homology with the pilins (Hapfelmeier *et al.*, 2000), suggesting a possible

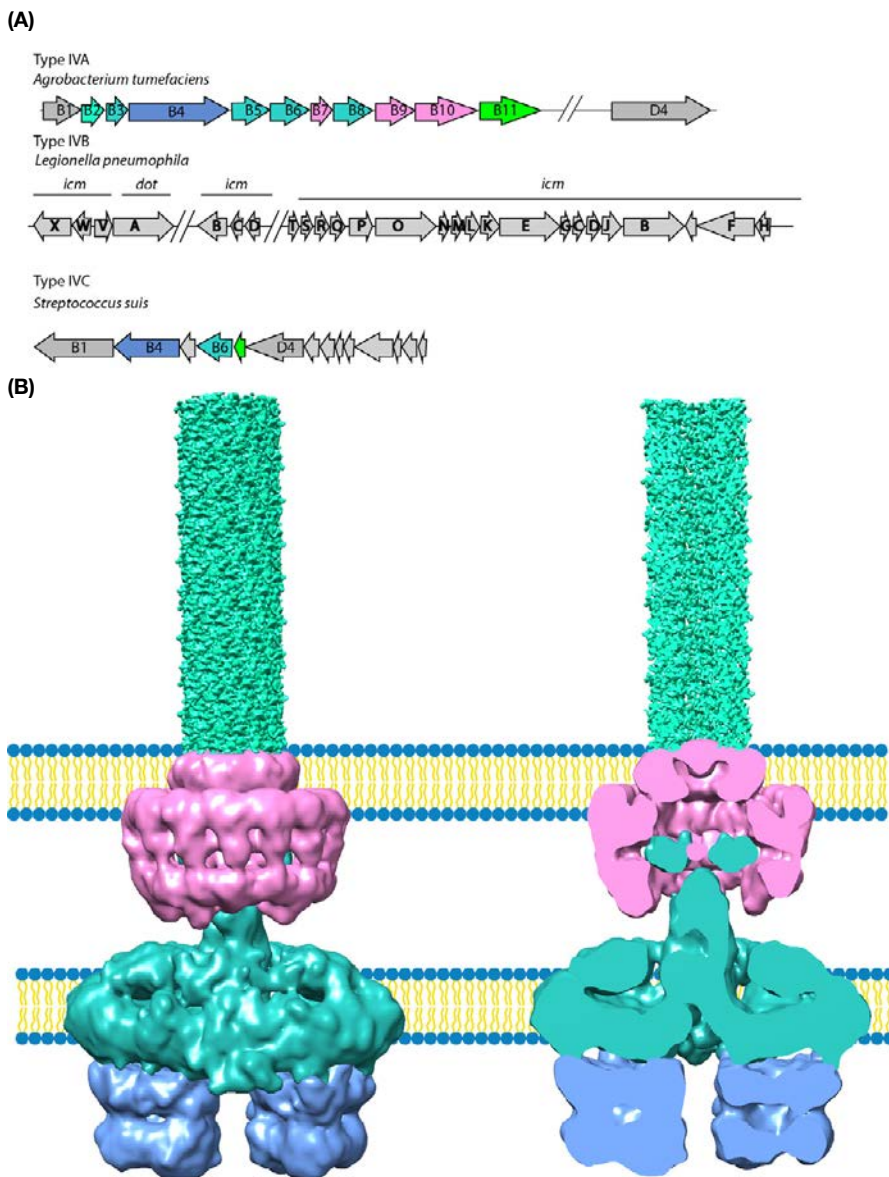


Figure 1.6 Type IV secretion systems arrangement and structure. (A) Genomic arrangement of the three different types of Secretion system IV. The proteins are coloured according to their special arrangement in the EM structure. (B) Structure of the type IV secretion system as solved by negative stain EM (EMDB 2567) with its pilus (EMD-4042). The colour scheme is maintained from section (A).

role in the assembly of the pilus. The VirB4 ATPase forms two hexameric barrels on either side of the complex (Low *et al.*, 2014) and likely mediates the dislocation of the virB2 pilin from the membrane (Kerr and Christie, 2010). VirB6 is a polytopic membrane protein that interacts with the substrate and the virB9 component of the outer membrane core complex

(Jakubowski *et al.*, 2004). Two ATPases, DotB and DotO, belong to the same class as the VirB11 and VirB4 ATPases (Segal *et al.*, 2005).

Outer membrane 'core' complex (OMC)

The outer membrane core complex is a large barrel that extends between the two membranes and it is composed of 14 subunits of VirB7, VirB9, and VirB10. It forms a central cavity through which the pili can extend (Fronzes *et al.*, 2009). In the Dot/Icm complex, DotF and DotG are located in the periplasm (REF) DotC and DotD are two lipoproteins like VirB7 and DotH is an outer membrane protein that is inserted by the two lipoproteins (Vincent *et al.*, 2006).

Cell surface pili or adhesins

The conjugative or secretion pilus is composed of polymerized VirB2 protein–phospholipid complex. It mediates the interaction of the secretion system with its target and provides a channel, 28Å large, through which macromolecules can be transported across the bacterial membranes directly to the cytoplasm of the target cell (Costa *et al.*, 2016).

The type IVb system is more complex than the type IVa and is composed of at least 25 proteins (Segal and Shuman, 1997). Only one of its components, DotG, shares sequence homology limited to its C-terminus with part of VirB10, the OMC component (Kubori and Nagai, 2016).

The secretion process differs between proteins and DNA, and begins with the assembly of the T4SS complex and the pilus at the cell poles (Judd *et al.*, 2005). For the process of conjugation, several proteins bind to the origin-of-transfer (OriT), a specific region of the DNA to be secreted (Fu *et al.*, 1991), create a nick and unwind the double-stranded DNA (Byrd and Matson, 1997). This complex, called a relaxosome, is then specifically recruited to the T4CP by a chaperone (Disqué-Kochem and Dreiseikelmann, 1997). On the contrary, proteins are directly recruited to VirD4 through a well conserved signalling sequence at the N- or C-terminus and are maintained in a secretion-ready state by chaperones (Alvarez-Martinez and Christie, 2009). Some species such as *B. pertussis* do not have a TC4P, but they exploit the Sec-dependent translocation system for the movement of the substrate from the IM to the periplasm (Farizo *et al.*, 2002). The effectors are likely transported in an unfolded manner directly to the cytoplasm of the target cell where they exert their function. The macromolecules secreted by T4SS increase the fitness of the bacterium by allowing spreading of antibiotics resistance, switching of surface antigens, the killing of neighbouring bacteria (Souza *et al.*, 2015) and the subversion of host cell response.

The type VI secretion system evolved as a killing machine

The type VI secretion system (T6SS) is present only in Gram-negative bacteria and consists of a two membrane–spanning complex that requires the assembly of at least 13 proteins encoded in one genomic locus (Boyer *et al.*, 2009) (Fig. 1.7). Two of the genes, *tssL* and *tssM* are homologous to *icmH* and *icmF* of the type Vb secretion systems and several proteins share structural, but not sequence, homology with bacteriophage contractile tail machinery (Cascales and Cambillau, 2012). The T6SS system is used as a defensive and offensive weapon against competitors and is activated upon contact with another bacterium or a host cell. The bacterium secretes toxins indiscriminately of its target and is protected from self-harm by the presence of immunity proteins that are not expressed in other species (Hood

(A)



(B)

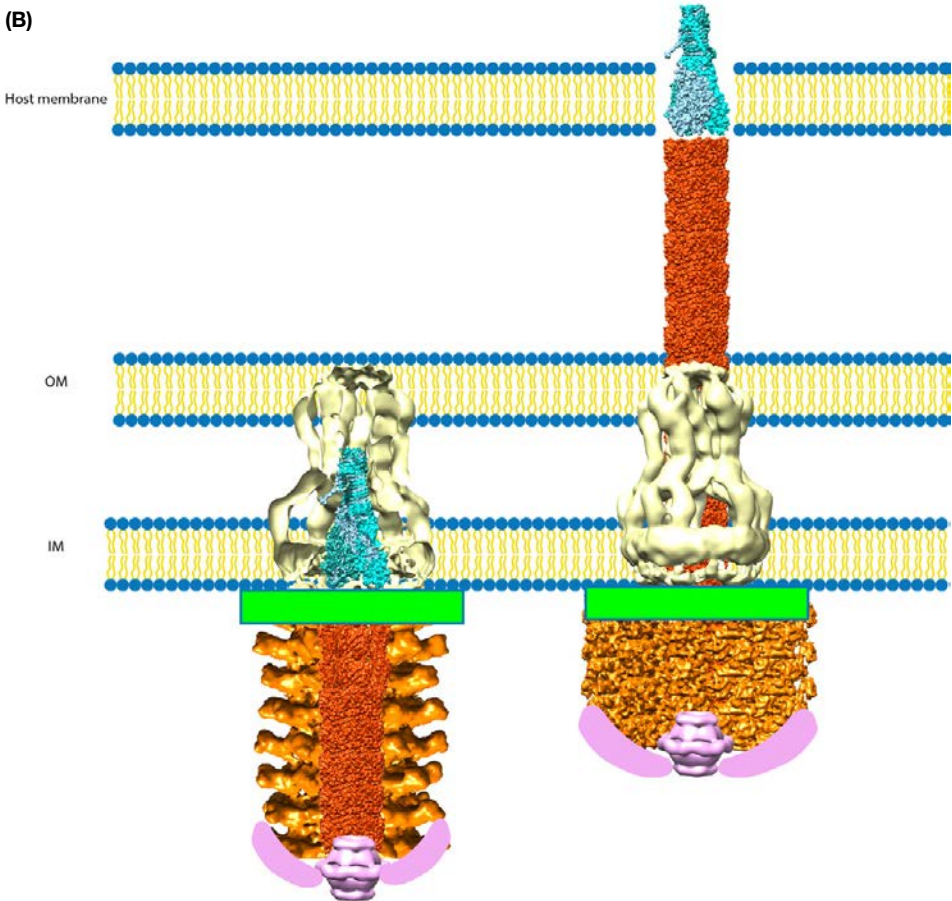


Figure 1.7 Mode of action of the type VI secretion. (A) Type VI secretion operon coloured on the basis of its structure. (B) Structure of the extended (left) and contracted sheath (right) showing the predicted mechanism of secretion. The type VI complex in yellow (EMD-2927), the VgrG trimer in blue (4UHV), the tail tube in dark orange, (hcp analogue, 1Y12), the extended and the contracted sheath of a homologue of VirB/C in orange (EMD-1126 and EMD-2524 respectively) and TssA in pink (EMD-3282).

et al., 2010). The T6SS is required both for virulence against eukaryotic hosts (Mougous *et al.*, 2006) and for intra-bacterial competition in the reproductive niche (Hood *et al.*, 2010).

The secretion apparatus can be divided into two substructures (Fig. 1.7).

The membrane complex (MC)

The MC is needed to anchor the baseplate complex to the membrane. It is composed of three proteins, TssJ, TssL and TssM that form a 'rocket-shape' structure of 1.7MDa that

spans both the outer and the inner membrane (Durand *et al.*, 2015). TssJ is the first one to be recruited to the outer membrane, followed by TssM, an elongated protein that spans the periplasm and the IM. TssL is the last protein to be added to the complex and is a single pass inner-membrane protein that interacts with TssK and other cytoplasmic components (Durand *et al.*, 2015; Zoued *et al.*, 2013).

The baseplate complex (BC)

The BC assembles independently of the MC and one of its main components is VgrG a protein that forms a spike needed to break the membrane of the target cells (Brunet *et al.*, 2014). VgrG recruits HCP1, a protein that oligomerizes into hollow tubes formed by homohexamers stacked head-to-tail as in the phage tail (Leiman *et al.*, 2009). The tail tube is surrounded by a sheath formed by oligomers of TssB (VipA) and TssC (VipB), an assembly that is attached to the baseplate through interaction with TssE. The sheath forms an extended micrometre-long contractile structure that contracts to power secretion (Basler *et al.*, 2012; Kube *et al.*, 2014). Once contracted the sheath is disassembled by ClpV through ATP hydrolysis (Kudryashev *et al.*, 2015). Not much is known about the other components of the BC. TssE is homologous to the gp25 protein in the phage base plate and co-purifies with Hcp1 (Leiman *et al.*, 2009). TssF and TssG were demonstrated to interact with each other and with TssE *in vitro* (Brunet *et al.*, 2015). TssA is recruited to the MC and mediates the priming and assembly of the base plate complex and tail (Zoued *et al.*, 2016).

Once the type VI secretion system assembles, the contraction of the sheath causes the injection of the effector in the cytoplasm of the target cell. The protein VgrG forms a trimeric spike, sharpened by the PAAR (proline-alanine-alanine-arginine repeat) protein, and is capable of piercing the membrane of the target cell. The effectors are bound to either protein and are released into the cytoplasm by a mechanism not yet clear (Shneider *et al.*, 2013).

Gram-positive mechanism of secretion

In Gram-positive bacteria some toxins can be secreted through the Sec pathway and they enter the cell through the SLO (haemolysin streptolysin O), a cytolysin that forms pores in the host membrane and allows the targeting of toxins, of even large size, to the membrane of the host (Madden *et al.*, 2001). In some Gram-positive bacteria and in mycobacteria some effectors are secreted through the type VII secretion system.

Type VII is the unique secretion system in Gram-positive bacteria

The type VII secretion system (T7SS) is exclusively found in Gram-positive bacteria. Initially identified in a virulence island in *Mycobacterium tuberculosis* (Gey Van Pittius *et al.*, 2001), homologues were later found in *Firmicutes* such as *Bacillus* and *Staphylococcus* species (Pallen, 2002). There are seven conserved genes that are required for secretion: two are in the cytoplasm (EspG and EccA) and the remaining five are in the membrane (EccB, EccC, EccD, EccE, and MycP). All the proteins that are not conserved and that are specific to each system are most likely ATPases, substrates or specific chaperones. In *Bacillus* and *Staphylococcus* species only homologues of EccC and of the secreted substrates are present, and for this reason it is considered as an alternative type VIIb system (Fig. 1.8). The type VIIa system is divided into five classes (ESX-1 to 5), depending on the conservation of the subunits.

In the case of cytoplasmic proteins, EspG does not interact with any of the other

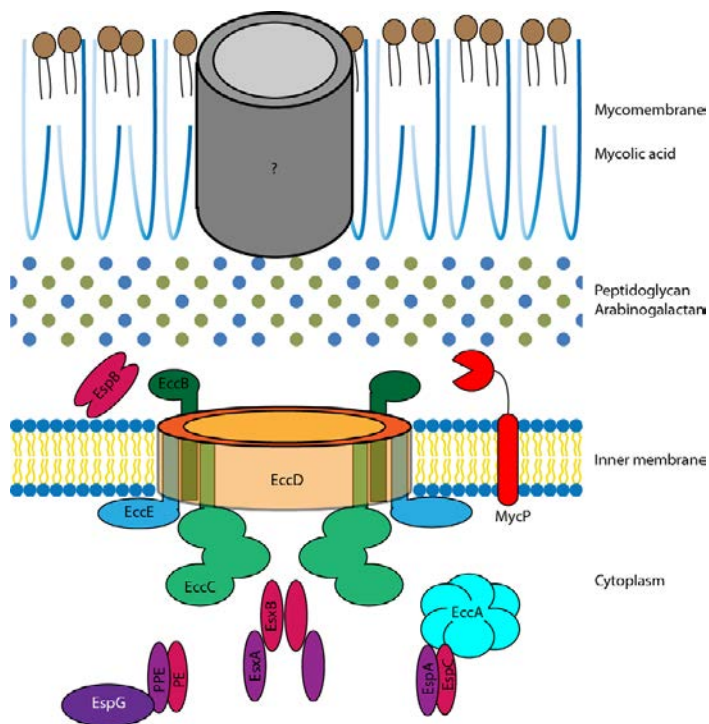


Figure 1.8 Type VII secretion system structure. Proposed assembly of the type VII secretion system across the inner membrane of Gram-positive bacteria. So far no transporter protein has been identified that would be active in the mycomembrane.

components of the system except the secreted substrates PE and PPE (Daleke *et al.*, 2012). EspG is necessary to prevent the aggregation of the substrate and is not present in the ESX-4 system probably because of the solubility of the secreted proteins (Korotkova *et al.*, 2014). EccA is the hexameric AAA+ ATPase that provides the energy for secretion. It has two distinct domains: the C-terminal domain that exhibits ATPase activity (Luthra *et al.*, 2008) and the N-terminal domain, homologous to the pilF of type IV pili, which contains several tetratricopeptide repeats that are involved in protein–protein interaction (Wagner *et al.*, 2014).

The membrane proteins, which form a 1.5MDa complex, have large cytosolic or periplasmic domains and one single transmembrane helix (Houben *et al.*, 2012). EccD is the exception; it is a polytopic membrane protein expected to have 11 transmembrane helices and is thought to be the pore required for secretion (Wagner *et al.*, 2016). It contains a ubiquitin-like domain in its N-terminal cytoplasmic domain and might form dimers (Wagner *et al.*, 2016). EccB, whose function is yet unknown, has a small cytoplasmic domain and contains a functional ATP binding site in its large periplasmic C-terminal domain (Wagner *et al.*, 2016; Zhang *et al.*, 2015). EccC is a member of the tsK/SpoIIIE-like ATPase family to which T4CP also belongs. It has three distinct nucleotide binding domains (NBD1–3) each with a specific function, NBD3 is required for substrate binding and NBD1 hydrolyses

ATP (Rosenberg *et al.*, 2015). EccE is a protein not present in all secretion systems and thus is thought to be peripheral to the membrane complex (Houben *et al.*, 2012). Mycosin P (MycP) is a membrane protein with a large periplasmic, subtilisin-like, protease domain (Brown *et al.*, 2000). Its presence is essential to secretion but its function as a protease is not (Ohol *et al.*, 2010). Nevertheless, the cleavage of the C-terminus of EspB by MycP causes a change in the quaternary structure of the secreted substrate and might be important to its function (Solomonson *et al.*, 2015).

The mechanism by which all these proteins can act in concert to modulate substrate secretion remains to be uncovered. Moreover, mycobacteria are contained in an outer membrane composed of mycolic acids linked to an arabinogalactan-peptidoglycan matrix embedded in unique lipids, proteins, polysaccharides and lipoglycans that differ from species to species (Daffé *et al.*, 2014). The transporter necessary to guide the substrate across the mycomembrane remains to be identified (Fig. 1.8).

Virulence factors in host cell interaction

The effectors released into the cytoplasm of the target host, have a profound effect on the physiology of the cell, mostly on cellular processes that respond to bacterial infections: cytoskeletal reshuffling, actin-dependent motility, autophagy evasion and phagosomal escape, adhesion, inhibition of signalling, obstruction of the immune response, (de)-ubiquitination, induction of apoptosis and formation of multinucleated cells.

Many of the effectors have one or more of these functions. Here we will separate the effector based on the secretion system responsible for their release into the extracellular space or the target's cytoplasm. Most of the best-characterized effectors are secreted by type III and type IV secretion systems, but a few are present in all other systems. The number and nature of effectors known is very large and a comprehensive list of all of them is beyond the scope of this review. We will be mentioning a few of the best-characterized effectors based on their function, hoping that it will give an overview of the diversity of mechanisms that bacteria have evolved to maintain their supremacy over other forms of life.

Type I secretion system secretes unfolded effectors

The T1SS secrete, in a single step through the double membrane, proteins of the repeats-in-toxin (RTX) family. RTX proteins vary in size (40 to more than 600 kDa) and in function (Linhartová *et al.*, 2010). They are characterized by the presence of several repeats of the nonapeptide X(L/I/F)XGGXG(N/D)D, a calcium-binding motif that forms a parallel β -roll (Baumann *et al.*, 1993). The proteins are secreted unfolded and are able to fold thanks to the high concentration of calcium ions present in the extracellular space (Thomas *et al.*, 2014).

The best-characterized function of RTX toxins is the pore forming and membrane disrupting activity of leukotoxins such as HlyA from *E. coli* (Welch *et al.*, 1992), LktA from *Mannheimia haemolytica* (Chanter, 1990), and CyaA from *Bordetella species* (Benz *et al.*, 1994). To be active these proteins require fatty acylation before secretion (Stanley *et al.*, 1994). Once in the extracellular space they interact with the host target membrane non-specifically, or through β_2 integrin surface receptors in leucocytes (Lally *et al.*, 1997), and glycophorin in erythrocytes (Cortajarena *et al.*, 2003). RTX toxins form pores though

which calcium and potassium can enter and through which important metabolites, such as ATP, can exit the cells (Bhakdi *et al.*, 1989). Depending on the amount of toxin released by the bacteria, the host cells will undergo necrosis or apoptosis (Russo, 2005).

Additionally to the pore-forming function the *Vibrio cholerae* MARTX toxin also contains an actin cross-linking domain (Sheahan *et al.*, 2004) that prevents actin polymerization (Kudryashov *et al.*, 2008) and a Rho GTPase-inactivation domain that causes cell rounding through the inhibition of the Rho GTPase family (Sheahan and Fullner Satchell, 2007). The *cyaA* toxin in *Bordetella* spp. also contains an additional domain with an adenylate cyclase function. Once inside the host leucocytes, *cyaA* is activated by calmodulin and produces excess cAMP that abolishes immune functions such as phagocytosis, chemotaxis and interleukins release (Vojtova *et al.*, 2006).

Type II effectors are mostly housekeeping proteins

The proteins secreted through the T2SS vary in terms of function and are mostly required for survival in the environment. The presence of T2SS nevertheless was shown to reduce the response of macrophages to infection through a yet unidentified mechanism (McCoy-Simandle *et al.*, 2011). HapA in *Vibrio cholerae* processes the HlyA secreted from type I secretion system, thus enhancing the effect of the toxin on the host (Nagamune *et al.*, 1996). The chitinase domain-containing exoprotein *chiA* has also been linked with persistence of *Legionella* in the lung (DeRoy *et al.*, 2006). The T2SS has also been associated with intracellular growth in amoebas of *Legionella pneumophila* (Hales and Shuman, 1999). Recently a yet unidentified effector secreted by T2SS was shown to be required for retention of a Rab1B GTPase to the *Legionella*-containing vacuoles (LCV), a process required for the successful intracellular growth of *L. pneumophila* (White and Cianciotto, 2016). Another secreted protein from T2SS was also found to affect cytokine secretion during infection in macrophages and inhibit IL-6 production through interference with the myeloid differentiation primary response (MyD88), TANK binding kinase 1 (TBK1) and the Toll-like receptor 2 (TLR2) pathways (Mallama *et al.*, 2017). More work remains to be done to identify such T2SS effectors and to better our understanding of the influence of these exoproteins on the host interaction and evasion.

Type III secreted effectors affect several host functions

Type III secretion systems target effectors directly to the cytoplasm of the host cell. Most share a common secretion signal, a chaperone-binding, and an active domain. We can classify the exoproteins on the basis of the changes elicited on the host metabolism.

Focal adhesion/tight junctions modulators

YopH in *Yersinia* species exhibits a protein tyrosine phosphatase (PTP) activity that, once inside the cytoplasm, targets proteins in focal adhesion complexes and blocks integrin-mediated phagocytosis in epithelial cells and macrophages (Rosqvist *et al.*, 1988). Its targets include paxilin, a protein involved in Fc receptor-mediated phagocytosis in macrophages (Greenberg *et al.*, 1993), p130^{Cas}, a focal adhesion-associated protein (Black and Bliska, 1997), the focal adhesion kinase (FAK) (Persson *et al.*, 1997), Cas and Fyn-binding protein (Hamid *et al.*, 1999), all of which when dephosphorylated disrupt focal adhesion complexes.

To colonize the epithelium effectively, *Shigella flexneri* secretes OspE1/2, an effector that binds to the integrin-linked kinase (ILK) leading to an increase in $\beta 1$ integrin concentration

on the host plasma membrane and a decreased phosphorylation of paxilin and FAK. The resulting decrease in focal adhesion turnover prevents cell detachment and promotes cell-to-cell dissemination of the bacterium (Kim *et al.*, 2009).

The gut of the mammalian host has an epithelial cell barrier that specifically selects the contents to absorb and those to keep out. The *E. coli* effectors NleA/EspI (Kim *et al.*, 2007), EspF (McNamara *et al.*, 2001) and Map (Dean and Kenny, 2004) affect the tight junctions between intestinal epithelial cells to allow the bacterium to enter through the gaps between the cells. NleA inhibits, upon binding, COPII, the protein required for ER vesicles trafficking (Kim *et al.*, 2007) while EspF and Map action is dependent on the interaction with the bacterial surface receptor intimin in a yet unidentified mechanism (Dean and Kenny, 2004).

In *Salmonella enterica* SipA, SopB, SopE and SopE2 decrease the expression of ZO and other proteins involved in tight junction formation, leading to an increased permeability of the epithelial layer (Boyle *et al.*, 2006).

Cytoskeleton modulators: actin microfilaments

Intracellular facultative and obligate pathogens release effectors that affect the stability of the actin cytoskeleton. YopE (Black and Bliska, 2000; Evdokimov *et al.*, 2002) in *Yersinia* spp., ExoS (Goehring *et al.*, 1999) and ExoT (Krall *et al.*, 2000) in *Pseudomonas aeruginosa*, SptP in *Salmonella* spp. (Fu and Galán, 1999) and AexT in *Aeromonas salmonicida* (Burr *et al.*, 2003; Litvak and Selinger, 2007) are all proteins that target Rho GTPases and mimic the guanidine exchange activation protein (GAP). Rho GTPases regulate actin polymerization and cytoskeleton dynamics (Hall, 1998). Their activity is dependent on their nucleotide state. Nucleotide exchange factors activate the protein by exchanging GDP for GTP. GAPs promote nucleotide hydrolysis by providing an arginine finger required for positioning of GTP in the binding site (Vetter and Wittinghofer, 2001). The activation of Rho GTPases causes the ruffling of the membranes and the internalization of the bacterium by micropinocytosis. The inactivation of Rho GTPases inhibits phagocytosis, providing another mechanism of host defences evasion. All the effectors mentioned above except YopE, contain an ADP-ribosyltransferase domain that targets Ras GTPase proteins for ADP ribosylation leading to a decrease in downstream signalling and alteration of several cellular processes (Vincent *et al.*, 1999). SopE (Hardt *et al.*, 1998), SopE2 (Stender *et al.*, 2000) in *Salmonella* spp. are Guanidine exchange factors that interact with Rho GTPases and induce actin filament formation and stabilization. This induces cell entry into the host through membrane ruffling and intracellular replication through a mechanism not yet clear (Vonaesch *et al.*, 2014). SipA (Kenny, 1999) and SipC modulate actin directly through binding. SipA stimulates actin bundling by increasing the activity of T-plastin (Zhou *et al.*, 1999) and inhibiting the depolymerizing cofactor ADF/cofilin (McGhie *et al.*, 2004), while SipC directly induces actin nucleation (Chang *et al.*, 2005) and bundling (Myeni and Zhou, 2010). The translocated intimin receptor (Tir) is one of the most studied effectors to hijack host systems. It is secreted by enterohaemorrhagic and enteropathogenic *E. coli* (EHEC and EPEC) and it inserts into the host membrane and acts as a receptor for intimin to lead to actin rearrangements (Kenny, 1999). Another EPEC/EHEC effector that alters actin recycling leading to the formation of filipodia is the mitochondrial associated protein (Map). Map contains a GEF and a PDZ domain that polarizes the actin control protein cdc42 to the membrane (Orchard *et al.*, 2012).

Cytoskeleton modulators: microtubules

EspG from EHEC/EPEC, and VirA in *Shigella* destabilize microtubules (Shaw *et al.*, 2005; Yoshida *et al.*, 2002) leading to the release of several factors, one of them being GEF-H1 the activator of RhoA. Such activity is thought to contribute to actin stress fibre formation and ruffling of the host membrane to facilitate bacterial entry (Matsuzawa *et al.*, 2004). EspG also localizes to the Golgi, where it binds to the Golgi proteins GM130, ADP-ribosylation factor GTPase (ARF) and p21-activated kinases (PAK), leading to the inhibition of protein secretion by the host (Clements *et al.*, 2011; Selyunin *et al.*, 2011).

Immune response modulators

YopJ is an acetyl transferase in *Yersinia* spp. that inhibits the innate immune response by reducing the expression of pro-inflammatory signals. It acetylates and thus inhibits IKK β (Zhou *et al.*, 2004), Mitogen-activated protein kinase (MAPK) signalling (Mittal *et al.*, 2006) and TGF β -activated kinase TAK (Paquette *et al.*, 2012), and deubiquitinates key proteins in the NF- κ B signalling cascade (Zhou *et al.*, 2005).

NleE and NleB from EPEC and OspZ from *Shigella* also affect the same signalling cascade by inhibiting the nuclear translocation and the activation of NF- κ B. This leads to the inhibition of IL-8 production and of the host inflammatory response (Newton *et al.*, 2010).

YopM (Cullinane *et al.*, 2008) inhibits platelet aggregation and the production of interleukin 10 by binding to α -thrombin, α 1 anti-trypsin, Rsk1 and Prk2 (Leung *et al.*, 1990; McPhee *et al.*, 2010). YopJ is not essential for virulence, rather it activates the caspase-1 cascade and induces the release of IL-1 β /IL18, balancing the effect of YopM in the host cells (Ratner *et al.*, 2016).

SptP in *Salmonella* spp. has a similar function to YopH in Mast Cells (MC). Through its phosphatase domain it dephosphorylates the protein responsible for vesicle fusion N-ethylmaleimide-sensitive factor (NSF) and Syk. This results in the blockage of MC degranulation and the blockage of neutrophils recruitment to clear the bacteria (Choi *et al.*, 2013).

Caspase inhibitors

Cells respond to infection by activating the caspase cascade that leads to pyroptosis: cell death and release of cytokines that direct the host defences to the site of infection (Ashida *et al.*, 2011). YopM in *Yersinia* spp. binds to caspase-1 and inhibits the pyroptosis, leading to an increase in virulence (Larock and Cookson, 2012).

Vacuole reorganization/autophagy evasion

Inc proteins from *Chlamydia* are secreted by the type III secretion system. IncA is homologous to SNAREs, vesicular scaffold proteins that mediate vesicle fusion, and induce vacuole re-organization by recruiting them (Delevoeye *et al.*, 2008). IcsB in concert with virA in *Shigella* and BopA of *Burkholderia pseudomallei* (Cullinane *et al.*, 2008) are linked to autophagy escape through a yet unidentified mechanism that might involve cholesterol binding and the inhibition of the VirG effector (Kayath *et al.*, 2010; Ogawa *et al.*, 2005).

Type IV effectors vary depending on the replication niche of the bacterium

The effectors secreted by the type IV secretion system aim at transforming a hostile environment into an optimal ecological niche for the bacteria. The effectors secreted by T4SS are similar in function to the T3SS effectors but in this section we will, rather, characterize them based on the pathogen's reproductive cycle: extracellular, obligate or facultative intracellular, since the requirements for each niche differ.

Extracellular pathogens

In *B. pertussis* the T4SS secretes exclusively the pertussis toxin (PT) through a Sec-mediated mechanism. Once secreted, it is able to enter the cells through receptor-mediated endocytosis (Plaut and Carbonetti, 2008). Once inside the cell, PT ADP-ribosylates a trimeric signal-transducing G protein, which locks it into an inactive state (Katada *et al.*, 1983). This results in an increase of cyclic AMP (cAMP) intracellular concentration and subsequently in the disruption of several signalling cascades required for function and recruitment of immune cells (Carbonetti, 2010).

Another well-characterized toxin of extracellular pathogens is the cytotoxin-associated antigen A (cagA) from *Helicobacter pylori*. cagA is phosphorylated (Selbach *et al.*, 2002) and then secreted through the T4SS directly into the cytoplasm of the host cell through the integrin $\alpha 5 \beta 1$ (Kwok, 2007). Once inside the cell, cagA and other effectors secreted by the T4SS mimic eukaryotic proteins leading to alterations in cell shape, signalling cascades and immune responses. They might affect phosphorylation, protein-protein interaction, ubiquitination, guanidine nucleotide exchange and other cellular processes (Gonzalez-Rivera *et al.*, 2016).

Facultative intracellular pathogens

Several bacterial species can evade host detection by hiding inside cells temporarily. One such species, *Bartonella* spp., infects erythrocytes and once inside the cells secretes up to seven *Bartonella* effector proteins (Beps) (Schulein *et al.*, 2005). Beps contain a Bep intracellular delivery (BID) domain required for secretion and function (Schulein *et al.*, 2005), a filamentation induced by cAMP (FIC) domain that post translationally modifies proteins through addition of a metabolite and an EPIYA motif, both of unknown function in *Bartonella* effectors. BepC and BepF interfere with Rho GTPases (Truttmann *et al.*, 2011) and BepG interrupts endocytosis by promoting F-actin rearrangements (Rhomberg *et al.*, 2009). BepE prevents apoptosis of its host by binding to the adenylyl cyclase and inducing cAMP production (Pulliainen *et al.*, 2012) and targets the Rho pathway, thus promoting normal migration of dendritic cells which facilitates dissemination of the infection (Okujava *et al.*, 2014).

During infection, *Brucella* species enters the host cells in an unknown site and is incorporated in *Brucella*-containing vacuoles (BCVs) that fuse with lysosomes. The fused vacuoles reach the endoplasmic reticulum (ER) where the bacteria are able to replicate and exit the cell through autophagy-like vacuoles (Pizarro-Cerdá *et al.*, 1998; Starr *et al.*, 2012). To induce toxicity in macrophages, the type IV effector VceC secreted by the T4SS induces ER

stress via the unfolded protein response (UPR) and induction of inflammation possibly by binding and inactivating the ER chaperone Bip (de Jong *et al.*, 2013). Two effectors, BtpA and BtpB, translocate into the cytoplasm of host cells and contain a toll/interleukin-1 receptor (TIR) that mediates the NF- κ B signalling pathway and both cause the failed maturation of dendritic cells (Salcedo *et al.*, 2013). TIR domains are also present in the effector proteins of other species such as TlpA of *Salmonella enterica* (Newman *et al.*, 2006), Tdp in *Yersinia pestis* (Rana *et al.*, 2011), TcpC in *Escherichia coli* (Yadav *et al.*, 2010), PdTLP in *Paracoccus denitrificans* (Low *et al.*, 2007) and TirS in *Staphylococcus aureus* (Askarian *et al.*, 2014). Recently identified effectors BspA, BspB and BspF prevent membrane trafficking and thus inhibit host protein secretion through mechanisms not yet elucidated (Myeni *et al.*, 2013).

Legionella pneumophila infects phagocytic cells such as macrophages and amoebas, and enters the cells through phagocytosis in *Legionella*-containing vacuoles (LCVs). Over 300 effectors are secreted through its type IVb secretion system (Hubber and Roy, 2010). The phosphatase SidP and the phospholipase VipD promote inactivation or removal of phosphatidylinositol-3-phosphate (PI3P) from the surface of endosomes thus blocking their fusion with the LCVs (Gaspar and Machner, 2014; Toulabi *et al.*, 2013). DrrA/SidM and RalF are GEFs that modulate the fusion of LCV with ER vesicles by activating the GTPases Rab1 and Arf1 respectively (Murata *et al.*, 2006). SidE effector family can also ubiquitinate without ATP Rab1 GTPase thus altering its function (Qiu *et al.*, 2016). LseA, YlfA and YlfB mimic the soluble NSF attachment protein receptor (SNARE) and mediate membrane fusion of LCVs to Golgi-associated vesicles (Campodonico *et al.*, 2016; King *et al.*, 2015). LepB has a Rab modulating function as a Guanidine activating protein (GAP) that disrupts vesicles trafficking in the cells (Mishra *et al.*, 2013). To escape killing by autophagy, *Legionella* releases RavZ, a protease that cleaves Atg8, a protein essential for the correct autophagosome maturation (Choy *et al.*, 2012). The effector sphingosine-1 phosphate lyase (LpSpl) also limits autophagy by blocking sphingosine biosynthesis (Rolando *et al.*, 2016). Organelles and vesicles trafficking can also be mediated by actin remodelling effectors to prevent lysosomes from fusing with the LCVs; LegK2 is a kinase that phosphorylates the ARP2/3 complex that recruits nucleation promoting factors (Michard *et al.*, 2015), and VipA accelerates actin polymerization and binds to endosomal organelles (Franco *et al.*, 2012). LubX is an E3 ligase that ubiquitinates Clk1, a protein required for optimal growth of the host (Kubori *et al.*, 2008) and AnkB (Lpp2082) and mimics the ubiquitin-binding site of parvin B, stabilizing the protein and leading to caspase-3 activation and apoptosis (Lomma *et al.*, 2010). *L. pneumophila* is unique in that it can also control the expression of host innate immune genes through methylation of chromatin by the effector RomA (Rolando *et al.*, 2013).

Obligate intracellular pathogens

Obligate intracellular pathogens require the host to remain viable throughout the infection process, thus they contain several effectors that inhibit apoptosis. In *Coxiella* species, AnkG, CaeA and CaeB all have anti-apoptotic effects through interaction with the mitochondria or the nucleus and interfering with several signalling cascades (Eckart *et al.*, 2014; Klingenberg *et al.*, 2013).

CvpA is thought to recruit clathrin from the host to allow its assembly in the *Coxiella*-containing vacuoles (CCV) (Larson *et al.*, 2013). Vesicular fusion leads to the formation of large CCVs where the bacterium can modulate and it is modulated by two effectors: cirA

which stimulates the activity of the Rho GTPase (Weber *et al.*, 2016) and CvpB that prevents the degradation of PI3P leading to membrane aggregation and fusion (Martinez *et al.*, 2016).

Type V effectors have multiple functions

Type V secreted exoproteins are mainly involved in adherence, but a few are also involved in pathogenicity. The IgA protease in *Neisseria* spp. is an effector that cleaves the region between the Fab and the Fc domains of the IgA antibody thus preventing clearance of the bacterium by the immune cells (Plaut *et al.*, 1975). Moreover the Fab fragment is still capable of binding to the antigen on the surface of the bacterium, thus masking it from other immunoglobulins (Mansa and Kilian, 1986).

Another important autotransporter (type Va) required for host manipulation by *Shigella* is IcsA (VirG), an effector that mediates actin-based motility (ABM) (Lett *et al.*, 1989; Suzuki *et al.*, 1995). ABM is the mechanism by which the bacterium, having escaped from the vacuole onto the cytosol of the host, rearranges actin filaments to form a comet-like tail and promote bacterial movement (Goldberg, 2001). IcsA passenger domain is released from the surface by the protease IcsP (Shere *et al.*, 1997) and it is then free to bind N-WASP, an actin regulator protein. Arp2/3 complex is activated by N-WASP and leads to actin polymerization that allows the bacterium to gain propulsion (Egile *et al.*, 1999). *Burkholderia* species require BimA, a trimeric autotransporter for ABM, but the mechanism of action differ between species. *B. thailandensis* BimA activates Arp2/3 complex that leads to branched actin filaments as with the IcsA of *Shigella*. BimA in *B. pseudomallei* and *B. mallei* instead acts independently of Arp2/3, rather it acts like a human Ena/VASP protein leading to the nucleation, elongation and formation of bundles of F-actin, rather than branching as in the case of BtBimA (Benanti *et al.*, 2015). LepA from *Pseudomonas aeruginosa* is a two-partner secreted protease that is capable of activating through cleavage PAR-1, -2 and -4 leading to increased NF- κ B and thus modulation of the immune response (Kida *et al.*, 2008).

Type VI effectors are mainly anti-bacterial but some have an effect on the eukaryotic hosts

The anti-bacterial function of the T6SS has been very well characterized and has been the focus of most research in the field. Little is known about its function in host-pathogen interactions. The presence of T6SS on the surface of pathogens has been linked to increased virulence, but the effect might be secondary due to its ability to outcompete other species. Nevertheless several effects in the eukaryotic host have been linked to the surface expression of T6SS and a few effectors have been recently identified (Hachani *et al.*, 2016).

The spike protein required for piercing the membrane of the host cell is very often also a toxin that contributes to pathogenicity. The VgrG5 in *Burkholderia* spp. mediates membrane fusion of neighbouring macrophages and the creation of multinucleated giant cells, a process that is essential for cell-to-cell spread and ultimately pathogenicity (Schwarz *et al.*, 2014; Toesca *et al.*, 2014). *Aeromonas hydrophila* secretes, in the eukaryotic host, VgrG1, the spike protein that contains an ADP ribosyltransferase site. VgrG1 ribosylates actin, preventing filament formation and leading to cell rounding and death of the host (Suarez *et al.*, 2010). *Pseudomonas aeruginosa* is a facultative intracellular pathogen that secretes VgrG2b into non-phagocytic cells to promote invasion. VgrG2 interacts with the γ -tubulin ring complex (γ TuRC) and precedes microtubule- and actin-dependent phagocytosis by

the host cell (Sana *et al.*, 2015). *Vibrio cholerae* secretes into the cytoplasm of the host cell the spike protein VgrG1, which not only serves as a puncturing device, but also has an actin cross-linking domain similar to the MARTX toxin of type I secretion systems (Pukatzki *et al.*, 2007). The cross-linking action causes the permanent polymerization of actin and eventually leads to cell death (Pukatzki *et al.*, 2007)

Vibrio cholerae also secretes into the extracellular space VasX, an effector with a pleckstrin homology (PH) domain. VasX binds to phosphoinositides on the eukaryotic host membrane and disrupts signalling (Miyata *et al.*, 2011). The two effectors PldA and Pldb, secreted by two different TYPES of T6SS in *Pseudomonas aeruginosa*, exhibit a phospholipase D (PLD) activity. PldA and Pldb target the lipids of competing bacteria and when injected into the cytosol of a eukaryotic host they bind to Akt1 and Akt2 to induce PI3K activation, a common mechanism for pathogen host invasion (Jiang *et al.*, 2014). *Yersinia pseudotuberculosis* secretes the effector YezP that is unique in its capacity to bind Zn²⁺ and thus provides the bacterium at the same time with a potential source of Zn²⁺ and protection against reactive oxygen species that are released by macrophages (Wang *et al.*, 2015).

Many effectors are known, but many more need to be identified to understand better the influence of T6SS in host–pathogen interactions. The identification of a secretion signal common to all the effectors would in all likelihood increase the identification of the effectors that have shown unique mechanisms of action compared to other secretion systems.

Type VII secreted proteins elicit a plethora of effects on the host

The expression of type VII secretion systems is not always associated with pathogenicity; rather they can sometimes be associated with development (Fyans *et al.*, 2013), DNA transfer (Coros *et al.*, 2008), and have a general role in physiology (Serafini *et al.*, 2009). The ESX-1 system has been mainly associated with phagosomal rupture as a means to escape phagocytosis by macrophages (van der Wel *et al.*, 2007), inflammation (Majlessi *et al.*, 2005), apoptosis and bacterial dissemination (Simeone *et al.*, 2012). EsxA/EsxB, a heterodimer of secreted effectors, creates pores in the lipid bilayers in red blood cells and thus allow the bacterium to escape phagocytosis (Smith *et al.*, 2008). In *Staphylococcus aureus* EsxA acts as an inhibitor of apoptosis and EsxB prevents the exit of the bacterium from the host cells to evade detection by the immune system (Korea *et al.*, 2014). The ESX-5 system secretes mainly substrates of the PE and PPE families that modulate the inflammatory response in the host (Bottai *et al.*, 2012). EspB, released by ESX-1, is composed of two domains homologous to PE and PPE and is involved in the dissemination of *M. tuberculosis* in mice (Ohol *et al.*, 2010).

The complex of EsxG and EsxH is secreted by ESX-3 in *Mycobacterium tuberculosis* and it targets the host factor Hrs, a component of the ESCRT complex. Secretion of the complex thus prevents the fusion of the mycobacteria-containing vacuoles with the lysosomes through the impairment of the ESCRT complex (Mehra *et al.*, 2013).

A better understanding of the molecular details of the effectors action on the host and the discovery of new effectors will greatly aid in the development of new drugs targeted at the elusive mycobacteria and other Gram-negative pathogens.

Gingipains are the effectors secreted by type IX secretion systems

Gingipains are the main effectors secreted by type IX secretion systems and they are Arg-X and Lys-X specific proteases that accumulate around the cell surface (Narita *et al.*, 2014).

Their targets include cytokines and complements that hinder the normal inflammatory response and cell surface molecules to prevent clotting (Fitzpatrick *et al.*, 2009).

Recent bioinformatics analysis of the genome of the *Bacteroidetes* phylum to which *Porphyromonas gingivalis* belongs, showed that the CTD is present in hundreds of proteins with different functions: proteases, glycosidases, adhesins, haemagglutinins and internalins (Veith *et al.*, 2013). Future work will be needed to understand better the effectors secreted by type IX secretion systems and their mechanism of pathogenesis.

Conclusion

Secretion systems are fascinating machines that have finely evolved to target eukaryotic and prokaryotic adversaries. The study of secretion systems has been accelerating in the last few years, thanks to advances in the understanding of the structural assembly of these well-evolved and efficient machineries of protein translocation. A lot is now known about how they assemble and the mechanism by which they are capable of secreting a plethora of effectors.

In recent years the emergence of antibiotic resistance in highly pathogenic bacteria has led to the exploration of new targets for the development of antimicrobial agents. Secretion systems would be an ideal target since they are mostly expressed in pathogenic bacteria and would leave the healthy commensal microbiota intact. Much work has been done to understand these systems, but more has to be done to be able to fully exploit them as a means to control the most dominant form of life.

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