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REVIEW

Protein secretion systems in bacterial pathogens

Li XU (✉)[1], Yancheng LIU (✉)[2]

[1] *Weill Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY 14853, USA*
[2] *Department of Microbiology and Immunology, Cornell University, Ithaca, NY 14853, USA*

Abstract **Many bacterial pathogens utilize specialized secretion systems to deliver virulence factors into the extracellular milieu. These exported effectors act to manipulate various processes of targeted cells in order to create a suitable niche for bacterial growth. Currently, seven different types of secretion system have been described, of which Type I – VI are mainly present in Gram-negative bacteria and the newly discovered Type VII system seems exclusive to Gram-positive species. This review summaries our current understanding on the architecture and transport mechanisms of each secretion apparatus. We also discuss recent studies revealing the roles that these secretion systems and their substrates play in microbial pathogenesis.**

Keywords bacterial pathogen, secretion system, virulence factors

Introduction

Protein secretion is a fundamental process that takes place in all type of cells where selected proteins are transported from within the cell to the extracellular milieu. This process is a critical step in pathogenesis and interbacterial competition for many bacterial pathogens. However, transporting macromolecules across bacterial envelope is not an easy task. The cell envelope of Gram-negative bacteria is composed of two membrane layers and a periplasmic space in-between, whereas the envelope of Gram-positive bacteria consists of a cytoplasmic membrane followed by a very thick peptidoglycan layer. To overcome these complex barriers, bacteria have evolved sophisticated membrane-bound machineries called secretion systems to facilitate protein transport. Here we provide a general overview on the seven secretion systems described so far, with emphases on their structural organization and their functions during infection.

Type I Secretion System

The Type I Secretion System (T1SS) is a one-step pathway during which substrates are secreted by a three-component protein translocon that spans both the inner and outer membranes, as well as the periplasmic space. The complex is composed of an ATPase binding cassette (ABC) transporter, a membrane fusion protein and a pore-forming outer membrane protein (Delepelaire, 2004; Holland et al., 2005). The inner membrane located ABC transporter serves as an energy pump for protein transport at the expense of ATP hydrolysis. The membrane fusion protein contains a small N-terminal cytoplasmic domain, a lipid binding domain for anchoring to the inner membrane, and a large periplasmic domain that connects the inner and outer membrane components. The outer membrane protein normally contains a transmembrane beta-barrel domain which forms a water filled channel through the outer membrane. Substrates of the T1SS are recognized by a C-terminal signal sequence, which is not cleaved during secretion. A variety of proteins have been reported to be secreted by this system, including proteases, phosphatases, glucanases, nucleases, lipases and toxins. Those exported proteins vary greatly in size, ranging from 19 kDa to 800 kDa, highlighting the remarkable flexibility of the translocon (Delepelaire, 2004; Holland et al., 2005).

The T1SS is first identified in uropathogenic *E. coli* (UPEC) for the secretion of an extracellular RTX (repeats in toxin) known as hemolysin A, which is involved in the pathogenicity of UPEC (Welch et al., 1981; Mackman and Holland, 1984). The hemolysin A (HlyA) T1SS is composed of three components: an ABC transport HlyB, a membrane

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Correspondence: [a]Li XU; [b]Yancheng LIU
E-mail: [a]lx83@cornell.edu; [b]yl949@cornell.edu

fusion protein HlyD and a pore-forming outer membrane protein TolC (Holland et al., 2005). Similarly, adenylate cyclase toxin (CyaA) of *Bordetella pertussis* is transported by the T1SS and serves as a primary virulence factor that inhibits innate immune responses and promotes bacterial colonization. The cyaA operon encodes an ABC transporter CyaB, a membrane fusion protein CyaD and a pore-forming protein CyaE (Shrivastava and Miller, 2009). Moreover, the T1SS is also encoded by other pathogenic Gram-negative bacteria such as *Vibrio cholera, Pseudomonas aeruginosa* and *Salmonella enterica* for the transport of RTX toxins, proteases and other virulence factors (Thomas et al., 2013; Wille et al., 2014). *Legionella pneumophila* also expresses a T1SS called Lss system, encoded by the lssXYZABD locus, among which LssB is an ABC transporter and LssD possibly constitutes the outer membrane pore (Jacobi and Heuner, 2003). However, this secretion system appears to be dispensable for bacterial virulence and no substrate has been identified yet (Jacobi and Heuner, 2003).

Type II Secretion System

Unlike the T1SS, the Type II Secretion System (T2SS) secretes proteins in two separate steps. First, proteins are translocated across the inner membrane through the general secretion system Sec or Tat and then the proteins are folded in the periplasm and subsequently transported across the outer membrane through the T2SS.

The general secretion system Sec functions to translocate many unfolded proteins across the bacterial cytoplasmic membrane. This system is composed of a molecular chaperon SecB which helps to stabilize unfolded substrates, an ATPase SecA which serves as a motor to drive translocation, a transmembrane channel comprised of the heterotrimeric complex SecYEG, and several accessory proteins (Robinson and Roy, 2006). Proteins destined for the Sec secretion system are labeled with an N-terminal signal sequence of about 20~30 amino acids including a positively charged N terminus followed by a stretch of hydrophobic residues, and an uncharged polar C-terminal region. This signal peptide is cleaved off by an inner membrane signal peptidase once the translocated protein emerges in the periplasm (Lammertyn et al., 2004; Geukens et al., 2006).

Different from the Sec system, the Tat (twin arginine translocation) pathway has the ability to translocate folded proteins. The signal peptide for the Tat system shares the same overall amino acid composition of that for the Sec system, with the exception that it possesses a highly conserved Arg-Arg motif followed by two uncharged residues near the N terminus (Berks, 1996). This signal peptide is recognized by an inner membrane complex, TatBC, which then targets it to a channel formed by TatA and allows the translocation through the cytoplasmic membrane. The signal peptide is also removed by a peptidase when translocation is complete (Voulhoux et al., 2001; Berks et al., 2005).

Both Sec and Tat systems are present in *L. pneumophila*. The Tat pathway has been shown to be important for growth under iron stress conditions, biofilm formation and intracellular replication of the bacteria in both human macrophages and *Acanthamoeba castellanii* (De Buck et al., 2005; Rossier and Cianciotto, 2005). Substrates secreted by this pathway include phospholipase C, cytochrome c oxidase, LvrE, and components of the flagellar biosynthesis system (De Buck et al., 2004; De Buck et al., 2005; Rossier and Cianciotto, 2005; De Buck et al., 2008). On the other hand, more than 500 potential Sec substrates have been identified based on bioinformatic studies suggesting that the Sec system plays a major role in the transport of *L. pneumophila* proteins across the inner membrane (DebRoy et al., 2006).

Once in the periplasm and properly folded courteous of general or specific chaperones, proteins are recognized and secreted into the extracellular milieu by the T2SS. The T2SS consists of at least 12 proteins: T2SC and T2SD make up the outer membrane complex (secretin) and T2SEFGLMO together form a pilus-like structure in the inner membrane which is thought to function as a piston to propel substrates through the secretin (Filloux, 2004; Johnson et al., 2006). In the case of *L. pneumophila*, proteins can be further exported into the extracellular milieu through a type II secretion system (Lsp) encoded on the genome. The Lsp system consists of 12 components, including an outer membrane secretin LspD, pseudopili components LspFGHIJK, a pre-pilin peptidase PilD, a coupling protein LspC, an ATPase LspE, and inner membrane accessory proteins LspFLM (Liles et al., 1999; Lammertyn and Anné, 2004; Cianciotto, 2009). More than 20 proteins have been identified as Lsp substrates which account for a variety of the extracellular enzymatic activities of *L. pneumophila* (Cianciotto, 2009). A functional Lsp secretion apparatus itself is required for growth of *L. pneumophila* in amoebae hosts, macrophages and A/J mice (Hales and Shuman, 1999; Rossier et al., 2004). However, most of the substrates except the zinc metalloprotease ProA are dispensable for bacterial intracellular replication, suggesting that functional redundancy and host specificity commonly exist among the substrates (DebRoy et al., 2006; Rossier et al., 2008).

Since its first discovery in *Klebsiella* in the late 1980s (d'Enfert et al., 1987), the T2SSs has been identified in many pathogenic and environmental strains (Nivaskumar and Francetic, 2014). These bacteria also utilizes T2SS to transport degradation enzymes, such as lipases, phosphatases, cellulases and amylases, to the surrounding milieu for the generation and acquisition of nutrient (Cianciotto, 2005). Some bacterial toxins are also found secreted by the T2SS, such as the heat-labile enterotoxin from enterotoxigenic *E. coli,* and the cholera toxin from *V. cholerae* (Sandkvist et al., 1997; Tauschek et al., 2002). The substrate recognition sequence for the T2SS is still unclear, however, it is

speculated that the signal is coded in distal regions of the primary sequence which can only be brought together when protein is properly folded (Sandkvist, 2001; Filloux, 2004).

Type III Secretion System

The Type III Secretion System (T3SS) is first identified in the early 1990s in *Yersinia* for delivering Yop proteins into host cells independent of known secretion systems (Michiels et al., 1991). The T3SSs are ancestrally related to the bacterial flagella export machinery (Journet et al., 2005; Cornelis, 2006). It has the ability to translocate substrates across three biologic membranes (bacterial inner and outer membrane and host cell plasma membrane) using a characteristic needle-like structure termed the injectosome (Blocker et al., 2001; Cornelis, 2006). The T3SS is composed of approximately 20 proteins. The architecture of the T3SS resembles that of the flagella basal body with an extended needle attached to two joined ring-like structures embedded in the bacterial double membranes (Cornelis, 2006; Galán and Wolf-Watz, 2006). The length of the needle structure is about 60 nm and the inner diameter is estimated to be 2–3 nm (Cornelis, 2006).

Unlike T2SSs, which are prevalent in all classes of bacteria, T3SSs are present exclusively in bacterial strains that live in close association with their eukaryotic hosts (Burkinshaw and Strynadka, 2014). The T3SSs are essential for the virulence of many plant and animal pathogens including *Pseudomonas* spp., *Erwinia* spp., *Salmonella* spp., *Yersinia* spp., *Shigella* spp., and some pathogenic *E. coli* species (Cornelis, 2006). Other symbiotic bacteria such as *Rhizobium* spp., *Bradyrhizobium* spp. and *Mesorhizobium loti* also contain T3SSs (Marie et al., 2001). For example, *Salmonella* has two T3SSs encoded by two pathogenicity islands: SPI1 and SPI2 (Galán, 2001). Although coordination of these two T3SSs has been reported (Deiwick et al., 1998), they mainly response to different environmental cues and function at different stages of infection. The SPI-1 T3SS is activated and required upon the initial contact and invasion of host cells. It translocates effector proteins targeting various host pathways, such as cytoskeleton rearrangement (Zhou et al., 1999), ubiquitination (Zhang et al., 2006) and modulation of Rho GTPase signaling (Rodríguez-Escudero et al., 2011) to promote bacterial entry. On the contrary, the SPI-2 T3SS is only induced after the bacterium is phagocytosed into the host cells. It translocates many SPI-2 effectors that are able to covalently modify host proteins (Figueira and Holden, 2012). Unlike bacterial toxins, protein effectors delivered by T3SSs often have more sophisticated modes of actions: reversibly, simultaneously and sometimes antagonistic with each other to establish a fine-tuned control of various host processes for bacterial proliferation (Burkinshaw and Strynadka, 2014).

Proteins recognized by the T3SS have a signal sequence located within the N-terminal amino acids. Although the exact nature of the signal is not clear, it is believed that T3SS substrates have a separate translocation signal located downstream near the secretion signal (Stebbins and Galán, 2001; Galán and Wolf-Watz, 2006). In addition, these proteins often require chaperones for delivery through the T3SS which are typically small acidic proteins. These chaperones recognize the N-terminal domain of their cognate substrates and keep them in a secretion and translocation competent form (Birtalan et al., 2002; Page and Parsot, 2002; Dai and Zhou, 2004; Higashide and Zhou, 2006).

Type IV Secretion System

The Type IV Secretion System (T4SS) is evolutionarily related to the bacterial conjugation apparatus (Backert and Meyer, 2006). The T4SS spans both membranes and directly transports macromolecules including DNA or protein from the bacterial cytoplasm to other bacteria or the host cell cytosol (Christie et al., 2005). Depending on sequence homologies, the T4SS can be further divided into two major classes, IVA such as the VirB/D4 system in *Agrobacterium tumefaciens,* and IVB, such as the Dot/Icm system in *L. pneumophila* (Backert and Meyer, 2006).

The VirB/D4 system is by far the most well characterized T4SS and serves as a prototype for the T4SSs. *A. tumefaciens* causes tumors in plants by using its T4SS to translocate oncogenic T-DNA and accessory proteins into plant cells. The T4SS of *A. tumefaciens* is composed of 11 VirB proteins, VirB1–11, and one VirD4 protein (Christie and Cascales, 2005). The VirB/D4 translocon is made up of three distinct complexes: core complex, pilus, and coupling protein. The core complex consists of VirB6–10 and constitutes the transfer channel across the inner and outer membranes. The pilus is composed of the major component VirB2 and the minor component VirB5, and it is linked to the core complex protein by interacting with VirB7 which acts to contact the host cell plasma membrane. Coupling protein VirD4 recognizes the substrates by a C-terminal localized signal and delivers them to the transfer channel. Three ATPases, VirB4, VirB11 and the coupling protein VirD4, energize the whole machinery by hydrolyzing ATP (Atmakuri et al., 2004; Cascales and Christie, 2004).

L. pneumophila encoded two T4SSs in its genome. The type IVA secretion apparatus Lvh is not required for bacterial infection under normal conditions. However, the *lvh* mutants display significant defect in entry and intracellular replication when the bacteria are pretreated with low temperature, water stress, or amoeba encystment (Ridenour et al., 2003; Bandyopadhyay et al., 2007). The type IVB secretion system Dot/Icm plays a central role for the virulence of *L. pneumophila* in both amoebae and macrophage hosts. A functional Dot/Icm apparatus has been shown to be required for the optimal growth throughout *L. pneumophila* intracellular life cycle (Liu et al., 2008). This T4SS system is encoded by 26 *dot* or *icm* genes organized in two different regions on

the chromosome, with *dotABCD* and *icmVWX* located in region I and other 19 genes located in region II (Segal et al., 1998; Vogel et al., 1998;Matthews and Roy, 2000). Because of its ability to support plasmid transfer among bacterial cells, the Dot/Icm system is thought to be ancestrally related to bacterial DNA conjugative transfer system. In support of this notion, 18 *dot/icm* genes show similarity to those of the IncI conjugal plasmids ColIB-P9 from *Shigella flexneri* and R64 from *S. enteric* (Komano et al., 2000). Accumulating evidence demonstrates that the Dot/Icm system has the ability to penetrate the entire bacterial cell envelop and host cell membrane although the exact architecture of the translocon remains unsolved. Nevertheless, recent studies suggests that five proteins constitute the core complex, whereby DotFG make up the inner membrane pore and DotH forms the outer membrane channel with the help of DotCD (Vincent et al., 2006). Three Walker box motif containing proteins, DotBLO, are hypothesized to provide the energy to the translocation machinery (Sexton et al., 2004; Buscher et al., 2005). A protein with the pore-forming activity, IcmQ, may have the ability to form the translocation pore on host cell membranes in the presence of its chaperone IcmR (Duménil and Isberg, 2001). Similar to the T3SS, the Dot/Icm employs cytoplasmic chaperones, such as IcmS and IcmW, for the delivery of at least a subset of substrates to the transporter (Coers et al., 2000; Zusman et al., 2003; Bardill et al., 2005).

Protein effectors translocated by the Dot/Icm system are essential for the virulence of *L. pneumophila* during infection (Xu and Luo, 2013). Many genetic, bioinformatic and cell biological screenings were designed to screen for novel effectors and almost 300 substrates have already been experimentally confirmed to be substrates of the Dot/Icm system (Luo and Isberg, 2004; Zhu et al., 2011). These effectors are shown to be involved in manipulation of many host processes, such as phagosome maturation (Xu et al., 2010; Gaspar and Machner, 2014), translational initiation (Shen et al., 2009), membrane trafficking (Nagai et al., 2002; Machner and Isberg, 2006; Murata et al., 2006; Liu and Luo, 2007) and innate immunity (Hubber and Roy, 2010; Zhu et al., 2013), although mitochondria recruitment during early infection by *L. pneumophila* seems to be independent of the Dot/Icm system (Sun et al., 2013).

Type V Secretion System

Similar to the T2SS, the Type V Secretion System (T5SS) also secrets proteins in a two-step process (Henderson et al., 2004). Substrates of T5SS possess an N-terminal Sec signal and are translocated across the inner membrane by the Sec system. These substrates either mediate the translocation across the outer membrane by themselves or through a pore formed by their cognate partners. Thus T5SSs can be further divided into two subtypes: the autotransporters and two-partner secretion systems (van Ulsen et al., 2013).

For the autotransporters, the substrate contains an N-terminal Sec signal followed by a passenger domain, and a C-terminal β-barrel domain which is able to form a translocation pore after inserting into the outer membrane (Henderson and Nataro, 2001). After secreted through the translocation pore from N to C terminus in a hairpin conformation, the passenger domains are cleaved proteolytically at the cell surface. The passenger domain may either remain attached to the outer membrane via non-covalent interaction or be released into the extracellular milieu (van Ulsen et al., 2003; Henderson et al., 2004; Thanassi et al., 2005). Unlike autotransporters, the passenger module and the pore-forming module in the two-partner secretion system are expressed as two separate proteins. After both proteins are delivered into the periplasm by the Sec system, the pore-forming protein forms a 16-stranded β-barrel in the outer membrane, recognizes the passenger protein by its N-terminal conserved domain and facilitate the translocation of latter across the outer membrane. Similarly, the secreted protein is then either released or still attached to the outer membrane by non-covalent linkage (Henderson et al., 2004; Thanassi et al., 2005).

The T5SS, as a relatively simple secretion system, has been found in a large variety of Gram-negative bacteria including both environmental and pathogenic species. The substrates of T5SSs including adhesions, proteases and toxins that are usually important for bacterial survival and virulence (van Ulsen et al., 2013). For example, VacA from *Helicobacter pylori* is an autotransporter protein that contains a signal sequence, a passenger domain and a transporting domain. Asa multifunctional bacterial toxin, VacA causes severe vacuolation of eukaryotic cells, alters mitochondrial membrane permeability and also inhibits the proliferation of T cells (Cover and Blanke, 2005). Additionally, the autotransporters IcsA from *Shigella* spp. is indispensable for pathogenesis of bacterial infection by promoting actin-based motility and icsA mutants are greatly attenuated and fail to cause *Shigella*-mediated diarrhea (Bernardini et al., 1989). Moreover, surface protein adhesins transported by the two-partner secretion system, such as the HWM adhesin from *Hemophilus influenzae* and filamentous haemagglutinin from *B. pertussis*, are involved in facilitating the microcolony and biofilm formation of pathogens in upper respiratory track (St Geme and Yeo, 2009; Serra et al., 2011).

Type VI Secretion System

The Type VI Secretion System (T6SS) is the most recently identified protein transport system present in Gram-negative bacteria (Mougous et al., 2006; Pukatzki et al., 2006). Although it is initially discovered in *V. cholerae* and *P. aeruginosa*, database search suggests that T6SS is widespread among almost 100 different bacterial species including *Rhizobium leguminosarum*, *Yersinia pestis*, *Salmonella enterica*, and *E. coli*. (Filloux et al., 2008). This secretion system injects protein substrates from the bacterial cytoplasm into recipient eukaryotic or bacterial cells in a one-step, cell-

contact dependent manner. By targeting various aspects of the recipient cell, these effector proteins function to contribute to both pathogenesis and inter-bacterial competition.

The T6SS is a multi-component secretion complex encoded by a minimal set of 13 core component genes (Zheng and Leung, 2007; Boyer et al., 2009; Zheng et al., 2011; Lin et al., 2013). A basic functional T6SS comprises two distinct complexes: a cytosolic cylinder structure that resembles the tail of a contractile bacteriophage, and a membrane-associated structure which anchors the cytosolic tail to the cell envelop (Kanamaru, 2009; Leiman et al., 2009; Basler et al., 2012; Silverman et al., 2012). The membrane-associated complex is made up of four proteins (TssJ, TssK, TssL and TssM). Among them, two proteins (TssL and TssM) are homologous to components (IcmH and IcmF) of the T4SS, suggesting possible divergent evolution of the two secretion systems. The phage tail-like complex consists of three subassemblies: a sheath formed by TssB-TssC, an inner tube made by Hcp, and a cytoplasmic baseplate-like structure composed of VgrG, TssE, and other possible components (Bönemann et al., 2009; Kanamaru, 2009; Leiman et al., 2009; Lossi et al., 2011; Basler et al., 2012).The current model suggests that during secretion the energy generated by sheath contraction propels protein substrates through the inner tube, and then a tip structure formed by VgrG punctures the target membrane and guides substrate delivery. Interestingly, VgrG and Hcp were readily detected in the extracellular milieu, suggesting additional roles that these proteins could play as T6SS effectors (Mougous et al., 2006; Pukatzki et al., 2006; Murdoch et al., 2011; Silverman et al., 2012; Zhou et al., 2012; Shneider et al., 2013; Silverman et al., 2013). Besides these 13 core components, the T6SS also consists of various numbers of accessory proteins (Cascales, 2008; Murdoch et al., 2011). These accessory components usually function to facilitate proper assembly of the secretion apparatus or play roles in regulation or host adaptation (Aschtgen et al., 2010; Silverman et al., 2011; Coulthurst, 2013).

Substrates translocated by the T6SS machinery have various biochemical activities. Mekalanos and colleagues have shown that the C-terminal domain of *V. cholerae* VgrG-1 is able to cause actin cross-linking in both amoeba and mammalian hosts (Pukatzki et al., 2007; Ma et al., 2009). In the mouse model, this actin-cross-linking activity led to a diarrheal response and contributed to bacterial growth in the intestine (Ma and Mekalanos, 2010). Another VgrG homolog, VgrG-1 from *Aeromonas hydrophila* has a C-terminal VIP-2 domain and shows an ADP-ribosylation activity toward actin in mammalian cells (Suarez et al., 2010). Besides targeting eukaryotic cells,T6SS effectors can also be delivered into competitor bacterial cells (Hood et al., 2010). Two groups of antibacterial substrates, the T6SS amidase effector (Tae) proteins and the T6SS glycoside hydrolase effector (Tge) proteins, function to degrade a common target-cell wall structural component peptidoglycan (Fischetti, 2008; Russell et al., 2012; Brooks et al., 2013; Srikannathasan et al., 2013; Whitney et al., 2013). Lipids component on the bacterial membrane is another common target for a group of T6SS lipase effector (Tle) proteins (Russell et al., 2013). In addition, recent studies provide evidence that T6SS also transport nucleases to degrade nucleic acids in recipient cells (Poole et al., 2011; Fritsch et al., 2013; Koskiniemi et al., 2013; Wenren et al., 2013).

Type VII Secretion System

The paradigm of Type VII Secretion System (T7SS), the ESX-1 multi-protein complex, was first identified in the notorious pathogen *Mycobacterium tuberculosis* (Hsu et al., 2003; Stanley et al., 2003;Guinn et al., 2004). *M. tuberculosis* mutant lacking the secretion system fails to replicate in macrophages and shows growth defects in the early phase of mouse infection (Stanley et al., 2003). More interestingly, the system is lost in the avirulent vaccine strain *M. bovis* bacillus Calmette-Guerin (BCG) and artificial deletion of the gene cluster encoding the ESX-1 in *M. tuberculosis* attenuates the pathogen (Mahairas et al., 1996; Pym et al., 2002; Lewis et al., 2003; Pym et al., 2003).

Despite intense research efforts by many groups, our understanding of the molecular mechanisms of ESX-1 is still in its infancy. The system consists of a putative cytoplasmic membrane spanning channel which is composed of at least five components EccB, EccC, EccD, EccE and MycP (Stanley et al., 2003; Brodin et al., 2006; Houben et al., 2012). EccD, a highly hydrophobic protein with 11 predicted transmembrane domains, together with EccB and EccC, may constitute the central path for substrate translocation, whereas EccE and the serine protease MycP are likely localized at the periphery of the channel (Stanley et al., 2003; Houben et al., 2012). The FtsK/SpoIIIE type ATPase EccC may serve dual roles in the secretion process by recognizing substrates as well as providing the energy for their transport across the cytoplasmic membrane (Converse and Cox, 2005; Champion et al., 2006). Two accessory components of the system, the chaperon-like protein EspG and the AAA+ type ATPase EccA, are localized in the cytosol and may also involve in substrate binding and energy production (Daleke et al., 2012; Wagner et al., 2014).

Two families of proteins are found to be secreted by the T7SS: the WXG100 proteins with a highly conserved Trp-Xaa-Gly motif and have an average size of 100 amino acids, and the PE/PPE proteins characterized by the presence of the Pro-Glu and Pro-Pro-Glu repeats near their N-termini (Sørensen et al., 1995; Pallen, 2002; Abdallah et al., 2006; Abdallah et al., 2009; Daleke et al., 2011; Ilghari et al., 2011). An intriguing phenomenon about T7SS substrates is that they are always secreted in pairs and dependent on each other for secretion. For example, the substrate pair ESAT-6 and CFP-

10 must form a tight heterodimer before EccC recognizes the YxxxD/E secretion signal located at the C terminus of CFP-10 (Champion et al., 2006). One possible explanation for this mutual dependence is that the substrates may function as part of the secretion system themselves (Ize and Palmer, 2006).

Future perspectives

Recent years have seen significant progress in the characterization of various protein secretion systems in bacterial pathogens. However, many important questions remain to be answered. How is ATP binding and hydrolysis converted to energy required for substrate delivery in T3SSs and T4SSs? How does VirB/VirD4 in *A. tumefacien* establish productive contact and transport substrates to diverse target cells including bacterium, plant and human? What is the energy force driving the translocation of T5SSs? The translocation channel of autotransporters formed in the β-barrel domain is very narrow (~1 nm) (Oomen et al., 2004). How do the stably folded proteins get through this tiny channel? How are T6SSs activated when the donor bacteria sense the recipients? How is a substrate selected for secretion through the T6SS? No Sec-dependent signal has been detected on the N-terminal of the substrates suggesting that the T6SS is Sec independent, but the exact nature of the recognition signal remains unknown (Pukatzki et al., 2006). Perhaps more attractively, is it possible to develop novel antimicrobial therapeutics based on the antibacterial potential of the T6SS? Regarding to the Esx systems in mycobacteria, although recent studies have shed lights on the mechanism of translocation across the cytoplasmic membrane, how substrates are subsequently transported over the mycolic-acid-rich outer membrane remains a mystery. Furthermore, biochemical functions of most of the T7SSs and their roles in virulence are still unknown.

Finally, while we keep revealing the fascinating details of the seven secretion systems, one question always surfaces: are there more novel secretion systems yet to be discovered? Most likely the answer will be yes. Recently, new secretion systems have been described in some Gram-postitive pathogens such as *Staphylococcus aureus, Bacillus anthracis* and *Listeria monocytogenes* (Burts et al., 2005; Burts et al., 2008; Garufi et al., 2008; Anderson et al., 2011; Baptista et al., 2013). Those systems also transport WXG100 family proteins and are therefore deemed as "T7SS-like." However, except the FtsK/SpoIIIE-type ATPase, they contain no homologs to the canonical T7SS components identified in mycobacteria. Moreover, these low-GC bacteria maintain very different membrane structures which completely lack the outer mycolic-acid-rich membrane as compared to mycobacteria. Further investigation into the secretion systems of these pathogens will likely reveal a distinctive mechanism of protein secretion.

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Compliance with ethics guidelines

Li Xu and Yancheng Liu declare that they have no conflict of interest. This article does not contain any studies with human and animal subjects performed by any of the authors.

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