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Minireview

The type III secretion injectisome, a complex nanomachine for intracellular 'toxin' delivery

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Abstract

The type III secretion injectisome is a nanomachine that delivers bacterial proteins into the cytosol of eukaryotic target cells. It consists of a cylindrical basal structure spanning the two bacterial membranes and the peptidoglycan, connected to a hollow needle, eventually followed by a filament (animal pathogens) or to a long pilus (plant pathogens). Export employs a type III pathway. During assembly, all the protein subunits of external elements are sequentially exported by the basal structure itself, implying that the export apparatus can switch its substrate specificity over time. The length of the needle is controlled by a protein that it also secreted during assembly and presumably acts as a molecular ruler.

Keywords: microbial pathogenesis; protein secretion; virulence; *Yersinia*.

Introduction: type III secretion

More than 25 different species of Gram-negative bacteria that interact with live animals, plants, nematodes or insects are endowed with a special protein export pathway called type III secretion (T3S). The T3S apparatus or injectisome allows bacteria docked at the surface of a cellular membrane to deliver effector proteins across this membrane, either in the cytosol or at the cytosolic face of the membrane (Cornelis and Wolf-Watz, 1997; Galan and Collmer, 1999; Cornelis and Van Gijsegem, 2000). Animal pathogens generally deliver from approximately six to more than 20 different proteins called effectors. These effectors display a large repertoire of biochemical activities and modulate the function of crucial host regulatory molecules. This allows bacteria to invade non-phagocytic cells or inhibit phagocytosis by phagocytes, downregulate or promote pro-inflammatory responses, induce apoptosis, prevent autophagy, or modulate intracellular trafficking (Mota and Cornelis, 2005). In plant cells, T3S effectors suppress host defenses but they also often inadvertently affect the pathogen by eliciting plant defenses (Alfano and Collmer, 2004; Grant et al., 2006). Bacteria endowed with T3S often have more than one system, that plays its specific role at a different stage of the infection, or possibly in a different host.

Approximately 25 proteins are needed to build the injectisome. Most of these are structural components but some are ancillary factors that are only involved during the assembly process and either shed afterwards (e.g., the molecular ruler) or kept in the cytosol (e.g., chaperones). In *Yersinia*, these proteins are called YscA to YscY (Yop secretion). The *Yersinia* letter code is used to designate the conserved injectisome proteins in many systems (Bogdanove et al., 1996) but not in the other archetypal systems from *Salmonella enterica* SPI-1 and from *Shigella flexneri*. In contrast to the large diversity observed among effectors, the injectisomes themselves are more conserved. In particular, a core of nine proteins (YscC, J, N, Q, R, S, T, U, V) are highly conserved in all known injectisomes.

Injectisomes are related to the bacterial flagellum and evolved into seven families

Among the nine conserved proteins, eight are shared with the flagellum (Fields et al., 1994; Woestyn et al., 1994; Van Gijsegem et al., 1995), suggesting a common evolutionary origin, which is also evidenced by the similarity of their basal body structure (Kubori et al., 1998). Injectisomes have evolved into seven different families (Gophna et al., 2003; Pallen et al., 2005; Troisfontaines and Cornelis, 2005). The ones found in most free-living animal pathogens belong to only three families. The Ysc injectisome of *Yersinia spp.* represents the archetype of the largest one, which includes, among others, injectisomes from *Pseudomonas aeruginosa* (Roy-Burman et al., 2001) and the fish pathogen *Aeromonas salmonicida* (Burr et al., 2003). The injectisomes from *S. flexneri* and *S. enterica* SPI-1 represent archetypes of another family, which is largely distributed among animal pathogens. The main representatives of the third family are from enteropathogenic (EPEC) and enterohemorrhagic (EHEC) *E. coli* and from *S. enterica* SPI-2. The injectisomes from plant pathogens belong to two families. The last two families are found in *Chlamydia* and *Rhizobiaceae.*

An electron microscopic view of the injectisome: the needle complex

After gentle bacterial lysis, several injectisome proteins copurify as a complex cylindrical structure, resembling the fla-

gellar basal body. This structure, called the needle complex (NC) or injectisome basal body, consists of two pairs of rings that span the inner (IM) and outer (OM) bacterial membranes, joined together by a narrower cylinder and terminated by a needle, filament or pilus (Kubori et al., 1998; Blocker et al., 1999; Kimbrough and Miller, 2000; Daniell et al., 2001; Jin and He, 2001; Sekiya et al., 2001; Morita-Ishihara et al., 2006; Sani et al., 2007; Hodgkinson et al., 2009).

Single particles of NCs from *S. enterica* SPI-1 (Marlovits et al., 2004, 2006) and from *S. flexneri* (Blocker et al., 2001; Sani et al., 2007; Hodgkinson et al., 2009) were characterized by cryo-electron microscopy (cryo-EM). These reconstitutions show that an inner rod with a central channel of approximately 2–3 nm is anchored at the basis of the chamber by a socket-like structure and traverses the chamber of the NC. This channel extends all the way to the tip of the needle. Some sample heterogeneity was reported among the *S. enterica* SPI-1 NCs. The lower rings of the majority of the complexes have a rotational symmetry of 20 or 21 but some are smaller (19-fold) or larger (22-fold). By contrast, the three-dimensional (3D) reconstruction of the *S. flexneri* NC, reveals an homogeneous 12-fold symmetry (Hodgkinson et al., 2009). The external diameter of the lower double ring in both structures is 21–22 nm (Marlovits et al., 2004; Hodgkinson et al., 2009). This value is significantly smaller than the diameter measured by transmission EM (TEM; approx. 40 nm; Kubori et al., 1998). It is also significantly smaller than the diameter of the C-ring of the flagellar basal body (40 nm; Macnab, 2003). This discrepancy indicates that the NC structures reconstructed have no C-ring, most probably because it breaks off during the purification procedure. Actually, the extracted NC not only misses the C-ring but also the ATPase and the transmembrane (TM) proteins thought to form the export apparatus (Figure 1). Electron microscopy has thus provided excellent information about the global structure of the injectisome but the determination of the precise localization of most structural components of the injectisome base and their secondary structure still requires a large effort.

The OM-rings

A triple ring spans the OM layer (Hodgkinson et al., 2009). It consists of a 12–14-mer of a protein belonging to the YscC family of secretins (Figure 1) (Koster et al., 1997; Kubori et al., 2000; Tamano et al., 2000; Blocker et al., 2001; Burghout et al., 2004b). Secretins are found not only in injectisomes but also in the type II secretion apparatus, in type IV pili (Collins et al., 2004; Chami et al., 2005) as well as in filamentous phages (Russel, 1994). They have a conserved C-terminal membrane spanning domain, predicted to be a β-barrel, and a variable N-terminal domain extending in the periplasm. The proper insertion of secretins in the OM requires the assistance of a lipoprotein (YscW family) anchored in the OM (Crago and Koronakis, 1998; Daefler and Russel, 1998; Burghout et al., 2004a). The crystal structure of the N-terminal domain of EscC, the homolog of YscC in EPECs was recently solved (Spreter et al., 2009).

The IM-ring and the connector

In the flagellum, the ring spanning the IM is called the MS-ring. It is made of FliF and spans the outer leaflet of the plasma membrane (Ueno et al., 1994). The homologs of FliF are lipoproteins YscJ (*Yersinia*)/PrgK (*S. enterica*)/MxiJ (*S. flexneri*)/EscJ (EPEC). The crystal structure of EscJ, the EPEC ortholog of YscJ (Crepin et al., 2005; Yip et al., 2005) allowed building of a 24-subunit ring model (Yip et al., 2005). However, attempts to dock the EscJ structure into the recent cryo-EM map of the *S. flexneri* NC, suggest that MxiJ might rather be a 12-mer (Hodgkinson et al., 2009). A protein from the less-conserved YscD (*Yersinia*)/PrgH (*S. enterica*)/*MxiC* (*S. flexneri*) family is proposed to participate in the IM-ring formation (Kubori et al., 2000) and possibly to

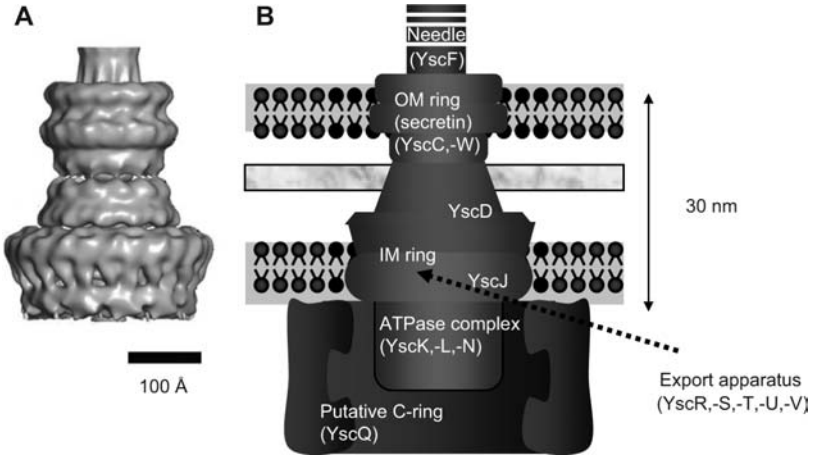

Figure 1 Needle complex (NC) structure.
The NC is modeled by cryo-EM and single particle analysis (Hodgkinson et al., 2009) (left) and a cartoon of the basal body is shown for comparison with the flagellar basal body, identifying the main substructures and their constituents. The fact that several elements of the basal body are missing in the NC is illustrated.

connect the IM- and OM-rings (Spreter et al., 2009). The structure of the periplasmic domain of PrgH reveals strong similarity to the EscJ/PrgK IM-ring component and to the periplasmic domain of the secretin (Spreter et al., 2009). This strong similarity in fold and architecture between the three proteins suggests the conservation of a fold that potentially provides a common ring-building motif for the assembly of the symmetrical ring structures that constitute the T3SS basal body (Spreter et al., 2009).

The C-ring

In the flagellum, the most internal part of the basal body is the 45–50 nm C-ring (cytosolic), made of 31–38 copies of Fli(MN_3) (Driks and DeRosier, 1990; Khan et al., 1992; Kubori et al., 1997; Young et al., 2003; Thomas et al., 2006). A 3D reconstruction of the C-ring has been proposed (Thomas et al., 2006). It is an essential component of the switch complex reversing the rotation of the motor but it also acts as a non-essential affinity cup-like structure during flagellar T3S to enhance the specificity and efficiency of the secretion process (Macnab, 2003; Erhardt and Hughes, 2010). As mentioned before, there is no C-ring visible in the cryo-EM reconstructions of the *S. enterica* SPI-1 and *S. flexneri* NCs (Marlovits et al., 2004, 2006; Hodgkinson et al., 2009) but proteins of the YscQ family have a significant similarity to FliN and FliM and genetic data indicate that such proteins are essential components in all injectisome families. In *Pseudomonas syringae*, the ortholog of YscQ even appears as two products called $HrcQ_A$ and $HrcQ_B$, which interact with each other. The overall fold of $HrcQ_B$ is remarkably similar to that of FliN (Fadouloglou et al., 2004). In agreement with this, immunogold-labeling experiments have shown that the *S. flexneri* ortholog of YscQ, localizes to a lower portion of the injectisome via interaction with the orthologs of YscD and YscJ that form the IM-ring (Morita-Ishihara et al., 2006). Together these data indicate that there is a C-ring in the injectisome even though it could not be visualized by cryo-EM thus far. It would form a platform at the cytoplasm/inner membrane interface for the recruitment of the ATPase complex (see below) (Jackson and Plano, 2000) and substrate-chaperone complexes (Morita-Ishihara et al., 2006; Spaeth et al., 2009).

The export apparatus

Among the eight proteins that are highly conserved between flagella and injectisomes, five (YscR, S, T, U, V) are TM α-helical proteins (Plano and Straley, 1993; Allaoui et al., 1994; Fields et al., 1994). They are thought to form the translocation channel(s) embedded in a patch of IM enclosed within the IM-ring but this view awaits biochemical confirmation. Two of them (YscU, -V) have a large C-terminal cytosolic domain. In YscU, this domain is autocleavable (Zarivach et al., 2008; Wiesand et al., 2009) and involved in the selection of the export substrates (Sorg et al., 2007). The stoichiometry of all these components is not known.

One of the conserved proteins is an AAA^+ ATPase (YscN family) whose integrity is essential to T3S (Woestyn et al., 1994). InvC from *S. enterica* SPI-1 has been shown to unfold the exported proteins in an ATP-dependent manner and to detach some T3S substrates from their cytoplasmic chaperones before their export (Akeda and Galan, 2005). It is also probable that the ATPase energizes export but the proton motive force is also involved (Wilharm et al., 2004; Minamino and Namba, 2008; Paul et al., 2008). HrcN, the ATPase from *P. syringae*, forms hexamers and dodecamers that are activated by oligomerization and peripherally associated with the cytoplasmic side of the inner membrane (Pozidis et al., 2003). According to cryo-EM, it has a 2.0–3.8 nm wide inner channel (Muller et al., 2006). This structure compares to that of the flagellar ATPase FliI. As for HrcN, its oligomerization and enzyme activity are coupled (Claret et al., 2003). Recently, the structure of the catalytic domain from EscN (50% identical to YscN) from EPEC was solved by crystallography at 1.8 Å resolution. Along with *in vitro* and *in vivo* mutational analysis, these data show that, in spite of the expected similarity with the F1 ATPase, there are important structural differences that dictate their unique secretory role (Zarivach et al., 2007). YscN was shown to interact with YscQ, YscK and YscL (Jackson and Plano, 2000; Blaylock et al., 2006). Likewise, in *S. flexneri*, the ATPase Spa47 was shown to interact with MxiK and MxiN, the probable orthologs of YscK and YscL (Jouihri et al., 2003). These observations fit with previous data showing that the flagellar ATPase FliI interacts with FliH (the so-called ATPase regulator), the ortholog of YscL and that the FliHI complex interacts with FliN from the C-ring (Minamino and MacNab, 2000; Gonzalez-Pedrajo et al., 2002). Given that YscL interacts also with YscQ, the component of the putative C-ring, YscL could be the protein tethering the ATPase to the export channel (Minamino and MacNab, 2000; Blaylock et al., 2006). The stoichiometry of YscK and YscL is still not known. Interestingly, FliH/YscL-like proteins represent fusions of domains from the b and δ subunits of the second-stalk components of the F_oF_1 ATPase (Pallen et al., 2006), pointing to some similarity between this nanomachine and the injectisome.

A rod?

In the flagellum, FlgBCF and -G form the so-called rod, a tube extending the hook inside the basal body. Some indirect biochemical and EM evidence suggests that, in *S. enterica* SPI-1, PrgJ forms such an 'inner rod' structure (Kubori et al., 2000; Sukhan et al., 2003; Marlovits et al., 2004). There is no *Yersinia* Ysc homolog of PrgJ but YscI has properties similar to those of the needle subunit. Based on this, some authors propose that YscI forms a rod inside the basal body of the *Yersinia* injectisome (Wood et al., 2008) but this awaits some direct biochemical evidence.

The needle

The needle is a straight hollow tube with an inner diameter of approximately 2.5 nm. It is constructed via the helical polymerization of approximately 150 subunits of the YscF family (Kubori et al., 2000; Hoiczyk and Blobel, 2001).

Although the needle subunits (approx. 9 kDa) are significantly smaller than flagellin (approx. 45 kDa), the helical parameters are similar (5.6 units per turn, helical pitch of 2.4 nm; Cordes et al., 2003). The crystal structure of MxiH, the *S. flexneri* needle subunit consists of two extended and bent antiparallel α-helices connected by a short turn (Deane et al., 2006), a structure similar to that of the D0 portion of flagellin (Yonekura et al., 2003). The structure of MxiH, combined to the EM 3D reconstructions, allowed the building of an atomic model (Deane et al., 2006). Scanning-TEM (STEM) analyses of *Y. enterocolitica* needles showed that they end with a distinct tip structure consisting of a homopentamer of the protein LcrV (Mueller et al., 2005; Deane et al., 2006; Broz et al., 2007) the crystal structure of which is known (Derewenda et al., 2004). An active injectisome in contact with a target cell terminates with a translocation pore that is inserted in the plasma membrane of the target cell (Hakansson et al., 1996; Blocker et al., 1999; Neyt and Cornelis, 1999). The assembly of this pore, upon cell contact, requires two hydrophobic proteins called YopB and YopD (Cornelis and Wolf-Watz, 1997). The needle-tip structure is thought to serve as a scaffold or assembly platform for the translocation pore (Goure et al., 2004).

Needle length control and substrate specificity switch

The final length of the needle varies between different bacteria or even strains but the length of the needle is regulated as is the case for the flagellar hook (55 nm). In *Y. enterocolitica* strain E40, the needle length is 65±10 nm (Journet et al., 2003) whereas in other *Y. enterocolitica* strains it varies from 49±6 to 87±11 nm (Wagner et al., 2009). During morphogenesis, the needle components, as with the hook and the filament of the flagellum, are sequentially exported by the T3S apparatus itself (Sukhan et al., 2001), traveling through the growing structure and polymerizing at its distal end (Li et al., 2002; Macnab, 2003). There is no clear hierarchy in the synthesis of the injectisome components and substrates. Thus, the export apparatus is expected to switch its substrate specificity over time so that needle subunits (early substrates) are exported before the tip structure subunit LcrV (intermediate substrate) and the effectors (late substrates). This substrate specificity switch presumably leads to the arrest of needle growth (Ferris and Minamino, 2006). The switch to export late substrates is triggered by the protein FliK, in the flagellum system (Hirano et al., 1994; Minamino et al., 1999, 2004) and YscP in the injectisome (Journet et al., 2003). The 515-residue YscP (Figure 2) is itself an early substrate of the machine driven by two independent N-terminal export signals (Agrain et al., 2005b). The switch activity is exerted by residues 405–500 (Agrain et al., 2005a), a domain called T3S4 for type 3 secretion substrate specificity switch. This domain is thought to interact with YscU (FlhB in the flagellum), a component of the basal body that is also involved in setting the hierarchy of export (Hirano et al., 1994; Sorg et al., 2007). The central domain of YscP is predicted to be helical and there is a linear correlation between the number of residues in the protein and the needle length,

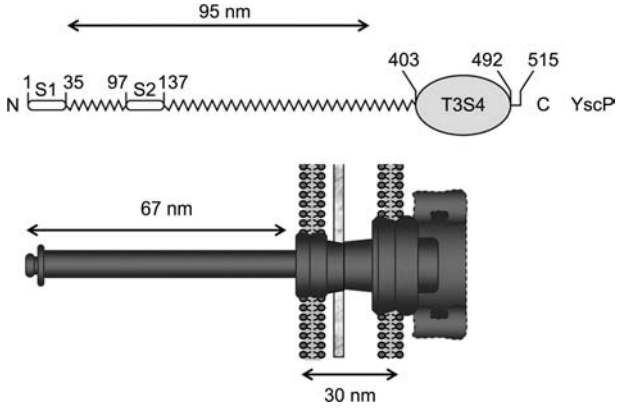

Figure 2 Illustration of the molecular ruler concept.
Above, a schematic representation of the YscP molecule with the two export signals (S1 and S2) at the N-terminus and the T3S4 domain at the C-terminus is shown. The ruler domain is represented by the zigzag line. Below is a schematic representation of the injectisome. The protein is aligned with the nanomachine in the way it is proposed to operate: the N-terminus towards the tip and the T3S4 domain in the cytosolic part of the basal body.

suggesting that YscP acts as a molecular ruler or a molecular timer. There is also an inverse correlation between the helical content of this central domain and the needle length, indicating that the functional ruler is partially helical (Wagner et al., 2009). The calculated length when the helical content is preserved correlates strikingly with the measured needle length, with a constant difference of approximately 29 nm, which corresponds to the size of the basal body (Figure 2). These data support the ruler model and show that the functional ruler has a helical structure (Wagner et al., 2009).

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