The type III secretion injectisome

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Abstract | The type III secretion injectisome is a complex nanomachine that allows bacteria to deliver protein effectors across eukaryotic cellular membranes. In recent years, significant progress has been made in our understanding of its structure, assembly and mode of operation. The principal structural components of the injectisome, from the base located in the bacterial cytosol to the tip of the needle protruding from the cell surface, have been investigated in detail. The structures of several constituent proteins were solved at the atomic level and important insights into the assembly process have been gained. However, despite the ongoing concerted efforts of molecular and structural biologists, the role of many of the constituent components of this nanomachine remain unknown.

Injectisome
The injectisome is a nanomachine that evolved for the delivery of bacterial proteins, by type III secretion, across eukaryotic cell membranes. In the present stage of knowledge, it consists of a basal structure, which resembles the basal structure of the flagellum, surmounted by either a needle, a needle and a filament or a long pilus.

Flagellum
The flagellum is a motility organelle consisting of a rotating long filament connected to a rotary motor by a short curved structure called the hook. The motor is powered by the flow of ions down an electrochemical gradient across the cytoplasmic membrane into the cell. The ions are typically H^+ (protons) in *Escherichia coli* and enterobacteria and Na^+ in alkalophiles and marine *Vibrio* species.

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doi:10.1038/nrmicro1526

Over twenty-five species of Gram-negative bacteria that interact with other organisms, either as pathogens or as symbionts, are equipped with a special protein-export apparatus called a type III secretion system (T3SS) or an injectisome (FIG. 1). The 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[1–3]. Over 100 different effector proteins are known and, undoubtedly more are yet to be discovered. Animal pathogens equipped with the T3SS generally deliver from six to over twenty different effector proteins into their target cells. These effector proteins display a large repertoire of biochemical activities and modulate the function of crucial host regulatory molecules. In animals, the targets of effector molecules include small GTP-binding proteins, mitogen-activated protein kinases (MAPKs), IκB-α and phosphoinositides. The activity of T3SS effector proteins allows bacteria to invade non-phagocytic cells or to inhibit phagocytosis by phagocytes, to down-regulate pro-inflammatory responses, to induce apoptosis, to prevent autophagy or to modulate intracellular trafficking. For a complete list of effector proteins from animal pathogens, and their function, see REF. 4. In plant cells, T3SS effector proteins suppress host defences but they can also betray the pathogen and elicit plant defences. Some effector proteins possess cysteine protease or protein tyrosine phosphatase activity. For recent reviews, see REFS 5,6. In contrast to the large diversity observed among the effector proteins, the injectisomes themselves are relatively conserved. In this article, I review our current understanding of the structure, assembly and operation of the injectisome, in light of new observations over the past few years. For a review article focusing on the structures of the proteins involved in T3SS, see REF. 7.

The injectisome is related to the flagellum. Early genetic studies showed that a core of eight proteins from the injectisome share significant similarity with components of the flagellum[8–10] (FIG. 1a; TABLE 1). The flagellum contains a built-in export apparatus for the sequential export of the hook and filament components[11]. In some instances, this export apparatus also exports non-flagellar proteins[12].

The common evolutionary origin of the injectisome and the flagellum became even more apparent when the needle complex (NC), encoded by the *Salmonella* pathogenicity island 1 (SPI-1), was purified from *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), and visualized under the electron microscope[13]. Although, it is often assumed that the injectisome derives from the flagellum, some phylogenetic studies do not endorse this view, indicating instead that the injectisome is as ancient as the flagellum and that both share a common ancestor[14].

Therefore, the T3SS is a protein export pathway used by two different nanomachines; however, in practice, the term T3SS often refers to the system used for the secretion or translocation of effector proteins, in the context of a host–microorganism interaction.

Different families of injectisomes. Phylogenetic analyses of four conserved proteins indicate that injectisomes have evolved into seven different families[14–16] (TABLE 2). Interestingly, the evolutionary trees of bacteria and of injectisomes are completely different, indicating that the genes encoding injectisomes have been distributed horizontally among bacteria, late in bacterial evolution. One family is limited to the Chlamydiae phylum and another to the Rhizobiales order. The injectisomes that occur in most free-living animal pathogens belong to

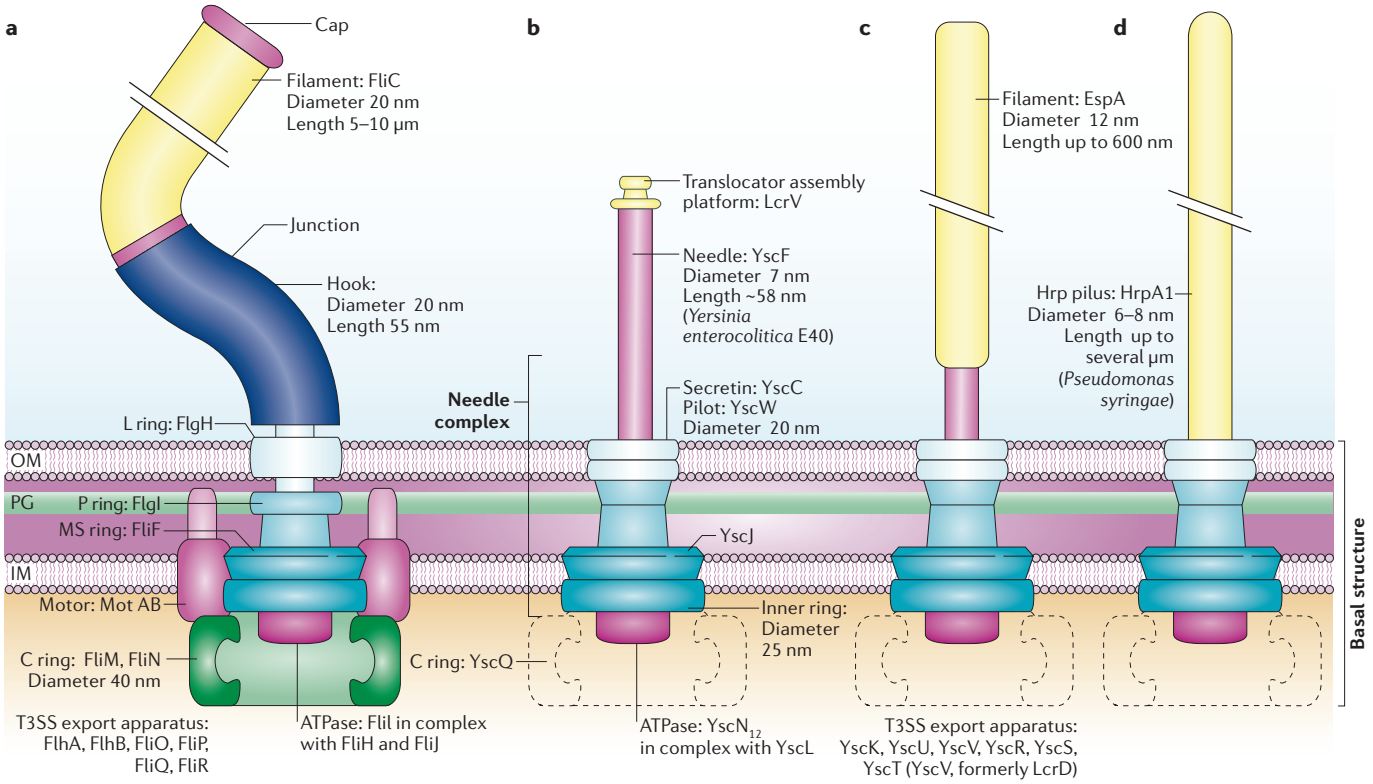


Figure 1 | **Structure of the flagellum and the injectisomes.** Schematic representation of the flagellum (**a**), Ysc injectisome (**b**), injectisome from EPECs (**c**) and the injectisome from plant pathogens (**d**). For the injectisomes, the C ring is represented by a dashed line as information on this component is still scarce. IM, inner membrane; OM, outer membrane; PG, peptidoglycan.

only three families: the Ysc injectisome of *Yersinia* spp. is the archetype of one of these families, and similar injectisomes are found in *Pseudomonas aeruginosa*[17] and in the fish pathogen *Aeromonas salmonicida*[18]. The injectisomes from *Shigella flexneri* and *S. typhimurium* (encoded on SPI-1) are archetypal examples of a second family whose members are distributed primarily among the animal pathogens. Examples of the third family are found in enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) *Escherichia coli*, and in *S. typhimurium* (encoded on SPI-2). Injectisomes that occur in plant pathogens belong to two families (called Hrp1 and Hrp2 in TABLE 2). It is not uncommon for the same pathogenic bacterium to encode two injectisomes belonging to different families, each having a role at a different stage of the infection process. The best studied example is *S. typhimurium*; as alluded to above, this pathogen harbours two pathogenicity islands (SPI-1 and SPI-2), each of which encodes a different T3SS[19,20].

Structure of the injectisome

Overview. The visualization by transmission electron microscopy (TEM) of the NC from *S. typhimurium* was a landmark achievement in the field[13]. Since then, the NCs from *S. flexneri* and EPEC have also been visualized using TEM[21–27]. Not surprisingly, the NCs from *S. typhimurium* SPI-1 and from *S. flexneri* seem very similar. The NC derived from the EPEC injectisome also looks similar, albeit smaller, despite the fact that this example belongs to a different family[21]. This finding indicates that the global architecture of NCs is conserved among the different injectisomes.

The NC consists of a cylindrical structure, similar to the flagellar basal body, composed of two pairs of rings that span the inner and outer bacterial membranes, joined together by a rod (FIG. 1). The discovery of this structure was consistent with previous observations indicating that the export of the effector proteins across the bacterial membranes and translocation through the eukaryotic membrane occurs in a single step. Depending on the family of injectisomes, a hollow stiff needle (FIG. 2b), a filament (FIG. 2c) or a pilus (FIG. 2d) terminate the structure.

Single particle analysis using cryo-electron microscopy (cryo-EM) allowed Blocker *et al.* to determine the structure of a purified NC from *S. flexneri* at high resolution[28]. This structure revealed, for the first time, a central channel of approximately 2–3 nm that extends from the bottom set of rings to the tip of the extracellular needle (FIG. 2b). This channel size is too small to allow folded proteins to pass through the tube, and is consistent with previous observations indicating that proteins need to be unfolded to be exported[29]. Using cryo-EM, Marlovits *et al.* analysed the NC encoded on SPI-1 of *S. typhimurium* and observed some sample heterogeneity among the NCs[30]. The lower rings of the complexes in most of the samples had a rotational

Needle complex
The needle complex is the part of the injectisome that was characterized in great detail by cryo-EM[28,30,85]. This structure contains neither the ATPase nor the putative C ring.

Table 1 | **Injectisome proteins (basal structure) that share sequence similarity**

Flagellum (*S. typhimurium*)	Injectisomes				
	Yersinia spp. Ysc	*S. typhimurium* SPI-1	*Shigella* spp.	Plant pathogens	Structure/function
FliF	YscJ	PrgK	MxiJ	HrcJ	MS ring Lipoprotein (EscJ in EPECs)
FliI	YscN	InvC/SpaL	Spa47	HrcN	ATPase Ring with 6-fold symmetry
FliN/FliM	YscQ	InvK/SpaO	Spa33	HrcQ ($HrcQ_A$/$HrcQ_B$)	Putative C ring
FliP	YscR	InvL/SpaP	Spa24	HrcR	Basal structure Inner-membrane protein
FliR	YscT	InvN/SpaR	Spa29	HrcT	Basal structure Inner-membrane protein
FlhB	YscU	SpaS	Spa40	HrcU	Basal structure Inner-membrane protein involved in substrate specificity switching
FlhA	YscV*	InvA	MxiA	HrcV	Basal structure Inner-membrane protein
(FliQ)	YscS	SpaQ	Spa9	HrcS	Basal-structure Inner membrane protein
No secretin	YscC	InvG	MxiD	HrcC	Secretin Outer-membrane protein

*Initially described as LcrD. EPEC, enteropathogenic *Escherichia coli*; SPI-1, *Salmonella* pathogenicity island 1; S. *typhimurium*, *Salmonella enterica* serovar Typhimurium.

symmtry of 20- or 21-fold; however, some samples were smaller (19-fold symmetry) or larger (22-fold symmetry). The images were sorted into symmetry classes before averaging, which allowed an increase in the resolution of the averaged structure (FIG. 2b). It should be mentioned here that a variable symmetry is not unprecedented in nanomachines. For example, the symmetry of the C ring (see below) of the *S. typhimurium* flagellum was shown to vary between 31- and 38-fold[31]. The analysis of Marlovits *et al.* also revealed that an inner rod traverses the chamber of the NC and that this rod is anchored at the base of the chamber by a socket-like structure[30] (FIG. 2b).

The external diameter of the lower double-ring of the NC modelled by Marlovits and colleagues is approximately 20 nm[30]. This diameter is significantly smaller than that measured previously using TEM (approximately 40 nm)[13]. It is also significantly smaller than the diameter of the C ring of the flagellar basal body (40 nm)[11]. This discrepancy is unlikely to be a result of the inaccuracy of the TEM measurements versus those of the cryo-EM measurements. Rather, it suggests that the NC described by Marlovits *et al.* has no C ring, because it is loosely associated and detaches during the purification procedure. Independently of the question of the C ring, the characterized NC does not represent the entire basal structure as it does not contain the conserved ATPase (YscN family), known to be essential for the injectisome function (FIG. 2c). As such, it is possible that additional components could be lost during the purification procedure.

Electron microscopy (EM) has provided excellent insight into the global structure of the injectisome; however, the precise localization of most structural components contained in the base of the injectisome, and their secondary structure, are yet to be determined.

Approximately 25 proteins are required to build the injectisome. Most of these are structural components but some are ancillary components that are only involved during the assembly process and are then either discarded (for example, molecular ruler) or are retained in the cytosol (for example, chaperones; see BOX 1). The core of injectisome proteins, whose sequence is conserved in all examples, does not exceed nine proteins (TABLE 1). For the injectisomes encoded by plant pathogens, these proteins are termed Hrc (for hypersensitive response conserved) followed by the letter used for the corresponding *Yersinia* Ysc protein[32]. These proteins include the eight proteins that are shared with the flagellum, and a protein from the secretin family.

The basal structure. The outer pair of rings, associated with the outer bacterial membrane and the peptidoglycan layer, is a 12–14 -mer of the YscC family of secretins[22,28,33–35] (FIG. 1). Secretins are a large family of proteins that form pores in the outer membrane (OM). They are found not only in injectisomes but also in other microbial nanomachines, including the type-II secretion apparatus and type IV pili[36,37], and in filamentous phage[38]. The proper insertion of secretins into the OM requires the assistance of a lipoprotein (YscW family) (TABLE 3), anchored in the OM[39–41].

In *S. typhimurium*, the inner rings are composed of two proteins called PrgH and PrgK[24,33] (TABLE 3). PrgH is not a conserved protein but PrgK belongs to the highly conserved YscJ family of periplasmic lipoproteins. It is believed that these proteins are anchored to the outer leaflet of the inner membrane.

Basal structure
Here, the basal structure is defined as the injectisome without its needle, filament or pilus.

Type II secretion apparatus
The type II secretion apparatus is a complex nanomachine that translocates proteins across the outer membrane. This machine involves a secretin in the outer membrane and a dynamic short pilus that functions as a piston.

Type IV pili
Type IV pili are retractable pili involved in adherence and motility and found on diverse bacteria. They are related to the piston of the type II secretion apparatus.

Lipoprotein
A Lipoprotein (LP) is a protein that is synthesized with a signal peptide followed by a cysteine onto which a diacylglycerol is covalently attached by a thioether bond during export. LPs insert either in the plasma membrane or in the outer membrane.

Table 2 | **List of bacterial species equipped with injectisomes**

Injectisome family	Species	Description	Taxon
Chlamydiales	*Chlamydia trachomatis*	Obligate intracellular human pathogen (trachoma, genital infections)	Chlamydiaceae
	Chlamydia pneumoniae	Obligate intracellular human pathogen (acute respiratory disease)	Chlamydiaceae
Hrp1	*Pseudomonas syringae*	Plant pathogen	γ-proteobacteria
	Erwinia amylovora	Plant pathogen	γ-proteobacteria
	Pantoea agglomerans (formerly *Enterobacter agglomerans*)	Environmental and human commensal, rarely pathogenic.	γ-proteobacteria
	Vibrio parahaemolyticus	Human pathogen (seafood-borne gastroenteritis)	γ-proteobacteria
Hrp2	*Burkholderia pseudomallei*	Human pathogen (meloidosis)	β-proteobacteria
	Ralstonia solanacearum	Plant pathogen	β-proteobacteria
	Xanthomonas campestris	Plant pathogen	γ-proteobacteria
SPI-1	*Salmonella enterica*	Human pathogen (gastroenteritis)	γ-proteobacteria
	Shigella flexneri	Human pathogen (dysenteria)	γ-proteobacteria
	Burkholderia pseudomallei	Human pathogen (meloidosis)	β-proteobacteria
	Chromobacterium violaceum	Emerging human pathogen (evoking meloidosis)	β-proteobacteria
	Yersinia enterocolitica	Human pathogen (gastroenteritis, mesenteric adenitis)	γ-proteobacteria
	Sodalis glossinidius	Tse-tse fly symbiont	γ-proteobacteria
SPI-2	*Escherichia coli* EPEC	Human pathogen (gastroenteritis)	γ-proteobacteria
	Escherichia coli EHEC	Human pathogen (uremia, hemolysis)	γ-proteobacteria
	Salmonella enterica	Human pathogen (gastroenteritis)	γ-proteobacteria
	Citrobacter rodentium	Mouse pathogen, model for EPEC	γ-proteobacteria
	Chromobacterium violaceum	Emerging human pathogen (evoking meloidosis)	β-proteobacteria
	Yersinia pestis	Rodent and human pathogen (plague)	γ-proteobacteria
	Yersinia pseudotuberculosis	Rodent and human pathogen	γ-proteobacteria
	Edwardsiella tarda	Human pathogen (gastroenteritis)	γ-proteobacteria
Rhizobium	*Mesorhizobium loti*	Plant symbiont (Nitrogen fixation)	α-proteobacteria
	Rhizobium sp	Plant symbiont (Nitrogen fixation)	α-proteobacteria
Ysc	*Yersinia pestis*	Rodent and human pathogen (plague)	γ-proteobacteria
	Yersinia pseudotuberculosis	Rodent and human pathogen	γ-proteobacteria
	Yersinia enterocolitica	Human pathogen (gastroenteritis, mesenteric adenitis)	γ-proteobacteria
	Pseudomonas aeruginosa	Animal, insect and human (cystic fibrosis, burned, immunocompromized patients) pathogen	γ-proteobacteria
	Aeromonas salmonicida	Fish pathogen	γ-proteobacteria
	Photorhabdus luminescens	mutualistic with entomophagous nematodes	γ-proteobacteria
	Vibrio parahaemolyticus	Human pathogen (seafood-borne gastroenteritis)	γ-proteobacteria
	Bordetella pertussis	Human pathogen (whooping cough)	β-proteobacteria
	Desulfovibrio vulgaris	Sulphate reducing environmental bacteria	δ-proteobacteria

EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*.

The structure of a representative member of this family (the EscJ protein encoded by EPEC) was solved[42,43], and a 24-subunit ring model was constructed[43]. This ring is probably the functional counterpart of the flagellum MS ring, composed of the protein FliF.

In *S. typhimurium*, numerous indirect biochemical and structural studies indicate that the inner rod is composed of a protein termed PrgJ[30,33,44] (TABLE 3). As there is no clear orthologue of PrgJ in other injectisome families, the identity of the rod in these nanomachines remains unknown.

In the flagellar basal body, beneath the MS ring, is the C ring composed of the proteins FliN and FliM. As discussed above, there is no cryo-EM image that indicates the presence of a C ring in the injectisome. However, proteins of the YscQ family — one of the conserved injectisome protein families — do have significant similarity to the flagellar proteins FliN and FliM. Additionally, in

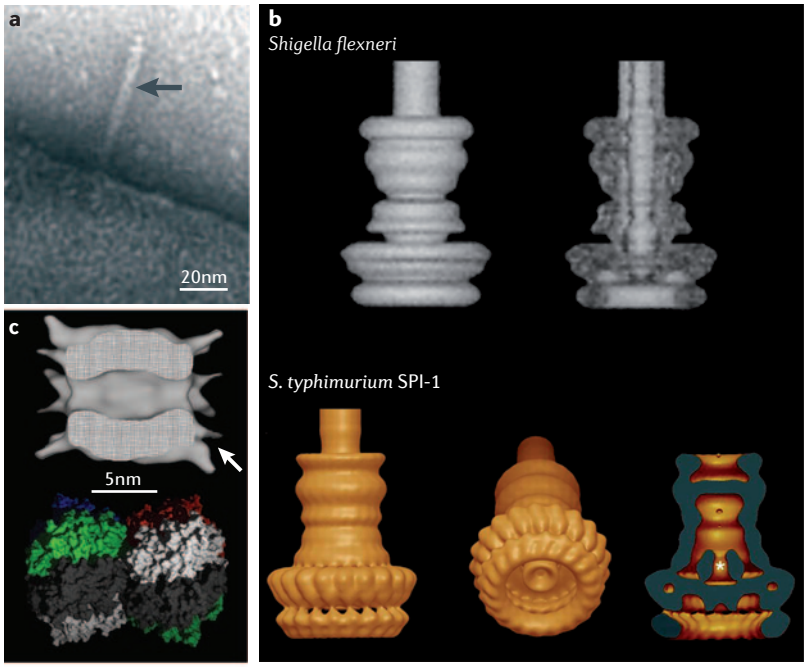


Figure 2 | **Structure of the injectisome. a** | Transmission electron micrograph of *Yersinia enterocolitica* E40 negatively stained with 2 % uranyl acetate. One needle (arrow) protrudes from the cell surface. **b** | 3D structure of the needle complex (NC) encoded by *Shigella flexneri* [28] (top) and by *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) pathogenicity island 1 (SPI-1) (REF. 30) (bottom), reconstructed by averaging cryo-electron micrograph images. Surface and sections are shown. In S. *typhimurium*, the structure shown is that of a structure with a 20-fold symmetry, averaged after the different images have been sorted according to symmetry. The asterisk shows the socket-like structure which serves as an anchoring point for the inner rod. Note that the needle is truncated and that the ATPase is not present in these structures. Please refer to the discussion regarding the C ring in the main text. **c** | 3D reconstruction and model of the ATPase HrcN from *Pseudomonas syringae* (section and side view)[52]. Two hexameric HrcN complexes are attached face to face at their carboxy-terminal ends. Part **b** reproduced with permission from REF. 28 © (2001) Blackwell Publishing and from REF. 30 © (2004) American Association for the Advancement of Science. Part **c** reprinted with permission from REF. 52 © (2006) Blackwell Publishing.

AAA+
(ATPases associated with various cellular activities) The AAA+ family is a large and functionally diverse group of enzymes that can induce conformational changes in a wide range of substrate proteins. The defining feature of the family is a structurally conserved ATPase domain that assembles into oligomeric rings and undergoes conformational changes during cycles of nucleotide binding and hydrolysis. AAA+ are associated with several ATP-dependent bacterial proteases, including ClpXP and ClpAP. They unfold proteins and translocate the unfolded polypeptide into the proteolytic chamber for degradation. See REF. 152 for a review.

the injectisome of *Pseudomonas syringae*, there are two YscQ homologues, termed $HrcQ_A$ and $HrcQ_B$, which interact with each other. The overall fold of $HrcQ_B$ is similar to that of FliN[45], suggesting that proteins from the YscQ family form a multimeric ring at the base of the *Yersinia* injectisome structure. Consistent with this assertion is the finding that Spa33, the *S. flexneri* orthologue of YscQ, localizes to a lower portion of the injectisome by interaction with the orthologues of PrgK and PrgH, forming the MS ring[27]. Furthermore, Sasakawa and colleagues demonstrated that Spa33 interacts with numerous proteins from the injectisome, including the ATPase (YscN family of core proteins). In conclusion, these data indicate that YscQ proteins form a platform at the cytoplasm–inner membrane interface for the recruitment of other proteins, including the ATPase. In agreement with this conjecture, FliN, a major component of the flagellar C ring, interacts with the FliH (ATPase regulator)–FliI (ATPase) complex[46]. It is likely that this YscQ platform has the shape of the flagellar C ring but confirmation by cryo-EM is required.

One of the conserved injectisome proteins is an ATPase (YscN family), the integrity of which is essential to the T3SS (REF. 10). HrcN, the ATPase from *P. syringae*, forms hexamers and dodecamers that are activated by oligomerization, and are peripherally associated with the cytoplasmic side of the inner bacterial membrane[51]. The structure of dodecameric HrcN was recently determined to 1.6-nm resolution by cryo-EM. $HrcN_{12}$ appears as two hexameric rings that are stacked face to face. The structure is 11.5 ± 1.0 nm in diameter, and the width of the inner channel is 2.0–3.8 nm[52] (FIG. 2c). The structure is comparable to the flagellar ATPase (FliI) that was also shown to form a ring structure with a six-fold symmetry, an external diameter of approximately 10 nm and a central cavity of 2.5–3.0 nm. Oligomerization and enzyme activity are coupled, as is the case for HrcN[53].

The main function of the ATPase molecule is to energize the export process. However, another function, characterized with InvC from *S. typhimurium* SPI-1, involves detaching some T3SS substrates from their cytoplasmic chaperones before export, a function that is similar to that done by the bacterial AAA+ (ATPases associated with various cellular activities) disassembly machineries[54]. In addition, InvC has been shown to unfold the exported proteins in an ATP-dependent manner[54].

Using yeast two-hybrid assays, YscN was shown to interact with YscQ, YscK and YscL[47] (TABLE 3), and associations between YscN and YscL, and YscL and YscQ were also confirmed in *Yersinia enterocolitica*[48]. In *S. flexneri*, the ATPase, Spa47 was shown to interact with MxiK and MxiN, the probable orthologues of YscK and YscL[49]. Each of these recent observations are consistent with previous data showing that the flagellar ATPase, FliI, interacts with FliH, the orthologue of YscL[50] (TABLE 3). Given that YscL also interacts with YscQ, the component of the putative C ring, it is possible that YscL could be the protein tethering the ATPase to the export channel[48,50].

So far, the stoichiometry and exact location of several other inner-membrane components of the basal structure of the injectisome (including YscR, YscS, YscT, YscU and YscV) has not been elucidated.

The needle, the filament and the pilus. The injectisome needle is a straight hollow tube, approximately 60 nm in length, with an inner diameter of approximately 25Å. It is constructed from the helical polymerization of 100–150 molecules (subunits) of the YscF family[33,55]. Although these subunits (9 kDa) are significantly smaller than those that make up the flagellar hook and filament (flagellin is approximately 45 kDa), the helical parameters are strikingly similar (5.6 units per turn, 24 Å helical pitch)[56]. The crystal structure of MxiH, the *Shigella* spp. needle subunit, was recently solved and was shown to consist of two anti-parallel, extended bent α-helices connected by a short turn[57]. This structure is similar to the structure of the D0 portion of flagellin[58], and EspA, the subunit forming the filament on top of the needle in EPEC (see below). The structure of MxiH (TABLE 3), in combination with EM-derived 3D

Box 1 | **The T3SS chaperones**

The assembly and operation of the injectisome requires the assistance of a series of small bacterial cytosolic proteins called the T3SS chaperones. They form three different classes that assist the effector proteins (class I), the pore forming proteins (class II), and subunits of substructures polymerizing outside the cytosol of the bacterium (class III). These chaperone proteins are never exported out of the bacterial cell and are likely to be recycled.

Class III

The extracellular, helical components of structures like the needle of the T3SS injectisome must be prevented from premature polymerization during their cytosolic stage. This is achieved by class III chaperones that bind the subunits and mask the domains that are involved in polymerization. In the flagellum, FlgN functions as a chaperone for FlgK and FlgL, the two hook-associated proteins; FliT functions as a chaperone for the flagellar filament cap protein FliD[123], and FliS functions as a chaperone for flagellin (FliC)[124]. In each case, the chaperone binds to the carboxyl terminus of its partner protein[125]. The helical components of the injectisome are also chaperoned by specialized cytoplasmic proteins that maintain these components in a monomeric state, preventing their premature self-association and possibly also assisting their delivery to the export channel. For example, the *Pseudomonas aeruginosa* needle component PscF, is trapped in the cytosol in a 1:1:1 complex with two small cytoplasmic proteins, PscE and PscG[87]. A similar process occurs with YscF in *Yersinia* spp. (I. Sorg and G.R.C., unpublished data). In enteropathogenic *Escherichia coli* (EPEC), CesA functions as a chaperone for EspA[152].

Class II

The hydrophobic translocators are toxic to the bacterial cell[126], and this toxicity is neutralized by class II chaperones[69,126,127]. Little is known about their mechanism of interaction with their partner protein but, generally, one chaperone (SycD in *Yersinia* spp., IpgC in *Shigella* spp. and SicA in *S. typhimurium*), serves the two hydrophobic translocators (YopBD in *Yersinia* spp., IpaBC in *Shigella* spp. and SipBC in *S. typhimurium* SPI-1). Interestingly, SicA has been shown to have an important regulatory role in the synthesis of effector proteins[128]. This observation makes sense as the presence of free translocator chaperones is a 'warning light' for the bacterium, signalling that the pore is made and the injectisome is functional[129].

Class I

Surprisingly, many effector proteins also have a dedicated chaperone (class I chaperones). There is a considerable body of literature describing their structure and how they interact with their partner proteins[69,130–133]. The genes encoding class I chaperones are often, but not always, adjacent to the gene encoding their partner protein. They are acidic (pI 4–5), dimeric proteins that interact with their cognate effector protein through a chaperone-binding domain (CBD) located within the first 100 amino acids of the effector protein, downstream from the short amino-terminal export signal (FIG. 3). Class I chaperones have a low sequence similarity, but do possess a conserved 3D structure (FIG. 3). They use a similar fold consisting of five β-strands and three α-helices[120,122,134–140]. The CBD of effector proteins wrap around chaperone homodimers in an extended, horseshoe-like conformation with some secondary structure organization in α-helical structures[121,122,138]. The presence of hydrophobic surfaces on the chaperone homodimers are responsible for the binding of the cognate effector protein[120,121,134].

Although several chaperones of T3SS effector proteins have been intensively studied and characterized, their essential function remains a matter of debate. Some studies indicate that chaperones could be 3D targeting factors or that they have a role in establishing a temporal export hierarchy[121,141]. Other observations indicate that they are required for the storage of effector proteins in a export-competent state prior to export[142] but chaperone binding does not prevent the folding of the whole effector protein[121,134,143,144]. Recently, it was shown that the CBD in YopO, YopE and (probably) YopT coincides with the membrane localization domain (MLD) targeting these effector proteins to the host cell membrane[143,145]. The CBD/MLD results in intrabacterial Yop insolubility and the binding of the Syc protein prevents this insolubility; however, it does not prevent folding and activity[143]. Therefore, SycO, SycE and (probably) SycT mask a domain in their cognate effector protein that is required for the correct localization of the protein when it is exported into the host target cell. Masking a cellular localization domain might therefore be the essential function of T3SS effector chaperones[143]. If this targeting domain was simply a hydrophobic domain, class I chaperones could serve several effector proteins but this is generally not the case although it should be mentioned that Spa15 (*Shigella* spp.), InvB (*S.typhimurium*) and CesT (EPEC) were reported to function as chaperones to several different effector proteins[142,146,147]. In any event, these findings indicate that the targeting domain of effector proteins is more subtle than simply a hydrophobic domain. The proposal that class I chaperones hide a cellular targeting domain does not exclude the possibility that they facilitate export of the effector protein. Indeed, chaperones bound to the effector protein could also function as a 3D export signal, as was suggested for SycE[121], or they could facilitate the exposure of the export signal located immediately upstream from the CBD. The presence of the chaperone could also promote the interaction of the effector protein with the ATPase, thereby facilitating the unfolding of the effector protein[54]. In *S. typhimurium*, it was shown that the chaperone prevents two effector proteins from being exported by the flagellum (instead of the injectisome)[148]. This observation is consistent with the proposal that the ATPase removes class I chaperones from their effector protein prior to the export of the protein[54], as the finding implies that the injectisome ATPase has evolved to recognize effector protein chaperones. Finally, some of the class I chaperones, in their free state, also have a regulatory role in the synthesis of effector proteins[128,149,150].

It is now clear that the T3SS involves different chaperones. If the primary (but not exclusive) function of class I chaperones is to mask a cellular targeting domain present in effector proteins, this indicates that the three chaperone classes have the same global function: to mask in the bacterial cytoplasm, polymerization or 'toxic' domains, the functions of which are exerted outside the bacterial cell.

Table 3 | **Orthologous proteins with low sequence conservation***

Flagellum (*Salmonella typhimurium*)	Injectisomes				
	Yersinia spp. Ysc	*Salmonella typhimurium* SPI-1	*Shigella* spp.	Others	Structure/function
FliK	YscP	InvJ	Spa32	–	Control of the hook/needle length (all). Molecular ruler (shown for YscP) Substrate specificity switch (all) Socket stabilizer (InvJ)
–	YscW	InvH	MxiM	–	Lipoprotein Assists the proper insertion of the secretin in the outer membrane
–	?	PrgH	MxiG	–	MS ring (inner membrane)
–	?	PrgJ	MxiI	–	Putative inner-rod subunit
–	YscK	–	MxiK	–	Interacts with the ATPase
FliH	YscL	–	MxiN	–	Interacts with the ATPase and the putative C ring. Could be tethering the ATPase in the export channel
–	YscF	PrgI	MxiH	PscF (*Pa*) EscF (EPEC)	Needle subunit
–	LcrV	SipD	IpaD	EspA filament subunit (EPEC)	Needle tip complex subunit Scaffold for pore formation
–	YopBD	SipBC	IpaBC	PopBD (*Pa*) EspDB (EPEC)	Hydrophobic pore formers

*Proteins listed are those cited in the text. EPEC, enteropathogenic *Escherichia coli*; *Pa*, *Pseudomonas aeruginosa* ; SPI-1, *Salmonella* pathogenicity island 1; S. *typhimurium*, *Salmonella enterica* serovar Typhimurium.

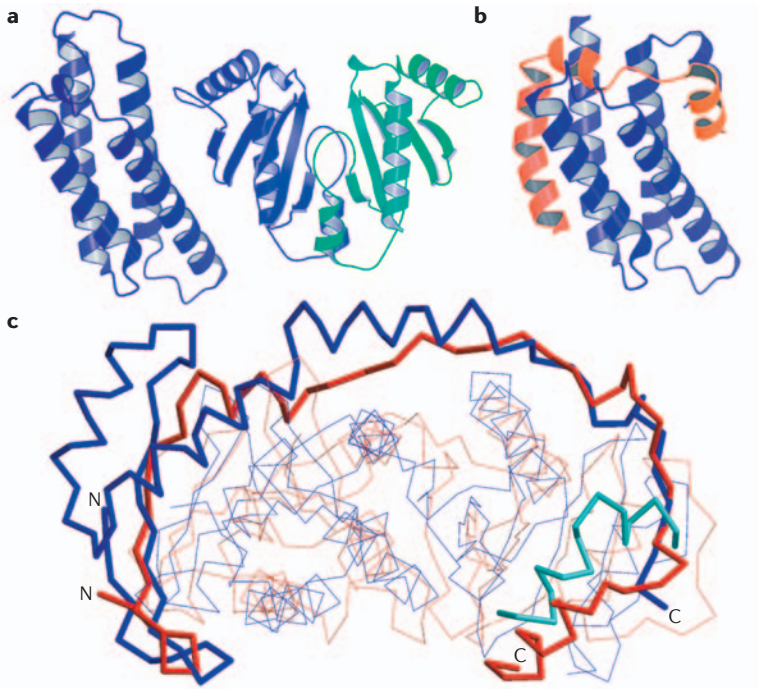


Figure 3 | **Class I and class III chaperone structures. a** | Class III chaperone FliS from *Aquifex aeolicus*[119] (left), and class I dimeric chaperone SycE (right) (each monomer is in a different colour)[120]. **b** | Structure of FliS (blue) in complex with residues 464–518 of flagellin FliC (orange). **c** | Chaparone-binding domain of effector proteins YopE (red) and SptP (blue) encircle SycE and SicP dimers, respectively[121,122]. Part **a**, **b** reproduced with permission from *Nature Structural Biology* REF. 119 © (2003) Macmillan Publishers Ltd. Part **c** reprinted with permission from REF. 121 © (2002) Elsevier.

reconstructions, has allowed the construction of the first atomic model of an injectisome needle[57].

Scanning-transmission electron microscopy (STEM) analysis of purified *Yersinia* spp. needles showed that the end of the structure contains a distinct tip complex composed of the protein LcrV, one of the translocators[59] (see below) (FIG. 4a,b). This observation is consistent with the crystal structure of LcrV (FIG. 4c) which, interestingly, also contains two extended α-helices[60]. Polymerization of the LcrV structure at the tip of the needle, as modelled by Deane *et al.* would result in the formation of a pentamer[57].

In the injectisome from EPEC, a long flexible structure — called the EspA filament (TABLE 3) — extends the needle[21,25]. In the injectisomes used by plant pathogens, a long, thin structure, termed the Hrp pilus, replaces the needle[61]. The reason for these different structures is because of the different functional constraints these nanomachines must operate under in their host organisms; in the intestine, the EPEC cells are separated from their target cells, the enterocytes, by a mucus layer, whereas plant pathogens must deliver their effector proteins across a thick plant cell wall.

The translocation pore. 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[23,62,63]. The assembly of this pore, on cell contact, requires a set of proteins termed the translocators, which are also T3SS substrates[23,64–67]. Generally, there are three translocators in each T3SS (YopB, YopD and LcrV in *Yersinia* spp.; IpaB,

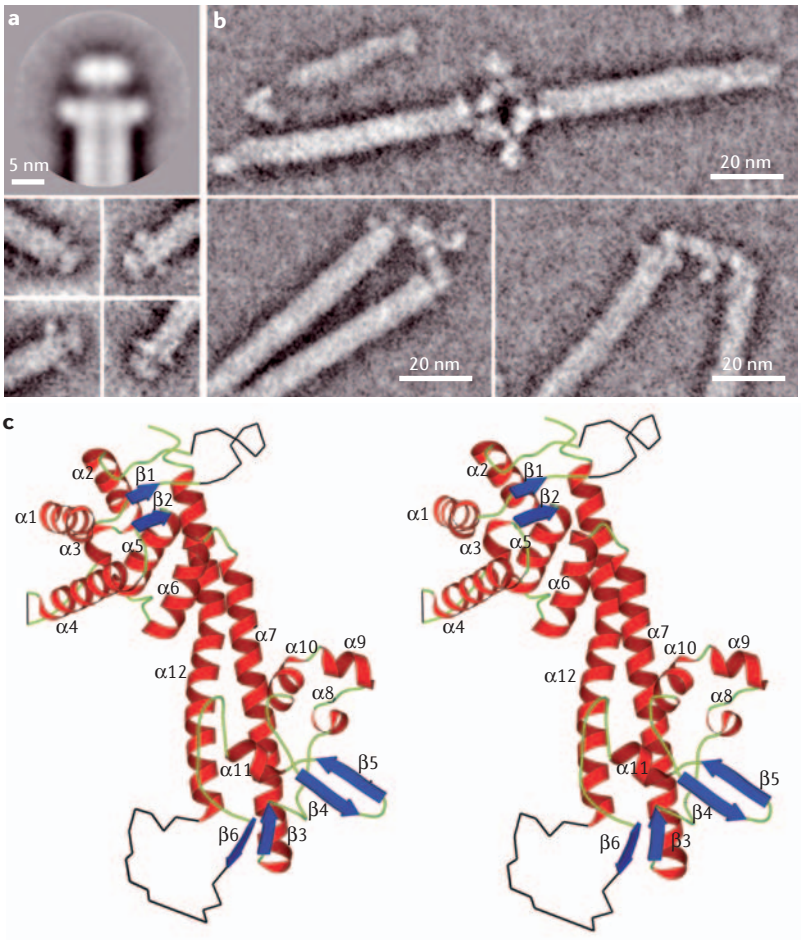


Figure 4 | **LcrV forms a structure at the tip of the needle. a** | Projection average (top) and typical single scanning transmission electron microscopy (STEM) images (bottom) of the complexes formed by LcrV at the tip of the needle of the injectisome of *Yersinia enterocolitica* E40 (resolution average 1.5 nm). A central channel permeates both the needle and the tip complex. **b** | STEM images of wild-type needle structures incubated with anti-LcrV antibodies and negatively stained. In general, the antibodies attached to the head domain of the tip complex. **c** | The crystal structure of *Y. pestis* LcrV with α helices and β strands coloured red and blue, respectively. Parts **a**, **b** reprinted with permission from REF. 59 © American Association for the Advancement of Science (AAAS). Part **c** reprinted with permission from REF. 60 © (2004) Elsevier.

General secretory pathway (Sec pathway) The General Secretory pathway is the most essential bacterial export pathway. It is involved in the assembly of inner membrane proteins and it translocates many proteins across the plasma membrane. The Sec machine recognizes its substrates by an amino-terminal signal peptide that is cleaved off during translocation.

IpaC and IpaD in *Shigella* spp.) (TABLE 3). Two translocators have hydrophobic domains (YopB, YopD; and IpaB, IpaC) whereas the third translocator (LcrV; IpaD) is hydrophilic. The three proteins have been shown to interact with each other[68,69]. The purified hydrophobic translocator proteins can interact with artificial membranes[70,71] and, under some experimental conditions, can also form pores[72,73]. However, using live bacteria and nucleated eukaryotic cells or erythrocytes, the formation of pores requires all three translocators to be present[64–67,74,75].

Pore formation by the injectisome was first demonstrated by incubating live *Yersinia* spp. with erythrocytes[76] and, subsequently, nucleated cells[63]. Although erythrocytes are not a natural target for T3SS, they are a convenient model to study pore formation[23,77]. Following infection of the erythrocytes by the live bacteria, the purified membranes of erythrocytes have been shown to contain the two hydrophobic translocators but not the hydrophilic protein[23,77,78]. The hydrophobic translocators insert into the membrane, even in the absence of the hydrophilic protein[77,78], but pores are only formed in the presence of the hydrophilic translocator[77,78]. So far, it has not been possible to use EM to directly visualize pores that are naturally inserted by an injectisome. In addition, the structure and stoichiometry of a naturally formed translocation pore is unknown.

The fact that LcrV forms a structure at the tip of the injectisome needle[59] (FIG. 4a,b), even before contact with a target cell, suggests a model in which the hydrophilic translocator functions as a scaffold or assembly platform for the pore (FIG. 5). This model is supported by similar observations that were made in *Shigella* spp.[23,79,80] and in EPEC[81]. Interestingly, genome analysis showed that, in EPEC, the orthologue of LcrV is EspA, the filament that extends the needle. Therefore, in EPEC, the tip protein has evolved to polymerize as a filament rather than a simple ring, but it has probably kept its scaffolding characteristics for the two hydrophobic translocators.

The fact that the hydrophobic translocator proteins are not present at the tip of the needle prior to contact with a target cell is expected, given their hydrophobic nature, and it implies that they are secreted immediately on contact with the host cell. However, as yet, the export of YopB and YopD, prior to effector protein export, has not been demonstrated experimentally.

Injectisome assembly

The assembly of the flagellum is a well-known sequential process[11]. The general secretory pathway (Sec) assembles both the base components and the export apparatus. The more distal components are exported by the nascent T3SS channel, and they assemble at their exit point from the channel. A cap at the tip of the growing flagellum ensures the efficient polymerization of the different subunits as they reach the tip[82]. The length of the flagellar filament is probably limited by capillary action on the filament subunits.

Considering the functional, structural and sequence similarities between the flagellum and the injectisome structures, one might expect the distal structures of the injectisome to grow by polymerization of the subunits at the tip of the nascent structure. Consistent with this hypothesis, the Hrp pilus was shown to elongate through the addition of pilin subunits at the tip of the growing pilus[83]. Similarly, using an elegant approach consisting of the sequential expression of different EspA proteins, Crepin *et al.* showed that EspA filaments also elongate by the addition of subunits to the tip of the growing structure[84]. For the needle structure, however, this process has not been demonstrated, possibly because this structure is much shorter and is therefore predicted to assemble in a very short period of time.

The general view of the field is that the components of the injectisome do assemble sequentially, as has been demonstrated for the flagellum. It should be pointed out, however, that an *S. typhimurium* Δ*invj*

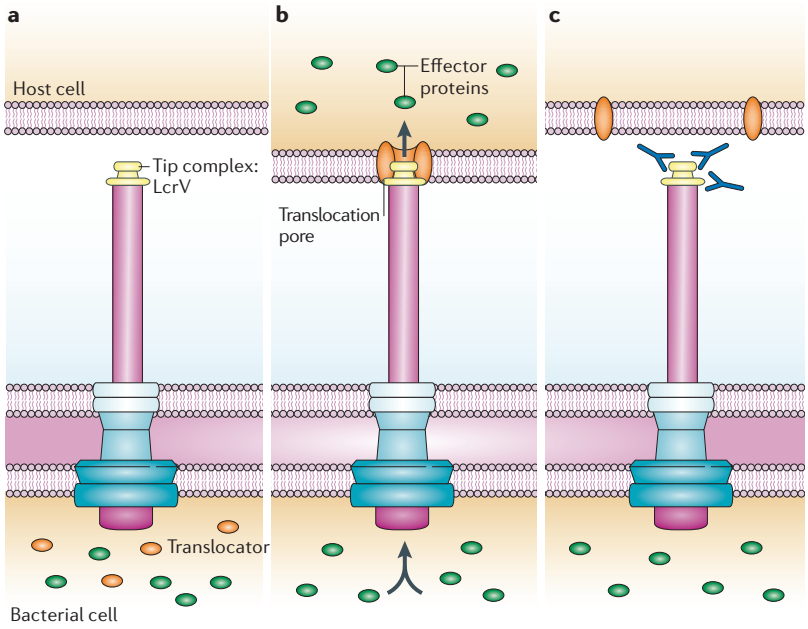


Figure 5 | **Hypothetical model of the function of the LcrV tip complex. a** | No contact with host cell: LcrV forms the complex at the tip of the needle. **b** | Contact with the host cell membrane: the tip complex assists with the assembly of the translocation pore, serving as an assembly platform. **c** | Anti-LcrV antibodies are protective because they prevent the formation of the translocation pore[59].

mutant (see below) still assembles the needle structure although it does not assemble the inner rod[85]. This is a noteworthy finding because it shows that an assembled rod is not necessary in order to assemble the needle. In other words, there is no checkpoint between the assembly of the rod and the needle.

In the flagellum, polymerization of the hook and filament subunits at the tip of the growing structure requires the presence of a special tip at the end of the structure, termed a cap. In the absence of the cap, the subunits are exported normally but are shed into the external environment rather than undergoing polymerization[11,86]. Given the structural and mechanistic similarities between the flagellar hook and the T3SS needle, it was expected that the needle also had a cap structure to ensure subunit polymerization. However, as yet, no injectisome mutant has been reported to have the phenotype expected for a needle-cap mutant. In addition, so far, the only tip structure analysed consists of LcrV and the *Yersinia* injectisome needle can polymerize *in vivo* in the absence of this protein, indicating that LcrV is not a polymerization cap[59]. Moreover, the injectisome needle expressed by *P. aeruginosa* was shown to self polymerize *in vitro*[87]. Therefore, the question of whether a cap structure is required in injectisome needle formation remains open; indeed, it is possible that needle subunits can polymerize without the presence of a cap at the tip of the growing structure.

Interestingly, the phenotype of *S. typhimurium* Δ*invJ* mutant strains corresponds to that expected for an inner-rod cap mutant. Indeed, in the absence of InvJ, PrgJ (the putative inner-rod subunit) is secreted but is not polymerized[85]. Nevertheless, there is no direct evidence that InvJ favours polymerization of PrgJ.

The fact that the injectisome exports its own distal components before it exports the effector proteins implies that the T3SS apparatus can recognize and sequentially export different categories of substrates. To achieve this, it is proposed that the apparatus can switch its substrate specificity when the assembly process has been completed. Export of the effector proteins is then triggered by contact with a eukaryotic target cell. In some systems, export of the effector proteins can also be triggered artificially by the chelation of Ca^{2+} ions (*Yersinia* spp. and *Pseudomonas* spp.), or by the addition of Congo red (*Shigella* spp.).

Needle length control

The final length of the needle varies between different bacteria species, or even bacterial strains, but the length is a regulated process. In *Y. enterocolitica* E40, the needle length is 58 nm ± 10 nm[88]. In the flagellum, the length of the hook is also controlled at approximately 55 nm. In both systems, the loss of one protein — FliK in the flagellar system of *S. typhimurium* and YscP in the *Y. enterocolitica* injectisome (TABLE 3) — leads to uncontrolled growth of the hook and needle, respectively[22,33,88–90]. Different mechanisms have been proposed to explain the control of the length of the needle and of the hook.

The cup model proposes that hook subunits accumulate in the cavity of the flagellar basal body, prior to their export, and so the volume of the cavity determines the hook length[91] (FIG. 6a). Once the cup is emptied, FliK can interact with the newly exposed, cytoplasmic face of the export system and change its specificity from hook-type proteins to flagellin-type export substrates. As shown in Fig 6a, this model implies that the injectisome has a C ring.

The ruler model has been proposed to explain how needle length is controlled in the *Y. enterocolitica* injectisome[88]. Deletions and insertions in *yscP*, were shown to lead to shorter and longer needles, respectively[88]. Furthermore, there is a linear correlation between the size of the YscP protein and the length of the needle structure, suggesting that the protein functions as a molecular ruler. In the proposed model, export of needle subunit proteins is allowed until the needle structure reaches the length of an extended YscP protein. At this point, YscP triggers a substrate specificity switch, which terminates needle subunit export and the YscP protein itself is secreted (FIG. 6b). In this model, the ruler needs to span both the needle and the basal body structure, a distance of approximately 75 nm. The ruler part of YscP comprises approximately 400 residues[92,93] (FIG. 6c). If these 400 residues were stretched to 75 nm, every residue would need to span 1.9 Å, which is more than the distance between two residues in an α-helix (1.5 Å) but is significantly below the theoretical distance between two residues in an extended chain (3.8 Å)[94]. This model is consistent with the requirement for YscP protein secretion to determine the needle length but not necessarily to switch the substrate specificity[92,93].

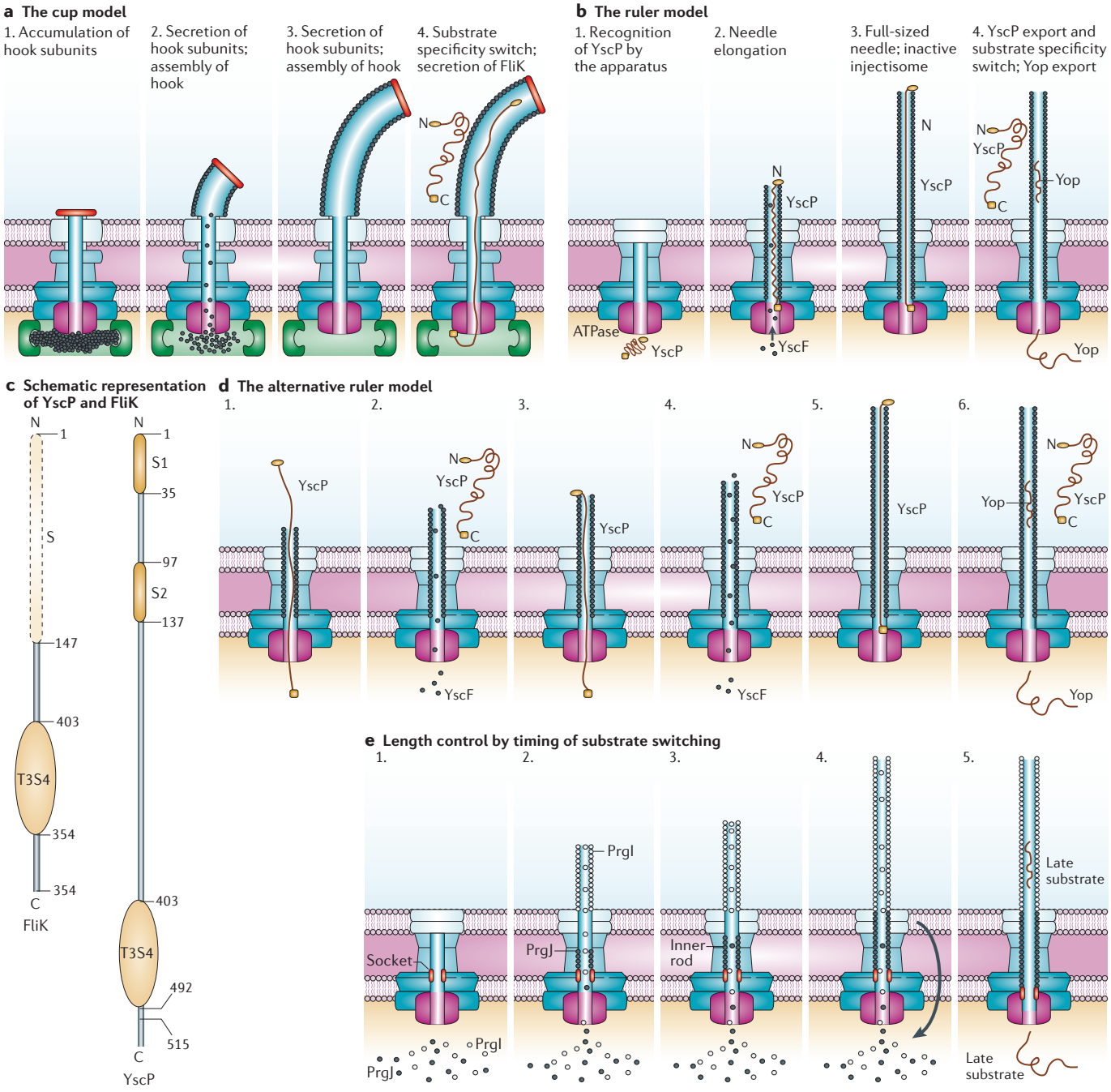


Figure 6 | **Control of the length of the needle and the hook. a** | The cup model[91]: first, the C ring of the basal body is filled with hook subunits (1); the subunits are exported and the hook elongates (2); FliK switches the substrate specificity (3); flagellin is exported and the filament grows (4). **b** | The ruler model[88]: YscP enters the channel after completion of the basal structure (1); the needle subunits are exported and polymerize, leading to the extension of the ruler component of YscP (2); the needle assembly is completed (3); the T3S4 domain of YscP switches the substrate specificity to effector secretion and the ruler is released (4). **c** | Schematic representation of YscP and FliK, showing the export signal(s) localized in the amino-terminal part of the proteins, and the T3S4 domain localized in the carboxy-terminal part of the proteins[118]. **d** | An alternative ruler model. In this, more dynamic model, ruler and subunit molecules are exported alternately (1 to 4). The ruler checks the length while travelling through the channel (1 and 3). When the exact length is reached, the ruler switches the substrate specificity (5), leaving the injectisome ready for translocator and effector proteins (Yop) export (6). **e** | Needle length control by timing of substrate switching[85]. In this model, InvJ stabilizes the socket substructure, shown in red (1), which is necessary for the assembly of the inner rod (black subunits composed of PrgJ). Note that the needle (white subunits, composed of PrgI) assembles simultaneously (2, 3). Termination of the inner rod results in conformational changes that lead to substrate switching (4). Late substrates can then be exported (5). C, carboxyl terminus; N, amino terminus; S, signal; T3S4, type III secretion substrate specificity switching; Yop, *Yersinia* outer protein. Part **c** reproduced with permission from REF. 118 © (2006) Elsevier.

The proposal that FliK and YscP terminate subunit export by functioning as a substrate specificity switch mediated by an interaction with the flagellum/injectisome export apparatus is common to both models. In both cases, this function is supported by two observations. First, *fliK* and *yscP* mutants are unable to export late substrates (flagellin or *Yersinia* spp. outer proteins (Yops)). Second, suppressor mutations of *fliK/yscP* null alleles map to *flhB*[95,96] or *yscU*[97], respectively, two genes that encode a transmembrane protein that is part of the export apparatus. In both cases, the domain of FliK or YscP that is responsible for switching the substrate specificity has been localized in the carboxy-terminal domain of the protein[92,98] (FIG. 6c). These domains, which show a significant degree of conservation, have been referred to as the T3S4 (for type III secretion substrate specificity switching) domain[92,98,99]. To summarize, both the cup and the ruler models are consistent on how YscP and FliK terminate the growth of the needle structure; however, the models differ on the mechanisms used by these proteins to determine the length of the needle.

In the case of FliK, although the ruler model was initially considered, it was subsequently discarded as experiments indicated that all *fliK* deletion mutants studied produced long hooks (termed polyhooks). In these studies, shorter hooks were not observed, although one explanation for this finding was that the deletions interfered with either the export signal or the T3S4 domain of FliK. Interestingly, in a recent study in which new deletions and insertions were introduced into FliK, there was a correlation between shorter or longer FliK proteins and the generation of shorter or longer hooks, respectively (K. Hughes and S. I. Aizawa, unpublished data). In conclusion, these findings indicate that the ruler model does apply to the flagellar hook. However, this does not mean that the ruler model is entirely correct, as proposed in the original version (FIG. 6b). In particular, this version of the model implies that the ruler component and the secreted subunits occupy the channel at the same time, which is questionable given the inner diameter (2–3 nm) constraints of the channel. As a consequence, a more dynamic version of the ruler model can be proposed, in which the ruler component and subunits travel sequentially and more than one ruler component is exported per needle assembled (FIG. 6d). Indeed, a dynamic model, with FliK being occasionally exported during hook assembly, was recently proposed for hook-length control[100]. The validation of the dynamic ruler model will require the determination of a number of parameters of the system, including the number of rulers exported per assembled needle.

The Ysc injectisome is, so far the only one for which needle length has been shown to be correlated with the length of a protein. The conservation of the T3S4 domain makes it likely that the model also applies to other members of the Ysc family of injectisomes. However, there is no additional published data that validates or refutes the ruler model in these systems.

Investigation of the *S. typhimurium* SPI-1 injectisome has lead to the proposal of a third model. Mutations in *invJ* from *S. typhimurium* (SPI-1) and in *spa32* from *Shigella* spp. (the putative orthologues of *fliK/yscP*) resulted in needle structures of indefinite length and the absence of substrate export[33,90,101]. To investigate the mechanism of substrate switching, Marlovits *et al.* compared the structure of NCs obtained from wild type and Δ*invJ* mutants of *S. typhimurium* using cryo-EM. They observed no significant difference in the external structure of the basal body but did note that the Δ*invJ* mutant lacks the inner rod and has no socket-like structure[85]. In addition, these researchers compared the structure of the assembled NC with the structure of the base alone, missing the rod and needle, and observed a significant structural change of the more distal inner ring[30]. Based on these observations, Marlovits *et al.* proposed a mechanism for how substrate specificity changes from the subunits that are required for the inner rod and needle structures to the substrates that are secreted at a later stage. This model (FIG. 6e) is based on the proposal that completion of the inner rod determines the timing of substrate switching and therefore the needle length. According to the model, the function of InvJ is to promote the assembly of the rod by stabilizing the socket in a conformation that is permissive for the anchoring of the rod. The model also explains the finding, observed with both *S. typhimurium* and *Shigella* spp., that overexpression of the needle subunit leads to unregulated needle length [22,33], although recent experiments indicate that this does not occur in *Yersinia* spp. (I. Sorg and G.R.C., unpublished data).

The three models that have been proposed to explain how needle length is controlled are clearly different from each other. The similarities between FliK and YscP, and between the experimental data obtained for both the flagellum and the *Yersinia* spp. injectisome, both indicate that the length of the flagellar hook and the *Yersinia* needle are controlled by a similar mechanism and that the cup and ruler models are incompatible. By contrast, InvJ and Spa32 have no obvious similarity to YscP and FliK. This finding is perhaps not surprising for their hypothetical ruler domains but is more surprising for the T3S4 domains as they fulfill their switch function by interacting with a protein that belongs to the core of conserved proteins (YscU/ FlhB). The fact that InvJ and Spa32 have no clear similarity to YscP or FliK raises the possibility that the mechanism used by *S. typhimurium* and *Shigella* spp. to control the length of the needle structure could differ from that used by *Yersinia* spp. However, the overall similarity between bacterial injectisomes makes it unlikely that the mechanism to control length is radically different. Of course, it is possible that the actual mechanism is more complex than the models proposed and, indeed, could combine characteristics and properties from both models. To add to the complexity, recent experiments on the flagellar hook suggest that a timer might also be involved in the mechanism[100].

Why is the needle length controlled? For a given bacterial strain, the length of the needle structure is constant, with a variation of about 20%. However, this length varies from one bacterial species to another and sometimes even from one biotype to another one (L. Journet and G.R.C., unpublished data). For example,

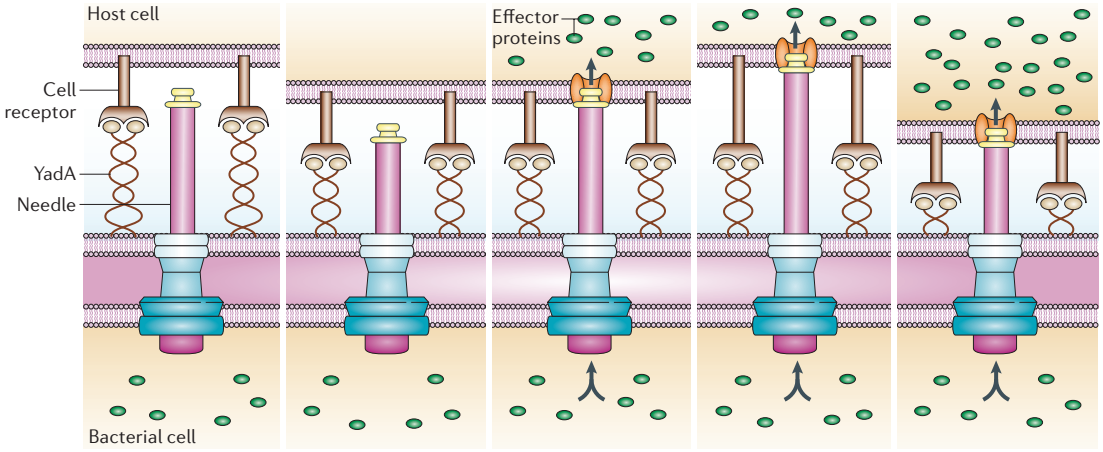
in *S. typhimurium* the length of the needle is 80 nm for SPI-1 (REF. 13) and 150 nm for SPI-2 (REF. 102). For *S. flexneri* it is 45 nm[22], and for *Yersinia* spp. it was shown to be 58 nm for *Y. enterocolitica* E40 and predicted to be 45 nm for *Y. pestis*[88]. This obvious differential control of NC length indicates that it is a crucial physical parameter that impacts on function. Indeed, it seems that the needle length has been evolutionary adjusted relative to the dimensions of other structures on the host cell surface and on the bacterial cell surface[103,104]. When the needle is too short to bridge the distance between the two cytosols, the effector proteins are not lost in the extracellular space. Rather, they are not exported, indicating that the needle structure itself can function as a sensor to trigger export[103] (FIG. 7).

Triggering T3SS export

The activity of the T3SS injectisome is tightly regulated, and export only occurs when a specific signal is triggered, which in turn boosts the expression of T3SS genes. Based on research on the *Yersinia pseudotuberculosis* system[105], the signal that triggers T3SS is commonly viewed as direct contact with a host cell. The mechanisms by which the activity of the injectisome remains blocked in the absence of a triggering event are not understood. In *Yersinia* spp., at least five proteins (YopN and its chaperones SycN and YscB[106,107], TyeA[108] and LcrG[109]) are required for this control. In the absence of any of these proteins, export is constitutive and the secreted effector proteins are lost in the external environment instead of being translocated into target cells. YopN, SycN, YscB and TyeA form a complex, probably located in the bacterial cytoplasm, which is proposed to block the T3SS in the absence of the appropriate trigger[110]. LcrG is a cytosolic protein that interacts with LcrV[111], and its regulatory role is likely to be related to its action on LcrV. However, as each of these five proteins is required to achieve a tight control of protein export through the T3SS, they all must contribute to a single complex control mechanism.

In *S. flexneri*, the T3SS seems to be triggered by the presence of cholesterol in eukaryotic cell membranes[112]. It was also shown that IpaB and SipB, the hydrophobic translocators of *S. flexneri* and *S. typhimurium* SPI-1, respectively, are cholesterol-binding proteins and that effector-protein delivery by the T3SS of *Shigella* spp., *Salmonella* spp. and EPEC does not occur when the membranes of their target eukaryotic cells are depleted of cholesterol[113]. Although this dependence on cholesterol might not apply to other bacteria (such as *Yersinia* spp.), these observations suggest that the lipid environment of the host cell membrane could trigger the formation of the pore complex around the tip of the needle structure. This hypothesis is consistent with the observation that the formation of a functional pore on contact between the needle tip and the host cell membrane is a necessary step to trigger the export of effector proteins[103].

It has also been hypothesized that the needle could transmit a signal back to the bacterium by altering its helical state[114]. Recently, Blocker and colleagues



YadA	Long	Normal	Normal	Normal	Short
Needle	Normal	Short	Normal	Long	Short
Cell receptor	Normal	Normal	Normal	Long	Normal

Figure 7 | **The role of the needle structure.** The docking of the bacterial cell to a target host cell occurs by contact between an adhesin (here YadA) and a receptor on the surface of the host cell (the identity of the receptor for YadA is currently unknown). Other structures are present at the bacterial surface, including lipopolysaccharide (not shown). The bacterial and target cell membranes are separated by a gap and the needle structure bridges this gap. This allows the bacterial effector proteins to be exported, in one step, from the bacterial cytosol to the cytosol of the target cell. In addition, the needle functions as a sensor. If the needle is too short, effector protein export is not triggered. In this experiment, the length of the needle and the adhesin from *Yersinia enterocolitica* E40 have been increased or decreased by genetic engineering, and the translocation of a reporter protein (based on the YopO kinase) was monitored[103].

isolated a number of MxiH mutants that lock the *S. flexneri* injectisome into different export states[114]. EM analysis failed to detect any alterations in the helical architecture of the functionally altered needle structures[56]. However, the position of amino-acid residues that are crucial for the control of protein export in the crystal structure indicates that the transmission of an export signal from the tip of the needle to the base could occur through subtle changes in the packing of the needle without the need for alterations in the helical parameters[57].

Phagocytes engulf their bacterial prey in less than a minute. As one of the principal roles of the T3SS in *Yersinia* spp. is to protect the bacterium from phagocytosis, it is assumed that the activation and functioning of a pre-assembled injectisome is a rapid process. Within 30 seconds of the onset of infection, wild-type *Y. pseudotuberculosis* caused a YopH-dependent dephosphorylation of phosphotyrosine proteins in J774 macrophages[115]. More recently, by using time-lapse microscopy, Hardt and colleagues have shown that *S. typhimurium* initiates delivery of the effector protein SipA into target cells within 10–90 seconds following cell contact, and that the injection process continues for 100–600 seconds until the bacterial SipA pool is exhausted[116]. In a study of the T3SS in *S. flexneri*, it was observed that entire pools of the effector proteins IpaB and IpaC were released concurrently on contact with the host cell, and that 50% of each protein was secreted within 240 seconds[117].

Conclusions and future perspectives

There is no doubt that the T3SS is a vibrant research field and that significant progress has been made over the past three years. In particular, we can now visualize the entire NC structure and the ATPase at a resolution of approximately 1.6–1.7 nm; however, a detailed structure of the C ring is still not available. Some significant constituents of the T3SS, including the needle and the MS ring, can be modelled at a higher resolution, based on available crystal structures. The discovery that LcrV forms a structure at the tip of the needle in the Ysc injectisome, and that it also functions as a scaffold or platform for the assembly of the translocation pore has explained how the pore is linked to the injectisome structure. From a mechanistic viewpoint, it is now known why the injectisome has a needle. It has also been established that the ATPase is responsible for unfolding the proteins destined for export and for removing their chaperone prior to the export process. The mechanism that underlies the control of the needle length was shown to involve a protein that seems to function as a molecular ruler but also as a substrate-specificity switch, a process that could also trigger a conformational change inside the basal structure of the injectisome. Some conformational changes were visualized, suggesting a possible mechanism for the switching process. The imaging of protein translocation into host cells was another recent breakthrough in this field.

What are the next challenges in our ongoing efforts to understand the T3SS process? For the injectisome structure, the precise location of many of the constituent proteins remains unknown. Structurally speaking, the long-term goal is the elucidation of the complete structure at an atomic level; however, this is an ambitious goal, and to achieve it will undoubtedly require several more years of concerted effort. A shorter term goal will be the solution of the pore structure. Deciphering the hierarchy of export during the assembly of the injectisome and, on contact with the host cell, the assembly of the pore, remains a major challenge. Understanding how direct contact with a host cell triggers bacterial protein export is another challenge for the coming years. Finally, and perhaps the greatest challenge of all, as we gain a greater understanding and appreciation of the T3SS and its role in microbial pathogenesis, there is an onus on the research community to exploit this knowledge to develop new anti-infective strategies that target this system and the dangerous pathogens that use it.

Recent reports that a small molecule that is known to block the T3SS of *Y. pseudotuberculosis*[153] efficiently inhibits the intracellular replication and infectivity of *Chlamydia trachomatis* at micromolar concentrations[154,155], demonstrates the validity of this new approach.

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Acknowledgements
This review does not claim to be comprehensive. For the sake of coherence, the *Yersinia* Ysc archetype was taken as a unifying thread, which means that there is a bias in favour of work on this organism. I apologize to colleagues whose work could not be cited for these two reasons. I sincerely thank P. Broz and M. Letzelter for help in the conception of the illustrations and for challenging discussions. I am grateful to P. Broz, M. Letzelter and I. Sorg for discussions, information and critical assessment of the manuscript. I also thank J. Galan for exchange of information. Work in my laboratory is supported by the Swiss National Science Foundation.

Competing interests statement
The author declares no competing financial interests.

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