REVIEWS

Protein delivery into eukaryotic cells by type III secretion machines

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Bacteria that have sustained long-standing close associations with eukaryotic hosts have evolved specific adaptations to survive and replicate in this environment. Perhaps one of the most remarkable of those adaptations is the type III secretion system (T3SS)—a bacterial organelle that has specifically evolved to deliver bacterial proteins into eukaryotic cells. Although originally identified in a handful of pathogenic bacteria, T3SSs are encoded by a large number of bacterial species that are symbiotic or pathogenic for humans, other animals including insects or nematodes, and plants. The study of these systems is leading to unique insights into not only organelle assembly and protein secretion but also mechanisms of symbiosis and pathogenesis.

Made up of more than 20 proteins, T3SSs are among the most complex protein secretion systems known in bacteria. Although the components of type III secretion machines are generally conserved, comparison of their amino acid sequence reveals the existence of different 'clades' of related T3SSs[1]. Despite this conservation, the actual arsenal of bacterial proteins that they deliver (collectively known as 'effectors') is unique to each system. T3SSs are thought to be evolutionarily related to the bacterial flagellum, although this relationship has been the subject of some debate[2]. In this article, however, we will discuss the most salient features of T3SSs, but we will not discuss the related flagellar system[3]. We have focused on what we believe are the general principles that govern the function of these biological machines. Readers can consult other reviews on this topic for more details on specific systems[4,5].

The assembly of a protein-delivery machine

A central component of T3SSs is a supramolecular structure known as the needle complex, which mediates the passage of the secreted proteins through the multi-membrane bacterial envelope. Although the needle complex was originally identified in *Salmonella typhimurium*[6], it has been subsequently detected in several other bacteria and it is therefore believed to be a core component of all T3SSs[7–9]. It is composed of a multi-ring base, which anchors the structure to the bacterial envelope, and a needle-like projection that protrudes several nanometres from the bacterial surface (Fig. 1). The base is traversed by the inner rod, which is a cylindrical substructure that connects the needle to the basal side of the base substructure. The entire needle complex is traversed by a narrow channel (~28 Å in diameter), which most probably serves as the conduit for proteins travelling through this secretion pathway. *Salmonella typhimurium* assembles heterogenous needle complexes of varying symmetries, ranging from 19- to 22-fold, on the vertical axis[10]. However, this heterogeneity may not be functionally significant, but may simply reflect the intrinsic difficulty in maintaining 'quality control' during the assembly of such a many-fold symmetry structure.

Assembly of the needle complex proceeds in a stepwise manner, in which the assembly of the base substructure precedes the assembly of the inner rod and needle (Fig. 2; refs 11–13). In *S. typhimurium* the base is composed of equimolar amounts of three proteins: InvG, a member of the secretin family of proteins that makes up the outer rings; and PrgH and PrgK, which are thought to form the rest of the structure[10]. Homologues of these proteins can be detected in other T3SSs, suggesting that the composition and assembly pathway are likely to be conserved. Proteins destined to make up the base are delivered to the bacterial envelope by the universal Sec protein secretion machinery, which is consistent with the observation that these proteins posses signal sequences for this common secretion pathway. In some T3SSs, assembly of the outer rings requires an accessory outer membrane protein that serves as a chaperone to presumably

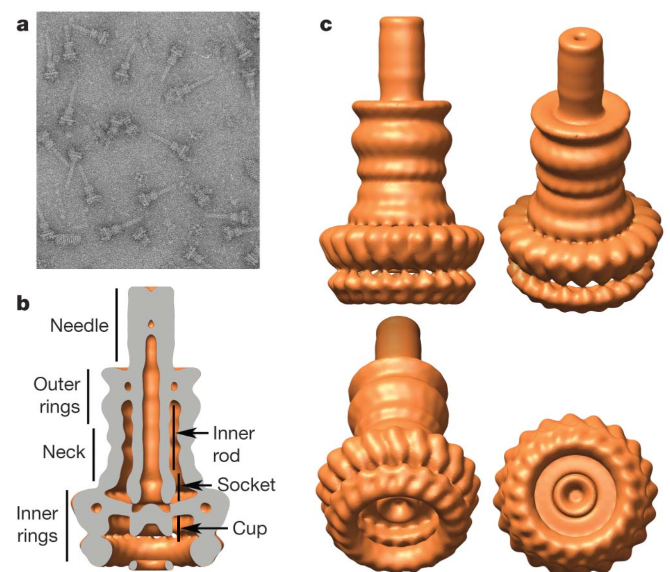

Figure 1 | Needle complex of *Salmonella typhimurium*. **a**, Electron micrographs of negatively stained isolated needle complexes. **b**, Cross-section of the structure of the needle complex indicating the location of its different substructures. **c**, Surface rendering of the structure of the needle complex. Shown here are different views of the structure of a 20-fold complex with 20-fold symmetry imposed.

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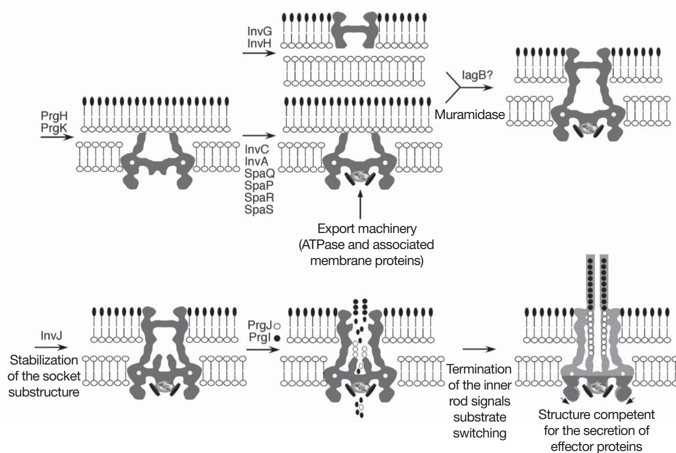

Figure 2 | Model of the assembly pathway of the needle complex. Assembly of the base substructure occurs in discrete steps, and its completion is followed by the recruitment of accessory proteins including an ATPase (InvC) that is involved in the recognition and unfolding of substrates of the type III secretion machine. A muramidase (IagB) may help passage of the structure through the peptidoglycan layer. Completion of the base initiates secretion of needle (PrgI), inner rod (PrgJ) and regulatory (InvJ) proteins. InvJ stabilizes the socket substructure of the base to allow assembly of the inner rod. Termination of the inner rod results in conformational changes on the cytoplasmic side of the base, which leads to reprogramming of the secretion machine to begin the secretion of effector proteins. InvH chaperones the assembly of InvG to form the outer ring but does not form part of the final structure. InvA, SpaP, SpaQ, SpaR and SpaS are polytopic inner membrane proteins that form part of the 'export apparatus'. The nomenclature of relevant proteins is from *S. typhimurium*. See text for details.

facilitate the proper folding and multimeric arrangement of the outer ring subunit[14,15]. Determination of the atomic structure of EscJ (the orthologue of *S. typhimurium* PrgK in enteropathogenic *Escherichia coli*) and the use of molecular modelling, indicate that this protein could form a large multi-subunit 'ring' that may serve as an initiator of needle complex assembly[16,17]. However, more experiments will be required to validate this attractive hypothesis.

Assembly of the base substructure presumably leads to the recruitment of a subset of highly conserved, accessory, inner membrane proteins, which are thought to facilitate the passage of secreted proteins through the inner membrane. By analogy to the flagellar apparatus, it has been proposed that another compartment, the so-called 'C ring', may be assembled in the bacterial cytoplasm immediately beneath the needle complex[18]. In *Shigella* spp., this compartment may be formed at least in part by the T3SS protein Spa33, which was shown to be associated with the needle complex base[19]. However, homologues of Spa33 in other T3SSs are secreted into the culture supernatant[20], which would not be consistent with this proposed function. More studies will be required to ascertain the role of this protein in type III secretion and better define the putative substructure that it may form.

Once fully assembled, the base substructure begins to function as a 'type III protein secretion machine'. However, this submachine is exclusively devoted to the secretion of proteins that are components of, or necessary for the assembly of the inner rod and needle substructures. In *S. typhimurium* these proteins are PrgJ (the putative inner rod protein), PrgI (the needle protein) and InvJ (a regulatory protein that is required for appropriate and efficient assembly of the needle complex, but that does not form part of the final structure; see below)[10,12]. On completion of the needle complex assembly, the secretion machine changes specificity and becomes competent for secreting effector proteins[12,21]. Substrate switching and reprogramming of the export machinery is also observed during flagellar assembly[3]. In this case, once the hook substructure reaches a certain length, the export machinery switches substrate specificity to export proteins necessary for the assembly of the flagellar filament. Substrate switching in the T3SS and flagellar systems is largely dependent on the function of a family of somewhat related accessory proteins (for example, InvJ (ref. 21) and YscP (ref. 22) in the case of the *Salmonella* spp. and *Yersinia* spp. T3SSs, respectively, and FliK (ref. 23) in the case of the flagellar hook). Mutant strains lacking these proteins assemble needle complexes with much longer needles or flagella with much longer hooks. The mechanisms by which these secretion machines change substrate specificity are incompletely understood. Comparison of the 17 Å resolution structure of the *S. typhimurium* fully assembled needle complex, which is competent for the secretion of effector proteins, with that of the base alone, which is competent for the secretion of the needle and the inner rod proteins, revealed intriguing differences that may provide the structural basis for this secretion reprogramming[10]. Very significant changes were observed on the cytoplasmic side of the base, which is the surface of the needle complex that would be available for interaction with the secreted proteins themselves or accessory proteins involved in substrate recognition (see below). In particular, a cup-like structure, situated at the centre of the cytoplasmic face of the base (see Fig. 1), undergoes a marked 'downward' movement on completion of the assembly of the needle substructure. Furthermore, the more distal inner ring, which is presumably located within the bacterial cytoplasm, undergoes a 'clumping' and 'downward' movement, further redefining the shape of the cavity that surrounds the area of the needle complex that is likely to be involved in substrate recognition. How these conformational changes affect substrate recognition, however, is not known. Furthermore, the mechanisms by which the InvJ, YscP and FliK proteins mediate substrate switching are very poorly understood.

At least three seemingly incompatible models to explain the mechanisms of substrate switching during the assembly of these organelles have been proposed. One model put forward for the flagellar system proposes that a flagellar substructure, the cytoplasmic C ring, acts as a 'measuring cup' that, once filled with hook proteins, permits their secretion and the assembly of a hook of defined length[24]. When the C-ring is emptied, the FliK protein (perhaps in conjunction with other regulatory proteins, for example, FlhB) interacts with the secretion apparatus and triggers substrate switching. Although attractive, there is little mechanistic and experimental evidence in support of this hypothesis.

The second model, formed to account for the mechanisms of needle-length control in the *Yersinia* spp. T3SS needle complex, proposes that the YscP protein works as a 'molecular ruler'[22]. In this model, YscP is proposed to exert its function as an extended polypeptide that is anchored to both the tip of the growing needle and the base of the needle complex. The full extension of the YscP 'ruler' would signal that the needle has reached the appropriate length and would somehow convey the information to the secretion apparatus to switch substrates. In support of this model, lengthening or shortening YscP by inserting or removing sequences at a discrete domain resulted in longer or shorter needles. In all likelihood, the needle substructure grows by addition of subunits that travel through the central channel and are added at the distal growing tip[25]. Therefore, in the context of this model it is not clear how these subunits could travel through the narrow central channel of the needle complex when it is occupied by the 'measuring ruler'.

A third model was proposed based on the observation that needle complexes isolated from the *S. typhimurium* Δ*invJ* mutant lack the inner rod substructure[26]. This observation indicates that assembly of the needle and inner rod substructures can occur independently, although firm anchoring of the needle does require the presence of the inner rod because needles isolated from the Δ*invJ* mutant easily detach from the base. The high-resolution structure of the needle complex showed that in the wild type, the inner rod is anchored to

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a 'socket-like' structure located on the basal side of the base[10]. This 'socket' is thought to serve as a symmetry adaptor between the helical inner rod and the *N*-fold symmetric base. The Δ*invJ* mutant lacks the socket[26], indicating that this structure may be required to nucleate the assembly of the inner rod, which is missing in this mutant. Furthermore, the cytoplasmic side of the base isolated from the Δ*invJ* mutant is very similar to that of the wild-type base before needle assembly, which is consistent with the fact that both structures have essentially identical substrate specificity. These observations led to the proposal of a model for the mechanism of substrate-specificity switching in which the completion of the inner rod would determine the timing of substrate switching (and hence needle length)[26]. In this model, the termination of the inner rod and the firm anchoring of the needle would lead to conformational changes on the cytoplasmic side of the base, resulting in the reprogramming of the secretion apparatus and rendering it competent for the secretion of effector proteins. Consistent with this model, changes in the relative concentrations of PrgJ and PrgI, which would presumably lead to relatively faster or slower assembly of the inner rod, led to needles of different lengths (that is, shorter in the case of overexpression of PrgJ and longer in the case of overexpression of PrgI). In the context of this model, the InvJ protein would control the assembly of the inner rod by stabilizing a conformation of the socket of the needle complex base that is permissive for the anchoring of the inner rod, in a manner similar to that proposed for the flagellar 'capping' proteins. These flagellar proteins, which like InvJ control flagellar assembly but are not part of the final structure and are discarded into the culture supernatant, are thought to work as scaffolds or 'kinetic traps' to facilitate the nucleation or anchoring of different flagellar substructures[3]. More studies will be required to reconcile these different models. In any case, it is clear that these models are incomplete because they do not account for the function of other accessory proteins (for example, the flagellar FlhB or its T3SS homologues) known to be important for substrate switching[27]. It is also evident that it is important for bacteria to assemble needles of a minimum length so that other bacterial surface structures do not interfere with type III secretion function[28,29].

Multiple signals to ensure substrate specificity

Type III secretion machines must be able to select the rather small number of substrate proteins that are destined to travel this pathway. In addition, some bacteria encode more than one T3SS, which are often expressed simultaneously. Therefore, the mechanisms of substrate recognition must warrant a level of specificity capable of ensuring that the correct substrates are targeted to the appropriate machine. The narrow opening of the channel that traverses the needle complex and serves as the conduit for the type III secreted proteins also dictates that, once recognized by the secretion machine, substrates must be unfolded before their secretion. Furthermore, evidence is beginning to mount indicating that the secretion process follows a hierarchy with a predetermined order in which different proteins are engaged and secreted by these machines[21,30,31]. Therefore, it is not surprising that the mechanisms of substrate recognition are complex, involving multiple signals and accessory proteins[32] (Fig. 3).

Most, if not all, type III secreted proteins posses a secretion signal located within the first ~20–30 amino acids[33,34]. Unlike Sec-dependent signal sequences, however, T3S signals are not cleaved on secretion. In general, these signals do not seem to have conserved features that are shared even among substrates of the same type III secretion machine. Many studies have even shown that introduction of frameshift mutations within the secretion signals of at least some type III secreted proteins does not prevent their secretion. In fact, this observation led to the proposal that the 'secretion signal' may be located in the coding messenger RNA and not in the polypeptide[35]. However, persuasive evidence has been presented that, at least in some type III secreted proteins, the secretion signal does indeed reside within the amino acid sequence and not the mRNA[36–38].

How can the demonstrated specificity of these secretion machines be reconciled with the obvious tolerance for change in the secretion signal? Although the answer to this question is still a matter of debate, it is conceivable that the following considerations may provide the basis for an explanation. First, it is possible that the presence of non-structured flexible segments at the amino terminus may be all that is required for a sequence to function as a type III secretion signal. However, it is unlikely that this feature alone could ensure specificity, as it is likely that many proteins that are not substrates of these machines exhibit this feature. Therefore other elements must ensure the specificity of substrate selection. Second, an additional layer of specificity may be conferred by accessory proteins such as a family of customized cytosolic chaperones that specifically bind at least some of the type III secreted proteins[39]. These type-III-secretion-associated chaperones are small, acidic, dimeric proteins, which unlike other chaperones, lack ATP-binding or ATP-hydrolysing activities[40]. Although in general type-III-secretion-associated chaperones do not

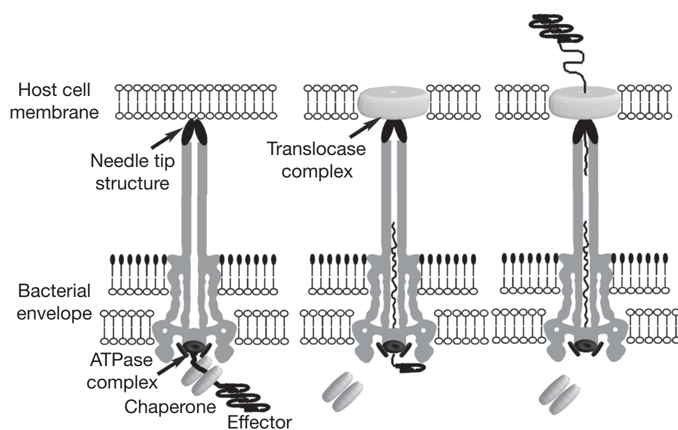

Figure 3 | Model for substrate recognition and delivery of proteins by type III secretion machines. The effector–chaperone complex is recognized by the secretion machinery, including a type-III-secretion-associated ATPase. The ATPase 'strips' the chaperone from the complex, which remains within the bacterial cell, and mediates the unfolding and 'threading' of the effector protein through the central channel of the needle complex. A 'translocator complex' made up of proteins also secreted by the T3SS is assembled on the host cell membrane and mediates the passage of the effector proteins through the target cell membrane. The translocated effectors re-fold within the host cell to carry out their function. See text for details.

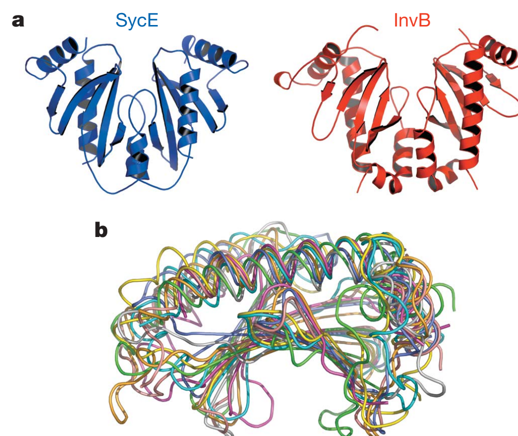

Figure 4 | Crystal structures of type III secretion chaperones. a, Ribbon representation of the crystal structures of type III secretion chaperones SycE from *Yersinia pseudotuberculosis* and InvB from *Salmonella typhimurium*, which bind to one of multiple effector proteins. **b**, Structural alignment of multiple type III secretion chaperones. The structures of AvrPphF (green), InvB (cyan), SigE (magenta), Spa15 (yellow), SicP (grey), SycH (purple) and YscB (orange) are shown.

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share significant primary amino acid sequence similarities among themselves, the crystal structures of several of these chaperones have clearly shown that they are highly related molecules[41] (Fig. 4). In general, these chaperones bind a ~50–100 amino acid domain of the secreted protein, located immediately downstream from the N-terminal secretion signal[34,42]. Although most often a given chaperone is specific for a single secreted protein, there are chaperones that can bind multiple target proteins. The co-crystal structures of several of these chaperones bound to their cognate secreted protein have provided very valuable insight into their function[43–45]. These structures showed that these chaperones maintain the chaperone-binding domain of their cognate secreted proteins in a non-globular conformation that nevertheless maintains secondary structure. This observation has led to the proposal that at least one of the functions of these chaperones must be to 'prime' the secreted proteins for rapid unfolding before secretion[41]. Another proposed function is to prevent the premature and undesirable interactions of the cognate secreted protein with other components of the type III secretion machinery within the bacterial cell[42]. In addition, it is clear that these chaperones play a key role in targeting the secreted protein to the cognate type III secretion apparatus. Consistent with this hypothesis, removal of the chaperone-binding domain of at least some type III secreted proteins prevents their secretion through their associated T3SS. Intriguingly, in some cases these deletion mutants are targeted to the flagellar export apparatus[38,45]. These results indicate that the N-terminal signal sequence may be a 'generic' type III secretion signal, but other sequences and accessory proteins such as the type-III-secretion-associated chaperones may confer the specificity.

It has been suggested that the overall three-dimensional path followed by the chaperone-binding domain when associated to its cognate chaperone could serve as a conserved 'universal' signal for recognition by T3SSs[44]. However, this is unlikely as comparison of the available co-crystal structures of several chaperone–effector complexes indicates that the polypeptide paths are too different to serve as universal signals[45]. It is therefore more probable that other structural features common to all chaperones may serve as recognition signals. Given the high degree of three-dimensional structural relatedness among these chaperones, this possibility seems likely. In addition a common feature has been identified in the chaperone-binding domain of effector proteins[45]. However, although this motif seems to be important for targeting the effector proteins to their cognate chaperones, there is no evidence to support a role for this domain in substrate recognition by the secretion apparatus.

In addition to recognizing secretion signals on the chaperone–effector complex, the secretion machine must 'strip' the chaperone from the effector protein because type-III-secretion-associated chaperones remain in the bacterial cytosol after delivery of the effector proteins to the secretion apparatus. The chaperone–secreted-protein interface is substantial (up to 6,200 $Å^2$ in some cases), therefore this presents a significant challenge for the secretion machine. Furthermore, the effector domains of the secreted protein, which are usually located carboxy-terminal to the chaperone-binding domain, are indeed folded when bound to the chaperone[46]. The limitation in size of the secretion channel (estimated to be ~28 Å) dictates that this domain must be unfolded before secretion. Recently, it has been shown that a highly conserved ATPase associated with the type III secretion apparatus has a critical role in all these steps. *In vitro* studies showed that the ATPase binds both the chaperones as well as the chaperone–effector complexes[47,48]. More importantly, addition of the ATPase to the chaperone–effector complex *in vitro* results in the dissociation of the complex and the unfolding of the effector domain of the effector protein[48]. Therefore the T3SS-associated ATPases play a critical role in the recruitment and unfolding of the T3SS substrates, a function consistent with the structure and proposed localization of the ATPases in close proximity to the secretion machine[49]. Furthermore, this unfolding activity may be critical for energizing the secretion process. These observations suggest intriguing parallels between T3SS-associated ATPases and AAA^+ ATPases. The members of the AAA^+ ATPase protein family are involved in a large variety of biological processes and they exert their function by dissociating protein complexes or unfolding specific protein substrates[50]. More remarkably, the similarity may extend to some essential features of the substrate recognition process. For example, AAA^+ machines, such as the ClpA/ClpP protease, select substrates by recognizing poorly conserved unstructured short peptide signals often located at the N- or C-terminus of the target proteins. In addition, substrate specificity is often ensured through the activity of accessory proteins. Therefore type III secretion and the translocation of substrates into triple AAA^+ machines may share some mechanistic features.

Protein delivery into target cells is not a needle stick

Addition of type-III-secreted effector proteins to the culture medium is not sufficient to promote their translocation into cultured host cells and the bacterial 'delivery device' is absolutely essential for protein translocation. However, the mechanism by which type III secretion machines carry out this function remains one of the least understood aspects of type III secretion. A model was proposed in which the needle itself, through the activity of the needle protein, would 'punch' a hole in the host cell membrane placing its 'tip' inside the cell and thereby delivering the proteins into the target cell cytosol[51]. Attractive as this model may seem, it is clearly incorrect. Indeed, the needle complex alone is not capable of mediating protein injection but needs the activity of a subset of somewhat conserved proteins that are themselves secreted by the T3SS[52,53]. This group of proteins (known as 'translocators') are thought to insert in the target cell membrane forming a channel through which the effector proteins can pass on their way to the target cell cytosol[53,54]. Consistent with this model, strains lacking the translocators are able to secrete the effector protein but are unable to deliver them into the target cells[52,53]. These mutants can assemble the needle complex, indicating that the needle itself is insufficient to mediate translocation. A possible scenario is that the needle actually 'docks' onto the pore or channel made up by the translocators thereby allowing the direct delivery of effector proteins into the target cell (Fig. 3). Such docking may be facilitated by accessory structures located at the tip of the needle. One of these structures, recently identified in *Yersinia enterocolitica*, is formed by a single protein, LcrV (ref. 55). Another more complex structure has been visualized in the T3SS of enteropathogenic *E. coli* and some plant pathogenic bacteria[56,57]. This structure, which is also formed by a single protein (for example, EspA in the case of the *E. coli* T3SS), takes the form of a long appendage that extends from the tip of the needle and presumably serves as a 'bridge' linking the needle with the bacterial translocators on the target cell membrane. It is not known how the insertion of the translocators in the target cell plasma membrane, the assembly of the accessory structures at the tip of the needle, the putative docking of the secretion machine to the translocator channel, and the actual delivery of the effector proteins are ultimately coordinated.

Sensing the target to deliver goods at the right time

T3SSs are highly regulated to ensure that they function at the appropriate time. In their simplest form, the regulatory mechanisms ensure that the secretion machine is deployed in the bacterial envelope when the appropriate cues are present. These regulatory mechanisms are largely transcriptional and rather specific for each T3SS (ref. 58). In addition, in a subset of T3SSs there are regulatory mechanisms to ensure that the secretion machine is activated only on bacterial contact with the target cell[30,59]. This regulatory mechanism is presumably in place to prevent the premature, non-productive release of the effector proteins from the cell. Although this activation step can be triggered *in vitro* with some specific treatments (for example, lowering the calcium concentration or adding Congo red triggers the *Yersinia* spp. and *Shigella* spp. T3SSs, respectively), the physiological

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signals must derive from target cell contact. The nature of the stimulating signal is unknown, but it is unlikely to involve a soluble factor because it is difficult to envisage how such a stimulant could ensure that the activation occurs only on bacterial cell contact. More likely, the bacteria or the secretion machine must be able to sense contact with the cell, leading to the activation of the secretion apparatus.

How could this mechanism work? An attractive hypothesis is that the needle, the outermost component of the secretion machine, may be able to sense cell contact and somehow transduce a signal to the cytoplasmic side of the secretion machine to trigger its activity. Although there is no experimental support for this hypothesis, it is intriguing that discrete mutations have been found in the needle protein that result in constitutive type III secretion[60,61]. It is therefore conceivable that these mutations lead to the assembly of needles whose conformation resembles the 'stimulated' state. Because monomers of the needle protein assemble into a helical structure, it has been proposed that sensing by this structure may occur through changes in the helical packing. However, mutants of the needle protein that showed altered secretion did not exhibit altered helical packing[62]. Whatever the sensing mechanism may be, the signal must be transduced to the cytoplasmic side of the secretion apparatus to 'open' the secretion machine. A candidate platform to transduce the activating signal within the bacterial cytoplasm is a multiprotein complex that in *Yersinia* spp. is composed of the YopN, SycN, YscB and TyeA proteins[63–65]. This complex is essential to prevent the secretion of effector proteins in the absence of stimulatory signals. Homologues of components of this platform that perform similar functions have been identified in other T3SSs[66,67], indicating that this platform is presumably conserved in all T3SSs.

Once the effector proteins are released, evidence suggests that regulatory mechanisms are in place to ensure that the system is 'reset' and the secretion machine is 're-loaded' either with the same arsenal of effectors or, in some cases perhaps with new ones[68]. Although the regulatory systems seem specific for each T3SS, a common mechanism involves the use of regulatory proteins that themselves are substrates of the T3SS (refs 69–71). Therefore, by removing either activators or repressors through secretion, T3SSs can couple the actual secretion process with the regulation of expression of effector proteins. A similar mechanism has been described in the flagellar system[72].

Mimicry as a strategy to modulate cellular functions

It is clear that each T3SS delivers a unique arsenal of effector proteins, which have presumably been assembled and optimized through evolution to suit the specific needs of the bacteria that harbour them. Therefore, it is not surprising that proteins delivered by different T3SSs can modulate or interfere with a vast array of cellular functions including actin and tubulin dynamics, gene expression, vesicular trafficking, programmed cell death and cell cycle progression. The function of only a very small number of effector proteins has been characterized in some detail. Space limitations, however, prevent us from discussing the individual functions of these effector proteins in any detail. However, one general theme that emerges from the functional characterization and the atomic structures of a handful of these effector proteins is one of 'mimicry' as a central strategy to modulate cellular functions[73]. Unlike other bacterial toxins that exert their function by introducing covalent, non-reversible modifications of their target host cell proteins, T3SS effectors seem to act by mimicking the function of host cell proteins. Indeed, this strategy seems appropriate to have been adapted by bacteria that have type III secretion systems as a central element for the establishment of a close functional interface that is often symbiotic in nature.

Another theme that has emerged from these studies is that these 'eukaryotic protein mimics' often seem to be the product of convergent evolution rather than the result of horizontal 'hijacking' of mammalian cell genes. For example, the *Salmonella* SPI-1 T3SS effector protein SopE is a Rho-family GTPase exchange factor (GEF) that shares no sequence or structural similarity with eukaryotic GEFs[74]. However, the crystal structure of the complex of SopE with its target Rac1 showed that the interaction leads to an outcome (that is, conformational changes in the critical switch 1 and switch 2 regions of Rac1) that is nearly indistinguishable from that of the interaction of a bona fide eukaryotic GEF and the same target[75]. A similar example is that of a family of related GTPase activating proteins (GAPs) for Rho-family GTPases encoded by *Salmonella* spp., *Yersinia* spp. and *Pseudomonas aeruginosa* (SptP, YopE and ExoS, repectively)[76–78]. Despite the utterly different amino acid sequence and structure of these GAPs in comparison with eukaryotic GAPs, they carry out their enzymatic activity using even the same chemistry as that used by their eukaryotic counterparts[79,80]. A corollary of this observation is that it is very difficult to predict the actual function of most T3SS effector proteins from amino acid sequence analysis or even from the solution of their atomic proto-structures (that is, the atomic structures of the effectors in the absence of their host cellular targets). Indeed, most known or predicted T3SS effector proteins share no obvious amino acid sequence similarity to other proteins, except their putative orthologues and paralogues. Therefore, in most cases, understanding of the function of these effector proteins will require systematic functional analysis.

Yet another important theme that has emerged from the, so far, limited studies of T3SS effector proteins is that often the activities of different effectors delivered by the same machine are carefully coordinated and temporally regulated[81,82]. Therefore, an understanding of the biology of a given T3SS will require an understanding of the biology of the function of all or most of the proteins that the system delivers into host cells. Indeed, understanding of the function of a given effector protein outside of the context of the function of the other effectors delivered by the same T3SS, may be incomplete, at best, or even entirely misleading. For example, the GAP activity of the *S. typhimurium* protein SptP by itself was originally interpreted as an activity aimed at disrupting the actin cytoskeleton of the target cell; however, in the context of its delivery along with activators of Rho-family GTPases, the function of SptP in *S. typhimurium* proved to be the preservation of the actin cytoskeleton rather than its disruption[76]. Therefore, a comprehensive understanding of the function of most effectors delivered by a given T3SS may be required to ultimately know the actual function of that T3SS.

Future perspectives

The discovery of type III secretion machines has arguably been one of the most significant discoveries in bacterial pathogenesis of the past few years. The widespread distribution of such a macromolecular machine and its use in rather diverse biological contexts is a testament to the success of the evolutionary forces working to shape the complex functional interface between pathogenic or symbiotic bacteria and their eukaryotic hosts. Its central role in the interaction of many pathogenic bacteria opens up the possibility of developing new anti-infective strategies[83]. In addition, a detailed understanding of these machines is allowing them to be harnessed to deliver heterologous proteins for therapeutic or vaccine purposes[84]. The past few years have seen a rather remarkable increase in the understanding of these machines. There is no doubt that the importance and intrinsic beauty of these fascinating machines will continue to attract the attention of scientists and therefore progress is likely to continue at an even faster pace.

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