

## Timing is everything: the regulation of type III secretion

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**Abstract** Type Three Secretion Systems (T3SSs) are essential virulence determinants of many Gram-negative bacteria. The T3SS is an injection device that can transfer bacterial virulence proteins directly into host cells. The apparatus is made up of a basal body that spans both bacterial membranes and an extracellular needle that possesses a channel that is thought to act as a conduit for protein secretion. Contact with a host-cell membrane triggers the insertion of a pore into the target membrane, and effectors are translocated through this pore into the host cell. To assemble a functional T3SS, specific substrates must be targeted to the apparatus in the correct order. Recently, there have been many developments in our structural and functional understanding of the proteins involved in the regulation of secretion. Here we review the current understanding of protein components of the system thought to be involved in switching between different stages of secretion.

**Keywords** Type III Secretion · Regulation · Gram-negative bacteria · Virulence

### Introduction

Many Gram-negative bacterial pathogens use Type Three Secretion Systems (T3SSs) to inject bacterial virulence proteins directly into eukaryotic host target cells. T3SSs are complex macromolecular machines that consist of the structural components of the apparatus itself, secreted proteins (including pore-forming translocators and effectors), chaperones and cytoplasmic regulators. The apparatus is made up of a basal body that spans both bacterial membranes and an extracellular needle that protrudes from the bacterial surface. The basal body possesses rotational symmetry and is made up of several rings of integral membrane and periplasmic proteins (reviewed in [1, 2]). During morphogenesis, the components of the transmembrane rings are handled by the Sec machinery while subsequent components are sequentially exported by the T3S apparatus itself [3, 4]. The needle projects from the basal body and is assembled by the polymerization of a single, small needle subunit (reviewed in [5, 6]). Both the basal body and needle are hollow, containing a channel that is thought to act as a conduit for protein secretion [7, 8]. The needle terminates in a tip structure made of several copies of the tip protein (reviewed in [5, 6, 9]). Upon contact with a host-cell membrane, the T3SS exports two distinct categories of proteins: the translocators that form a pore in the target membrane and the effectors that traffic through this pore into the host cell.

In order to build a functional T3SS, there are distinct stages during assembly that have specific requirements regarding the substrates to be exported and the timing of the switch to the next stage. These stages are briefly outlined below and illustrated in Fig. 1. Early substrates such as the proteins that make up the rod (the periplasmic portion of the needle that lies within the basal body) and

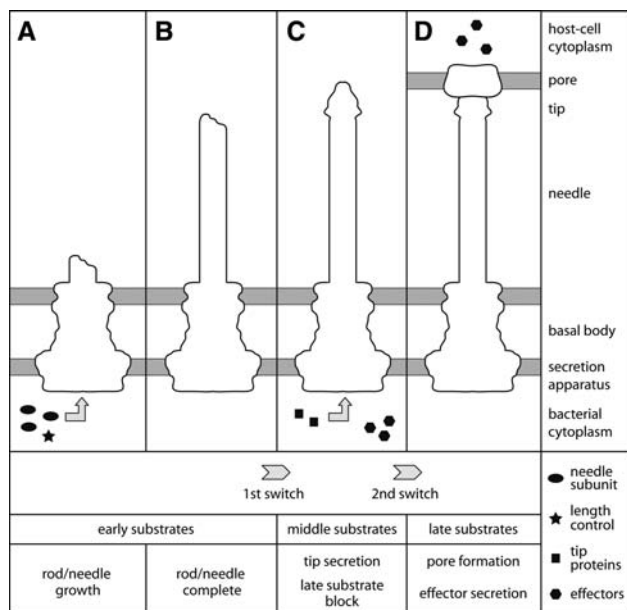
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**Fig. 1** Schematic diagram illustrating different secretion states of pathogenic Type Three Secretion Systems. **a** Following assembly of the basal body (*black outline*) into the inner and outer bacterial membranes (*grey*), early substrates including the needle subunit (*black oval*) and the needle-length control protein (*black star*) are targeted to the apparatus for secretion. **b** The secreted needle subunits assemble to form a hollow extracellular needle. Upon completion of the needle, the secretion apparatus undergoes the first substrate specificity switch to secrete the tip proteins. **c** Tip proteins (*black squares*) are secreted and assemble at the tip of the needle, and further secretion is blocked until host-cell contact. **d** Host-cell contact triggers the second switch at which point the pore-forming translocator proteins are inserted into the host-cell membrane, and effector proteins (*black hexagons*) are targeted to the host cell

needle must be targeted to the secretion apparatus after the membrane rings are formed (Fig. 1a) [3]. This stage must be monitored as it is known that the length of the needle is well controlled [10–12]. Thus, the substrate specificity of the apparatus must be switched at the appropriate point to arrest needle growth (Fig. 1b) [13]. The trigger for this switch involves a component of the inner membrane and an early substrate of the export apparatus, a cytoplasmic protein known as the length-control protein [14, 15]. Following completion of the needle structure, the tip proteins are exported, and secretion must be blocked until contact with a host-cell membrane (Fig. 1c) [16]. This block is mediated in part by a cytoplasmic protein that is thought to act as a gatekeeper and that may be able to respond to specific signals following host-cell contact [17–20]. Upon contact, translocators must be secreted and inserted into the host cell membrane to form a pore prior to secretion of late substrates, the effectors (Fig. 1d) [21, 22]. In this way, effectors will be exported directly into host cells rather than into the extracellular milieu.

Clearly, pathogens require several mechanisms to ensure hierarchical and temporal control over the secretion of

T3SS components. Regulation of secretion is controlled at several levels: transcriptional, post-transcriptional, translational as well as via the conformational switching and secretion of T3SS components (reviewed in [23, 24]). In this review, we will address the most recent work relating to the protein components of the system thought to be involved in the switching between different stages of assembly and secretion. Component names from representative families are provided in Table 1. Although there are significant homologies between pathogenic T3SS and flagellar secretion, especially in the early stages of assembly, we will not include a detailed discussion of flagella components in this review as this has been covered by other recent reviews [25–27].

### The secretion apparatus: components and targeting

Reconstructions of the basal body reveal the presence of a socket structure within the inner membrane ring [28, 29]. This structure is visible from the cytoplasmic face of the basal body and extends into the hollow chamber of the T3SS basal body. The socket is thought to act as an anchor for the inner rod that traverses the length of the basal body and connects to the extracellular needle. Although the proteins that make up the socket structure have not been identified, it is likely to be formed by at least some of the domains of the five well-conserved inner membrane proteins that are associated with the basal body. In *Shigella* these are: Spa9, Spa29, Spa24, Spa40 and MxiA (Table 1). The roles these inner membrane proteins may play in export are not well understood. However, two of these components (Spa40 and MxiA) possess cytoplasmic domains [30–32] that are likely to be accessible to cytoplasmic proteins such as the secretion substrates, their chaperone-complexes and cytoplasmic regulators.

As protein production and secretion are not always coupled, some T3SS proteins must be stored in the bacterial cytoplasm in a secretion-competent state, but blocked from interacting prematurely with partner proteins or the apparatus itself. Specialised chaperones play this role by reversibly binding one or several partner proteins. Several different classes of chaperones can be distinguished based on structural and functional analysis as reviewed in [33–36].

Recognition and targeting of proteins to the T3S apparatus requires that secreted proteins possess a secretion signal. Several studies have identified a signal in the N-terminus of effector proteins either encoded in the mRNA [37, 38] or the peptide sequence [39–42]. Recently, several groups have used machine learning techniques to identify cryptic secretion signals within the protein sequence of the N-terminus of T3SS effector proteins [43–45]. Although these studies indicate that most secreted

**Table 1** Equivalences between representative families for components mentioned in the text (adapted from [87])

	<i>Shigella flexneri</i>	<i>Escherichia coli</i> LEE	<i>Yersinia</i> sp.	<i>Salmonella</i> sp. SPI-1
Early substrates				
Needle subunit	MxiH	EscF	YscF	PrgI
Rod subunit	MxiI	EscI	YscI	PrgJ
Length-control	Spa32	Orf16 <sup>a</sup>	YscP	InvJ
Middle substrates				
Tip	IpaD	EspA <sup>b</sup>	LcrV	SipD
Pore	IpaB/C	EspD/B	YopB/D	SipB/C
Secretion apparatus components				
Inner-membrane	Spa9	EscS	YscS	SpaQ
Inner-membrane	Spa29	EscT	YscT	SpaR
Inner-membrane	Spa24	EscR	YscR	SpaP
Inner-membrane	MxiA	EscV	YscV	InvA
Switch	Spa40	EscU	YscU	SpaS
ATPase	Spa47	EscN	YscN	InvC
ATPase-associated	MxiN	EscL	YscL	OrgB
ATPase-associated	MxiK	Orf4	YscK	OrgA
ATPase-associated	Spa33	EscQ	YscQ	SpaO
Regulator/gatekeeper	MxiC	SepL	YopN/TyeA	InvE

<sup>a</sup> Based on its position within the type III secretion gene cluster and its predicted secondary structure, Pallen et al. identify Orf16 as a candidate for the needle-length control protein [87]

<sup>b</sup> The *Escherichia coli* extension to the basal body is very different from that of other T3SS as it possesses an additional filament structure beyond the needle. Thus, the polymerization of EspA is quite different from that of the other tip proteins (reviewed in [5])

substrates possess a protein-encoded secretion signal, they do not preclude the role of mRNA as some effectors are surprisingly tolerant to frameshift mutations [44]. The secretion signals of early substrates appear to be different from that of the effectors. In *Pseudomonas syringae*, N-terminal truncations of the needle-length control protein, HrpP, were still translocated, suggesting this substrate possesses an atypical secretion signal [46]. In *Yersinia*, it has been shown that the needle-length control protein possesses two independent secretion signals, only one of which is at the N-terminus [47]. In addition, studies of the *Yersinia* tip protein reveal that an N-terminal His tag does not block secretion and that the C-terminus is also required for export, suggesting that the signal sequences differ between early and late substrates. However, there is currently very little data available on the specific targeting of early substrates to the T3S apparatus.

### The first switch: needle length control via substrate specificity switching

The substrate specificity switch from early substrates (needle and rod subunits) to later substrates must only occur once the needle has reached its full length. This is important since the length of the needle must match

specific structures at the bacterial and host-cell surfaces [48, 49]. Needle length varies between species, but is well controlled within species: ~50 nm in *Shigella*, ~60 nm in *Yersinia enterocolitica* and ~55 nm in SPI-1 of *Salmonella* [10–12, 21, 50]. At its simplest, length control involves two steps: the measurement of needle length and the switch of substrate specificity, i.e., halting the secretion of needle subunits. The needle-length control proteins are located in the same region of their respective genomes, but they possess very little sequence identity, and large regions are predicted to be highly unstructured. Knockout mutations of Spa32 in *Shigella*, InvJ in *Salmonella* or YscP in *Yersinia* lead to abnormally long needles and defects in effector secretion [10–13, 15, 51–54].

Several recent reviews have discussed in detail the different models for needle-length control in T3SS and flagella [55–58] as well as the homology of these control mechanisms to those used by bacteriophage-tail and flagella-hook length control [55]. For this reason, we will restrict this discussion to the data and models relevant to understanding the most recent work done in this field.

### Needle length: the ruler/tape-measure model

In *Yersinia*, insertions and deletions were engineered into the *yscP* sequence and the length of needles measured [10].

This work identified a linear correlation between the number of residues of YscP and the mean needle length, suggesting that the extended protein might act as a molecular ruler. Mutation of the secretion signals of YscP reduces or abolishes its secretion, and length control is dramatically impaired [47]. This result suggests that the export of YscP is coupled to its measuring activity, leading to the proposal that YscP may measure needle length directly. This model proposes that YscP would function as an extended polypeptide anchored to the tip of the growing needle and the base of the needle complex. Its full extension would signal that the needle has reached the appropriate length. A recent paper by Wagner et al. has shown that in *Yersinia*, the needle length changed in inverse function of the helical content, indicating that not only the number of residues but also their structure controls length [59]. These data support the ruler model and indicate that the functional ruler in this system possesses some helical structure. In some species the needle-length control protein is much shorter than in *Yersinia*, for example, Spa32 from *Shigella* possesses only 292 residues compared with 515 residues for YscP. As the *Shigella* needle is only ~15% shorter than in *Yersinia*, this suggests that Spa32 would possess almost no helical content while measuring the needle length. Future studies altering the amount of helical content in different members of this family will help establish the importance of secondary structure in determining needle length across species.

This model for needle-length control implies that the length-control protein is either present in the needle channel while needle subunits are being secreted or that it can be secreted without triggering the substrate switch. This latter model was originally proposed in the flagella field and was named the “infrequent export” model [60]. In this model, the needle length-control protein is periodically secreted during needle assembly to “check” whether the needle has reached full length. This would require that the mechanism by which it signals completion of the needle must not be prematurely triggered (see the substrate switch section below). The possible mechanisms by which this may work remain unclear. Alternatively, if the length-control protein remains in the needle channel, there must be sufficient room to fit both the ruler protein and the needle subunits being secreted. Several different methods have been used to estimate the diameter of the bore of the needle. The electron microscopy reconstruction of the *Shigella* needle resulted in a bore of 2.5 nm [61]. The modelling of the needle assembly within this reconstruction using the X-ray structure of the needle subunit from *Shigella* gave a bore size of 1.8 nm [62]. This difference is likely due to the uncertainties inherent in each method, for example, the relatively low resolution of the reconstruction and the ambiguity in positioning of the helices lining the

needle channel [61, 62]. Since a folded  $\alpha$ -helix (including side chains) has a diameter of  $\sim 1.2$  nm, the question of whether one or two folded helices can simultaneously fit in the needle channel remains uncertain. If the ruler remains in the needle channel during assembly, the narrower bore size would require the complete unfolding of either the ruler or the needle subunit protein. If the ruler protein is unfolded, then the secondary structure content becomes irrelevant in the measurement, and so the results of Wagner et al. require that the needle subunit be completely unfolded.

Recent work in *Shigella* has shown that large deletions within *spa32* do not produce shorter needles than those generated by variations with minimal deletions [14]. Furthermore, Spa32 variants lacking internal residues 37–94 or 130–159 retained full Spa32 function indicating that, in *Shigella*, control of needle length is not via a YscP-like molecular ruler mechanism. Botteaux et al. also showed that Spa32 and InvJ or YscP are functionally interchangeable (including needle-length control and translocator secretion) despite very different protein sizes. In contrast, insertion of just the YscP ruler domain into Spa32 produced longer needles, supporting the role of YscP as a molecular ruler [10, 14]. As discussed previously this may be subtly affected by the proportion of helical content in these proteins, but does suggest that the measuring function may be subtly different between species and that the current ruler/tape-measure models may be oversimplified.

#### Substrate specificity switching: the Spa32-like proteins

Despite the low sequence homology of the needle-length control proteins, a small domain near the C-terminus of these proteins was identified by hydrophobic cluster analysis and named the type III secretion substrate specificity switch (T3S4) domain [63]. This domain is predicted to possess a globular structure, an  $\alpha\beta$ -fold and a Pro-x-Leu-Gly signature. In *Yersinia*, the over-expression of this domain in strains lacking *yscP* restores secretion of effectors, but needle length remains variable, suggesting the T3S4 domain plays a role in the substrate switch. Together with the results discussed above, this suggests that this family of proteins may be bifunctional: they determine needle length and trigger the substrate switch.

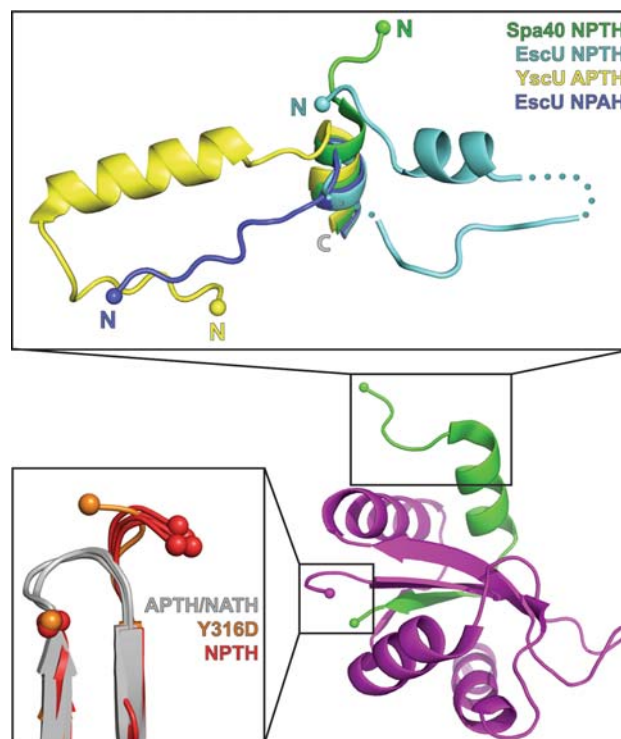
#### Substrate specificity switching: the Spa40-like proteins

The switch of substrate specificity is also controlled by one of the inner membrane components of the system, the highly conserved Spa40 family [55, 58, 64, 65]. These proteins possess a cytoplasmic domain that has been shown to undergo an autocatalytic cleavage event to produce a



small C-terminal domain that remains tightly associated with the membrane-bound N-terminal fragment. Cleavage occurs between the Asn and Pro within the highly conserved Asn-Pro-Thr-His (NPTH) sequence within the cytoplasmic domain. Mutants of the N and P prevent cleavage and interfere with the substrate switch. Non-cleavable mutants in *Salmonella* SpaS and *E. coli* EPEC EscU block secretion of translocators and filament components [64]. Similarly, mutation of the N or P to alanine (N263A or P264A) in the *Yersinia* homologue YscU results in bacteria that do not export the tip protein or translocators (LcrV, YopB and YopD) [65]. These non-cleaving mutants do form needles, although longer and less controlled than the wild type, and are capable of secreting late effectors [65]. In the YscU N263A mutant strain, export of the tip protein can be restored if a hybrid protein is constructed that possesses at its N-terminus the signal sequence from an effector protein (residues 1–15 of YopE) [65]. This implies that the tip/translocators have a specific type of export signal and a status distinct from the late effectors regarding export. This led to the proposal that YscU cleavage is required to adopt the conformation allowing recognition of the translocators.

In the last year several structures have been determined for the cytoplasmic domain of proteins belonging to this family: EscU<sub>C</sub> of *E. coli*, SpaS<sub>C</sub> of *Salmonella*, Spa40<sub>C</sub> of *Shigella* and YscU<sub>C</sub> of *Yersinia* [64, 66–68] (Fig. 2). These structures revealed that the NPTH sequence lies on a surface-exposed loop and is cleaved in the absence of any other factors (Fig. 2, main panel). Structures of cleaved and uncleavable mutants of these proteins reveal that there is a distinct conformation for this loop in each state and that the cleaved loop is stabilised by surrounding residues (Fig. 2, bottom inset). Analysis of the uncleavable mutants suggests that the protein fold presents the loop in a conformation that is primed for the cleavage event to occur. It has therefore been proposed that cleavage occurs immediately upon folding and that the switching event is a consequence of partner proteins interacting with the cleaved protein. In artificial systems where the cleavage event is blocked by mutation, the surface properties of the protein are altered and thus may not present the necessary structure for substrate switching rather than the structure of the uncleaved form playing an active role in inhibiting the switch. If, as has been proposed in the flagella field, cleavage of this loop acts as a trigger for substrate switching, then the timing of this cleavage event becomes critical, and these proteins must be maintained in an uncleavable state until the needle is completed. Such a state could be attained by the specific chemistry of the protein fold itself, or by external factors such as pH or inhibitory proteins. It should be noted that in wild-type *Y. enterocolitica* with YscU expressed at natural levels, the cleavage



**Fig. 2** Structures of the cytoplasmic domain of the substrate specificity switch protein. *Main panel:* Ribbon diagram of *Shigella flexneri* Spa40<sub>C</sub> illustrating the N-(green) and C-(magenta) terminal regions of this domain (PDB: 2VT1). *Left panel:* Overlay of the NPTH-loop region from cleaved and non-cleavable mutants of EscU, SpaS, Spa40 and YscU (PDB: 3BZL, 3BZP, 3BZY, 3C01, 2VT1, 2V5G, 2JLJ, 2JLI). *Top panel:* Views of the N-terminal regions of EscU, Spa40 and YscU overlaid via the C-terminal region to illustrate the lack of defined structure at the N-terminus (PDB: 3BZL, 3BZV, 2VT1, 2V5G)

at the NPTH site was not complete, i.e., full length uncleaved protein was detected in purified membranes [65]. This is therefore an area that requires further investigation.

The structures also revealed that the N-terminal portion of the cytoplasmic domain is able to adopt very different conformations (Fig. 2, top inset). This flexibility may be due to different crystallisation conditions or may be the result of separating this domain from the membrane-bound portion of the protein. It is also possible that this region is genuinely flexible and that the position of this domain within the secretion apparatus may play a role in regulation. As shown previously for flagella, deletions or point mutations in this region of EscU abolish secretion of both translocators and effectors [64, 69]. This suggests a distinct role for this region in the export capability of the apparatus rather than its substrate specificity. Further experiments are required to address this question, including a structure of the intact, full-length protein including the N-terminal integral membrane region.

### Substrate specificity switching: working together

In addition to lack of translocator export, the YscU N263A mutant exported reduced amounts of YscP and made longer needles [65]. Overexpression of *yscP* in the N263A strain resulted in increased export of YscP, resulting in needles of normal length, but did not restore translocator secretion. Therefore, failure in needle length control of the N263A mutant was due to the failure of YscP export rather than the failure to switch substrate specificity. Furthermore, the recovery of needle-length control indicates that the N263A mutation does not affect the capacity of the T3S export apparatus to switch off the export of needle subunits. Since the uncleavable variant of YscU can give rise to different needle lengths depending on the amount of YscP exported, this supports the hypothesis that the cleavage event itself is not the trigger.

Much work has been done in the flagella field looking at the interplay between the hook-length control protein, FliK, and the substrate switching protein, FlhB (reviewed in [57, 58]). Specific mutations within the cytoplasmic domain of FlhB were identified that rescue the *fliK* null mutant [70, 71]. Equivalent mutations were made in *Yersinia* YscU and were shown to restore effector secretion (i.e. are capable of switching) in *yscP* null mutants [15]. These data suggest that YscU and YscP work together to control both needle length and switch the substrate specificity of the secretion apparatus. Recently, Botteaux et al. have identified a direct interaction between the cytoplasmic domain of Spa40 and a short C-terminal region within the predicted T3S4 domain of Spa32 [14]. Removal of these residues from Spa32 resulted in a non-functional protein suggesting that the interaction with Spa40 is crucial for the switch from early to later substrates.

Recent work from Riordan et al. has identified interactions between YscU and ATPase-regulator complexes. When purified from the cytoplasm of *Yersinia*, cleaved YscU<sub>C</sub> bound to YscL/K/Q but lacked interaction with YscN or YscP or LcrV [72]. It was also shown that overexpression of any *yscU* allele (including wild-type) abolished type III secretion, suggesting that YscU may bind and sequester an essential component of the apparatus [72]. Furthermore, a GST-fusion protein of the cytoplasmic domain of YscU was found in the cytoplasm not associated with the T3S apparatus, but was still able to block secretion [73]. These results suggest a role for YscU in the recruitment of ATPase complexes to the T3S apparatus in order to initiate secretion. Similarly, impassable GST fusions of YscP have been shown to block secretion and co-purify with ATPase complexes [73].

### Needle length: the rod assembly model

There is little dispute that the Spa32 family of proteins is responsible for needle-length control and substrate switching. However, as briefly mentioned earlier, there is some disagreement in the literature about its role as a ruler/tape measure. Alternative models for needle-length control have been proposed in both pathogenic and flagella T3SS (reviewed in [55–58]). One model that we discuss here involves a component known as the rod protein that is thought to be the periplasmic portion of the needle. In *Salmonella*, it has been shown that the needle-length protein, InvJ, is required for formation of the inner rod structure, and reconstruction of a  $\Delta$ *invJ* needle complex revealed significant structural differences within the base [74]. Although, InvJ is not detected as an integral component of the base [11, 75], the  $\Delta$ *invJ* reconstruction supports a role for InvJ in stabilisation of the socket and inner rod structures. The model proposed by Marlovits et al. suggests that completion of the inner rod anchors the needle to the base and triggers a conformational change in the base that switches substrate specificity.

Marlovits et al. also showed that altering the stoichiometry of the inner rod and needle proteins has a profound impact on the length of the needle substructure [74]. Recent work has shown that in *Yersinia* the rod protein, YscI, plays a critical role in substrate specificity switching [76]. Wood et al. suggest that YscP and YscU exert their effects on substrate export by controlling the secretion of YscI [76]. Together these observations support a role for the rod protein in needle-length determination.

The abundance of data available relating to needle length control in several species and in pathogenic and non-pathogenic T3SS has allowed the proposal of many different mechanisms for length control. It seems likely that the fundamental mechanism of needle-length control will be conserved across species, but at this stage these models cannot yet be reconciled and in some cases appear to be incompatible.

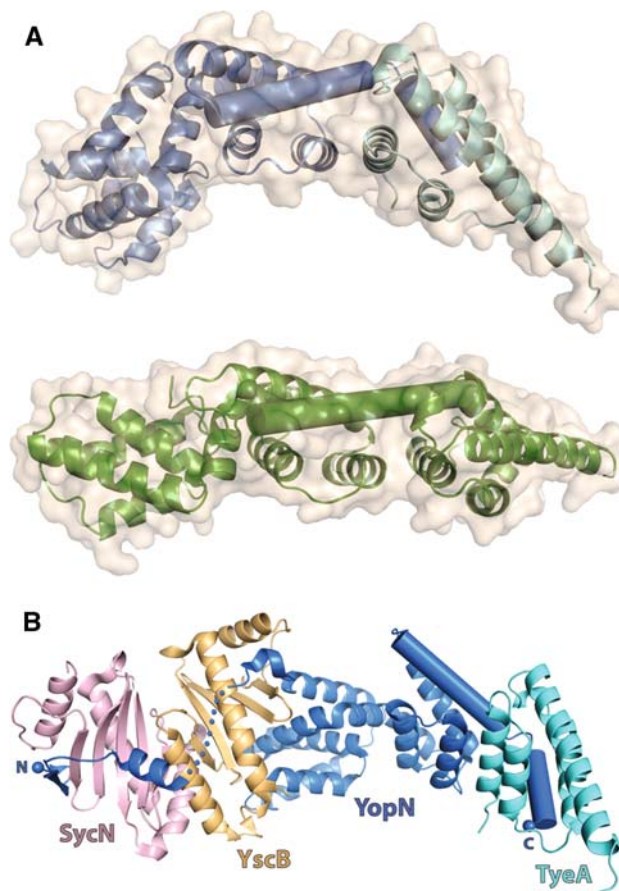
### The second switch: assembly of the tip and late substrate block

After completion of the needle structure, the tip complex is assembled at the end of the needle, and the secretion of effector proteins must be blocked. In *Yersinia*, a tip complex has been visualised that is assembled at the tip of the needle [77]. This complex is made up of several copies of the protein LcrV and is present even in the absence of effector secretion [77], suggesting that the secretion of these two classes of substrates, tip protein and effectors, is differently controlled.

In *Shigella*, IpaD has been identified at the tip of the needle [78, 79], has been shown to interact directly with the needle subunit, MxiH, in vitro [80], and is required for the assembly of the translocators [81]. These data suggest that the tip proteins are exported after the needle is assembled but before the later substrates. In *Shigella*, the translocator protein IpaB has also been identified at the needle tip prior to membrane contact and without IpaC recruitment [81–83]. Although the lack of detection of IpaC may be due to technical limitations, these data suggest that there is a stepwise secretion of the proteins that sit at the tip of the needle. Whether there are distinct triggers for the secretion of each of these components remains uncertain. However, it is clear that the exact conditions under which these experiments are conducted are critical in determining which components are identified. For this reason, there is some ambiguity about whether the translocator proteins are a distinct secretion category from both the tip proteins and the late effectors.

The transition from blockage to activation of effector secretion can be considered the second switch of the T3SS (Fig. 1c, d). A family of proteins, of which MxiC is the *Shigella* homologue, has been identified that act as regulators at this stage. Functional knockouts of members of this family have no effect on needle formation or stability, but do result in enhanced secretion of effector proteins [17–19, 84, 85]. It has been proposed that these proteins play an active role in repressing the secretion of effectors and that they may function by interacting with the T3SS apparatus in a manner that blocks secretion [84, 86]. In support of this hypothesis, Botteaux et al. recently identified an interaction between MxiC and the ATPase, Spa47 [17].

The *Yersinia* homologue of MxiC differs from that of other species in that it exists as two separate polypeptide chains: YopN and TyeA [87]. However, the structure of the YopN–TyeA complex is very similar to that of the *S. flexneri* homologue MxiC (Fig. 3a) [88, 89]. The structures reveal that these proteins are rod-shaped with a highly disordered N-terminus that possesses the secretion signal and chaperone-binding region [90]. The rod shape provides these proteins with a large surface area potentially allowing for simultaneous interactions with several partner proteins. Despite their similar fold, consisting of multiple bundles of  $\alpha$ -helices, these proteins possess almost no sequence conservation on their surfaces, suggesting that the sequences of the proteins they interact with are also not highly conserved. Interestingly, the overall shape of these two structures is quite different due to twisting of some domains and the kinking of a long, central helix (Fig. 3a). Specifically, YopN–TyeA has a more curved shape, whereas MxiC is quite straight. The conditions in which the crystals of these proteins grew had very different pH



**Fig. 3** Structures of YopN complexes and MxiC. **a** Surface representations (*wheat*) and ribbon diagrams of: YopN (*blue*) in complex with TyeA (*cyan*) and MxiC (*green*). The central helix discussed in the text is displayed as a *cylinder*. **b** Ribbon diagram of the complex formed by YopN (*blue*), TyeA (*cyan*) and the chaperones SycN (*pink*) and YscB (*yellow*). The N- and C-terminal residues of YopN are highlighted (*balls*), and an unstructured region of YopN is displayed as a *dashed line*

values: MxiC crystals were grown at pH 6.5–7.5, whereas YopN–TyeA crystals grew at pH 10.5. Whether the differences in these structures provide a glimpse of a functionally important, potentially pH-dependent, conformational change or whether they are artefacts of crystallisation will require further study.

The structure of YopN in complex with its heterodimeric chaperone, SycN–YscB, has also been determined (Fig. 3b) [89]. Although homologues have been identified for most other chaperones within the T3SS, the homologues of SycN and YscB have not yet been clearly identified in other species. The binding faces for TyeA and the chaperone complex lie at opposite ends of YopN and are structurally independent. This observation suggests that the different functions this family of proteins possess may be structurally separated. Indeed, recent work with the *E. coli* homologue, SepL, has demonstrated that its



different functions, including membrane localisation, translocator export and effector secretion regulation, can be separated [91].

The effects of knockouts in this family on tip and translocator secretion seem to differ between species. In *E. coli* and *Salmonella*, knockout of SepL or InvE, respectively, reduce or abolish the secretion of the tip protein and translocators [19, 20, 84, 92]. This phenotype is similar to that observed for the N263A mutant of YscU, namely the absence of tip and translocator secretion while retaining the ability to secrete effector proteins. However, an understanding of whether there is some co-ordination between the first and second switches in these species will require further study.

The secretion of tip and translocator proteins is not affected in *Shigella* and *Yersinia* strains knocked out for MxiC and YopN, respectively [17, 65, 93]. However, these strains are impaired in cell invasion and do not efficiently translocate effectors. YopN is secreted by the T3SS, and it has been suggested that TyeA suppresses the secretion of YopN prior to host-cell contact. The dissociation of YopN and TyeA has been proposed as a mechanism for the regulation of secretion, but clearly this cannot be a conserved mechanism in those species where the homologue is a single polypeptide chain [94, 95]. However, recently it has been shown that in *Shigella*, MxiC is secreted and that its secretion is required for its function [17]. In other species, secretion of this component has not been seen.

In *E. coli*, it has been suggested that SepL and its binding partner SepD control a switch from translocator to effector secretion by sensing certain environmental signals such as low calcium [84]. Similarly, in *Yersinia*, changes in the environmental calcium concentration are thought to relieve the TyeA-mediated blockage, triggering the secretion of YopN and activating secretion [96–98]. This response to low calcium levels is thought to involve both the YopN–TyeA complex and a pathway involving the negative regulator, LcrG, and its interaction with the tip protein, LcrV [99, 100]. Recently, LcrG has also been implicated in the negative regulation of transcription of T3SS genes [101]. The indirect role played by LcrG in transcriptional regulation is similar to that seen by Botteaux et al. in a MxiC mutant that showed increased expression of effector genes due to the increased secretion of the anti-activator OspD1 [17, 102]. The secretion of regulatory proteins is a convenient mechanism to control their levels in the cell and provides one means of co-ordinating secretion and switching. In flagella, this has been well studied for the regulatory protein FlgM (reviewed in [27]). In addition, there is evidence that post-transcriptional cleavage of transcripts plays a role in the regulation of protein production [103]. The mechanisms of this regulation are only beginning to be understood.

The data currently available for the regulation of type III secretion do not support any single model for each step. It is likely that there are subtle differences between species due to varying numbers and types of proteins present in the virulence repertoires of different species. Furthermore, the final trigger for effector secretion is likely to be unique to each species due to their different host specificities and local environments at infection.

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