

## Chapter 6

### Summary and concluding remarks

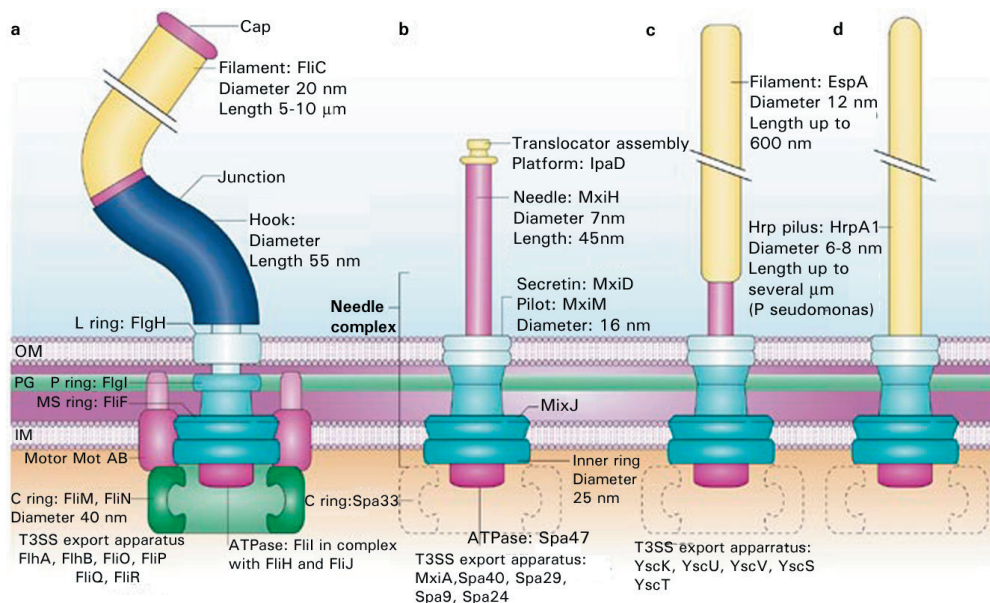
*Bacteria that have sustained long-standing close associations with eukaryotic hosts have evolved specific adaptations to survive and replicate in their host. An important mechanism that frequently reoccurs in this process of adaptation is that the pathogen and its host engage in complex molecular cross-talk that often dictates the outcome of an infection. This inter-kingdom communication allow the bacteria to deliver an arsenal of bacterial proteins (collectively known as 'effectors') directly to the cytoplasm of their eukaryotic hosts via the virulence-associated T3SS. These translocated effectors alter such basic host-cell structural and functional features as cytoskeletal architecture, signal transduction, membrane trafficking, and cytokine gene expression.*

*The system is widely used by more than 25 species of Gram-negative bacteria and is thought to be evolutionarily related to the bacterial flagellum (figure 1) (13). An essential component of this secretion system is the T3SA, which is made up of more than 20 proteins. Knowledge of the way in which an invading pathogen uses this system to interact with its host at a molecular level is an essential aid to understanding the nature and extent of disease caused. Recent genetic, biochemical and structural studies have led to the identification of specific protein components of the structural apparatus of this system (termed the needle complex) and have provided important insights into the complex assembly pathway required for the formation of this organelle. This chapter summarizes this thesis contribution to the current understanding of needle complex assembly and architecture.*

## The T3SA and the needle complex

### Structural organisation of the needle complex

The core of T3SA has been termed the needle complex (NC) because of its characteristic shape; it has been previously reported for several species, including *Shigella flexneri* (Kubori 1998, Tamano 2000, Blocker 2001, Sekiya 2001). In **Chapter 2** we describe purification and further structural identification of the NC. The use of a new, less stringent purification method not only generated acceptable yields of NCs, which displayed very high purity but also allowed detection of the MxiM subunit, which has never been observed to co-purify with the intact NC. Extensive single particle analysis has extended the resolution of the NC to 16 Å. The resulting EM averages revealed a needle with more noticeable helical architecture and a central channel of 20-30 Å that extends from the bottom set of rings to the tip of the extracellular needle. This observation implies that this passageway is far too narrow for most globular proteins to go through and thus effectors need to be partially, if not completely unfolded before translocation. In addition, the averages show a clear division of seven individual rings in the membrane associated basal body. The use of specific deletion mutants that lacks the needle MxiH and MxiI components to classify this basal body (BB) reveals the presence of an extra density termed the socket, transversing the inner cavity of the substructure. This suggests that the needle does not insert to the cytoplasmic end of the BB, and that components that form this socket most probably assemble first to provide a platform to nucleate the assembly of the needle. Conversely, the socket may also function as a symmetry adaptor between the helical needle and the rotational symmetric base. The rotational symmetry of the peripheral ring of the BB was determined to be 24-fold. The overall dimensions of this 24 subunit model are similar to the recent crystal map of EscJ, which is a MxiJ homolog in enteropathogenic *E. coli* and indicate that this protein could oligomerize to form a ring with 24 copies that is anchored to the inner membrane.



**Figure 1. Structural comparison between the flagellum and the T3SA.** Schematic representation of the flagellum (a), T3SA from *S. flexneri* (b), T3SA from EPECs (c) and the T3SA from plant pathogens (d). The C ring for the T3SA is represented by a dashed line as its existence has not been firmly established yet.

Proteins of this highly conserved family are thought to be anchored to the outer leaflet of the inner membrane by a lipid that is covalently linked to their N termini after cleavage of the signal peptide (27). Furthermore, by purifying the NC at showed different detergent concentrations, which disrupt the lipids around the NC to different extent, we also eluded to the position of the NC within the two membranes. The organization of the outer membrane ring is quite simple because this structure comprises only one main protein component: MxiD secretin. Secretins form a large protein family that function as portals for folded proteins to move across the outer membrane. In the T3SS, however, secretins seem to have a different role: the EM map of the NC shows that MxiD secretin appears as two stacked rings that ‘wraps’ around the needle structure that extends beyond the bacterial envelope. Thus, instead of directly mediating protein movement across the outer membrane as is observed in other secretion systems (2), it functions as an anchoring and stabilization device for the needle. Notably, the outer membrane channel formed by InvG secretin of *Salmonella* is closed by a ‘septum’-like structure before assembly of the needle (14).

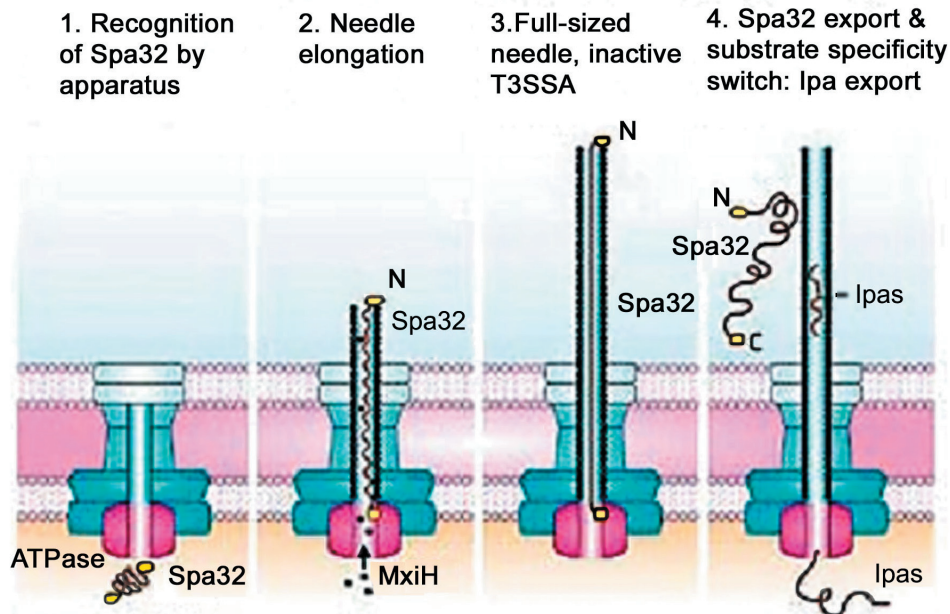
## Needle length control

Why is it important for the needle length to be controlled? Because bacteria making short needles are not efficient in invading host cells as compared to wt, suggesting that the needle must span a minimal distance above the surface of the bacterium. This is required to breach the outer surface layers of the bacterium to allow it to come into direct contact with the membrane of the target cell. This result is supported by data from studies showing that the glycosylation state of the lipopolysaccharide on the surface of *Shigella* affects not only the length and thickness of these bacterial outer membrane molecules but also the accessibility and function of the T3SA needle (26). Thus the control of needle length reflects an adaptation to fit the physical and chemical environment of the bacteria–host interface.

Variations in the length of the extracellular needle fall within a narrow range of ~50Å. Cornelis and colleagues [17] examined *Yersinia* YscP and discovered, through a series of deletion and insertion experiments, that the needle length is proportional to the number of residues in the middle region of YscP. They proposed that YscP acts like a ‘molecular ruler’. Studies using gene knockouts have identified *Shigella* Spa32 as a key regulator of needle length (25). The question of whether Spa32 also functions as a molecular ruler in the control of the needle length was determined by examining the phenotypes of Spa32 variants obtained by deletions in the N-terminal, central and C-terminal part of the full length *spa32* gene (**Chapter 3**). Small deletions in the central part did not interfere with Spa32 function, indicating that Spa32 tolerates limited alterations in its sequence without affecting its functions in needle length control, effector secretion, and bacterial invasion of epithelial cells. Most truncated Spa32 proteins engineered lead to longer needles rather than shorter needles. Because deletions affecting both N- and C-termini of Spa32 lead to a loss of length control, we hypothesize that the two ends of Spa32 act as anchors. One end would be attached to the basal body probably by its interaction to an inner membrane protein, whereas the other would be connected to the growing tip of the needle, similar to what was observed in YscP of *Yersinia* (12). The ability of the length control protein homologs from other T3SS that are hundreds of amino acids larger than Spa32 to restore needle length to wt size is presented as an argument that Spa32 cannot act as a molecular ruler. The fact that YscP (409 aa) and InvJ (336 aa) were able to restore needle length, though these proteins are larger in size than Spa32 (292 aa) demonstrates that these length controllers share intrinsic properties still to be discovered.

In the model we propose, Spa32 would control needle length by allowing its globular domain to interact with an inner membrane component of the base (figure 2.1), possibly Spa40, to stabilize the conformation of the socket, while its N-terminal containing the putative secretion signal would

serve as a capping domain attached to the growing end of the needle (figure 2.2). Once the full length of the needle is reached, signalled by the full stretch of the Spa32 (figure 2.3), its globular domain detaches from its interacting partner and is secreted (figure 2.4). The detachment of Spa32 from the socket leads to a profound conformational change that may serve as a signal for substrate specificity switch (figure 2.4).

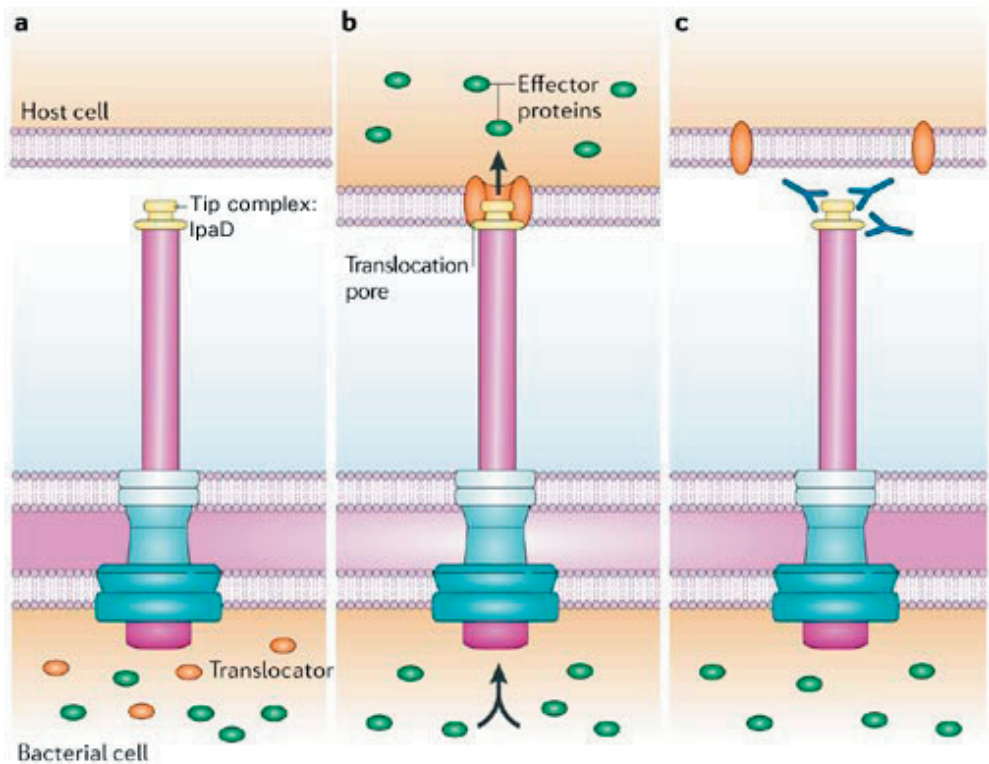


**Figure 2. Model for the needle length control.** Spa32 enters the channel after completion of the basal structure (1); the needle subunits are exported and polymerize, leading to the extension of the ruler component of Spa32 (2); the needle assembly is completed (3); the T3S4 domain of Spa32 switches the substrate specificity to effector secretion and the ruler is released (4). Modified from Cornelis (4).

## Needle tip

The needle is thought to function as the conduit for protein translocation. However, effector delivery also requires a set of type-III-secreted proteins called translocators. These proteins assemble into a translocation pore in the target-cell membrane (3, 19). Because effector injection is regarded as a one-step process, this implies that the needle and the pore must be connected. The assembly of this pore, on cell contact, requires two hydrophobic translocator proteins (IpaB and IpaC) and a hydrophilic protein (IpaD) (3, 5, 15, 21). All three proteins are also substrates of the T3SA and have been shown to interact with one another (5, 16). Though the purified hydrophobic translocator proteins can interact with artificial membranes (6, 9, 11, 20) and, under some experimental conditions, can also form pores (8, 24), the formation of pores using live bacteria requires all three proteins to be present (10, 15, 17). Thus although IpaD is not known to be a component of the pore, it never the less is involved in pore formation. Until now, IpaD has not been shown to co-purify with the NC. By optimizing the purification procedure to enable the enrichment of otherwise labile components, we were able to copurify the tip complex (henceforth referred to as the cap) with the NC (**Chapter 4**). By electron microscopy analysis of isolated NC with the tip complex and a combination of biochemical approaches, we demonstrated that a structure at the needle tip was formed by IpaD. The cap is assumed to assemble at the tip of the needle even before contact with a target cell (as observed for

LcrV of *Yersinia*) (18). This suggests a model in which the cap functions as an assembly platform for the hydrophobic translocator to form a pore (3, 7, 21, 23), and conversely might function as a connector to ensure continuity between the needle and the pore (figure 3). This is an important finding and provides a crucial clue towards the understanding of the poorly characterized molecular mechanisms of *Shigella flexneri* pathogenicity. In particular, the inhibition of invasion with anti-IpaD antibodies indicates that IpaD may be able to elicit cross protection against *Shigella* infection, thus elevating the status of IpaD as a candidate vaccine antigen (figure 3).



**Figure 3. Hypothetical model of the functioning of the IpaD tip.** In the absence of contact with a eukaryotic host-cell lipid membrane, the T3SA is assembled and the system is in a resting state. In this state, a ‘tip complex’ is assembled at the needle tip and prevents any ipa effector secretion (a). Upon host-cell contact, T3S is triggered, followed by IpaB-IpaC translocation pore assembly in the eukaryotic cell plasma membrane with the assistance of the IpaD tip complex (b). Anti-IpaD antibodies are protective because they prevent the formation of the translocation pore in the plasma membrane of host (c). Adapted from (4).

## Conclusion and future challenges

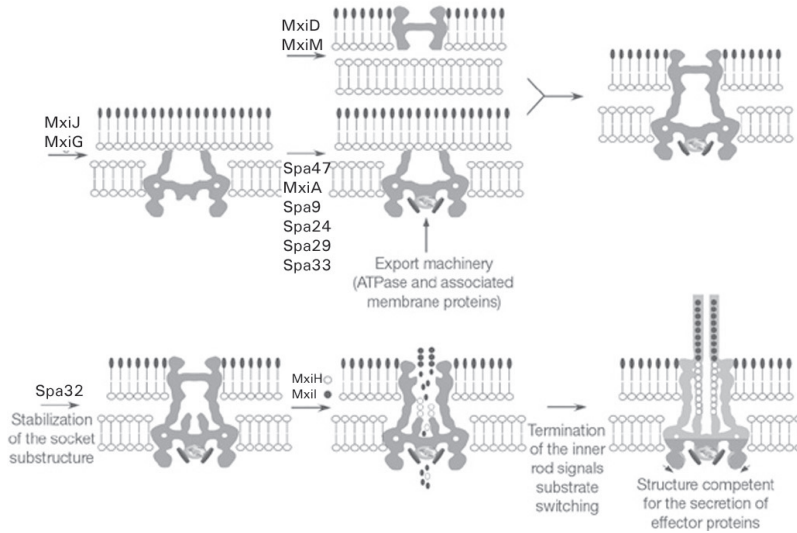
The enormous complexity of T3SSs constitutes a specific challenge. Accumulated data on the system in the past few years have transformed our view and shed new light on the assembly (figure 4) and functional pathway of the system (figure 5). In terms of structures, we now have intermediate-resolution EM maps of the core T3SA and extracellular structures including the needle, and high-resolution crystal structures of some of the components. As ongoing structural studies will undoubtedly continue to build a better resolved and more complete picture of the T3SA, we also can

address specific questions about functional aspects arising from these structures. The nature of the secretion signal or – more likely – of the different signals has to be elucidated. What are the specific roles of the different components in the activation and regulation of type III secretion? And what special characteristics enable proteins to move along it. There is a particular lack in understanding of how the structural components interact with one another in the context of the bacterial membranes because many of the components embedded in the inner membrane have evidently been lost during purification of the NC. How do chaperones perform their job and how are they perceived? Akeda *et al* (1) showed that InvC, an ATPase associated with a *Salmonella enterica* type III secretion system (Spa47 homolog), has a critical function in substrate recognition inducing chaperone release from its substrates and the unfolding of these proteins destined for secretion in an ATP-dependent manner (figure 5). What about the substrates without known chaperone assistance? Finally, developing methods to reconstitute the secretion and translocation subunits of the T3SA *in vitro* should enable us to understand how the different events that have been observed or postulated from the structural studies are coordinated. All this acquired knowledge will only lay the foundation for a deeper understanding of the infection process as a whole.

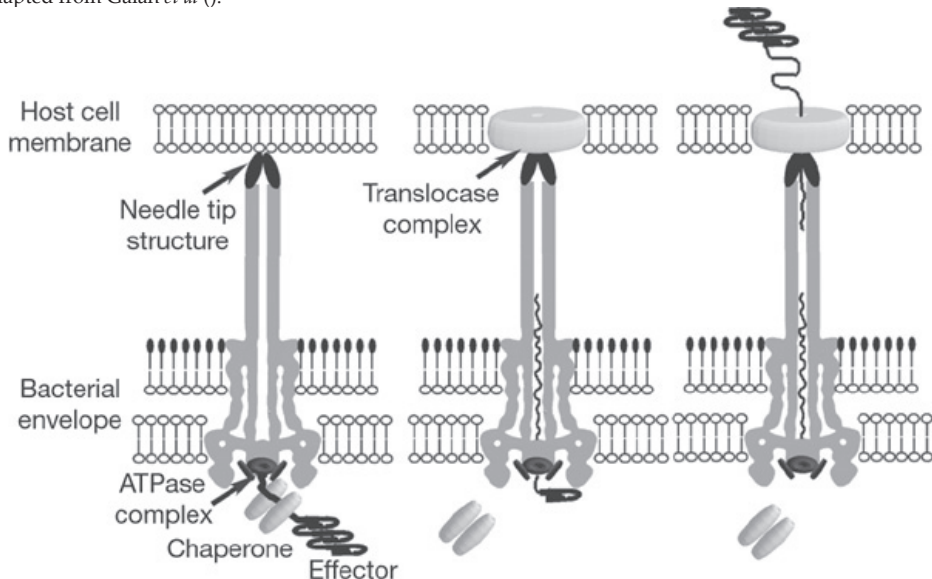
In conclusion, the widespread distributions of the T3SS and its use in diverse biological contexts is an evidence to the success of evolution working to shape the complex cross talk between pathogenic or symbiotic bacteria and their eukaryotic hosts. The central role of this system in the interaction of many pathogenic bacteria opens up the possibility of developing new vaccine strategies. In addition, a detailed understanding of these machines will allow them to be harnessed to deliver heterologous proteins for therapeutic or vaccine purposes (22).

## Part B

Archaea are diverse group of organisms found in extreme environments such as high temperatures, high salinity, and high or low pH. A number of strategies for efficient survival in this environment have evolved, including flagella dependent motility. In this way, cells benefit from being motile while avoiding lethal hot spots in their environment. In the last chapter (**Chapter 5**), we present studies on the flagellation of the thermoacidophilic crenarchaeon *Sulfolobus solfataricus*. We identify growth conditions at which the structural *flaB* gene is strongly induced when cells are depleted of carbon source. Furthermore, we present a structural model for the flagellar filament based on single particle electron microscopy and suggest that the structural subunits (flagellins) are assembled by a mechanism that resembles the biogenesis of the type IV pilus, than that known for the eubacterial flagella.



**Figure 4. Model of the assembly of the needle complex.** Assembly of the base substructure occurs in discrete steps. During this *sec*-dependent phase, the base components MxiG/MxiJ form the inner membrane rings while MxiM chaperones the assembly of MxiD to form the outer ring. The assembly of the base is complete by the recruitment of accessory proteins (MxiA, Spa9, Spa24, Spa29 and Spa33) including an ATPase (Spa47) that is involved in the recognition and unfolding of substrates of the type III secretion machine. Completion of the base initiates secretion of needle components (MxiH and MxiI), and regulatory (Spa32) proteins. Spa32 regulates the length of the needle by interacting with an inner membrane protein (Spa40) and the growing needle. Completion of needle assembly, signalled by the secretion of Spa32, 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. Adapted from Galan *et al* ().



**Figure 5. A current model for substrate recognition and delivery of proteins.** Upon activation by host contact, the effector-chaperone complex is recognized by the secretion machinery, including a type-III-secretion-associated ATPase. The ATPase causes the chaperone to dissociate 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’ also secreted by the T3SS is assembled on the host cell membrane and facilitate 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. Adapted from Galan *et al* ()

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