Assembly of the bacterial type III secretion machinery

Andreas Diepold1 & Samuel Wagner2,3

1Department of Biochemistry, University of Oxford, Oxford, UK; 2Interfaculty Institute of Microbiology and Infection Medicine (IMIT), University of Tübingen, Tübingen, Germany; and 3Partner-site Tübingen, German Centre for Infection Research (DZIF), Tübingen, Germany

Correspondence: Samuel Wagner, Interfaculty Institute of Microbiology and Infection Medicine (IMIT), University of Tübingen, Elfriede-Aulhorn-Str. 6, 72076 Tübingen, Germany. Tel.: +49 (0)7071 29 84238; fax: +49 (0)7071 29 5440; e-mail: samuel.wagner@med.uni-tuebingen.de

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Abstract

Many bacteria that live in contact with eukaryotic hosts, whether as symbionts or as pathogens, have evolved mechanisms that manipulate host cell behaviour to their benefit. One such mechanism, the type III secretion system, is employed by Gram-negative bacterial species to inject effector proteins into host cells. This function is reflected by the overall shape of the machinery, which resembles a molecular syringe. Despite the simplicity of the concept, the type III secretion system is one of the most complex known bacterial nanomachines, incorporating one to more than hundred copies of up to twenty different proteins into a multi-MDa transmembrane complex. The structural core of the system is the so-called needle complex that spans the bacterial cell envelope as a tripartite ring system and culminates in a needle protruding from the bacterial cell surface. Substrate targeting and translocation are accomplished by an export machinery consisting of various inner membrane embedded and cytoplasmic components. The formation of such a multimembrane-spanning machinery is an intricate task that requires precise orchestration. This review gives an overview of recent findings on the assembly of type III secretion machines, discusses quality control and recycling of the system and proposes an integrated assembly model.

Introduction

Various Gram-negative bacteria use type III secretion systems (T3SS) to inject effector proteins into host cells, promoting either mutual benefit or pathogenesis (Cornelis & Wolf-Watz, 1997; Galán & Collmer, 1999) (Box 1). Animal pathogens like Salmonella, Shigella, Yersinia and pathogenic Escherichia coli are attenuated and often rendered completely avirulent by a lack of their T3SS, demonstrating the importance of the system in pathogenicity (Hueck, 1998). While a high degree of diversity among the translocated effectors reflects the specific lifestyle of the bacterium (Tampakaki et al., 2004; Heijden & Finlay, 2012; Mota et al., 2005; Galán, 2009), the export machinery itself, which is also called injectisome, is conserved among species (Cornelis, 2006; Galán & Wolf-Watz, 2006). Moreover, when type III secretion genes were first identified, it appeared that some of them were also related to flagellar genes (Fields et al., 1994; Woestyn et al., 1994; Gijsegem et al., 1995), a notion supported by the first partial structure of an injectisome, the needle complex (NC) of Salmonella enterica pathogenicity island 1 (SPI-1), which was visualized by electron microscopy (EM) (Kubori et al., 1998). The similarity resides on the so-called basal body (or base) spanning both bacterial membranes (Blocker et al., 2003; Macnab, 2004; Erhardt et al., 2010). On the extracellular side, the flagellum possesses a filament that translates its rotation into propelling force, while the injectisome has an extracellular hollow needle that bridges the gap between bacterium and host cell to allow the direct translocation of effector proteins.

References that study the respective protein or mechanism in the bacterial flagellum are marked in the text with asterisks to allow for easier identification.
The last 15 years saw a large variety of structural studies on different type III secretion systems. After the seminal first visualization of an NC, the part of the structure that comprises the membrane rings and the needle, in *Salmonella* (Kubori et al., 1998), additional structures of NCs from *Shigella* (Tamano et al., 2000; Blocker et al., 2001) and EPEC (Daniell et al., 2001; Sekiya et al., 2001) were solved, and resolution was greatly increased by cryo-EM techniques and averaging (Marlovits et al., 2004; 2006; Sani et al., 2006; Hodgkinson et al., 2009; Schraadt et al., 2010; Kawamoto et al., 2013; Kudryashev et al., 2013). These techniques now even allowed visualizing secretion systems that enclose trapped translocation substrates (Radics et al., 2013). In recent years, the availability of high-resolution structures of several domains of components of T3SSs (Yip et al., 2005; Zarivach et al., 2007, 2008; Spreter et al., 2009; Wiesand et al., 2009; Lilic et al., 2010; Worrall et al., 2010; Loquet et al., 2012; Abrusci et al., 2013) has allowed to dock some of these structures into medium-resolution electron density maps obtained by EM. Therefore, parts of the system have been described at molecular-resolution (Deane et al., 2006; Moraes et al., 2008; Spreter et al., 2009; Schraadt et al., 2010; Schraadt & Marlovits, 2011; Fujii et al., 2012; Bergeron et al., 2013) (Fig. 1).

These images show a complex structure that displays a series of membrane-spanning rings and embedded substructures. As most of the structures were solved from purified NCs that lack components in the cytosol and inner membrane (IM) and their refinements were based upon circular symmetries, there is a strong bias in knowledge towards the stable ring-forming components of the injectisome. As we still lack structural information on the cytosolic components and the core of the export machinery, the complete structure of the operational injectisome will be even more complex than the current images suggest.

### Box 1. Classification of type III secretion systems

While the flagellum can export its distal components by means of an internal T3SS (Aizawa, 2001; Macnab, 2004), the term ‘Type III secretion system’ is generally used in reference to the systems translocating effector proteins into host cells. Based on phylogenetic analyses of conserved system components (Foultier et al., 2002; Gophna et al., 2003; Pallen et al., 2005), these type III secretion systems can be classified into different subfamilies. Well-studied systems are the injectisomes from *Salmonella* spp. SPI-1 and *Shigella flexneri* (SPI-1-like), the injectisomes from enteropathogenic and enterohaemorrhagic *E. coli* (EPEC, EHEC) and *Salmonella* SPI-2 (SPI-2-like), and the Ysc system present in *Yersinia* spp., some *Pseudomonas* spp., and *Aeromonas* spp. Plant pathogens can possess either of two types of T3SSs: Hrp1, which has mostly been studied in *Pseudomonas syringae*, and Hrp2 present in *Ralstonia solanacearum* and *Xanthomonas campestris* (Büttner & He, 2009). Further distinct subfamilies of T3SS are present in the nitrogen-fixating plant symbiont *Rhizobium* (Marie et al., 2001) and in *Chlamydiales* (Hisia et al., 1997; Hefti & Stephens, 2007). The latter appears to be most divergent from the other T3SS. Interestingly, in this system, interactions with flagellar components have been observed (Stone et al., 2010). In addition to variations on the protein sequence level, some subfamilies have additional essential, often cytosolic, components and can differ in the nature of their extracellular appendices. Often, T3SS genes are clustered in pathogenicity islands, as in *Salmonella* SPI-1 (Galán & Curtiss, 1989) and SPI-2 (Shea et al., 1996), and the Hrp1 and Hrp2 systems (Bonas et al., 1991; Alfano et al., 2000; Noël et al., 2002) or on virulence plasmids, as in *Shigella* (Maurelli et al., 1985; Watanabe & Nakamura, 1985) and *Yersinia* (Gemski et al., 1980; Zink et al., 1980; Ben-Gurion & Shafferman, 1981).

How does such an intricate system accomplish its efficient assembly into the final functional multi-membrane spanning complex? While early purification-based genetic studies outlined the rough order of assembly, the employment of emerging techniques in recent years has provided a more detailed understanding of the assembly process. As especially for the IM components, the order of assembly is likely to be closely linked to the function of the machinery, these studies also provide insight into the mechanisms of type III secretion. This review aims to summarize the latest findings and to point out general principles that govern the assembly of T3SSs. Further, we will propose an integrated assembly model and discuss quality control and recycling of the system.

### The main building blocks of the injectisome

The injectisome requires more than twenty proteins to assemble, at least fifteen of which are thought to be part of the functional protein complex (Cornelis, 2006; Büttner, 2012). The protein names for some of the best-studied T3SS families, the degree of conservation of their components, and flagellar homologues are listed in Table 1. In this review, we will use the ‘function names’ as well as the unified Sct names (secretion and cellular translocation; Hueck, 1998) for general statements, along with the name of the protein in the respective organism when referring to specific studies.

Many studies have revealed a network of interactions between these proteins (recently reviewed by Büttner (2012)). In combination with structural information, this allows to roughly divide the injectisome into the following substructures: needle, membrane rings (also termed ‘base’), export apparatus and cytosolic components (Fig. 1), which we will briefly outline below. Please
refer to dedicated reviews for details on structure (Moraes et al., 2008; Hodgkinson et al., 2009; Spreter et al., 2009; Erhardt et al., 2010; Izor et al., 2011; Schraidt & Marlovits, 2011; Chatterjee et al., 2013) and function (Cornelis, 2006; Galan & Wolf-Watz, 2006; Böttner, 2012).

**Needle**

The extracellular needle is generated by helical polymerization of a small hairpin protein (SctF) (Cordes et al., 2003; Deane et al., 2006; Fujii et al., 2012; Loquet et al., 2012). An oligomer of hydrophilic translocator proteins forms the needle tip (Mueller et al., 2005; Johnson et al., 2006; Broz et al., 2007), which is proposed to act as a scaffold for the assembly of hydrophobic translocator proteins that permeate the host cell membrane (Tardy, Häkansson et al., 1996; Blocker et al., 1999; Neyt & Cornelis, 1999; Goure et al., 2004; Picking et al., 2005; Montagner et al., 2011); reviewed in (Mattei et al., 2011). In attaching and effacing animal pathogens such as *E. coli* pathotypes EPEC and EHEC, and *Citrobacter rhodentium*, in *Bordetella*, and in plant pathogens, the tip is replaced by a protein forming a pilus or filament (Jin & He, 2001; Chen & Frankel, 2005; Medhekar et al., 2009; Bergstrom et al., 2012).

The needle is presumably anchored to the base by the alpha-helical inner rod protein SctI (Marlovits et al., 2004; Wood et al., 2008; Zhong et al., 2012).

**Membrane rings/the base**

The base consists of two membrane-spanning ring structures (Fig. 1). The outer membrane (OM) ring, which extends deeply into the periplasm, consists of 12–15 copies of a protein from the secretin family (SctC) (Koster et al., 1997; Kubori et al., 1998; Blocker et al., 2001; Spreter et al., 2009; Schraidt & Marlovits, 2011; Bergeron et al., 2013). Secretins were found to require dedicated pilot proteins for proper functioning. Pilots form a structurally dissimilar group of OM lipoproteins that are thought to passage secretins piggyback through the periplasm by use of the Lol system. At the OM, pilots are believed to facilitate the insertion, oligomerization, and assembly of their cargo (Koster et al., 1997; Crago & Koronakis, 1998; Daefler & Russel, 1998;
Table 1. Homologous proteins and their function in various families of T3SS and the flagellum (Hueck, 1998; Meyer et al., 2006; Holzer & Hensel, 2010; Abby & Rocha, 2012; Büttrner, 2012; Gazi et al., 2012; Monjarás Feria et al., 2012)

| T3SS family | Ysc | Inv-Mxi-Spa | Salmonella | Ssa-Esc | Hrp-Hrc 1 | Hrp-Hrc 2 | Degree of conservation (Alignment score) | Flagellar |
|-------------|-----|-------------|------------|---------|-----------|----------|----------------------------------------|----------|
| Functional name* | Sct | name | Yersinia | Shigella | SPI-1 | SPI-2 | E. coli | P. syringae | R. solanacearum |
| Secretin | SctC | YscC | MxiD | InvG | SsaC | EscC | HrcC | HrcC | High (75) |
| Outer MS ring protein | SctD | YscD | MxiG | PrgH | SsaD | EscD | HrpQ | HrpW | Low (33) FlG |
| Inner MS ring protein | SctU | YscJ | MxiJ | PrgK | SsaJ | EscJ | HrcJ | HrcJ | High (75) FlI |
| Minor export apparatus protein | SctR | YscR | Spa24 (SpaP) | SpaP | SsaR | EscR | HrcR | HrcR | High (91) FlP |
| Accessory cytosolic protein | SctK | YscK | MxiK | OrgA | – | – | HrpD | – | Low (38) |
| C ring protein | SctQ | YscQ* | Spa33 (SpaO) | SpaO | SsaQ* | EscQ | HrcQA-δ | HrcQ | Low* (38) FlM + FlN |
| Stator | SctL | YscL | MxiN | OrgB | SsaK | EscL (Orf5) | HrpE | HrpF | Low (45) FlH |
| ATPase | SctN | YscN | Spa47 (SpaL) | InvC | SsaN | EscN | HrcN | HrcN | High (92) FlL |
| Needle filament protein | SctF | YscF | MxiH | PrgJ | SsaG | EscF | HrpA | HrpY | Low (40) |
| Inner rod protein | SctI | YscI | MxiL | PrgJ | SsaI | EscI (Orf18) | HrpB | HrpJ | Low (47) |
| Needle length regulator | SctP | YscP | Spa32 (SpaN) | InvI | SsaP | EscP (Orf16) | HrpP | HpaP | Low (33) FlK |
| Hydrophilic translocator, needle tip protein | LcrV | IpaD | SipD | – | – | – | nd | – |
| Hydrophobic translocator, pore protein | YopB | IpaB | SipB | SscC | EspD | HrpK | PopF1, PopF2 | Very low (21) |
| Hydrophobic translocator, pore protein | YopD | IpaC | SipC | SscD | EspB | – | – | Very low (27) |
| Pilotin | YscW | MxiM | InvH | – | – | – | nd | – |
| Gatekeeper | SctW | YopN | MxiC | InvE | SsaL | SepL | HrpJ | HpaA§ | Low (36) |

Protein names are given for well-studied members of the respective T3SS families. The degree of conservation is represented by the similarity score of the consensus sequence determined by the multiple sequence alignment package M-Coffee (Wallace et al., 2006) for the respective homologues of Yersinia enterocolitica virulence plasmid pYVE227 (for Ysc), Salmonella enterica spp. enterica serovar Typhimurium SL1344 SPI-1 (for Inv-Mxi-Spa), enteropathogenic Escherichia coli O127:H6 str. E2348/69 (for Ssa-Esc), Pseudomonas syringae pv. tomato str. DC3000 (for Hrp-Hrc 1) and Ralstonia solanacearum GM11000 (for Hrp-Hrc 2). Protein sequence identifiers are listed in Supporting Information, Table S1.

Kimbrough & Miller, 2000; Crepin et al., 2005; Yip et al., 2005; Silva-Herzog et al., 2008), and a single-spanning transmembrane protein constituting the outside (SctD) (Kimbrough & Miller, 2000; Sprent et al., 2009; McDo-
well et al., 2011; Gamez et al., 2012). SctD also connects the two membrane rings through its periplasmic domain (Diepold et al., 2010; Sanowar et al., 2010; Schraidt et al., 2010; Ross & Plano, 2011), which is flexible in length (Kudryashev et al., 2013). The MS ring has been proposed to have a 12- or 24-fold symmetry (Yip et al., 2005; Hodgkinson et al., 2009; Spreter et al., 2009; Schraidt et al., 2010; McDowell et al., 2011; Schraidt & Marlovits, 2011).

**IM export apparatus**

In addition to the MS ring components, the five highly conserved IM proteins SctRSTUV are essential to secretion. These five proteins span the IM with up to eight transmembrane domains (Allaoui et al., 1994; Fields et al., 1994; Ghosh, 2004; Berger et al., 2010), as was shown for their flagellar counterparts (Ohnishi et al., 1997; Minamino & Macnab, 2000a). While the three minor export apparatus components (SctRST) consist mainly of transmembrane or periplasmic domains, the two major proteins (SctUV) each contains a large cytosolic C-terminal domain (Plano et al., 1991; Allaoui et al., 1994; Berger et al., 2010). All five proteins were proposed to be located within the membrane patch surrounded by the MS ring, where they are thought to select substrates and to form the substrate translocation channel (Minamino & Macnab, 1999*; Aizawa, 2001*; Tampakaki et al., 2004). Based on this proposed function, they have been termed ‘export apparatus’ or ‘IM export machinery’. Recent cryo-EM data showed that in the absence of the export apparatus, an electron density at the centre of the MS ring is missing that could be attributed to its minor proteins (Wagner et al., 2010). Moreover, a toroidal-shaped electron density between the MS ring and the ATPase has been assigned to the cytosolic domains of the two major export apparatus components (Chen et al., 2011*; Abrusci et al., 2013*; Kawamoto et al., 2013).

It has been shown that SctV forms oligomers (Wagner et al., 2010) and is present in multiple copies in the machinery (Diepold et al., 2011; Li & Sourjik, 2011*); a recently published crystal structure showed that the cytosolic part of this protein forms a circular nonamer (Abrusci et al., 2013). While the other components are likely to have a lower stoichiometry (Wagner et al., 2010; Diepold et al., 2011; Abrusci et al., 2013), their exact copy number is currently unknown.

Despite the central role in substrate export that is attributed to these proteins, very little is known about their interactions and how they function within the injectisome. The best-studied member of the export apparatus is SctU, which is cleaved autocatalytically (Minamino & Macnab, 2000b*; Lavander et al., 2002*; Ferris et al., 2005*). Autocleavage was linked to substrate selection (Sorg et al., 2007), and hence, the protein has been termed switch protein (see ‘assembly of needle and tip’ for details on substrate switching).

**Cytosolic components**

There are five essential cytosolic proteins that display homology to flagellar cytosolic components and are therefore thought to be part of the injectisome structure: An ATPase (SctN), a stalk protruding from the center of the ATPase and connecting it to the major export apparatus protein (SctO) (Ibuki et al., 2011, 2013*), a homologue of the flagellar C ring (cytosolic) components FlIM and FlN (SctQ), a stator that bridges the ATPase and the C ring component (SctL) (McMurry et al., 2006*), and an additional accessory protein (SctK). Interactions between these proteins have been studied by pull-down experiments (Morita-Ishihara et al., 2006, Riordan & Schnewind, 2008; Johnson & Blocker, 2008), yeast-two-hybrid (Y2H) (Jackson & Plano, 2000; Jouihri et al., 2003; Spath et al., 2009) and quantitative proteomics (Biemans-Oldehinkel et al., 2011). Except for the stalk, all of these proteins were shown to interact with each other and the Y2H experiments consistently showed a line of interactions SctK-SctQ-SctL-SctN, which is in agreement with additional data from a yeast-three-hybrid experiment (Jackson & Plano, 2000). Based on structural similarities of a part of SctV, and of SctN, SctO, and SctL with E, α/β, γ, and b/δ subunits of V- and F-type ATPases, respectively, it has been hypothesized that the overall structure of the cytoplasmic components of the T3SS resembles that of V/F-type ATPases (Pallen et al., 2006; Worrall et al., 2010; Ibuki et al., 2011*; Abrusci et al., 2013). The ATPase is thought to detach chaperones and unfold export substrates (Akeda & Galán, 2005), but the actual substrate export also depends on the proton-motive force (Wilharm et al., 2004). Furthermore, for the related flagellar T3SS, an elegant chain mechanism was just postulated, which harnesses the entropic force of the unfolded substrates in the secretion channel for substrate export (Evans et al., 2013*). Further details on energizing type III secretion have been presented in a recent review of the flagellar protein export pathway (Minamino, 2013*). While the stator might act as a negative regulator of the ATPase (Blaylock et al., 2006; Pallen et al., 2006), as has been shown for its flagellar homologue FlIH (Minamino & Macnab, 2000a*; González-Pedrajo et al., 2002, 2006*), the role of the C ring homologue SctQ and the accessory protein SctK is less clear. The function of the flagellar C ring in reversal of the rotation direction (Driks & DeRosier, 1990*; Khan et al., 1992*) is generally thought to be obsolete in the injectisome, despite recent evidence that the injectisome tip rotates (Ohgita et al., 2013). However, SctQ has been shown to localize to the...
proximal side of the injectisome in *Shigella* (Morita-Ishihara et al., 2006) and to co-localize with other injectisome components in *Yersinia* (Diepold et al., 2010). The *Chlamydia* C ring binds to a general cargo chaperone (Spaeth et al., 2009), and it was recently shown that in *Salmonella*, a cytotoxic ‘sorting platform’ consisting of at least the C ring protein, the stator, and the accessory protein (SpAO, OrgB, and OrgA) is involved in establishing the secretion hierarchy of export substrates (Lara-Tejero et al., 2011). The precise role of the stalk protein SctO is still unclear. It has been shown to selectively bind cognate chaperones of translocator proteins (Evans & Hughes, 2009), which is also the case for its flagellar homologue FliJ (Evans et al., 2006*). FliJ has also been suggested to promote hexamerization of the ATPase (Ibuki et al., 2011*) and to help coordinate the secretion of late flagellar building blocks together with the major export apparatus component FlhA (Bange et al., 2010*).

### Assembly of the flagellum

The assembly of the flagellum has been studied for 30 years, and extensively reviewed (Aizawa, 1996*; Aldridge & Hughes, 2002*; Macnab, 2003, 2004*; Apel & Surette, 2008*; Chevance & Hughes, 2008*; Minamino et al., 2008*). For the purpose of this review, we will mainly concentrate on the features that are relevant because of their homology to the injectisome-type T3SS.

In the flagellum, an approximate order of assembly is already suggested by the order of expression of the flagellar genes. Studies in *E. coli* (Komeda, 1986*; Kutsukake et al., 1990*; Chilcott & Hughes, 2000*) showed that the 14 flagellar operons are arranged in a regulatory cascade of three classes: The single class 1 operon flhDC controls expression of the class 2 operons, which encode for hook-basal body components and the transcriptional activator for the class 3 operons. Transcription of the class 3 operons does not occur before completion of hook-basal body assembly and leads to the expression of the rotor/stator proteins and the flagellin filament, as well as the linked chemotaxis system. Transcriptional control is subject to various regulatory and adaptation mechanisms, which are reviewed elsewhere (Jones & Aizawa, 1991*; Chilcott & Hughes, 2000*; Aldridge & Hughes, 2002*; McCarter, 2006*; Smith & Hoover, 2009*; Anderson et al., 2010*).

An early landmark paper in the study of flagellar assembly was published in 1992, when Kubori et al. examined the formation of flagellar precursors in a wide array of deletion mutants. They concluded that assembly of the flagellum starts at the IM and proceeds sequentially to more distal structures. The smallest detected structure was the MS ring, which did not require any structural protein other than its component FliF. In various deletion strains, different precursors of the basal body could be visualized, which allowed to establish an approximate pathway for flagellar assembly (Kubori et al., 1992*).

Further studies, mainly based on heterologous overexpression of proteins, helped to elaborate details of the assembly pathway: Immediately after assembly of the MS ring, the C ring switch complex, consisting of FlIG, FlIM, and FlIN, can be formed (Kubori et al., 1997*; Lux et al., 2000*; Macnab, 2003*). For the next observable steps, integration of the eight proteins proposed to form the export apparatus and the ATPase has to occur. Whether in wild-type conditions, these proteins require the MS ring and the C ring for their assembly, could not be determined at this point, which left the possibility that assembly of the export apparatus is an independent event (Macnab, 2003*; McMurry et al., 2004*). New data from Li & Sourjik (2011) showed that indeed, the major export apparatus component FlhA can assemble independently at the IM and that presence of FlhA promotes oligomerization of FliF (MS ring) at native FliF expression levels, which otherwise only occurs when FliF is overexpressed.

After completion of the hook-basal body, the flagellum grows outwards, subsequently forming the rod, the P and L rings in the peptidoglycan layer and in the OM, respectively, the hook, and the filament. Specific cap proteins guide the polymerization of the extracellular components (Ikeda et al., 1985*; Yonekura et al., 2000*).

### Assembly of the injectisome

Transcriptional control of the operons of the injectisome is simpler compared to the flagellum. As the virulence associated T3SS have no homologues of the proteins encoded in the class 3 operons in the flagellum, all structural injectisome components are encoded in operons corresponding to the flagellar class 2, and transcription is expected to occur simultaneously. It has been known for a long time that overall transcription levels can be subject to regulation and in many cases, expression of the T3SS genes is upregulated upon contact to the host cell (Rosqvist et al., 1994; Pettersson et al., 1996; Brutinel & Yahr, 2008). Nevertheless, all system components are likely to be present at the same time, and the assembly order will largely depend on affinities and kinetics of protein–protein interactions.

### Assembly of the membrane rings

The first genetic studies on the assembly of the injectisome were performed in *Salmonella* SPI-1. The *Salmonella* secretin InvG (SctC) and the MS ring components PrgH and PrgK (SctD, SctI) form the base of the NC even in the absence of the ATPase or the major export apparatus protein (Kubori et al., 2000). When PrgH and PrgK were overexpressed in...
E. coli, ring structures could be observed by EM (Kimbrrough & Miller, 2000). This suggested that injectisome assembly is initiated at the IM, like it is the case for the flagellum (Kimbrrough & Miller, 2002). Next, the MS ring would attach to the secretin ring in the OM, which has been shown in various bacteria to form a stable structure as well (Koster et al., 1997; Kubori et al., 2000; Tamano et al., 2000; Blocker et al., 2001; Schuch & Maurelli, 2001; Burghout et al., 2004a, b). A structure similar to the sum of these rings was found in several mutant strains (Kimbrrough & Miller, 2000). As all further cytolic and IM components of the injectisome were required for export of the needle subunit, which was the next observable event, their assembly order remained open. The idea that the two components of the MS ring form a somewhat stable and even functional complex is supported by the observation of secretin-less, but otherwise complete NCs when the secretin was either removed mechanically (Kimbrrough & Miller, 2002) or absent (Schraidt et al., 2010). Notably, much fewer complexes were observed in the latter case, suggesting a low assembly efficiency and/or decreased complex stability in the absence of the secretin. In line with this, an earlier study by Sukhan et al. (2001) found that all three ring-forming proteins of Salmonella Typhimurium SPI-1, the secretin InvG and the MS ring components PrgH and PrgK, were required to form the base efficiently. While low amounts of assembled secretin InvG could be purified from the membrane in mutants lacking any of the two MS ring proteins, multimers of the outer MS ring protein PrgH were found in low amounts in the InvG mutant strain, but not in a strain lacking the inner MS ring protein PrgK. Taken together, these studies suggest that all three base components are required for the efficient assembly of a sufficiently stable structure. Likewise, it was found that in EPEC, all three base proteins, EscCDJ were required for assembly of the T3S apparatus (Ogino et al., 2006).

Using a different approach, a study investigating the assembly of fluorescently labelled Y. enterocolitica injectisome components in vivo (Diepold et al., 2010) confirmed the independent assembly of the base. However, in this report, the secretin YscC, but not the inner MS ring component YscJ was required for assembly of the outer MS ring protein YscD. In line with this observation, YscC and YscD interacted in the absence of YscJ, whereas the interaction of YscJ with YscC or YscD required the presence of all three proteins. In conclusion, these data suggested an outside-in assembly starting from the stable secretin ring in the OM and stepwise assembly progression via the outer and inner MS ring components YscD and YscJ.

Taken together, there is agreement that the secretin ring assembles efficiently in the absence of the MS ring components and that secretin and MS ring can form a stable structure as well. As the secretin ring bridges the largest part of the periplasm, an outside-in assembly order would accomplish the necessary penetration of the peptidoglycan layer in the first assembly step, which could facilitate the attachment of subsequent components. Taking into account the ubiquitously observed inefficiency of MS ring assembly in the absence of the secretin ring, a model of assembly is conceivable where the secretin ring scaffolds the assembly of the outer MS ring protein [Fig. 2 (1 and 2)]. Notably, there is no functional imperative for the order of assembly of the membrane rings, which is therefore likely to be dictated by kinetics of the protein–protein interactions (Box 2). Whether outside-in is the rule for all classes of T3SS, especially in the presence of dedicated lytic transglycosylases that assist in the local degradation of the peptidoglycan layer in many T3SS (Miras et al., 1995; Koraimann, 2003), remains open.

**Box 2. The ring-building motif (RBM) and its role in formation of the circular substructures**

The majority of injectisome components form oligomeric substructures within the overall system, many of which have been shown to be ring-shaped. Crystallographic analysis of the inner and outer membrane base components by Strynadka and co-workers revealed a common modular domain within these proteins that was termed RBM according to its putative role in base assembly (Spreter et al., 2009). The motif comprises two helices on one side packing against a three-stranded beta sheet on the other side. RBMs were subsequently also identified in the cytoplasmic domain of the major export apparatus component (Lilic et al., 2010; Worrall et al., 2010), in secretins of type II secretion systems (Korotkov et al., 2009, 2011), and in the intercellular channel complex of Bacillus subtilis (Levdikov et al., 2012; Meisner et al., 2012). A recent analysis of the RBMs of the base components of the Salmonella SPI-1 system suggested that electrostatic interactions between the front and the back of the motifs are the driving force of self-association of RBMs (Bergeron et al., 2013). Although the evidence is compelling that RBMs drive ring formation, the RBM-containing purified domains of the base components are largely monomeric in solution and do not efficiently assemble into ring structures. The RBM of the major export apparatus component in Shigella does not participate in interactions between neighboring monomers (Abruscì et al., 2013). In addition, the RBM of the outer MS ring component in Yersinia was not essential for effector translocation (Ross & Plano, 2011). This suggests that other factors are also important for formation and function of the ring-forming T3SS components.
Assembly of the export apparatus and the cytosolic components

The proteins forming the export apparatus in the IM and the cytosolic components of the T3SS, thought to be responsible for substrate targeting, unfolding and finally export, are easily lost during the classical purification of the machinery used for electron microscopic studies (Wagner et al., 2010; Schraidt & Marlovits, 2011; Abrusci et al., 2013). Therefore, new techniques such as quantitative blue native polyacrylamide gel electrophoresis of membrane protein complexes and visualization of fluorescently labelled components in vivo had to be employed to investigate the assembly of these parts.

Export apparatus

It has long been presumed that the export apparatus is located within the membrane patch surrounded by the MS ring proteins (Aizawa, 2001; Tampakaki et al., 2004). Recently, this hypothesis was confirmed for some components in Salmonella SPI-1 (Wagner et al., 2010). The outside-in scaffolding model therefore appears to be at odds with the central localization of the export apparatus components within the MS ring. An overly efficient outside-in assembly leading to a premature permanent closure of the MS ring prior to integration of the export apparatus components would result in secretion-incompetent complexes. Although the base can form on its
own, it is not clear whether this occurs to a significant degree in vivo.

Combining complex purification and EM in a system that allows the consecutive expression of different T3SS components, it was demonstrated that some of the SPI-1 export apparatus components not only can form a complex on their own, but also that this subcomplex, composed of two of the minor export apparatus proteins, SpaP and SpaR, is required for the efficient assembly of the NC base (Wagner et al., 2010) [Fig. 2 (3)]. In addition, the third small component SpaQ, and to a minor extent the switch protein SpaS were required to reach the normal level of base assembly [Fig. 2 (4 and 5)]. In contrast, the large export apparatus protein InvA was not needed for this process and could apparently be incorporated into pre-existing bases upon delayed expression.

In Yersinia, the InvA homologue YscV has been shown to be present in multiple copies at the injectisome. This complex presumably forms a ring structure as recently shown for the Shigella homologue MxiA (Abrusci et al., 2013). It assembles in the absence of the structural ring proteins but requires the three small export apparatus components YscR, S, T (Diepold et al., 2011), consistent with an independent ‘IM assembly pathway’ [Fig. 2 (3–5)]. The dependence of oligomerization of the large export apparatus component on the small export apparatus proteins was corroborated for Shigella (Abrusci et al., 2013). YscV, probably as part of an YscRSTV complex, interacts with the inner MS ring component YscJ, even in the absence of the switch protein YscU and the other base components [Fig. 2 (6)]. While the complex forms in the absence of both MS ring components and the secretin, these components were required to stably localize the complex within the peptidoglycan layer (Diepold et al., 2011).

Based on these data, we can infer a bipolar scaffolding model of assembly, where two subcomplexes, the small export apparatus components and the secretin, nucleate assembly at the inner and OM, respectively [Fig. 2 (1 and 3)]. The small export apparatus proteins scaffold the assembly of the large export apparatus components in the IM [Fig. 2 (3–5)]. At the other end, the secretin ring scaffold the assembly of the outer MS ring protein [Fig. 2 (2)]. These two subcomplexes are subsequently integrated into a holo-complex through interactions with the inner MS ring protein [Fig. 2 (7)]. This assembly has to be orchestrated such that closure of the MS ring does not prevent the incorporation of the export apparatus – inner MS ring protein assembly [Fig. 2 (6)].

At this point, the precise roles of the two large export apparatus proteins in NC assembly are unclear: Lack of the switch protein universally did not impair incorporation of other export apparatus components into the base. However, in Yersinia, it was also not required for recruitment of the inner MS ring component to the export apparatus complex (Diepold et al., 2011), while in Salmonella SPI-1, the switch protein was required for efficient base assembly (Wagner et al., 2010). Lack of the large export apparatus component InvA neither had an effect on base assembly, nor on incorporation of the minor export apparatus protein SpaP into the base (Wagner et al., 2010), excluding a vital role for InvA in bridging the minor export apparatus components and the inner MS ring protein.

### Cytosolic components

Similar to the export apparatus, the cytosolic components are easily lost during purification and therefore difficult to assess in purification-based approaches. Visualization of NCs in a strain lacking the Shigella C ring component Spa33 showed that the C ring is required for formation of the needle, but not the membrane rings (Morita-Ishihara et al., 2006). This study also provided the first direct evidence that a cytosolic component actually assembles at the proximal side of the membrane rings, as is the case for the flagellum. The analysis of fluorescently labelled Yersinia T3SS components (Diepold et al., 2010) allowed to investigate the assembly of the cytosolic components in vivo. It showed that the ATPase YscN and the C ring component YscQ require the presence of each other and both interacting cytosolic components (accessory protein YscK and stator YscL) to assemble at the proximal side of the basal body [Fig. 2 (9)]. Assembly of this cytosolic complex does not require the stalk protein YscO (Diepold et al., 2012). How and when does the cytosolic complex interact with the membrane-bound injectisome components? The observation that C ring, stalk, and accessory proteins co-purify with the cytosolic domain of either wild-type or a noncleavable mutant of the export apparatus switch protein (Riordan & Schneewind, 2008; Bouteaux et al., 2010) suggests that the switch protein provides the docking site. However, the export apparatus is not absolutely required for the assembly of the ATPase – C ring complex, even though assembly of this complex was reduced in absence of some of the export apparatus components (Diepold et al., 2010). Similarly, the assembly of the Salmonella SPI-1 sorting platform consisting of the C ring component SpaO, the stator OrgB, and the accessory component OrgA does not require the presence of the export apparatus or ATPase either (Lara-Tejero et al., 2011). These data support the notion that the cytosolic components can assemble independently and possibly dock in a subsequent step onto the proximal side of the injectisome [Fig. 2 (8, 9)]. As the MS ring proteins...
and the accessory cytosolic protein (but not the export apparatus) were required for formation of the ATPase – C ring complex (Diepold et al., 2010), it is conceivable that the outer MS ring protein mediates binding to a protein of the ATPase – C ring complex. This interaction could then allow the subsequent attachment of the ATPase-C ring complex to the export apparatus. Befittingly, the cytosolic domain of the outer MS ring protein shows strong homology to a forkhead-associated (FHA) domain (Johnson & Mahony, 2007; McDowell et al., 2011; Bari son et al., 2012; Bergeron et al., 2013) which is often involved in protein interactions (Durocher & Jackson, 2002; Pallen et al., 2002).

**Needle and Tip**

The assembly of the T3SS culminates in the formation of the needle filament [Fig. 2 (10)]. Beyond the base, export apparatus and cytosolic components, which are required for substrate secretion per se, correct formation of the needle filament requires the co-secretion of other early substrates, including the inner rod protein and the needle length regulator [Fig. 2 (10)] (Kimbrough & Miller, 2000; Kubori et al., 2000). In some systems, secretion of needle subunits is assisted by dedicated chaperones that prevent premature filament formation in the bacterial cytosol (Quinaud et al., 2005, 2007; Sun et al., 2008; Chatterjee et al., 2011; Sal-Man et al., 2013). It was shown that secretion of the needle filament subunit is required for export of the other proteins (Kimbrough & Miller, 2000). Marlovits et al. (2006) subsequently observed that variations in the expression ratio of the *Salmonella* inner rod protein PrgI and the needle subunit PrgI resulted in changes in needle length and concluded that the actual assembly of the needle and the inner rod coincide. Recently, it was demonstrated that early export substrates in *Yersinia* can be translocated into the periplasm in the absence of the needle subunit, but not in the absence of the inner rod protein (Diepold et al., 2012). This suggests that ‘basic transport’ of substrates across the IM can occur without the needle, which is, however, needed to open the secretin channel (Marlovits et al., 2004, 2006; Diepold et al., 2012). Interestingly, inner rod mutants, which were not secreted, were shown to allow for the secretion of the needle filament subunit but not for filament assembly. However, in the absence of the needle length regulator, the same mutants were secreted and could support filament assembly (Wood et al., 2008).

The mechanism of elongation of the T3SS needle in vivo is technically difficult to resolve. In contrast to the flagellum, the injectisome is not known to employ a dedicated cap protein to facilitate multimerization. Blocker et al. (2008) hypothesized that the smaller size of the needle subunit might keep the respective domains close enough to self-assemble, a hypothesis supported by the results of a recent structural study (Fujii et al., 2012). Additionally, a secreted protein, *Yersinia* YopR/YscH, has been implicated in the control of needle formation (Blaylock et al., 2010).

Using a functional *Salmonella* needle filament subunit PrgI mutant that can be solubly expressed in *E. coli*, Poyraz et al. (2010) showed that nucleation is the rate limiting step of filament assembly in vitro. Furthermore, they demonstrated that needle elongation occurs by subunit polymerization at the distal end through partial refolding of the PrgI protomers from α-helix into β-strand conformation.

Needle length is controlled by a mechanism involving the secreted needle length control protein SctP and the switch protein SctU. Several models have been put forward to explain control of needle length and were discussed extensively elsewhere (Cornelis, 2006; Galán & Wolf-Watz, 2006). In brief, the ruler model and its derivatives (Journet et al., 2003; Shibata et al., 2007*; Erhardt et al., 2011*) presume that secreted SctP acts as a molecular ruler or tape measure that checks needle length. When needle length reaches a point in which the N-terminus of the extended SctP interacts with the growing tip while its C-terminus simultaneously interacts with SctU, the switch to secretion of later substrates is induced (Agrain et al., 2005; Botteaux et al., 2008; Shen et al., 2012). This in turn brings about the termination of needle polymerization. The ruler model is based on the observation that YscP (SctP) variants of increasing length result in correspondingly longer needles in *Yersinia* spp., and a similar correlation exists between the flagellar ruler FliK and the length of flagellar hooks. It has to be noted though, that while needle length correlates well with the length of SctP within species, the correlation is rather poor across homologues of different T3SS (Büttner, 2012). The second major model is based on the observation that NCs of InvJ (SctP) mutants lack an inner rod and show loosely attached needles besides having disregulated needle length in *Salmonella* Typhimurium (Marlovits et al., 2006). This model postulates that SctP chaperones the formation of the inner rod. Completed inner rod formation results in a conformational change of the base, which in turn is believed to translate into switching of substrate specificity. In essence, this model presumes that needle length is determined by the ratio of the rate of secretion of needle protein and inner rod protein. So far, this model has not accommodated a role for the switch protein SctU. As both models explain different experimental observations, further experiments are required until an integrated model can be distilled from the data.
The exact mechanism of substrate switching is still unknown, but some themes have been emerging. The export apparatus switch protein SctU possesses an autocleavage site in its C-terminal domain (Minamino & Macnab, 2000b*; Lavander et al., 2002*; Ferris et al., 2005*). The mechanism of autocleavage involves cyclization of the strictly conserved asparagine residue in the NPTH motive of the switch protein (Lavander et al., 2002; Deane et al., 2008; Zarivach et al., 2008; Wiesand et al., 2009). As autocleavage-deficient mutants cannot switch to the secretion of later substrates, it was postulated that the cleavage event manifests the switch (Ferris et al., 2005*; Riordan & Schneewind, 2008; Björnfor et al., 2009). However, as isolated SctU cleaves itself efficiently without the support of additional factors [in fact, the crystal structure of the cleaved C-terminal domain was solved (Deane et al., 2008; Zarivach et al., 2008)], it is also conceivable that autocleavage is unregulated and occurs when folding of the cytosolic domain is completed. Why then is autocleavage essential for switching of substrate specificity? It was postulated that the conformationally and electrostatically unique surface created after cleavage might be essential for the interaction with other T3SS components (Zarivach et al., 2008). Indeed, the interaction profile of the wild-type and cleavage site mutants has been shown to be different (Riordan & Schneewind, 2008; Botteaux et al., 2010), pointing out the possibility that the substrate switch involves proteins beyond SctU itself. Alternatively, cleavage may allow for conformational flexibility in the C-terminal domain that is critical for productive interactions. Frost et al. (2012) recently suggested that the switch is manifested by the release of the cleaved very C-terminal domain and its subsequent secretion through the T3SS; however, this new concept remains to be confirmed for other systems.

The substrate specificity switch that occurs at reaching the correct needle length results in the secretion of needle tip proteins, which subsequently attach to the distal end of the needle (Fig. 3). The needle tip is formed by an oligomer, most likely a pentamer of a generally hydrophilic protein (Mueller et al., 2005; Johnson et al., 2006; Broz et al., 2007). This protein is not required for needle formation or regulation of needle length, but thought to form a scaffold for the formation of a pore in the host cell membrane that is composed of two interacting translocator proteins. Formation of the needle tip also halts needle elongation by capping of the distal end (Poyraz et al., 2010).

Fig. 3. States of secretion and model of the events leading to substrate specificity switching. Secretion of early substrates leads to assembly of the needle filament until it reaches a specific length. The needle length is controlled by the needle length regulator, which switches substrate specificity to the secretion of intermediate substrates (needle tip protein), presumably through interaction with the switch protein (1). The needle tip complex senses contact with the host cell membrane. The contact signal is transduced through the needle filament down to the cytoplasmic side of the base, where it leads to dislodging of the gatekeeper (2). This frees the way for secretion of late substrates and their subsequent injection into the host cell cytoplasm (3).
Host cell sensing

After assembly of the needle tip, a ‘standby mode’ (Enninga & Rosenshine, 2009) is reached. The T3SS is ready, but remains inactive and does not secrete effector proteins efficiently. The standby mode requires the presence of a ‘plug’ protein complex including a ‘gatekeeper’ protein (SctW), which is thought to be located at the cytosolic interface of the injectisome (Yother & Goguen, 1985; Forsberg et al., 1991; Day & Plano, 1998; Iriarte et al., 1998; DeBord et al., 2001; Ferracci et al., 2005). Host cell contact releases this plug, and a second substrate switch takes place (Fig. 3). Two hydrophobic translocator proteins are then exported, which are thought to form a pore in the host cell (Håkansson et al., 1996; Blocker et al., 1999; Neyt & Cornelis, 1999; Tardy et al., 1999; Marenne et al., 2003; Goure et al., 2004; Picking et al., 2005) and the pool of effector proteins is translocated within few minutes (Schlumberger et al., 2005). Efficiency and timing of translocation are influenced by various proteins, which are mostly exported effectors themselves (reviewed in Dewoody et al., 2013).

Several models for host-cell sensing have been put forward and are reviewed elsewhere (Blocker et al., 2008). Host-cell contact is most likely sensed by the needle tip (Veenendaal et al., 2007; Roehrich et al., 2013). The signal could then be transmitted via structural changes in the SctF needle subunits through the bacterial cell envelope down to the cytosolic side of the NC (Davis & Mecas, 2006; Kenjale et al., 2005; Torruellas et al., 2005), triggering plug release (Martinez-Argudo & Blocker, 2010). It was recently suggested that the gatekeeper SctW interacts with the inner rod protein SctI, and that the signal of host-cell contact breaks this interaction, leading to dislodging of SctW (Cherradi et al., 2013).

Quality control of T3SS assembly

The assembly data reviewed above suggest the existence of an inherent quality control mechanism (Fig. 4). On the one hand, there is no evidence for a strict ‘assembly checkpoint’ that prevents the formation of incomplete or misassembled intermediates. In fact, it has been shown that absence of the secretin does not completely prevent formation of the MS ring (Kimbrough & Miller, 2002; Schraidt et al., 2010), and the export apparatus is not absolutely required for formation of both the MS ring (Diepold et al., 2010; Kimbrough & Miller, 2002; Sukhan et al., 2001; Schraidt et al., 2010) and the cytosolic complex (Diepold et al., 2010) (red arrows in Fig. 4). On the other hand, all these assembly products were present in low numbers or appeared to be instable (Diepold et al., 2010; Schraidt et al., 2010; Wagner et al., 2010), which might explain the ubiquitously observed low number of erroneously assembled complexes in vivo. The assembly of the functional holo-complex therefore appears to be much more efficient than the assembly of incomplete complexes; alternatively, incomplete assemblies could be turned over very quickly. Hence, the assembly pathway is optimized to lead to secreting (with export apparatus) and OM penetrating (with secretin ring) complexes. The underlying bipolar assembly initiation (black arrows in Fig. 4) is a prerequisite for assembly of functional holo-complexes and a showcase for inherent quality control of assembly.

In contrast, formation of needle and tip involve a true checkpoint for correct assembly. These proteins are exported by the T3SS itself and therefore depend on the correct function of the assembled base, export apparatus and cytosolic components.

The fate of the machinery after assembly

Recent findings, made possible by advances in fluorescence microscopy, show that most protein complexes, intriguingly including the closely related flagellum, are by no means the static entities they were thought to be but can rapidly exchange subunits, alter their overall conformation, and even their protein composition (Bai et al., 2010*; Daley, 2008; Leake et al., 2006*; Delalez et al., 2010*). This dynamic behaviour allows bacteria to adapt the function of the respective complex to their current needs and to changes in the environment. It is very likely that this holds true for the injectisome as well. In fact, within the host, regulating long-term activity of the system will be critical for the bacterium. Although the infection process greatly varies between T3SS-containing pathogens, it seems obvious that at least in persistent infections, secretion of effectors has to be downregulated after some time, as has been shown in some cases (Cisz et al., 2008; Dewoody et al., 2011; Mills et al., 2013). In other cases, secretion may need to be stopped and the system re-initialized for the next host cell contact. For example, in Salmonella, the SPI-2 T3SS is needed for the passage of the bacterium through the epithelial cells and subsequently for survival within macrophages. Are the same systems recycled or are T3SS disposable objects, only useable for one shot? If so, wasted systems must be disposed of or the mistargeting and loss of effectors is inevitable. The needle might be subject to strong shearing forces, and actually can be easily mechanically removed at least in some organisms (Hoiczzyk & Blobel, 2001). At present, it is unclear whether the export machinery can ‘switch back’ to needle formation after the needle has been detached, or if a new machinery has to be assembled for this purpose. Another open question is the behaviour...
of the cytosolic complex of T3SSs. The observations that all four components SctKLNQ are required for its assembly (Diepold et al., 2010), that it localizes both to the cytosol and to the IM (Lara-Tejero et al., 2011), and that it is easily lost during purification, raise the possibility of dynamic shuttling of the complex from and to the NC base. However, this behaviour has not been studied in detail so far and therefore currently little is known about the dynamics of the injectisome in or after action.

Concluding remarks

Using a variety of techniques to monitor different steps of T3SS formation, substantial insights into the assembly of this complex molecular machinery have been gained. The resulting tentative assembly model incorporates two independent points of origin – the secretin in the OM and the small export apparatus proteins in the IM. Most likely, these two nuclei unite at the MS ring, leading to a stable assembly fixed in the peptidoglycan layer, which then incorporates the cytosolic components at the same time, possibly as one complex. The resulting machinery is ready to export the needle protein, the translocators and the effectors.

Compared with the current model of flagellar assembly, the main difference is the proposed order of assembly of the membrane rings. Interestingly, recent results (Abrusci et al., 2013; Li & Sourjik, 2011*; Wagner et al., 2010) indicate that the assembly of the different T3SS families may follow a quite similar overall pathway, in line with the close evolutionary relation of these systems (Erhardt et al., 2010; Gophna et al., 2003).

While we have a good idea about the primary assembly pathway, the structural and functional changes that lead to and occur during and after the export of effector proteins are still largely obscure. Both the events at the actual
export gate and within the cytosolic interface of the injectosome are difficult to study and possibly require the combination of high-resolution structures and the observation of dynamic behaviour of these proteins. With the strong efforts towards structure determination and modelling and new tools to observe the dynamics of the working complex, we can expect new findings that will further deepen our knowledge about the assembly and, beyond this, function of the T3SS.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sequence identifiers of homologous proteins of T3SS used for the calculation of the degree of conservation (Table 1).