The Type III secretion system is a bacterial ‘injectisome’ which allows Gram-negative bacteria to shuttle virulence proteins directly into the host cells they infect. This macromolecular assembly consists of more than 20 different proteins put together to collectively span three biological membranes. The recent T3SS crystal structures of the major oligomeric inner membrane ring, the helical needle filament, needle tip protein, the associated ATPase, and outer membrane pilotin together with electron microscopy reconstructions have dramatically furthered our understanding of how this protein translocator functions. The crucial details that describe how these proteins assemble into this oligomeric complex will need a hybrid of structural methodologies including EM, crystallography, and NMR to clarify the intra- and intermolecular interactions between different structural components of the apparatus.

Introduction

Many Gram-negative bacterial pathogens share the remarkable ability to inject bacterial virulence factors directly into their eukaryotic host cells. These effector proteins subsequently subvert eukaryotic cellular processes in a variety of ways and enable the pathogen to directly modulate their host environment, making it susceptible for invasion and infection. The key component responsible for mediating the secretion of the bacterial virulence factors into the host cell is the Type III secretion system (T3SS). It has been identified in many Gram-negative organisms including Pseudomonas, Yersinia, Salmonella, EPEC and is essential for their virulence [1].

The T3SS is a structurally and functionally conserved, complex macromolecular machine, consisting of more than 20 different proteins [2–5]. Most of these proteins are involved in constructing a macromolecular complex that spans the inner bacterial membrane, the periplasmic space, the peptidoglycan layer, the outer bacterial membrane, the extracellular space and the host cellular membrane (Figure 1). This macromolecular assembly constitutes a regulated, continuous path for effector proteins from the bacterial cytosol into the host cell.

Since its first isolation in 1998, considerable progress in characterizing the T3SS apparatus has been made, but elucidation of the underlying principles of architecture and assembly of this macromolecular complex remains essential in understanding the processes of translocation and regulation. The complexity and membrane spanning nature of this sophisticated macromolecular assembly poses great challenges for structural biologists studying the molecular details of the individual components and the ultimate understanding of their individual functional roles within the context of the intact complex. In recent years, different approaches, including 3D EM reconstructions of the T3SS core at different stages of assembly and high-resolution crystal structures of some of the components, combined with molecular modeling techniques have advanced our understanding of the T3SS apparatus (Table 1).

In this review, we will highlight these recent structures and discuss their implications, in addition to a particular focus on the membrane spanning components of the needle complex.

Type III secretion system: the needle complex

The isolation of the core T3SS apparatus from native sources constituted a landmark achievement in the field and facilitated the characterization and visualization of the intact macromolecular complex [6]. 3D EM reconstructions of the purified complex revealed the so-called needle complex (NC) as the core T3SS assembly that spans both bacterial membranes and projects into the extracellular space (Figure 1) [7,8,9]. This NC has been proposed to present a central channel, allowing passage of unfolded proteins in one step from the bacterial cytosol into the host. Utilizing cryo-EM and a combination of particle sorting and symmetry particle averaging, Marlovits et al. [10,11] were able to further extend the resolution and show, that the NC consists of a cylindrical basal body and a hollow, extended needle like structure, which is incorporated into the base during assembly. Even though these EM reconstructions have provided excellent insight into the overall...
Overall architecture of the Type III secretion apparatus. Crystal structures of known individual protein components [accession codes: LcrV(1r6f), MxiH (2v6l), MxiM (1y9l), EscJ (1yj7) and EscN (2obl)] have been docked into a cryo EM Map of the S. typhimurium needle complex[3D EM Database accession code emd1100] alongside a model displaying the morphology of the T3SA. The predicted dimensions and locations of the three lipid bilayers and peptidoglycan layer are illustrated.

<table>
<thead>
<tr>
<th>Functional component—location</th>
<th>Characterized homologs</th>
<th>Available structures</th>
<th>Refs.</th>
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<tr>
<td>Translocon</td>
<td>EspB, EspD (EPEC)</td>
<td>AFM</td>
<td>Ide et al. [40]</td>
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<tr>
<td>Needle—tip</td>
<td>LcrV (Yersinia)</td>
<td>X-ray</td>
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<tr>
<td></td>
<td>IpAD (Shigella)</td>
<td>X-ray</td>
<td>Johnson et al. [32]</td>
</tr>
<tr>
<td></td>
<td>BipI (Burkholderia pseudomallei)</td>
<td>X-ray</td>
<td>Johnson et al. [32]</td>
</tr>
<tr>
<td>Needle</td>
<td>PrgI (S. typhimurium)</td>
<td>NMR</td>
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<td>MxiH (Shigella)</td>
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<td></td>
<td>BsaI (Burkholderiapseudomallei)</td>
<td>NMR</td>
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<tr>
<td>Outer membrane secretin</td>
<td>InvG (S. typhimurium)</td>
<td>EM</td>
<td>Crago et al. [22]</td>
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<td></td>
<td>YscC (Yersinia)</td>
<td>EM</td>
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<td>Outer membrane pilotin</td>
<td>MxiM (Shigella)</td>
<td>X-ray</td>
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<td>Inner membrane rings</td>
<td>PrgK (S. typhimurium)</td>
<td>EM</td>
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<td></td>
<td>EscJ (EPEC)</td>
<td>X-ray</td>
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<td>PrgH (S. typhimurium)</td>
<td>EM</td>
<td>Marlovits et al. [11**]</td>
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<td>ATPase</td>
<td>EscN (EPEC)</td>
<td>X-ray</td>
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architecture of the T3SS, the intermediate resolution does not allow unambiguous localization of individual components and their interpretation is limited by the observed sample heterogeneity and the relevance of symmetry averaging to extend the resolution. Twenty-fold symmetry averaging of the whole NC might lead to an incomplete picture, since the NC is likely to be built up by oligomers with differing internal symmetries. Furthermore, the purified NCs lack a number of the inner membrane components that are embedded in the basal body and are essential for T3 secretion (Figure 2).

The basal body
Remarkably, the basal body of the T3SS, in which the needle-like structure is anchored, consists of only three proteins. In *Salmonella typhimurium* this base is formed by the proteins PrgH and PrgK, that together form the inner membrane rings, and InvG, which is the only outer membrane component [6,7,12].

Inner membrane components
The inner membrane rings, constituted by the proteins PrgH and PrgK in *S. typhimurium*, exhibit a larger

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**Figure 2**

<table>
<thead>
<tr>
<th>Membrane Components of the T3SS:</th>
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<tr>
<td><strong>T3SS component</strong></td>
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<tr>
<td>Host Membrane Components:</td>
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<tr>
<td>SipE (YopB)</td>
</tr>
<tr>
<td>SipC (YopD)</td>
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</tbody>
</table>

**Outer Membrane Components:**

| InvG (YscC) | - | Outer membrane ring / Secretin | 12-14 | Sec |

**Inner Membrane Components:**

| PrgH | FilF | Inner membrane ring / basal body | 18 - 20 | Sec |
| PrgK (YscJ) | FilF | Inner membrane ring / basal body | 24 | Sec / N-terminal lipidation |
| SpaR (YscR) | FilP | Center of IM ring | ? | ? |
| SpaQ (YscQ) | FilO | Center of IM ring | ? | ? |
| SpaR (YscT) | FilR | Center of IM ring | ? | ? |
| SpaS (YscU) | FilB | Center of IM ring / substrate specificity switch | ? | ? |
| InvA (YscV) | FilA | Center of IM ring | ? | ? |

Integral membrane components of the T3SS. The table on the left side summarises the integral membrane proteins of the T3SS, including the components in the inner and outer bacterial membrane and the translocon, which inserts into the host membrane. The illustration on the right shows a schematic diagram of the membrane proteins and their proposed localization, highlighting the transmembrane segments. The dimensions of the individual domains represent only a rough estimate to exemplify the overall architecture.
diameter than the outer membrane component. The protein PrgK belongs to the highly conserved YscJ family of periplasmic lipoproteins, which are thought to be anchored to the outer leaflet of the inner membrane by N-terminal lipidation and for many of the members in addition by a C-terminal transmembrane helix [7,13]. The atomic structure of EscJ, the YscC homolog from EPEC, has recently been solved [13,14]. Intriguingly, in the crystal structure, individual molecules pack into a superhelical assembly with 24 subunits, indicating that EscJ might form corresponding symmetrical ring structures in the T3SS basal body. The proposed EscJ ring model has been supported by molecular modeling studies, confirming a symmetrical 24-subunit EscJ ring as a likely model [15]. This symmetrical 24 subunit EscJ model has similar overall dimensions to the IM rings, as visualized by EM and can be docked into the available EM map of the T3SS basal body from the homologous and highly conserved S. typhimurium (Figure 1). This appealing ring model is in agreement with biochemical data, since all of the typically C-terminal transmembrane anchors and N-terminal lipidation sites align in the same direction towards the membrane, allowing symmetrical anchoring of the complete ring to the inner membrane (Figure 3). The position of the model, lining the inside of the IM rings, is also supported by biotinylation data on the intact NC, indicating that the EscJ homolog PrgK is only partially accessible from the outside [13]. In the currently proposed model, the periplasmic YscJ proteins form a 24 subunit symmetrical ring-structure, that is anchored to the outer leaflet of the IM, occupying the inner part of the upper IM ring as illustrated in Figures 2 and 3. In this localization within the T3SS, the PrgK ring might function as an initiator of NC assembly and present

Figure 3

Comparison of the T3SS NC to the T1SS (Type I Secretion System) and Wza, the translocon for capsular polysaccharides. (1) Crystal structure of the octameric 340 kDa outer membrane protein Wza, the E. coli translocon for capsular polysaccharide export (pdb accession code 2j58). (2) Crystal structures and proposed orientation of the T1SS components TolC and AcrB (pdb accession codes 1ek9 and 1iwg). (3) Proposed orientation of the T3SS NC. Shown are the 3D EM reconstruction of the NC (3D EM database code emd1100) and the docked EscJ ring model (pdb accession code 1yj7). Indicated are the positions of the outer and inner bacterial membrane and the approximate dimensions of the peptidoglycan layer.
a specialized membrane patch for recruiting other putative inner membrane embedded components of the T3SS (Figure 2).

In *S. typhimurium*, the second protein, which together with PrgK, constitutes the inner membrane rings is PrgH. The exact stoichiometry of PrgK:PrgH:InvG in the basal body is still a matter of debate, but it has been proposed to be close to 1.7:1.3:1.0 or 22 ± 1.7:17.4 ± 1.4:13 ± 1, assuming InvG with 12–14 subunits as reference [11**,13**]. Recent single particle cryo-EM studies using symmetry averaging have suggested a variable 19–21-fold symmetry at the inner membrane rings of the NC, with the majority of purified NCs having 20-fold symmetry [11**]. It has to be noted though, that 20-fold symmetry averaging over the whole NC will most likely not correctly reflect the components that do not follow 20-fold symmetry, like PrgK, InvG and needle subunits. Nevertheless, the 20-fold averaged EM map shows clear separation of individual subunits on the outside of the inner membrane rings, implicating an approximate 20-fold symmetry of this particular part of the inner membrane rings. Interestingly this correlates with the proposed stoichiometry and localization for PrgH and the hypothesis that the PrgK ring is encompassed by a PrgH oligomer [15**].

Taken together these studies present an initial hypothesis for the architecture of the IM rings of the T3SS apparatus. Continuative biochemical and structural analysis will be important to allow for more homogeneous and complete NC preparations, to confirm the stoichiometry of the subunits and to unambiguously determine the localization of the individual components and their symmetries.

**Outer membrane component**

The outer membrane component of the T3SS belongs to the secretin family of outer membrane proteins. The secretins are a group of unique integral membrane proteins that assemble into homomultimeric ring structures with large central pores and that function as the outer membrane component of a variety of multicomponent export systems, including Type III secretion, Type II secretion, Type IV pilus biogenesis and filamentous phage release. Secretins from different systems have been studied by low resolution EM, all showing a remarkably similar overall structure and organization, with 12–14 secretin subunits depending on the bacterial species; and the translocation pore that consists of two membrane spanning proteins related to yopB and yopD family that hetero-oligomerize to create a channel in the host eukaryotic membrane [5].

The outer membrane secretin requires, for efficient localization and assembly, the assistance of a specific lipoprotein called pilotin [18,22,23]. The recent crystal structure of MxiM, the pilotin from *Shigella*, has shed more light on how this protein might assist in proper insertion and assembly of the secretin [24**]. The pilotin binds the C-terminal helix of the secretin displacing a lipid-filled pocket allowing the pilotin to mediate the localization of the secretin to the outer membrane [24**]. In the absence of its pilotin (PulS), the Type 2 secretion system secretin PulD assembles in the inner bacterial membrane suggesting a targeting function for the pilotin. Furthermore in the presence of its pilotin, PulD does not require the essential outer membrane assembly factor YaeT for proper multimerization into the outer membrane [25**]. Taken together this suggests that the secretins are properly assembled and embedded independent of the classical, β-barrel OMP pathway and may be more similar in structure to the α-helical *Escherichia coli* Wza protein involved in capsular polysaccharide secretion [26**].

**Extracellular components**

The extracellular components of the T3SS consists of three distinct parts: the needle, a helical polymer made of a few hundred subunits of the YscF family; the needle extension, that forms a bell shaped cap or an extension depending on the bacterial species; and the translocation pore that consists of two membrane spanning proteins related to yopB and yopD family that hetero-oligomerize to create a channel in the host eukaryotic membrane [5].

**The needle**

Although its polymerized nature has precluded direct crystallographic analysis of the needle itself, the crystal structure of the monomeric MxiH [27] and NMR models of monomeric BsaL (*Burkholderia pseudomallei*) and PrgI (*S. typhimurium*) [28] have provided recent insights into the atomic resolution structure of significant regions of the needle ‘building block’. All the needle component structures share a two-helix bundle coiled-coiled motif that is linked by a conserved PxxP turn. This coiled–coil motif was subsequently docked into ~16 Å EM reconstructions of intact *Shigella* needles [29] providing the first atomic predictions of the intermolecular interactions that facilitate the helical needle architecture (Figure 1). Interestingly, the mutations in MxiH that lead to a constitutive secretion state or serve as defects in hemolysis or invasion [30] all map to a common region of the needle—within the contact surface between the head of one subunit in the polymer and the tail of the monomer packing above it. This corroborates the notion that the sensing feature of the needle is only partially conserved among secretins of different export systems, but is highly conserved within each system and is thought to function in recognition and binding of other periplasmic or inner membrane components.
ecular contacts between subunits thereby regulating secretion.

**Tip/needle extension**

Tip proteins are thought to orchestrate the correct insertion of the translocon, linking the needle to the eukaryotic membrane. The docking of MxiH into the EM reconstruction provided a platform for modeling the needle and tip proteins. By superimposing the C-terminal helices of MxiH with LcrV helices, five molecules of LcrV could be placed at the tip of MxiH polymer needle [27] (Figure 1). This bell shaped complex at the tip of the *Yersinia pestis* needle has been shown to be LcrV [31] and recently NMR chemical shift mapping between MxiH and IpaD has further corroborated this interface [48]. Several structures of tip proteins (IpaD and BipD [32]) are now available to compare with the initial LcrV [33] and EspA [34] structures. High-resolution structures of both IpaD and BipD from *Shigella flexneri* and *Burkholderia pseudomallei* have recently been elucidated [32,35], illustrating that they share a central coiled–coil domain with both LcrV and EspA. Both the N and C terminal domains vary to give specificity for the needle and translocon that they interact with. Of interest are the alpha helical coiled–coil structure and the N-terminal four helix domain of BipD and IpaD that mimics the T3SS chaperones with structural homology to the chaperone FliS that prevents premature association and oligomerization of secreted proteins.

The equivalent tip protein in EPEC, EspA, is bound to its chaperone CesA before being secreted through the needle complex. This feature was exploited to trap a monomeric and homogeneous form of the protein for crystallography analysis [34]. Upon eruption from the needle, EspA forms a distinct and highly extended filament extension on the end of the EscF-needle, likely an adaptation to the required attachment of EPEC to host gut epithelial cells. Approximately 15 Å EM real space reconstruction of EspA filaments illustrates that the filament is polymorphic in its length, despite having a fixed twist of 5.6 subunits per turn, the axial rise varies between 3.6 and 5.8 Å [36]. The mechanism to transmit a sensing signal from the tip of the extracellular domain though to the needle and then to the base may employ this method of polymorphic axial rise (although mutant needles that are constitutively active have the same average axial rise as the wildtype protein [37]). We await more atomic resolution details of EspA in an oligomeric form to further probe the details of this tip structure.

**Translocon**

The construction of the tip of the needle and its interaction with the membrane spanning translocon was recently addressed with STEM and quantitative immunoblotting inferring that the tip of the needle structure is homo-pentameric [38]. Because of species variation, it remains unclear whether the tip of the needle is a homo-pentamer [38] or a heteropentameric where one of the tip protein IpaD molecules is replaced by a translocon protein such as IpaB. The heteropentameric complex of IpaD:IpaB(4:1) may serve to plug the end of the needle preventing the premature loss of translocon and effector proteins. Indeed there is evidence to demonstrate the regions of IpaD and IpaB are needed to form a functional needle complex [39].

What does this mean for the translocon’s architecture and oligomerization? YopB (one of the *Yersinia* translocon proteins) interacts with LcrV (using its N-terminal domain) and this interaction is required for proper insertion of YopB into the host membrane, but the oligomerization state of the pore and the stiochiometry remains unclear. To date, there is only one structural report describing EM and AFM of the EPEC translocon embedded in a membrane (EspB and EspD within infected sheep red blood cells) suggesting a non-symmetric 6–8 subunit hetero-oligomeric stoichiometry [40]. The predicted topology of the translocon proteins indicate key features including coiled–coiled domains, a single transmembrane domain in the SipB homologues and two transmembrane domains, coiled–coil and an amphipathic helix in the SipB homologues. The orientation of these two proteins with respect to the membrane is based on the interactions of the predicted coiled–coil domains with the needle and tip proteins (Figure 3). Obviously a huge void in determining how proteins are translocated across the eukaryotic host membrane is the lack of structural detail of any component of the translocation pore itself.

**Regulation of assembly and translocation**

The assembly of the T3SS is accomplished in a highly regulated stepwise manner, in which the formation of the basal body precedes the insertion of the needle [12,41]. The initial step is the separate assembly of the inner and outer membrane rings. How the outer and inner membrane components connect to form the basal body is still an unanswered question. After completion of the core basal body, the inner membrane rings are thought to provide a platform for localization of the essential inner membrane embedded proteins YscR, YscS, YscT, YscU, YscV (Figure 3) and associated ATPase.

**ATPase**

The conserved and essential ATPase is an inner-membrane component of the T3SS that serves to energize the export function and is involved in the initial stages of selective secretion. The recent structure of the EPEC ATPase, EscN [42], and the flagellar ATPase, FilI [43], illustrates their structural homology to the F1 ATPase rather than the AAA ATPase disassembly apparatus that had once been inferred [44]. The monomeric structures are composed of three functional domains; an N-terminal six stranded β-barrel which may aid in the overall hex-
meric formation; an ATPase domain with the typical nucleotide binding α/β Rossman fold, and a walker A and B motif; and finally a C-terminal domain which has less structural homology to the F1-ATPase. The variant C-terminal domain is of interest as it may provide a docking interface for chaperone/effecter complexes as first proposed in InvC through analysis of C-terminal mutants [44,45]. In comparison to the F1-ATPase, helices within interface for chaperone/effecter complexes as first proposed in InvC through analysis of C-terminal mutants [44,45]. In comparison to the F1-ATPase, helices within the C-terminal domain of EscN are truncated or missing [44,45]. In comparison to the F1-ATPase, helices within the C-terminal domain of EscN are truncated or missing and could be complimented by helices present within the helical chaperones such as those present in CesT or SycN—suggested by Zarivach et al. [42]. We believe that EscN sits as a hexamer in the C-ring aside from the main T3SS pore and that the ATPases’ central cavity is filled by a stator that links it to the base of the needle complex. A modeled form of the hexameric ATPase (as verified by mutational and docking analysis) might be docked into the recently described ~16 Å resolution cryo-EM of the double hexameric ring formed by HrcN [46], once the EM maps are made available. Linking this structure to membrane is also a critical venture in order to clearly dictate how a stator like protein and/or an effector chaperone would associate. This would finalize debate over how EscN functions to disassemble and unfold effector–chaperone complexes and pipe them through the needle complex.

Conclusions
Over the past couple of years additional atomic details of the T3SS have been placed into the topology presented by the EM reconstructions. Still much more information needs to be assembled in order to understand how this macromolecular machine functions to direct virulence factors across three membranes. In addition to the high-resolution structures provided by NMR and crystallography and better isolation methods of needle complexes yielding more complete and hopefully even higher resolution EM maps, further biochemical methods need to be employed to identify protein–protein interactions and binding sites used to piece together this molecular apparatus. Together with molecular docking strategies an overall structural representation can then be assembled and the answers to how this injectisome functions as a macromolecular machine can be fully addressed.

Acknowledgments
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Papers of particular interest, published within the period of review, have been highlighted as:
● of special interest
◆ of outstanding interest
11. Marlovits TC, Kubori T, Sukhan A, Thomas DR, Galan JE, Unger VM: Structural insights into the assembly of the type III secretion needle complex. Science 2004, 306:1040-1042. This article describes single particle cryo-EM studies of both the NC and the basal body from S. typhimurium. The EM reconstructions show differences between the assembled NC and the basal body in the interior of the complex, allowing further insight into the composition and assembly of the NC. Using a combination of particle sorting and 20-fold symmetry averaging the resolution of the EM maps could be extended to 17 Å. The approach of 20-fold averaging of the whole complex to extend the resolution is questionable though, since not all of the components of the NC have 20-fold symmetry and might therefore be underrepresented.
13. Yip CK, Kimbrough TG, Felise HB, Vuckovic M, Thomas NA, Pfuetzner RA, Frey EA, Finlay BB, Miller SI, Strynadka NC: Structural characterization of the molecular platform for type III secretion system assembly. Nature 2005, 435:702-707. This research paper describes the crystal structure of the protein EssJ, a member of the YscJ family. The crystal packing indicates that EssJ could form a 24-subunit ring structure, that is anchored to the outer leaflet of the inner membrane, lining the interior of the inner membrane rings of the NC. The hypothesis is supported by EM, labelling and mass spectrometry data analysing the stoichiometry, surface accessibility and membrane association of the homolog PrgK.
25. This paper presents the first atomic structure of a secretin pilus protein. A deep hydrophobic cavity within the structure of MxiM provides a specific binding domain for the acyl chains of bacterial lipids. Isothermal titration calorimetry shows that the C-terminal domain of the secretin, MxiD525-570, hinders lipid binding to MxiM suggesting a mechanism for binding and localization of the secretin.
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oligomeric form of the protein for function. The structure also suggests a binding site for chaperone/effector complexes that need to be dissociated by the ATPase before being translocated through the basal body.


