

The *Salmonella* Type III Secretion System Virulence Effector Forms a New Hexameric Chaperone Assembly for Export of Effector/Chaperone Complexes

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Bacteria hijack eukaryotic cells by injecting virulence effectors into host cytosol with a type III secretion system (T3SS). Effectors are targeted with their cognate chaperones to hexameric T3SS ATPase at the bacterial membrane's cytosolic face. In this issue of the *Journal of Bacteriology*, Roblin et al. (P. Roblin, F. Dewitte, V. Villeret, E. G. Biondi, and C. Bompard, *J Bacteriol* 197:688–698, 2015, <http://dx.doi.org/10.1128/JB.02294-14>) show that the T3SS chaperone SigE of *Salmonella* can form hexameric rings rather than dimers when bound to its cognate effector, SopB, implying a novel multimeric association for chaperone/effector complexes with their ATPase.

Gram-negative bacterial pathogens, including *Salmonella* (typhoid fever), enteropathogenic *Escherichia coli* (EPEC) (diarrhea), *Shigella* (bacillary dysentery), *Vibrio* (gastroenteritis and diarrhea), *Chlamydia* (sexually transmitted disease), *Pseudomonas* (pneumonia and wound infections), and *Yersinia* (plague) organisms, can export virulence effectors directly into target host cell cytoplasm via a type III secretion system (T3SS). The T3SS, often referred to as the injectisome, is a highly organized macromolecular machine that is evolutionarily related to bacterial flagella. T3SS consists of ~25 proteins that oligomerize to form an export apparatus, basal body, and extracellular needle (Fig. 1A) (1, 2). The export apparatus is assembled by a subset of inner membrane proteins and a soluble ATPase complex. A key component of the export apparatus is a cytoplasmic nonameric ring structure called the export gate, which localizes to the base of the basal body and is proposed to sequester effectors prior to secretion (3). The ATPase (called EscN in EPEC) forms complexes with its positive and negative regulators, EscO and EscL, respectively (4). EscN is one of the best-characterized T3SS ATPases and is suggested to form a hexameric ring structure, analogous to that of the F₀F₁-ATPase, with ATP bound between adjacent monomers (5). The EscN ATPase hexameric ring is proposed to attach to EscO and EscL far from the inner membrane T3SS export gate, which may represent an export-off state (6). The basal body is composed of an outer membrane-embedded secretin ring that spans the peptidoglycan layer and a pair of concentric oligomeric rings in the inner membrane (Fig. 1A). The inner rod forms a channel through the basal body and connects to the needle. The assembly of the inner rod appears to be critical for the switching of substrate specificity during T3S from downstream apparatus components to effector substrates (7, 8). Once this switch has occurred, a specific signal is triggered to allow export of the effector proteins by direct contact with the host cells. Protruding from the basal body of the injectisome is the needle, which is a hollow tubular structure, composed of a multicopy assembly of needle subunits. The length of the needle is specifically controlled and varies (between 45 and 150 nm) among bacterial species (1). The length of the needle is critical in order to effectively deliver the effectors into eukaryotic host cells and likely corresponds to the length of the

species-specific adhesins that bridge the bacterial and host cells (9). The diameter of the needle's central channel is ~2 to 3 nm, which is too small to allow secretion of fully folded effector proteins. A recent cryo-electron microscopy study of substrate-trapped injectisomes revealed density for the trapped effector protein inside the central channel of the needle, while the C-terminal green fluorescent protein (GFP) tag (which could not be unfolded) localized to the export apparatus (10). These data demonstrated requisite unfolding of effector proteins for secretion and further suggested their spontaneous refolding after exiting the needle filament.

Unfolding and secretion of effector proteins require the assistance of specialized chaperones (11). T3SS chaperones are small acidic proteins (pI ~4 to 5) that are often found as homodimers or heterodimers and interact with their cognate effectors through the chaperone-binding domain (CBD), which typically encompasses the first 15 to 150 amino acids of the effectors. Crystal structures of the chaperone/effector complexes such as SicP/SptP and InvB/SipA in *Salmonella* show that the CBD of an effector binds to the homodimeric chaperones as a nonglobular polypeptide and wraps around the chaperones primarily through hydrophobic interactions or their so-called β-motif (12, 13). While the extended CBD interacts with the chaperones, the C-terminal domain retains a globular fold (12). It has generally been thought that dimeric chaperones target the specific effector protein for direct interac-

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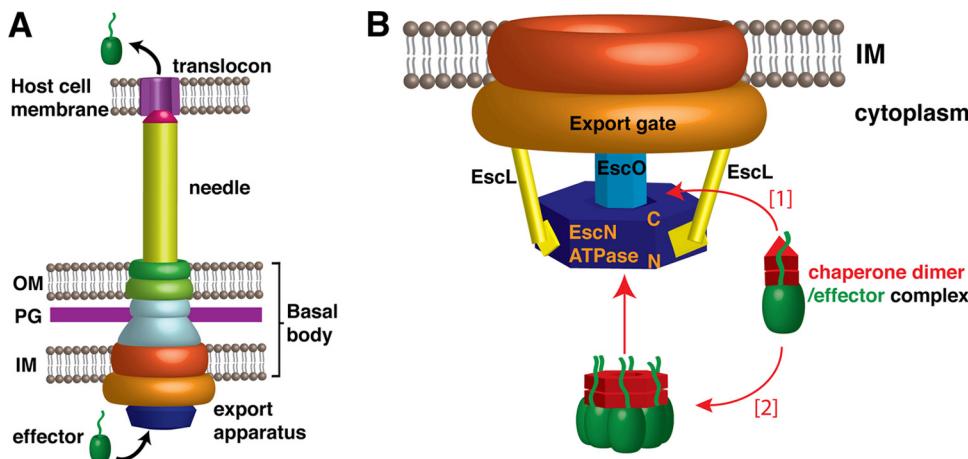


FIG 1 (A) Overall architecture of T3SS. The T3SS is composed of an export apparatus, basal body, and extracellular needle. When the T3SS contacts the host cell membrane, it secretes two translocon proteins, which oligomerize to form a pore in the host membrane. The effectors can then be transported from the bacterial cytosol through the needle and translocon into the host cell cytosol. (B) Proposed interactions of the chaperone/effectector complex with the T3SS ATPase. The peripheral hexameric EscN ATPase (blue), one of the best-characterized T3SS ATPases, is stabilized by positive ATPase regulator EscO (cyan) and interacts with the negative regulator EscL (yellow), which connects to export gate (orange) associated with the inner membrane. The chaperone/effectector complex could interact with the ATPase in two ways: (i) the chaperone dimer/effectector complex binds to C-terminal region of the ATPase, or (ii) the chaperone dimer/effectector complex assembles into a hexameric complex in a concentration-dependent manner and binds to the N-terminal region of the ATPase. After binding to the ATPase, the effector is unfolded and secreted by the ATPase and the chaperone is disassembled.

tion with the ATPase, which disassembles chaperone/effectector complexes and unfolds the effector proteins for secretion (11). Chaperones remain in the bacterial cytosol and are possibly recycled for the next round of effector binding. But is it really a dimeric form of the chaperone/effectector complex that interacts with ATPase? In this issue of the *Journal of Bacteriology*, Roblin et al. (14) provide evidence that the chaperone/effectector complex (SigE/SopB) from *Salmonella enterica* serovar Typhimurium forms a heterohexameric ring complex in solution.

To date, crystal structures of T3SS chaperone/effectector complexes indicate that a single effector binds a dimeric chaperone (12, 13); however, Roblin et al. (14) challenge the idea that this dimeric chaperone/effectector complex interacts with the ATPase. Rather, with their finding that SigE and SopB form a concentration-dependent heterohexameric assembly in solution with similar dimensions to the ATPase, they postulate that it is this hexameric chaperone/effectector complex that mediates the secretion-competent interaction with the ATPase. They investigate different SopB effector constructs to pinpoint the essential region required for formation of the hexameric complex. Assembly of the hexamer was monitored by small-angle X-ray scattering (SAXS) coupled with high-performance liquid chromatography (HPLC), a step that separates dynamic oligomeric states and provides monodispersed samples for SAXS measurements. Further, Roblin et al. generated oligomerized models of the SigE/SopB complex to fit the SAXS data. Their model shows a ring-like hexamer of SigE dimers, each bound to one SopB effector. The SopB N termini are in an extended conformation (perhaps accessible for translocation), and the SopB CBD wraps around SigE. The globular SopB C-terminal domains form a second layer beneath the SigE ring. Previous structural studies show that SigE is unstable to form a hexamer in the absence of SopB, or in the presence of the minimal SopB CBD (15, 16). Interestingly, successive truncation of the SopB N termini still results in a hexameric SopB/SigE assembly, with the SopB N termini remaining in an accessible, extended

conformation. In order for SopB N termini to remain accessible even after truncation, SigE is proposed to shift position to bind a subsequent region of the SopB chaperone binding domain. Roblin et al. propose that gradual truncation of the SopB N termini mimics liberation of the SopB N termini during translocation into the secretion apparatus. After release of the first ~150 SopB residues, the hexameric assembly is destabilized. As this is the first evidence that T3SS chaperone/effectector complexes can form hexamers in solution, it will be important to test if this hexameric assembly extends to other T3SS effector/chaperone complexes as well.

In order to investigate how a hexameric chaperone/effectector complex may interact with the ATPase, we first need to understand how the ATPase assembles as a hexamer and forms a complex with the central and peripheral stalk proteins (EscO and EscL in EPEC, respectively). Although one study has suggested that formation of the EscN ATPase hexameric ring may occur independently of ATP binding, as mutation of the ATP binding site does not affect oligomerization (5), separate studies on the homologous bacterial flagellar ATPase FliI and type IV pili ATPase Flai show that formation of the hexamer occurs in an ATP-dependent manner (17, 18). Based on homology to the γ subunit of F₁-ATPase and flagellar central stalk FliJ, EscO is predicted to form an extended coil-coil that penetrates into the center of the ATPase hexameric ring and connects to the membrane or export gate (4). Some studies suggest that EscO thus promotes the oligomerization of EscN and stimulates ATPase activity (4). The peripheral stalk, EscL, inhibits the EscN ATPase activity and may form a trimeric heterocomplex with EscN and EscO, resembling the homologous flagellar FliH-FliI-FliJ complex (19). Based on the structural and functional similarities of the T3SS and flagellar ATPase complexes, EscL is predicted to bind the N-terminal region of EscN, while EscO binds the C-terminal region of EscN (4, 20). EscL and EscO are linked to the export gate while holding the EscN ATPase far from the export gate in an export-off state (6) (Fig. 1B). A combination of atomic-level structural details and

solution X-ray scattering conformations with *in situ* cryotomography will be the key to further understanding how this dynamic complex assembles and disassembles during secretion.

How does the ATPase recognize effector/chaperone complexes? The first 25 residues of the N-terminal region of effector proteins encode a secretion signal; however, the sequence consensus is poor. Nonetheless, the prediction program EffectiveT3, which analyzes amino acid composition and secondary structure, can successfully predict ~70% of effectors (21). Besides this conserved N-terminal secretion signal, some effectors require a C-terminal secretion signal (22). When effectors bind chaperones through their CBD, these secretion signals are exposed to the ATPase for recognition. Some results suggest that the chaperone/effector docking site is situated on the C-terminal region of the ATPase (5, 23) (Fig. 1B). If the chaperone/effector complex assembles as a hexamer, interaction with the C-terminal region of ATPase, which is located close to the membrane, would face steric hindrance of the central and peripheral stalk. Instead, the hexameric chaperone/effector complex may dock to the N-terminal side of the hexameric ATPase, which faces the cytoplasm (Fig. 1B). The N-terminal region of the ATPase may be critical for optimal hexameric ATPase assembly (24) and possibly has an important role for chaperone/effector recognition. However, contrary to these findings, a genetic analysis of *Salmonella* ATPase InvC indicated that mutation of the N-terminal ATPase domain disrupted ATPase interaction with the membrane, suggesting that it is actually the ATPase N terminus that faces the membrane, with C-terminal region facing the cytoplasm (25), as opposed to the orientation depicted in Fig. 1B. Another possibility is that the ATPase forms a dodecameric or double hexameric ring complex that orients with one of the C-terminal sides facing the cytoplasm, as observed with the ATPase homolog (HcrN) in *Pseudomonas syringae* (26). Roblin et al. show that the diameter of hexameric SigE/SopB ring complex is ~115 Å (14), resembling that of the modeled ATPase hexamer (~105 Å) (5). This hexamer-hexamer docking could make secretion of effectors more efficient and is an interesting avenue for future studies. Clearly, the precise orientation of the ATPase and location of the chaperone-binding site need to be better defined before we can fully understand how the ATPase complex recognizes effector/chaperone complexes.

The T3SS secretes numerous effectors into host cells in various quantities and a predetermined order (27), underlining the complexity of recognition and transport of effectors by the ATPase. Translocation of the effectors begins 10 to 90 s after docking to host cells and can be completed in 10 min (28). Additionally, the translocation efficiency is affected by the concentration of the effectors (29). Roblin et al. (14) have revealed a new possibility that chaperone/effector complexes can assemble as a hexamer, at least in the case of *Salmonella* SigE/SopB. A high concentration of chaperone/effector complex triggers the hexamer oligomerization and may accelerate the translocation efficiency. However, it is still unclear how the ATPase would interact with a hexameric effector/chaperone complex. Would the ATPase unfold one effector at a time or six effectors sequentially or simultaneously? How would other components of the ATPase complex, such as the central and peripheral stalk, affect interaction between the ATPase and chaperone/effector complexes? As the T3SS is an amazingly efficient and highly organized nanomachine, it is particularly challenging to capture the numerous dynamic interactions that occur during secretion, particularly in the cytoplasmic export apparatus. Im-

portantly, Roblin et al. have captured a concentration-dependent hexameric chaperone/effector complex, similar in size to the ATPase. The next challenge will be to verify if hexamer formation of chaperone/effectors is a common theme in T3SS and investigate how such a hexamer could interact with the ATPase complex during secretion.

The relationship of T3SS to bacterial flagella and the relationship of the type II secretion systems to archaeal flagella and bacterial type 4 pili (T4P) (30) suggest analogous evolutionary developments of these two major microbial secretion machines from movement machinery. Also, as for T3SS, even an initial understanding of T4P-type machine assembly has required the combination of X-ray crystallography with cryo-electron microscopy (31). So evidence suggests that combined methods such as the biochemical and structural characterization of *Salmonella* SopB/SigE chaperone/effector complex by SAXS reported in this issue will be invaluable for gaining insights into functional assembly. Understanding T3SS structure and activity with the aim of using this knowledge to develop drugs specifically targeting bacterial pathogens has been an important goal of many research groups around the world for the last 20 years. These new results show the value of X-ray scattering for examining the assembly of machine components in solution under various conditions. SAXS is developing into both an accurate and high-throughput method to examine comprehensive conformations and assemblies in solution (32, 33). Investigations of the nature of T3SS assemblies and their interactions by combined structural, biochemical, and genetic efforts have made tremendous progress toward unveiling how this machine works, with implications for therapeutic developments. It will therefore be exciting to see the mechanistic basis for T3SS function continuing to emerge from the growing knowledge of its dynamic assembly, including whether other chaperone/effector complexes yield results resembling those obtained with the SigE/SopB chaperone/effector hexameric complex from *Salmonella*.

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