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Fueling type III secretion

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Abstract

Type III secretion systems are complex nanomachines that export proteins from the bacterial cytoplasm across the cell envelope in a single step. They are at the core of the machinery used to assemble the bacterial flagellum, and the needle complex many Gram-negative pathogens use to inject effector proteins into host cells and cause disease. Several models have been put forward to explain how this export is energized, and the mechanism has been the subject of considerable debate. Here we present an overview of these models and discuss their relative merits. Recent evidence suggests that the proton motive force is the primary energy source for type III secretion, although contribution from refolding of secreted proteins has not been ruled out. The mechanism, by which the proton motive force is converted to protein export, remains enigmatic.

Keywords

T3SS; needle complex; flagellum; proton motive force; ATPase

An overview of type III secretion system (T3SS) organization

Bacteria use a wide variety of secretion mechanisms to export proteins from the bacterial cell. Protein export is critical for the ability of the bacterium to colonize and shape its environment. Protein secretion is used to assemble structures on the bacterium, secrete proteins into the extracellular milieu, or deliver proteins into targeted host cells. Secreted proteins have to pass the membrane barrier that separates their point of synthesis from their final destination. One particular mechanism, type III secretion, relies on a complex apparatus to deliver proteins from the bacterial cytoplasm across the envelope in a single step [1]. Type III secretion systems are involved in assembling the bacterial flagellum

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(fT3SS), or promoting virulence by delivering proteins, called effectors, into targeted host cells (vT3SS). How this process is energized is the subject of this review.

The fT3SS and vT3SS are related and share significant homology when comparing cytoplasmic components, and the inner membrane export apparatus. The ATPase and associated proteins have been proposed to recruit secretion substrates to the apparatus, and unfold them for insertion into the export channel [2, 3]. YscQ/FliN and associated proteins, in turn, have been proposed to form a sorting platform that, together with export gate proteins FlhA/YscV and FlhB/YscU, help order the sequence of protein export [3–8]. Secretion substrates are delivered to the export apparatus, which is thought to move secretion substrates across the inner membrane (Figure 1)[9, 10]. The exact route, by which proteins are recruited to the export apparatus, and by which the sequence of export is ordered, remains to be elucidated. Beyond this structure, the flagellum and vT3SS diverge. The flagellum consists of a rod, connected to a hook structure, to which the flagellar filament is attached. The needle-complex, on the other hand relies on a secretin ring to pass through the outer membrane, which connects to the needle that projects from the bacterium. The needle terminates in a specialized tip structure (Figure 1)[10, 11]. The secretion channel is narrow and secreted proteins have to be unfolded by the T3SS-associted ATPase in order to pass through the secretion system [2, 12].

Several models have been proposed to explain how protein transport by these T3SSs is energized. On the one hand, energizing transport across the inner membrane has been proposed to involve pushing of the secretion substrate into the secretion channel by the ATPase as part of the unfolding reaction [2, 12], or conversion of proton motive force (pmf) into protein export [13, 14]. On the other hand, promoting movement of secreted proteins through the needle, or even longer flagellar filament, has been proposed to either rely on electrostatic repulsion within the secretion channel [15], or on potential energy stored in the unfolded polypeptides that pass through the T3SS, which is recouped by either assembling into the apparatus, or folding upon secretion [8]. We will examine these mechanisms in turn.

The relative role of the T3SS ATPase and the pmf in protein unfolding and transport across the inner membrane

Proteins destined to be exported via the T3SS are targeted via an amino-terminal secretion signal [16]. Export is usually also promoted by the association with a cognate secretion chaperone [17]. How these secretion signals are recognized by the T3SS, and the route secreted proteins take through the T3SS are as yet unclear. During the export process, secreted proteins are stripped of the export chaperone before being funneled into the secretion system. Stripping the chaperone off secreted proteins, as well as unfolding proteins for export, is a function of the ATPase that is associated with the T3SS [2]. Consistent with this model, export chaperones bind specifically to the ATPase, suggesting that they may help target exported proteins to the ATPase [2, 18]. However, whether engaging the ATPase is the first stop for proteins destined to be exported, or subsequent to recognition by another apparatus component, such as the export gate, is an open question. The ATPase subunits assemble into a hexameric ring [18, 19], with a central opening. By analogy to unfoldases, such as ClpA or ClpB [20], it was proposed that proteins are funneled through this central

cavity as part of the unfolding process [2]. By extension, this mode of unfolding secreted proteins also suggested that moving the protein through the ATPase could provide energy for pushing exported proteins through the secretion channel, thereby driving protein export (Figure 2A).

However, in recent years this model has been called into question. First, flagellar assembly can proceed even in strains lacking the ATPase FliI, if the associated FliH protein is also removed [13, 14]. Protein export in these strains is inefficient, but could be enhanced by suppressing mutations in *flhA* and *flhB*which encode subunits of the export gate [13]. In contrast, protein secretion could be blocked by collapsing the pmf, both in fT3SS and vT3SS, arguing that protein export relies on conversion of the pmf into protein export (Figure 2B)[13, 14, 21, 22]. Moreover, by analogy to F1/F0 ATPase, to which the T3SS ATPase is related, the chaperone binding site is actually thought to be located on the membrane-proximal side of the ATPase [18], which would suggest that proteins targeted to the ATPase, and funneled through the central cavity, would actually be moved away from the membrane, rather than towards the secretion channel. This proposed organization of the ATPase may be incorrect; the function of the F1/F0 ATPase is quite distinct from that of the T3SS-associated ATPase. Also, a prior genetic analysis of the InvC ATPase in *Salmonella* demonstrated that a V51E mutation (near the amino-terminus) prevents membrane association, which would argue for the opposite orientation of the ATPase [23]. The orientation of the ATPase relative to the membrane remains to be resolved conclusively. Electron cryotomographic analysis of the T3SS *in situ* has demonstrated that the ATPase is quite distant from the cytoplasmic membrane, ~10 nm [10, 24]. It is unclear how the ATPase could generate the force to push an unfolded protein through the secretion channel given the distance between the ATPase and the base of the apparatus. However, it was recently proposed that this distant location of the ATPase may be represent an inactive T3SS, and that the ATPase is brought into close proximity with the apparatus during active protein export [3]. As mentioned above, export of flagellar subunits in the absence of the ATPase is inefficient. Pseudorevertants in FlhB could restore protein export, but the efficiency with which export was suppressed varied largely among secreted proteins [13]. Differences in the requirement for the ATPase to promote efficient export could reflect variation in the need for ATPase-mediated unfolding to allow passage through the narrow secretion channel. Ensuring that secretion substrates are export competent may be the primary function of the ATPase.

Collapsing the pmf abolishes protein export via T3SS. Evidence suggests that this block in secretion is not attributable to a concomitant drop in cytoplasmic ATP levels, or block in the assembly of the apparatus [14, 22]. A discrepancy exists between the fT3SS and vT3SS with regard to the relative contribution of the two pmf components, ΔpH (the proton gradient) and Δψ (the membrane potential). While export in the wild-type fT3SS is primarily Δψ dependent [25], export via the vT3SS requires both Δψ and ΔpH [22]. Interestingly, flagellar export in the absence of the ATPase complex (FliHI) requires both Δψ and ΔpH, suggesting that ΔpH in this instance is required to overcome the absence of the ATPase, perhaps by providing energy to unfold secretion substrates that are being funneled into the export apparatus [25]. Evidence from both fT3SS and vT3SS suggests that the inner membrane

export gate component FlhA (YscV and homologs in vT3SS), as well as the cytoplasmic component FliJ (YscO) are involved in controlling the conversion of the pmf to protein export [22, 25, 26]. Asp208, located in a cytoplasmic loop of FlhA, was proposed to be directly involved in modulating the flow of protons via FlhA [26]. Similarly in the fT3SS, pmf-dependent protein export was linked to the interaction of FliJ with the export gate component FlhA [25, 27]. These data correlate well with data from the *Pseudomonas aeruginosa* vT3SS, where mutations in *pscO* (*yscO* homolog) could accelerate protein export via the T3SS, by increasing the efficiency with which the T3SS converts the pmf to protein export [22]. The latter result, in particular, is significant, since it argues that conversion of the pmf to protein export is the rate limiting step for protein export via the T3SS, and the pmf therefore the primary fuel for secretion via the T3SS. Despite some evidence pointing to FlhA serving as proton channel, further evidence will be needed to confirm this model. The mechanism of converting the pmf to protein export is likewise as yet unclear. A recent structural analysis of the vT3SS of *Salmonella enterica serovar* Typhimurium suggested that the inner rod segment of the apparatus is mobile when a secretion substrate is inserted into the apparatus [12]. Whether this mobility is indicative of pmf-induced conformational changes that drive protein export remains to be determined.

Models for movement of subunits through the needle or flagellum

Once the secreted protein has passed across the inner membrane, it still has to travel the length of the needle, or, in case of the flagellum, through the flagellar filament. First evidence that flagellar subunits travel through the filament derived from complementation experiments where bacteria making wavy or straight flagella were mixed. Neither bacteria showed a mixed flagellar phenotype, arguing that flagellar subunits incorporated into the flagellum were not derived from a secreted pool of subunits [28]. Structural analysis of the flagellar filament subsequently revealed a 10–15Å channel that was proposed to serve as the conduit through which flagellar filament subunits are transported [29, 30]. Most recently, a jammed export substrate was visualized in the central channel of the *Salmonella* SPI1 T3SS, demonstrating that secretion substrates exported through the central channel in the needle, and by extension the flagellar filament [12]. How this process is energized, or if it needs a specific energy source, is as yet unclear.

Three models have been proposed to explain movement of secretion substrates through the needle, or flagellar hook and filament, respectively. The first model is that secreted proteins diffuse in single file (Figure 2C)[31]. This process is thought to be driven by potential energy that is stored in the unfolded protein and is recouped, when the secreted proteins refolds to either be incorporated into the structure (e.g. flagellin), or refolds upon exiting the secretion channel [32].

Recently, a second model was proposed, whereby flagellar rod, hook, and filament subunits are pulled through the nascent flagellum by virtue of interactions between the C-terminus of a secreted protein with the amino-terminus of the subsequently secreted protein [8]. These interactions were proposed to pull secreted proteins through the structure in a process that is driven by the assembly of proteins at the tip of the nascent flagellum (Figure 2D). It was suggested that a similar pulling mechanism could also be responsible for export of proteins

via vT3SSs. Here, folding of the secreted substrate upon exit from the secretion channel was proposed to provide the energy for pulling effectors through the apparatus. The pulling mechanism for substrate secretion was bolstered by demonstrating subunit interactions *in vitro*as well as *in vivo* in the flagellar channel [8]. Moreover, it was argued that simply pushing subunits into the nascent flagellum by the export apparatus could not explain a constant rate of flagellum assembly. Resistance to moving subunits through the secretion channel should grow with channel length due to the cumulative effect of friction experienced by subunits being pushed through the growing flagellum. However, while a recent report did demonstrate a constant rate of flagellar elongation [33], it has also been argued, that single file diffusion of flagellin subunits is sufficient to allow for constant flagellar growth without proposing a pulling mechanism [31]. Moreover, earlier reports had demonstrated that the rate of flagellar elongation declines once flagella reach approximately 10 μm in length [34, 35]. The chain model is also not consistent with the observation that proteins that are not normally secreted via the T3SS can be fused to a T3SS export signal and efficiently exported [36–38]. This is also true for export via the flagellum [39]. Since these proteins would not be expected to bind to preceding proteins in the export channel, it is unclear how a pulling mechanism could explain their export. Regardless of chain- or diffusion mechanism, the contribution of folding of secreted proteins to the rate of export has not been explored directly, and could be addressed using folding mutants in export substrates.

A third model suggests that electrostatic repulsion of the secreted protein through charged residues within the secretion channel and on the surface of the secreted protein is involved in promoting protein export [15]. Repulsion would provide a barrier for introducing the secreted protein into the secretion channel (Figure 2E), most likely overcome through the action of the pmf-dependent transport of the secreted protein through the export apparatus. Conversely, repulsion would be predicted to then help propel the protein from the needle or flagellum upon exit from the secretion channel. The model, while interesting, was unfortunately based on an incorrect structure of the vT3SS needle, which had predicted a strong negative charge in the secretion channel. More recent models of the vT3SS needle suggest that the charge in the needle is mostly balanced (although charged residues, primarily positively charged, do project into the lumen of the needle in this model as well) [40].

Concluding remarks and future directions

Significant progress has been made in understanding how protein export via the T3SS is energized, but many questions remain (Box 1). The export apparatus that, presumably, is central to the pmf-dependent export of proteins is essentially a black box. While FlhA (YscV) represents a strong candidate for the channel through which protons flow to promote protein secretion, direct evidence for this activity is still lacking. Similarly, how FliJ (YscO) modulates the rate of secretion is unclear, although it is tempting to speculate that it could modulate the flow of protons through FlhA, for example, by influencing the conformation of the proton channel. More work will also be needed to understand how proton flow can be converted into protein transport. A better understanding of the structure of the export apparatus will be crucial for this endeavor. At this point, we do not know how the export

apparatus is organized, or which export apparatus components help form the secretion channel, and make direct contact with secreted proteins. A structural analysis of the export apparatus will therefore have to be paired with a clearer understanding of the route secreted proteins take through the T3SS.

Type III secretion systems are crucial protein export machines that help define the ability of bacteria to navigate their environment and survive in a host environment. Beyond the fundamental insights afforded by studying these powerful nanomachines, understanding how secretion is energized will also open the door for new avenues of therapeutic intervention.

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Highlights

1. The proton-motive force (pmf) energizes the rate-limiting step of type III secretion.
2. The type III secretion system (T3SS) can change the efficiency of pmf utilization to modulate protein export speed.
3. The ATPase likely renders secreted proteins export competent by unfolding them.

Box 1. Outstanding questions

- How are secreted proteins recognized and handed off to the export apparatus?
- How is the export apparatus organized?
- How is proton flux converted into protein export?
- What is the role of the ATPase?
- Does refolding of secretion substrates contribute to movement through the needle or flagellum?

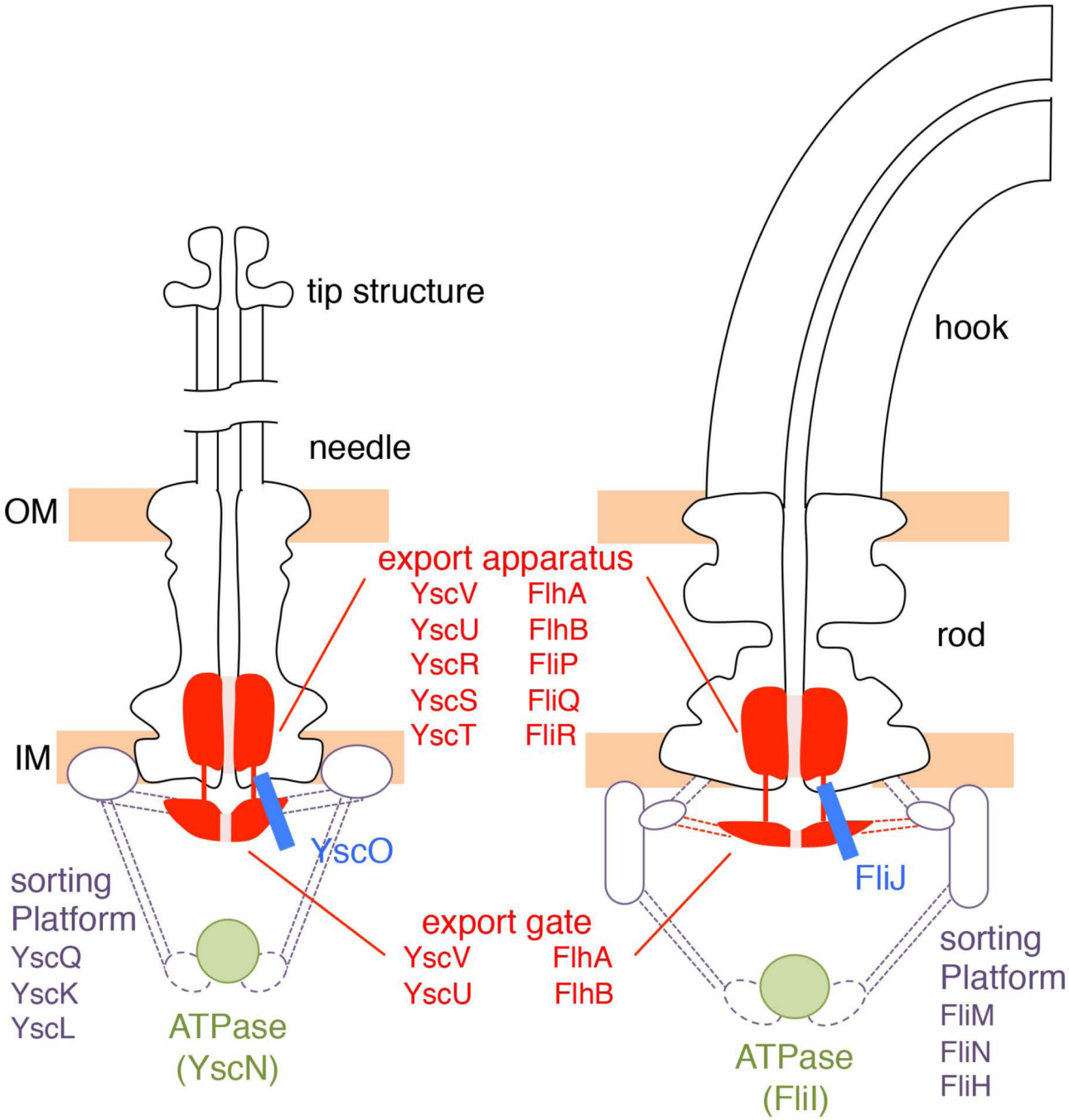

Figure 1. Organization of flagellar- and virulence-associated type III secretion systems

Schematic diagrams of virulence-associated (left) and flagellar type III secretion systems (right) is shown, based on the recent *in situ* cryoelectrontomography-based structural analysis of the *S. enterica* sv. Typhimurim flagellum and SPI1 T3SS. Export apparatus, sorting platform components, as well as the ATPase, and regulatory FliJ (YscO) protein are indicated following the nomenclature of the prototypical *Y. enterocolitica* vT3SS and *S.* Typhimurium fT3SS, respectively. Adapted from Kawamoto *et al.* [10]. Abbreviations: IM, inner membrane; OM, outer membrane.

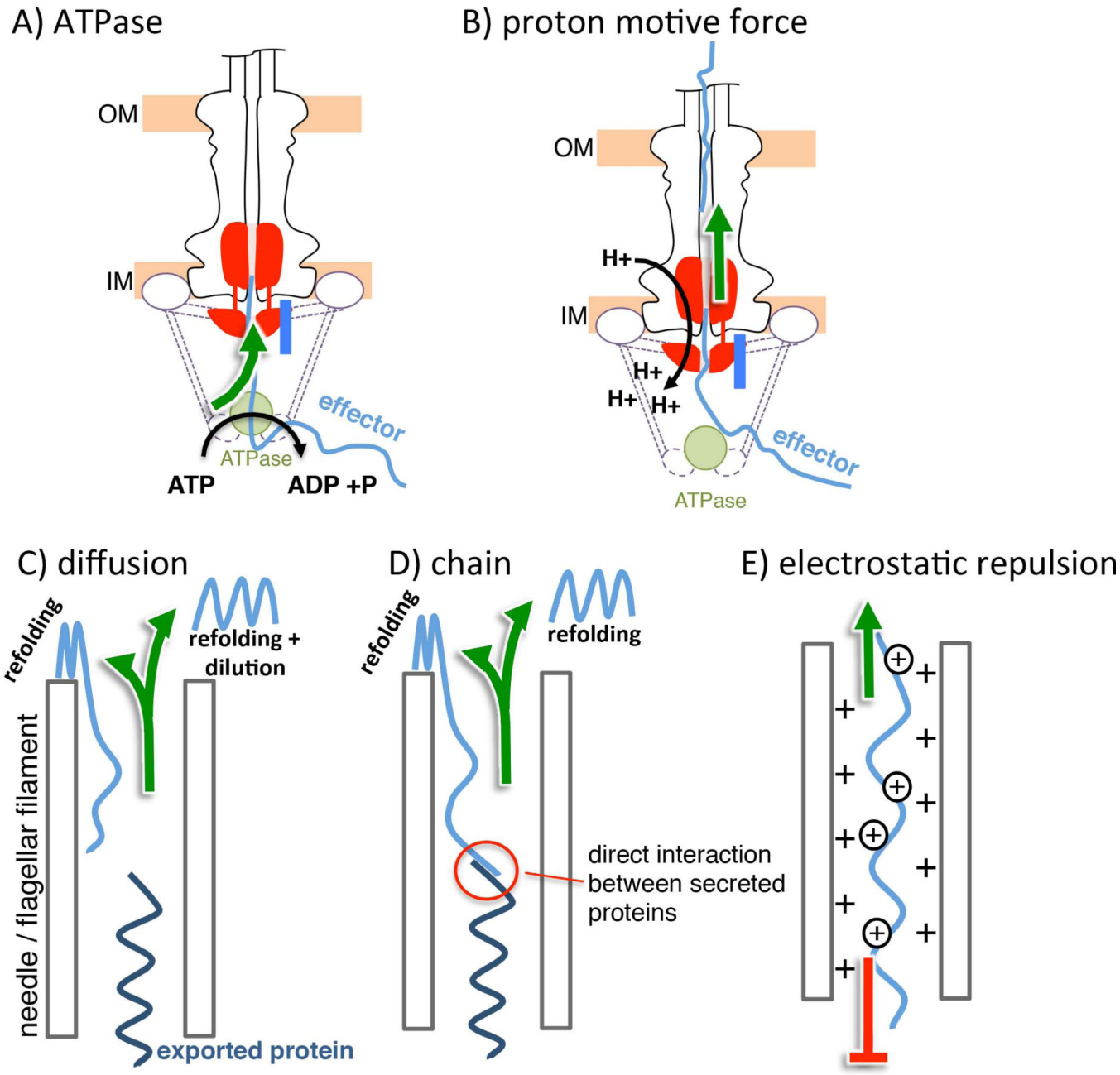

Figure 2. Models of protein export via the T3SS

Models are presented to explain how protein transport through the T3SS is energized. A) Pushing of the secretion substrate into the secretion channel as byproduct of unfolding of secreted proteins by the T3SS-associated ATPase. B) Proton motive force-dependent pulling of the secreted protein by the export apparatus. Transport relies on the flow of protons, most likely through FlhA (YscV), modulated by the interaction with FliJ(YscO). Movement of secreted proteins through the T3SS needle, or flagellar hook/filament structure, could be accomplished in one of three ways: C) Diffusion, whereby secreted proteins diffuse in single file and are removed from the secretion channel through assembly into the structure, or folding and dilution upon exiting the channel. D) Chain. Here secreted proteins interact

directly, the C-terminus of the preceding protein binding to the N-terminus of the following protein. Folding and incorporation of the secreted protein into the apparatus structure, or folding upon exiting the secretion channel, would pull following proteins through the secretion channel. E) Electrostatic repulsion of secreted proteins by charged residues facing the lumen of the secretion channel. Charge repulsion is proposed to both present a barrier to the entry of proteins into the secretion channel, but also to help propel proteins from the secretion channel upon exiting the T3SS. Abbreviations: IM, inner membrane; OM, outer membrane.