The tip of a molecular syringe

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The primary goals of pathogenic bacteria are to escape the host's defense mechanisms, multiply and then spread into the next host. To mediate or enhance their virulence towards the host organism, pathogenic bacteria produce virulence factors. These factors are generally found on the outer surface of the bacterium or they can be secretory proteins. Some plant and animal bacterial pathogens secrete virulence factors directly into the host cell but for different reasons: enteropathogenic *Escherichia coli* (EPEC) inject virulence factors into the host to adhere to epithelial cells after effacement of the brush border microvilli[1], whereas *Shigella* or *Salmonella* spp. do so to induce their uptake by cells that are not normally phagocytic[2,3], and some *Yersinia* spp. inject effectors to prevent bacterial uptake by macrophages[4]. In all of these cases, injection of bacterial virulence factors into the host cytoplasm requires a specialized apparatus called the type III secretion system, which has now been found in several Gram-negative bacterial plant and animal pathogens[5]. These secretion systems are independent of the Sec machinery and are well conserved, as one type III secretion system can export proteins normally secreted by another. Additionally, the secreted proteins do not have a periplasmic intermediate, but appear to be translocated directly from the bacterial cytoplasm to the host membrane or host cytoplasm, and therefore must cross three membranes consecutively. Considering that the translocation of a hydrophilic protein across a lipid bilayer is not a trivial problem[6], this phenomenon represents a great challenge for investigators.

The assembly of the type III secretion system requires an extracellular signal that is believed to be host cell contact. Electron microscopy has shown that a syringe-like structure is assembled that appears to span both the inner and outer bacterial membranes[7]. However, little is known about how the effector proteins of the type III apparatus actually cross the host plasma membrane. Genetic studies have, however, identified proteins that are required for the translocation of effectors into the host, including proteins containing hydrophobic regions that could potentially cross a lipid bilayer, such as EspD in EPEC, IpaB in *Shigella* and YopB in *Yersinia*. Although it has been shown that YopB has contact-dependent hemolytic activity[8], no direct evidence is available to indicate that these proteins actually insert into the host cell membrane.

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Diffusely adhering EPEC

Wachter *et al.*[9] have studied the translocation of the type III effector proteins of diffusely adhering EPEC. EPEC colonize the small-intestinal mucosa and, by triggering signal transduction and host cytoskeletal rearrangement, efface the brush border microvilli, thereby favoring intimate attachment with the host cell. The bacterium is then located on a pedestal-like structure[1]. EPEC rely on a type III secretion system to exploit host cell machineries. Wachter *et al.*[9] have shown that EspD inserts into the host cell membrane, as indicated by its presence in a non-cytoplasmic, Triton X-100 extract. Also favoring membrane insertion, rather than just membrane binding, is the discovery that high salt and carbonate washes cannot remove EspD. These results provided the first direct evidence that EspD or a related protein, such as YopB or IpaB, actually reaches the host plasma membrane.

The next question to be addressed is how does EspD enable the translocation of other effectors such as EspB? EspD and related proteins share, within their two putative transmembrane regions, homology with the predicted transmembrane regions of *E. coli* hemolysin (71% similarity for the first transmembrane domain and 41% for the second). This toxin belongs to the family of RTX toxins that are known to form channels in lipid bilayers[10]. Interestingly, RTX toxins are structurally related to the *Bordetella pertussis* adenylate cyclase, CyaA. CyaA is a modular toxin: it contains an amino-terminal, ATP-cyclizing, calmodulin-activated catalytic domain and a carboxy-terminal hemolytic domain[11]. The carboxy-terminal domain not only mediates the binding but also the translocation of the catalytic moiety into the host cell. CyaA has also been shown to mediate the translocation of exogenous peptides. These properties suggest that RTX toxins might have the capacity to translocate proteins across membranes – a property that could be shared with EspD.

Toxin translocation

Although the mechanism of translocation of the adenylate-cyclase domain of CyaA into the host is poorly understood from a structural point of view, the translocation of other toxins has been well documented, particularly diphtheria toxin and anthrax toxin, which use two different modes of translocation (reviewed in Ref. 12).

Diphtheria toxin is internalized by mammalian cells via receptor-mediated endocytosis, and is then cleaved into two disulfide-linked fragments: the catalytic A moiety

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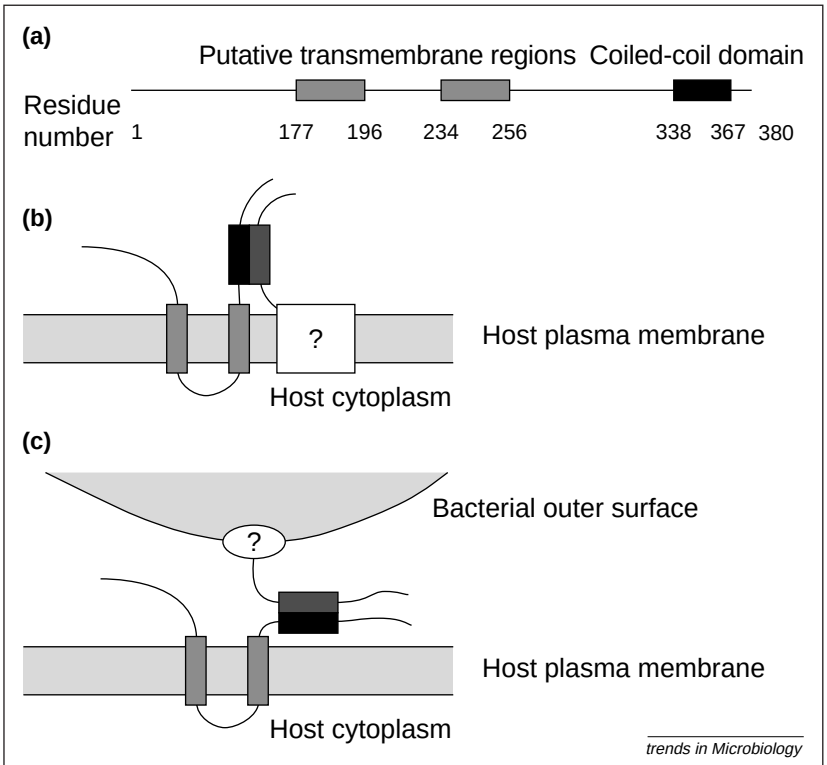

Fig. 1. Domain organization and possible mechanism of action of EspD. **(a)** Putative domains in EspD. There are two putative transmembrane regions (grey) and one putative coiled-coil region (black). Proteolysis experiments[9] indicate that the majority of EspD is extracellular, which, considering the positions of the putative transmembrane regions, suggests that the coiled-coil domain is extracellular. EspD could interact *in cis* with itself or other proteins in the host cell membrane **(b)**, or could interact *in trans* with proteins present on the bacterial outer surface **(c)**. These possibilities are not mutually exclusive, as the coiled-coil domain of EspD is predicted to assemble in trimers.

and the translocation B moiety. As the luminal pH decreases along the endocytic pathway, the B (and later the A) fragment partially unfolds and subsequently interacts with the endosomal membrane. With the help of the B fragment, the partially unfolded A fragment is translocated across the membrane and released into the cytoplasm where it refolds and can reach its cytoplasmic target. The current view on the translocation mechanism is that the B fragment forms a hydrophilic cleft that facilitates the translocation of the A fragment. In this so-called 'cleft model', the A fragment interacts with the B cleft via its hydrophilic regions, and with the lipids via its hydrophobic regions.

Anthrax toxin is composed of three separate proteins: protective antigen (PA), edema factor (EF) and lethal factor (LF), which act in binary combinations to generate two toxic responses. The role of PA is to translocate EF and LF into the cytoplasm, where their targets reside. Similar to diphtheria toxin, anthrax toxin is internalized by receptor-mediated endocytosis and only then are the catalytic subunits delivered to the cytoplasm. In contrast to the diphtheria toxin cleft model of translocation, a channel model is proposed for anthrax toxin. PA is proteolytically processed, thereby acquiring the ability to oligomerize into a ring-like structure that forms a transmembrane pore through which LF and EF are translocated, presumably in an unfolded state.

EspD interactions

At present, it is too early to state whether EspD allows translocation of other polypeptides by a cleft or a channel system. The presence of two hydrophobic regions suggests a greater similarity to diphtheria toxin than to anthrax toxin, but this clearly needs to be studied in more detail. With regard to the membrane insertion of EspD, it is interesting that EspD is stored in the bacterial cytoplasm in association with its chaperone. One can speculate that the chaperone keeps EspD in a partially unfolded conformation that will enable it to migrate through the syringe-like structure of the type III secretion system and then insert into the host plasma membrane. It has been demonstrated for a variety of proteins that membrane insertion or translocation requires partial unfolding[13].

EspD contains a predicted three-stranded coiled-coil domain. Wachter *et al.*[9] have shown that, within the host cell membrane, EspD can be degraded by externally added trypsin, indicating that the major part of the protein is extracellular. Considering the position of the two putative transmembrane regions (Fig. 1a), the coiled-coil domain should be extracellular. Once inserted into the host plasma membrane, EspD could interact *in cis* either with itself or with other coiled-coil-containing proteins in the same membrane, possibly forming an oligomeric pore (Fig. 1b). Alternatively, EspD could interact *in trans* with coiled-coil-containing proteins present on the bacterium (Fig. 1c) and form a complex that would favor close contact between the bacterium and the host in a manner reminiscent of the SNARE complex, which brings a donor vesicle and an acceptor membrane in close contact during membrane fusion in eukaryotic cells[14]. Interestingly, EspA has recently been found on filamentous organelles at the surface of EPEC during the initial interaction of the bacterium with the host cell[15]. As EspA has also been shown to contain a coiled-coil domain[16], one possibility is that EspA and EspD could interact to allow contact between the type III secretion apparatus and the host. Indeed, a strong interaction is probably required, as a weak interaction could lead to the loss of effectors destined for the host cytosol in the extracellular milieu. Also, if a channel was formed by

Esp proteins in the host cell membrane, a strong interaction would prevent host cell death from perforation of the plasma membrane.

Recent work has identified all the components of the type III secretion system. The function and molecular organization of the various components of the system are now being unraveled. Structural studies on the various components of the type III secretion system, as well as studies on the interaction of secreted proteins with biological membranes, will be required to further understand the mechanism of translocation of proteins across three consecutive membranes.

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The tip of a molecular syringe: Response

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We thank Drs Fivaz and van der Goot for their discussion of the fascinating machinery for the translocation and 'injection' of virulence factors into eukaryotic target cells, in the light of our recent work on the localization of the EspD protein[1]. We agree that EspD is an excellent candidate for being part of the translocation machine (translocator) for proteins secreted by the type III system. We have shown that EspD is involved in protein translocation in diffuse adhering enteropathogenic *Escherichia coli* (EPEC) and in the localized adhering EPEC strain 2348/69. Although homologues of EspD are probably also involved in translocation, whether these proteins are part of the translocator remains to be seen.

How the various effector proteins gain access to eukaryotic cells is central to the pathogenesis of microorganisms that utilize a type III secretory system. The recent observations concerning the functions of EspA, B and D (and their homologues) in other organisms emphasize that the type III systems involve not only a type III secretion system but also a 'type III translocation system'.

Drs Fivaz and van der Goot speculate on the function of EspD in protein translocation, based on its homology to RTX toxins, and also discuss a channel model and a cleft model of translocation. We think that a bacterial-toxin-like mechanism for protein translocation is unlikely, for two main reasons. Firstly, these toxins only translocate their effector proteins at a 1:1 stochiometry, whereas the translocation of effector proteins by EspD probably requires a catalytic mechanism. Secondly, the homology with the RTX toxins is restricted to the transmembrane regions and could, therefore, merely reflect the capacity of these regions for membrane insertion[2] and for the formation of, or contribution to, a pore. The existence of pore structures in target membranes is well documented in *Yersinia*[3,4] and we have also found that haemolytic activity in EPEC is mediated by a pore of restricted size (C. Wachter, S. Laarman and M.A. Schmidt, unpublished). In this context, an intriguing question concerns the plasticity of the type III secretion/translocation system; for example, the YscC transport channel[5] of *Yersinia* spp. has an inner diameter of ~5 nm, whereas the haemolytic (translocator) pore is supposed to be considerably smaller.

With reference to the relatedness of type III systems to flagellar systems[6], the idea of a 'molecular syringe' by which the effector proteins are introduced through a continuous tube directly into the target cell[7] is certainly a very attractive concept. However, one should keep in mind that the experimental evidence is still indirect and that other possibilities remain compatible with the available experimental data. In many laboratories – including ours – several of these exciting questions are currently under investigation. By studying different secretion/translocation systems, we hope to generate useful comparisons and look forward to the exploration of what has already presented itself as a fascinating solution to a specific biological problem.

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