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Front cover illustration: Coloured scanning electron micrograph of a macrophage cell (yellow) engulfing *Mycobacterium tuberculosis* (green) by phagocytosis. Courtesy Professor S. H. E. Kaufmann and Dr J. R. Golecki/Science Photo Library.

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The structure of the enteropathogenic *Escherichia coli* type III secretion apparatus

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INTRODUCTION

Most virulence factors of pathogenic bacteria are active outside the bacterial cell and must be actively secreted across the bacterial envelope. In the case of Gram-negative bacteria, the envelope includes two hydrophobic membranes hindering the passage of hydrophilic molecules. However, at least five protein secretion pathways have evolved to overcome this barrier (Thanassi & Hultgren, 2000). One Gram-negative secretion pathway, termed the type III secretion pathway, is associated with pathogenesis and allows the transfer of virulence factors from the bacterial cytoplasm directly to the host cell cytosol in a single, *sec*-independent, step (Hueck, 1998). The type III secretion apparatus or secreton is a complex multi-component organelle, which spans both bacterial membranes, links the bacterium to the host cell and forms a pore within the host plasma membrane (Hueck, 1998). Nine components of the secreton are highly conserved between bacterial species and several others share physical characteristics and roles although showing a lower degree of homology; some components are also shared with the flagellar apparatus (Aizawa, 2001). Examination of purified secretons from *Shigella* (Tamano *et al*., 2000; Blocker *et al*., 2001), *Salmonella* (Kubori *et al*., 1998) and enteropathogenic *Escherichia coli* (EPEC; Sekiya *et al*., 2001; Daniell *et al*., 2001b) has revealed a common structure composed of two pairs of membrane rings linked by a cylindrical structure and an external needle-like structure. In addition, the EPEC secreton has a long filament attached to the distal end of the needle (Wilson *et al*., 2001). Although the components of the secreton are highly conserved, the effector proteins secreted by them are highly variable, highlighted by the wide spectrum of diseases caused by type III secreting pathogens (Hueck, 1998). Effector proteins

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generally serve to subvert host cell functions, resulting in colonization, invasion and dissemination of the pathogen as well as the subversion of host defence mechanisms (Hueck, 1998). See the chapter by Kenny & Warawa, this volume, for additional details.

EPEC is an enteric pathogen of humans that utilizes a type III secretion system (TTSS). It is mainly associated with diarrhoeal diseases in young children (<2 years) and is an important cause of infant mortality in the developing world (Nataro & Kaper, 1998). Additionally, it is closely related to the emerging zoonotic pathogen enterohaemorrhagic *Escherichia coli* (EHEC), which causes acute gastroenteritis and haemorrhagic colitis (Nataro & Kaper, 1998). In adhering to intestinal epithelial cells, these bacteria subvert the cellular architecture to produce histopathological features known as attaching and effacing (A/E) lesions (Frankel *et al*., 1998), which are characterized by localized destruction of brush border microvilli and intimate attachment of the bacteria to the plasma membrane of the host epithelial cells. *E. coli* capable of forming A/E lesions has also been recovered from rabbits, pigs, calves and dogs with diarrhoea (Cantey & Blake, 1977; Drolet *et al*., 1994; Fischer *et al*., 1994; Zhu *et al*., 1994). In mice, the bacterium *Citrobacter rodentium* colonizes large intestinal enterocytes via formation of A/E lesions (Schauer & Falkow, 1993) which are ultrastructurally identical to those formed by EHEC and EPEC in animals and humans.

The capacity to form the A/E lesion is encoded on a pathogenicity island termed the locus of enterocyte effacement (LEE). The LEE is divided into three functional regions (Elliott *et al*., 1998). The 5′ end consists of three operons (*LEE1–3*) encoding a positive regulator, Ler (Mellies *et al*., 1999), and the main structural components of the bacterial TTSS (Esc and Sep proteins). The 3′ end of the LEE (*LEE4*) encodes additional TTSS structural proteins (EscD and EscF; Wilson *et al*., 2001), secreted translocators (EspA, EspB and EspD) and effectors (EspF; McNamara & Donnenberg, 1998) as well as proteins of unknown function. The central part of the LEE consists of an operon (*LEE5* or *eae*) encoding the outer-membrane adhesin intimin, the translocated intimin receptor (Tir) and CesT, the Tir chaperone (Abe *et al*., 1999; Elliott *et al*., 1999).

This chapter will describe the current understanding of the structure of TTSSs with particular reference to the EPEC system.

THE TYPE III SECRETION APPARATUS

The secretons of *Salmonella*, *Shigella* and EPEC have been isolated and examined by electron microscopy (Kubori *et al*., 1998; Tamano *et al*., 2000; Blocker *et al*., 2001; Sekiya *et al*., 2001; Daniell *et al*., 2001b); the observed structures are referred to as the needle complex and consist of stacked membrane rings connected via a central rod-like structure and a thin needle-like structure projecting from the cell. Although the overall

structures are very similar, the dimensions of the complexes vary slightly between species. The inner and outer rings of *Salmonella* are the largest, with diameters of approximately 40 nm and 20 nm, respectively (Kubori *et al*., 1998). In *Shigella*, the inner rings are also wider than the outer rings at 26 nm and 15 nm, respectively (Tamano *et al*., 2000). In contrast, the rings of the EPEC complex are approximately the same size, at 16–20 nm (Daniell *et al*., 2001b; Sekiya *et al*., 2001). The needle itself ranges from 40–45 nm in length in *Shigella* (Tamano *et al*., 2000), *Salmonella* (Kubori *et al*., 1998) and EPEC (Daniell *et al*., 2001b; Sekiya *et al*., 2001) to 60–80 nm for *Yersinia* (Hoiczyk & Blobel, 2001) and is 6–8 nm in width (Kubori *et al*., 1998; Hoiczyk & Blobel, 2001; Tamano *et al*., 2000). Plant pathogens such as *Pseudomonas syringae* also have needle-like structures of similar width (Roine *et al*., 1997) but displaying much longer length, often reaching several micrometres (Brown *et al*., 2001). Analysis of the components of the needle complex has revealed the presence of many of the conserved TTSS proteins including a member of the secretin superfamily, which is among the most highly conserved TTSS proteins. Secretins form multimeric rings in the outer membranes of Gram-negative bacteria, which are involved in various transport pathways, including the *sec*-dependent secretion pathway, the release and assembly of filamentous phage and DNA uptake (Genin & Boucher, 1994).

Associated with the needle complex is the inner-membrane type III machinery closely related to the flagellar apparatus. These components include conserved membrane proteins and an ATPase, which may be required to provide energy for protein secretion or for keeping substrates in a secretion-competent state. It is thought that these components are inserted in the central pore of the inner-membrane rings (Fan *et al*., 1997). The functions of this machinery are unclear but it does appear to play a role in switching from secretion of early to late substrates, as occurs in the flagellar system (Williams *et al*., 1996; Minamino & Macnab, 2000a).

Early substrates of TTSSs are the translocator proteins, so called because they are essential for the translocation of effector proteins into host cells but are dispensable for secretion (Blocker *et al*., 2000). Translocators are known to form translocation pores in lipid bilayers (Tardy *et al*., 1999; Holmstrom *et al*., 2001; Buttner *et al*., 2002) and have been observed to form such pores in the membranes of infected cells (Ide *et al*., 2001). Translocators in different systems show little sequence homology although some conserved features including membrane-spanning regions and coiled-coil domains, implicated in protein–protein interactions, are common (Buttner & Bonas, 2002; Delahay & Frankel, 2002).

The EPEC TTSS has all of the above features but is unique among the described TTSSs due to the presence of a filament attached to the distal end of the needle, which links the

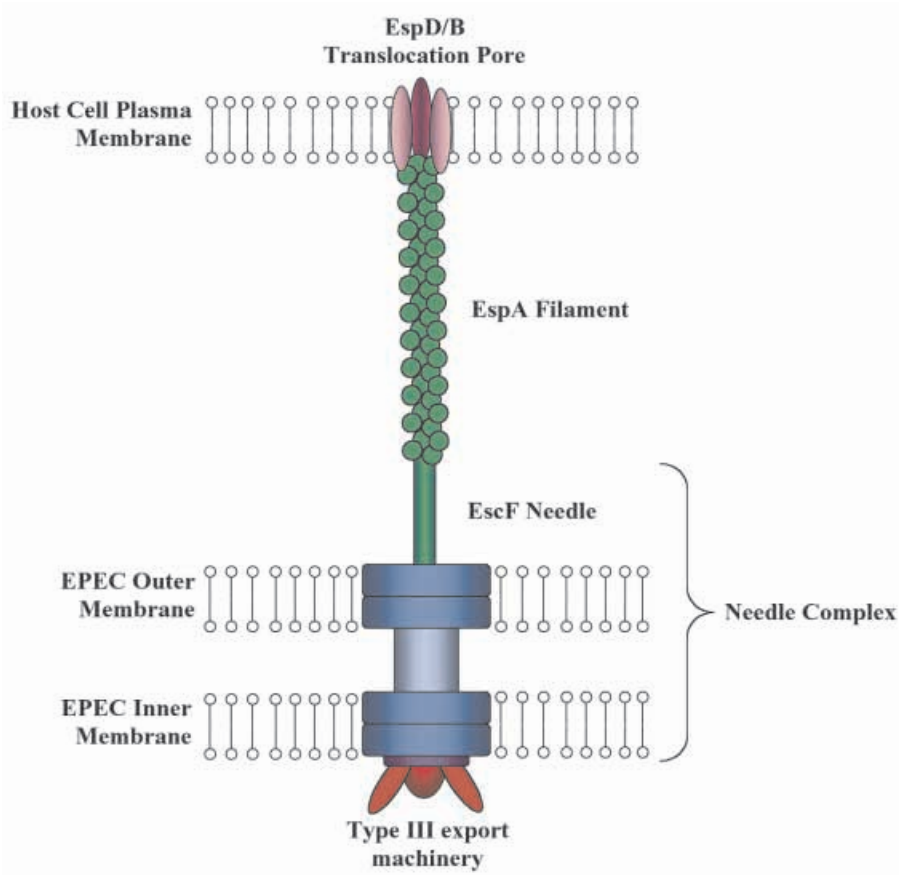

Fig. 1. Schematic representation of the type III secreton of enteropathogenic *E. coli* (EPEC).

bacterium to the host cell (Daniell *et al*., 2001b; Sekiya *et al*., 2001; Wilson *et al*., 2001). A diagrammatic representation of the EPEC TTSS is shown in Fig. 1.

THE EPEC TTSS NEEDLE COMPLEX

There are few experimental data on the structure of the EPEC needle complex but general information is inferred based on homology with other systems. EPEC has homologues of three proteins found in the needle complexes of *Shigella* and *Salmonella*: EscC, EscJ and EscF. These are highly conserved proteins found in all TTSSs and, in addition, EscJ is homologous to FliF of the flagellar system. EscC is a member of the secretin family and is therefore believed to form a pore in the bacterial outer membrane, as observed for other TTSS secretins (Koster *et al*., 1997; Crago & Koronakis, 1998). The archetype of this family is PulD of the pullulanase secretion pathway; electron microscopic studies of PulD complexes revealed a structure with two

membrane-bound rings and a domain extending into the periplasm (Nouwen *et al*., 1999, 2000). Hence it is likely that TTSS secretins represent the two stacked rings observed in the outer membrane. Many secretins, including PulD and type III secretins from *Shigella*, *Salmonella* and *Yersinia*, require a small lipoprotein 'pilot' for stabilization and/or correct localization (Koster *et al*., 1997; Crago & Koronakis, 1998; Daefler & Russel, 1998; Nouwen *et al*., 1999; Schuch & Maurelli, 2001). As yet, however, no such protein has been identified for the EPEC system. The inner-membrane rings are thought to be composed of EscJ as it is a homologue of FliF, which forms the MS rings and a part of the proximal rod in the flagellar system (Jones *et al*., 1990; Ueno *et al*., 1992). Purified FliF complexes show a pair of concentric rings with the inner ring corresponding to the proximal rod and the outer ring corresponding to the MS rings (Suzuki *et al*., 1998). Studies of the *Shigella* TTSS using the yeast two-hybrid system show that MxiJ, the EscJ homologue, interacts with MxiD, the EscC homologue linking the two pairs of stacked rings (Schuch & Maurelli, 2001). However, a yeast two-hybrid study of protein–protein interactions in the EPEC TTSS failed to demonstrate an equivalent interaction (E. A. Creasey and others, unpublished data), suggesting a possible structural difference between the *Shigella* and EPEC systems. The third protein, EscF, shown to be the main needle subunit (Wilson *et al*., 2001), is required for the secretion of both the translocator and effector proteins (Sekiya *et al*., 2001; Wilson *et al*., 2001). The *Yersinia* homologue of EscF, YscF, has been shown to puncture host cells (Hoiczyk & Blobel, 2001). However, it is unlikely that the EPEC needle performs a similar function because there is a filamentous extension to the distal end of the EscF needle (Wilson *et al*., 2001).

THE EPEC TYPE III EXPORT MACHINERY

EPEC has seven predicted inner-membrane components that are shared with the flagellar type III machinery: EscR, S, T, U, V, N and D, homologous to FliP, Q, R, FlhB, A, FliI and G, respectively. Genetic analysis of the *Salmonella* homologues of these proteins showed that all components of the export machinery are required for assembly of the needle structure but are not required for assembly of the membrane-bound rings (Sukhan *et al*., 2001). Although the type III export machinery is essential for assembly of the secreton and secretion of effectors, the precise roles of each protein remain unclear and there are very few experimental data about the EPEC machinery, but again, due to the high conservation of these components, some information can be inferred from other systems. EscN and its homologues are highly conserved and possess ATP-binding motifs (Walker boxes A and B) resembling the F_0F_1 proton translocase and related ATPases, identifying them as probable ATPases. Indeed, purified InvC of *Salmonella typhimurium* has been shown to catalyse ATP hydrolysis (Eichelberg *et al*., 1994), and disruption of the Walker A box in YscN of *Yersinia enterocolitica* impairs effector secretion (Woestyn *et al*., 1994). FliI of the *Salmonella* flagellar system interacts

with membrane-bound components of the export machinery (Minamino & Macnab, 2000b), indicating a direct role in secretion. All the remaining conserved components of the machinery are membrane-associated. Little is known about the roles of EscR, S and T homologues, although their flagellar counterparts have been localized to the membrane fraction of *S. typhimurium* (Ohnishi *et al*., 1997). FlhB, the flagellar homologue of EscU, has been implicated in switching substrate specificity from rod- and hook-type substrates to filament-type substrates (Williams *et al*., 1996; Minamino & Macnab, 2000a) and a similar role could be envisaged for EscU. EscU homologues possess a conserved NPTH sequence which is proteolytically cleaved (Minamino & Macnab, 2000a; Lavander *et al*., 2002). In *Yersinia pseudotuberculosis*, this is essential for bacterial survival and the cleaved domains have differing effects on Yop expression and secretion (Lavander *et al*., 2002), implying that cleavage has some role in determining substrate specificity. EscV homologues have a conserved secondary structure consisting of a hydrophobic N-terminus predicted to form six to eight transmembrane domains and a hydrophilic cytoplasmic C-terminus. The N-terminal domain is highly conserved whereas there is more variation in the C-terminal domain; indeed, a chimeric protein consisting of the N-terminal domain of YscV and the C-terminal domain of InvA could complement an *S. typhimurium invA* strain whereas YscV could not (Ginocchio & Galan, 1995). Therefore, the cytoplasmic region of these proteins may have a species-specific role possibly interacting with other cytoplasmic components of the secretion system. There is some evidence that FlhA, the flagellar homologue of EscV, interacts directly with the MS ring within which it is housed (Kihara *et al*., 2001).

A study on protein–protein interactions in the EPEC TTSS revealed several interactions between EscR and other membrane-spanning proteins, including EscS, EscU and SepZ, indicating that these proteins may form a complex within the membrane (E. A. Creasey and others, unpublished data). The interactions between EscR and EscU/S have implications for all TTSSs as these proteins are highly conserved; in contrast, the interaction between EscR and SepZ is specific to LEE-encoded TTSSs, as SepZ is not widely conserved. SepZ ranks among the most divergent LEE-encoded proteins with less than 70 % homology between different pathogens (Deng *et al*., 2001). However, the EHEC SepZ is required for the secretion of the translocators and the Tir effector protein (DeVinney *et al*., 2001), consistent with a role for this protein in the secreton machinery.

THE EspA FILAMENT

The EPEC TTSS and other LEE-encoded TTSSs are structurally unique in that they possess a long filamentous structure that extends from the distal end of the needle to the surface of the host cell (Knutton *et al*., 1998; Daniell *et al*., 2001b; Sekiya *et al*., 2001). This filament is known to be composed of EspA and is required for the

translocation of effector proteins (Knutton *et al*., 1998). Assembly of the filament is dependent on a predicted coiled-coil region in the C-terminal domain of EspA (Delahay *et al*., 1999) consistent with the role of coiled-coils in mediating protein–protein interactions. Assembly of the filament is also dependent on many other type III proteins, including components of the secretion apparatus and the EspD translocator protein (Kresse *et al*., 1999). EspD may perform a similar function to the flagellar cap protein FliD in promoting self-assembly of the EspA filament (Yonekura *et al*., 2000, 2001).

Although the EspA filament shows some similarity to the bacterial flagellum, visually they are quite different. EspA filaments are much narrower (12 nm compared to 20 nm) and, following negative staining, show diagonal rather than parallel striation (S. J. Daniell and others, unpublished data). Investigation of the 3D-structure of EspA filaments using computational analysis of EM images indicated that the structure comprises a helical tube with a diameter of 120 Å enclosing a central channel of 25 Å diameter. This is consistent with the size of the translocation pore inserted into the host cell membrane and suggests that effector proteins are transported through the EspA filament directly through the translocation pore into the host cell. The subunit arrangement may be considered as a one-start helix with 28 subunits present in five turns of the helix and an axial rise of 4·6 Å per subunit (S. J. Daniell and others, unpublished data).

THE TRANSLOCATORS

As well as EspA, two other secreted proteins, EspD and EspB, are required for the translocation of effector proteins (Donnenberg *et al*., 1993; Foubister *et al*., 1994; Lai *et al*., 1997), classifying them as translocators. EspD and EspB have weak homology to translocators from other systems, including YopB from *Yersinia* and IpaB from *Shigella*, and are similarly targeted to the host cell cytoplasmic membrane upon infection (Wolff *et al*., 1998; Kresse *et al*., 1999; Wachter *et al*., 1999). EPEC forms pores in erythrocyte membranes and causes haemolysis (Warawa *et al*., 1999). This is dependent on EspD and EspB (Warawa *et al*., 1999; Shaw *et al*., 2002), suggesting that they are the pore-forming proteins. However, this has not been directly demonstrated. Consistent with a pore-forming role for these proteins, both possess transmembrane domains, a C-terminal coiled-coil domain in EspD is required for haemolysis (Daniell *et al*., 2001a) and *in vitro* EspD–EspD homo protein interactions (Daniell *et al*., 2001a) and EspD and EspB hetero protein interactions (Ide *et al*., 2001) have been demonstrated. Analysis of the pores formed by diffusely adhering (DA)-EPEC strains by atomic force microscopy showed that the channels were composed of six to eight subunits. Moreover, osmoprotection studies revealed a minimal pore size of 3–5 nm (Ide *et al*., 2001).

The translocator proteins have a close relationship with the EspA filament. Indeed, EspA was originally classified as a translocator (Kenny *et al*., 1996). An interaction between EspA and EspB has been demonstrated, suggesting a direct interaction between the filament and the translocation pore (Hartland *et al*., 2000). A direct interaction between EspA and EspD has not been demonstrated. However, EspD is required for filament biosynthesis and cell attachment (Kresse *et al*., 1999; Daniell *et al*., 2001a). The role of EspD in filament biosynthesis appears to be distinct from its role in pore formation as the C-terminal coiled-coil domain essential for pore formation is not required for filament biosynthesis (Daniell *et al*., 2001a).

As well as being targeted to the membrane of infected cells, EspB is also found in the host cell cytoplasm, where it appears to have an effector function (Taylor *et al*., 1998, 1999). Expression of EspB within host cells causes dramatic changes in cell morphology and a marked reduction in actin stress fibres (Taylor *et al*., 1999). Also the N-terminal region of EHEC EspB interacts with α-catenin, a cytoskeletal-associated protein that is recruited to the site of attachment in both EHEC and EPEC infections (Kodama *et al*., 2002). See also the chapter by Kenny & Warawa, this volume.

INTIMIN

An important stage in A/E lesion formation is intimate attachment of the bacterium to the host cell, mediated by interaction of the outer-membrane adhesin intimin (Jerse *et al*., 1990) with its receptor in the host cell membrane (Rosenshine *et al*., 1996). This receptor is a bacterial protein that is translocated via the TTSS into the host cell membrane, and is therefore called Tir for translocated intimin receptor (Kenny *et al*., 1997). Intimin is not strictly part of the TTSS and is not required for translocation of effector proteins (Gauthier *et al*., 2000); however, it is encoded in the LEE and without it much of the histopathology of EPEC infection would not occur. Intimin is targeted to the outer membrane via the general secretion pathway (Gomez-Duarte & Kaper, 1995) and is known to bind host cells in both a Tir-dependent and Tir-independent mechanism (Hartland *et al*., 1999).

There are at least five distinct classes of intimin, α, β, γ, δ and ε, each associated with different subclasses of A/E-lesion-causing bacteria (reviewed by Frankel *et al*., 2001): EPEC clone 1 expresses intimin-α, whereas EPEC and EHEC clones 2 express intimin-β, intimin-γ is expressed by EHEC O157 : H7 and EPECs O55 : H7 and O55 : H–, intimin-δ is expressed by EPEC O86 : H34 and intimin-ε is associated with both EPEC and EHEC of serogroup O103. The expression of different intimin subclasses is associated with the tissue tropism of the strain, with EPECs able to colonize various regions of the small intestine mucosa and EHECs restricted to the follicle-associated epithelium (FAE) of Peyer's patches (Phillips *et al*., 2000). The N-termini of different intimin subclasses

are highly conserved whereas the C-termini show sequence variation (Adu-Bobie *et al*., 1998); the variable C-terminal 280 residues (Int_{280}) includes the receptor-binding domain of intimin (Frankel *et al*., 1994). It was hypothesized that the Tir-independent mechanism by which Int_{280} interacts with a host cell receptor may determine tissue tropism. Indeed, changing the intimin class of EPEC strain E2348/69 from intimin-α to intimin-γ changed the tissue tropism of the strain from small intestinal muscosa to FAE of Peyer's patches (Phillips & Frankel, 2000). Likewise, changing the intimin class of EHEC O157 : H7 from intimin-γ to intimin-α resulted in colonization spreading from the colon to the small intestine in gnotobiotic piglets (Tzipori *et al*., 1995). Site-directed mutagenesis has begun to reveal individual residues that affect the biological activity of intimin and provide initial clues to the molecular basis of how intimin mediates tissue tropism and host specificity (Reece *et al*., 2001).

CONCLUDING REMARKS

The application of TTSSs to subvert host cell functions is a common theme in the pathogenesis of Gram-negative bacteria. However, while the general mechanism of type III secretion is well understood, the exact structure of the secretion machinery is unclear. The work described here represents ongoing research to elucidate the structure of the EPEC TTSS. Because of the high level of conservation between TTSSs, the implications of this work extend much further than the EPEC TTSS. Despite the large amount of research done in this field, many questions remain to be answered. Foremost is unravelling the arrangement of subunits within the basal body of the secretion system and the roles of these individual components. Other unsolved questions relate to the function of the machinery, e.g. what energy requirements does secretion have? In other words, is energy required for secretion, assembly of the apparatus, maintenance of substrates in a secretion-competent state or for another reason? Is the secretion channel gated and if so how? How is secretion ordered? Detailed analysis of the components of the secretion apparatus will enable us to answer these questions and identify the differences between TTSSs from different pathogens. A thorough knowledge of the structure may also identify targets for drug and vaccine design.

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