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Enteropathogenic *Escherichia coli* (EPEC) – a crafty subversive little bug

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Overview

Enteropathogenic *Escherichia coli* (EPEC) is the most extensively studied member of the attaching and effacing (A/E) family of pathogens which, like its close relative enterohaemorrhagic *E. coli* (EHEC), is human specific whilst other members have adapted to species including rabbits, dogs, pigs, cattle and sheep. In most cases, it is the young and old that are most susceptible to infection, via the oral–faecal route, resulting in a severe watery diarrhoea that can be fatal if untreated. EHEC has recently become the focus of extensive research as low-level contamination of processed foodstuffs can induce diarrhoea that can be bloody in nature with added complications such as haemorrhagic colitis and renal failure linked to the additional expression of Shiga-like toxins (Nataro & Kaper, 1998). The A/E family of pathogens derive their name from their ability to attach intimately to gut epithelial cells and induce the localized loss (effacement) of absorptive microvilli (Moon *et al.*, 1983). Intimate attachment is followed by the reorganization of host cytoskeletal proteins beneath the adherent bacteria into distinct pedestal-like structures (Moon *et al.*, 1983). The onset and severity of A/E-induced diarrhoea is considered too rapid to result solely from the loss of absorptive microvilli, indicating that host secretory mechanism(s) may be activated.

The ability to define the molecular mechanisms by which A/E pathogens induce disease arose from the discovery that infection of tissue-cultured cells could produce lesions similar to those observed *in vivo*, which also permitted pedestal composition to be investigated (Finlay *et al.*, 1992; Knutton *et al.*, 1987, 1989). This discovery also facilitated screening programmes to identify mutants defective in pedestal formation, revealing that a bacterial outer-membrane protein, intimin, was required for intimate adherence, pedestal formation and disease (Donnenberg *et al.*, 1990, 1993; Jerse *et al.*, 1990). Another key finding came from the correlation of EPEC adherence with the tyrosine phosphorylation of a protein of ~90 kDa (Hp90) within the host membrane (Rosenshine *et al.*, 1992). Subsequent analyses revealed

that Hp90 served as a receptor for intimin, and moreover that Hp90–intimin interaction was essential for pedestal formation (Kenny & Finlay, 1997; Rosenshine *et al.*, 1996). A further important finding was that EPEC secreted a number of proteins (EPEC secreted proteins; Esp) whose release was linked to Hp90 phosphorylation and pedestal formation (Jarvis *et al.*, 1995; Kenny & Finlay, 1995; Kenny *et al.*, 1996). The genes encoding these secreted proteins were mapped to the same chromosomal locus as the gene encoding intimin (*eae*). These genes were themselves part of a ~35 kb region, named LEE for Locus of Enterocyte Effacement, that had a significantly lower GC content than the *E. coli* chromosome, implying that the region had been acquired via horizontal transfer (Elliott *et al.*, 1998; McDaniel *et al.*, 1995). LEE carries ~41 open reading frames encoding components of a type III secretion system, secreted proteins, chaperone molecules, regulatory proteins and intimin (Elliott *et al.*, 1998). Cloning of this region into K-12 strains conferred an ability to induce A/E lesions, suggesting that LEE encodes all the factors required for this process, though other chromosomal encoded factors undoubtedly contribute to the appropriate regulation of gene expression (Elliott *et al.*, 1998; Friedberg *et al.*, 1999; McDaniel & Kaper, 1997). The type III apparatus forms a super-molecular structure that spans the double membrane system of Gram-negative bacteria and in EPEC appears to be dedicated to the secretion of specific LEE-encoded proteins, including EspA, EspB and EspD (Kenny & Finlay, 1995; Kubori *et al.*, 1998; Sekiya *et al.*, 2001). EspA is the major constituent of a filamentous structure that forms an extension of the type III apparatus to facilitate the delivery of proteins, such as EspB and EspD, directly into the host cell (Knutton *et al.*, 1998; Sekiya *et al.*, 2001; Wilson *et al.*, 2001). EspD appears to play a role in EspA appendage elongation and has been detected in the plasma membrane, along with EspB, where together with EspA they appear to form a pore enabling EPEC molecules to be delivered into the host cell (Daniell *et al.*, 2001; Ide *et al.*, 2001; Knutton *et al.*, 1998; Shaw *et al.*, 2001; Wachter *et al.*, 1999; Warawa *et al.*, 1999; Wolff *et al.*, 1998). EspB has also been detected

in the cytoplasmic fraction, raising the possibility that it may have an effector function(s) (Taylor *et al.*, 1998, 1999). To date, three EPEC type III secreted effector molecules have been identified, with an additional protein, EspG (Elliott *et al.*, 2001), reported to be translocated into host cells, though its function remains undefined.

The first EPEC effector molecule to be identified was, surprisingly, the previously characterized intimin receptor molecule Hp90, leading to its renaming to Tir for Translocated intimin receptor (Kenny *et al.*, 1997). As stated above, studies with Hp90 had already revealed that this molecule becomes tyrosine-phosphorylated and inserted into the plasma membrane, where interaction with intimin mediates intimate bacteria–host cell contact and pedestal formation (Rosenshine *et al.*, 1992, 1996). Tir–intimin interaction can also trigger additional signalling responses such as the phosphorylation of PLC- γ 1 (Kenny & Finlay, 1997), and both Tir and intimin have been shown to be essential virulence determinants in a natural animal infection model (Marches *et al.*, 2000). The second effector, EspF, disrupts host intestinal membrane barrier function by an unknown mechanism (McNamara *et al.*, 2001), while the third, Map (Mitochondrial associated proteins; formerly *orf19*), is targeted to host mitochondria, where it interferes with their ability to maintain their membrane potential (Kenny & Jepson, 2000). This article will focus on recent data concerning the mechanism of action and function of the Tir and Map effector molecules within host cells, as they are the subjects of investigation within my laboratory.

Topology of Tir within the host plasma membrane

Secretion of Tir, like typical type III secreted proteins, appears to be dependent on an N-terminal signal sequence, as deletion of the first 50 amino acids abolishes secretion, whereas deletion of residues ~78 to ~478 does not (Abe *et al.*, 1999; Kenny, 1999). Tir, again like many other type III secreted proteins, is dependent on a chaperone molecule, CesT, for stability involving the first 100 amino acids of Tir (Abe *et al.*, 1999; Elliott *et al.*, 1999b). However, CesT is not absolutely essential for secretion by EPEC or by the homologous type III secretion apparatus of *Yersinia* (Elliott *et al.*, 1999b; Kenny & Warawa, 2001). Interestingly, CesT was not required for Tir stability within *Yersinia* but was required for efficient Tir secretion, suggesting that CesT may also play a role in directing Tir to the type III secretion apparatus.

Surprisingly, two secreted forms of Tir (sharing identical N-terminal sequences) have been detected migrating as ~72 and ~78 kDa proteins by SDS-PAGE analysis, although *tir* encodes 550 amino acids with a predicted molecular mass of ~55 kDa (Kenny *et al.*, 1997). This discrepancy may be (i) a function of the amino acid composition, (ii) indicative of bacterial modification or (iii) related to Tir adopting some form of SDS-PAGE-

resistant conformation/structure. Tir is predicted to possess two transmembrane regions (234–259 and 363–382), indicating that it may adopt a hairpin-like arrangement in the plasma membrane. This premise has been supported by topology analyses, which indicate that both termini are located inside the host cell, while the predicted central extracellular domain has been shown to mediate binding to intimin (de Grado *et al.*, 1999; Hartland *et al.*, 1999; Kenny, 1999). This reveals that Tir is divided into roughly three similarly sized domains, with the two terminal domains presumably available for interaction with host proteins and the extracellular domain for binding intimin. It was assumed that one of these terminal domains would carry the substrate site for tyrosine phosphorylation and studies have shown that a single tyrosine residue (Y474) within the C-terminal domain of EPEC Tir is phosphorylated (Kenny, 1999). More recently, interaction of intimin with the extracellular Tir domain has been determined at a molecular level, and is suggestive of Tir dimerization, with each molecule interacting with a lectin-like domain of intimin (Batchelor *et al.*, 2000; Luo *et al.*, 2000). It has also been suggested that a complex is formed of Tir dimers and intimin trimers to mediate the formation of symmetrically packed actin fibrils to aid in the generation of the observed pedestal-like structures beneath the adherent bacteria (Luo *et al.*, 2000).

Mechanism of Tir insertion into the plasma membrane

So how does the soluble Tir secreted protein become incorporated into the host plasma membrane? One possibility is that Tir is inserted directly into the plasma membrane during the translocation process or, conversely, it may be delivered into the cytoplasm and then inserted into the membrane. Current data favour the latter possibility as Tir modification intermediates can be detected within the cytoplasmic fraction, while an apparent delay between Tir delivery and interaction with intimin (to trigger pedestal formation) further supports this hypothesis (Kenny, 1999; Kenny *et al.*, 2002). The modification intermediates became evident following Tir expression from a multicopy plasmid, leading to increased expression and delivery to presumably saturate the modification process and subsequent accumulation of the intermediates (Kenny, 1999). Although the unmodified Tir (T^0) form was detected by Western blot analysis in all fractions, a second form (T' ; displaying an increased molecular mass of ~5 kDa) was only detected in the saponin-released cytoplasmic and Triton X-100 soluble membrane fractions. Moreover, a third form (T'' ; ~7 kDa larger than T^0) was observed in the Triton X-100 membrane and insoluble (latter contains adherent bacteria, host nuclei and cytoskeleton), but not cytoplasmic, fractions (Kenny, 1999). Treatment of the isolated membrane fraction with alkaline phosphatase revealed that increases in apparent molecular mass were due to the addition of phosphate groups (Kenny *et al.*, 1997). Indeed, the identification and substitution of the Tir

tyrosine-phosphorylated residue revealed that there was no role for this event in the shifts, implying that Tir is also phosphorylated on other residues within host cells, presumably on serine and/or threonine residues (Kenny, 1999). Identification of the cytoplasmic T⁰ and T' Tir forms, as stated above, is suggestive of a mechanism whereby Tir is first delivered into this compartment prior to insertion into the plasma membrane, though it is possible that increased Tir delivery could simply induce some mis-localization. Insertion from a cytoplasmic location is further supported by the finding that the *Yersinia* delivered Tir molecule is only modified to the T'-like form and is not available for interaction with intimin (Kenny & Warawa, 2001), suggesting that this species is not inserted into the membrane. Moreover, as the T'' form (not tyrosine-phosphorylated) can interact with intimin (Kenny, 1999), this links the T' to T'' modification step with Tir insertion into the plasma membrane. The inability of the *Yersinia* delivered Tir molecule to be modified to a T''-like form indicates that an additional EPEC factor(s) is required to facilitate this modification step. Such a putative factor(s) apparently needs to be co-expressed or co-delivered with Tir as the *Yersinia* delivered Tir molecule failed to be further modified during co-infection experiments with EPEC, although the EPEC delivered Tir molecule underwent normal modification (Kenny & Warawa, 2001). This putative factor(s) is presumably encoded within the EPEC LEE region as cloning LEE into K-12 strains conferred an ability to generate Tir-mediated pedestal formation (McDaniel & Kaper, 1997). The data to date are also consistent with Tir tyrosine phosphorylation being dependent on Tir's prior modification to the T' form, which itself appears to be dependent on prior modification to the T⁰ form (Kenny, 1999, 2001). We have proposed that these non-tyrosine-phosphorylation-mediated shifts in Tir molecular mass may represent conformational shifts, retained during SDS-PAGE analysis, that aid Tir's correct insertion into the plasma membrane and/or the presentation of kinase modification sites (Kenny, 1999).

Serine-phosphorylation-mediated changes to Tir structure

A possible role for such hypothetical phosphorylation-induced Tir conformational changes could be to provide energy to facilitate the energetically unfavourable process of translocating the intimin-binding extracellular domain across the lipid bilayer, probably in a manner dependent on both host and bacterial factors. Support for such a process comes from our recent finding that the cAMP-dependent enzyme protein kinase A (PKA) can modify Tir in a phosphorylation-dependent manner to induce shifts in molecular mass mimicking those observed within host cells (Warawa & Kenny, 2001). Tir has three putative PKA recognition motifs, of which only two participate in the PKA-mediated shifts. Modification at the RRXS434 (X is any residue) motif that is conserved among Tir homologues triggers the T⁰ to T'-like shift, whilst higher PKA concentrations trigger the

additional T' to T''-like shift following modification of the non-conserved putative PKA motif RXS463. As phosphate groups have a molecular mass of ~0.1 kDa, while the overall shift in Tir apparent molecular mass is ~7 kDa, this argues that the addition of single phosphate groups triggers sequential changes in Tir structure that are preserved during SDS-PAGE. It is possible that the addition of charged phosphate groups simply alters Tir-SDS interaction to affect its migration by SDS-PAGE, but this is unlikely as tyrosine phosphorylation of Tir does not contribute to detectable shifts in apparent molecular mass (Kenny, 1999).

Further support for phosphorylation-mediated conformational change in Tir stems from the finding that a T⁰ to T'-like shift could be mediated in a phosphorylation-independent manner by specific substitutions at the PKA-modification site linked with this shift (Warawa & Kenny, 2001). Such substitutions, like phosphorylation, are predicted to alter the charged nature of this region, suggesting that they may serve to destabilize one Tir structure in favour of an alternative one. As unmodified Tir migrates as a ~72/78 kDa protein, and not the predicted ~55 kDa, it may itself have already adopted some stable structure with the sequential addition of phosphate groups triggering the adoption of alternative conformations.

PKA modification of Tir within host cells

The identification of Tir as an *in vitro* substrate for PKA raised the possibility that it was also modified by this kinase within host cells. This was supported by the finding that pre-treatment of host cells with a PKA-selective inhibitor hindered the T⁰ to T' modification process, whilst an activator compound accelerated the process (Warawa & Kenny, 2001). However, at sub-lethal concentrations of the PKA inhibitor, a subpopulation of Tir was modified to the T'' form, suggestive of incomplete inhibition of the modification process and/or the existence of an alternative, less efficient kinase modification pathway(s). Evidence for the latter came from fractionation studies, which revealed the presence of two Tir-modifying activities. One activity fractionated with the membrane/insoluble fraction and was inhibited by the PKA-specific inhibitor PKI, while the other activity remained in the saponin-released cytoplasmic fraction, was refractory to PKI, and less efficiently modified Tir. This non-PKA-mediated modification was not dependent on the RRDS motif, revealing that phosphorylation of other residues can also trigger shifts in apparent molecular mass/conformation. The data also suggest that Tir T⁰ to T' modification occurs at the membrane, probably involving an adaptor protein to target the PKA holoenzyme to this site with the catalytically active kinase released following cAMP binding (Felicciello *et al.*, 2001).

However, PKA is not the only enzyme that can modify Tir at the RRDS motif to produce a T'-like species as *Yersinia*-mediated delivery of Tir into cells deficient in PKA activity did not prevent its modification to the T'

form, but this event was dependent on S434 (Warawa & Kenny, 2001). It is possible that non-PKA modification of Tir is a feature of immortalized cell lines and would not occur in polarized gut epithelial cells where more stringent control of kinase expression and their compartmentalization is expected. This may indeed be the case as preliminary data from EPEC infections of cultured polarized cell lines are suggestive of a significant reduction in Tir T'' levels within the plasma membrane when Tir carried a S434A substitution. On the other hand, it is also possible that an ability to modify Tir by alternative mechanisms may provide an advantage by increasing the likelihood of Tir modification/function within new hosts, helping it to spread to new species.

Phosphorylation on S434 is required for maximal Tir function

Surprisingly, the RRDS motif was found not to be essential for Tir modification to the actin-nucleating T''^{py} form (Warawa & Kenny, 2001). However, Western analysis revealed that the single S434A substitution resulted in an altered Tir modification pattern as evidenced by the presence of an additional membrane-associated species migrating between the T⁰ and T' forms (Warawa & Kenny, 2001). It is presumed that this new phosphorylation-related intermediate is a consequence of Tir phosphorylation on an alternative residue and serves to promote further modification to the fully functional T''^{py} form. Despite this alternative modification process, evidence was provided that modification on S434 is required for maximal Tir function. Thus, although EPEC expressing the Tir S434A-substituted molecule, from the LEE locus, could trigger similar numbers of actin-nucleating events as the parental strain, it was significantly less efficient at mediating pedestal elongation – a late event in Tir-intimin-triggered actin nucleation (Warawa & Kenny, 2001). Given that Tir is an essential virulence determinant (Marches *et al.*, 2000) and that this S434-independent pathway impacts negatively on Tir function, this argues that S434 phosphorylation may contribute significantly to the virulence capacity of EPEC. Furthermore, the identification of this substitution provides an opportunity to investigate the role of pedestal elongation in virulence.

Although an important role for the T⁰ to T' modification process in virulence remains to be formally proven, alternative support comes from the finding that Tir mutants could be isolated that display a T'-like molecular mass in the absence of phosphorylation (Warawa & Kenny, 2001). Because such molecules are delivered by EPEC into host cells and modified to the T''^{py} actin-nucleating form, this argues that strong selection pressure has retained the phosphorylation-mediated mechanism. Indeed, the conservation of the PKA recognition motif RRXS, implicated in the T⁰ to T' shift, among Tir homologues further supports an important role for modification at this site in Tir function.

In contrast to the RRDS motif, the second *in vitro*-identified PKA modification motif (RNS463), linked

with the T' to T''-like shift, is not conserved and does not appear to play a role in EPEC Tir function within host cells (Warawa & Kenny, 2001). At present, we believe that the *in vitro* PKA-mediated conformational shift simply mimics a phosphorylation event that occurs within host cells, suggesting that this second shift can, like the T⁰ to T' shift, be triggered by alternative mechanisms. As stated above, the current data indicate that the *in vivo* modification associated with the T' to T'' shift is linked with Tir's correct insertion into the plasma membrane by a process dependent on bacterial, and probably host, proteins. We are currently screening Tir, which carries 98 serine/threonine residues, for the residue(s) whose modification mediates the T' to T'' shift so that the role of this event in Tir membrane insertion, function and virulence can be assessed. A model representing the putative steps of EPEC Tir modification within host cells relating to its insertion into the plasma membrane is depicted in Fig. 1.

Tyrosine phosphorylation of EPEC Tir is essential for its actin nucleation function, unlike the EHEC (O157:H7 serotype) Tir molecule

The O157:H7 EHEC serotype is most commonly associated with EHEC disease and, like other A/E pathogens, carries an homologous LEE region. Comparison of the EPEC and EHEC LEE sequences revealed a high degree of identity between those genes encoding the type III secretion apparatus and chaperone molecules, while the genes encoding the secreted and effector molecules are more variable (Perna *et al.*, 1998). This variation translates into a higher degree of non-synonymous alterations, which was initially thought to reflect natural clonal divergence during adaptation to or evasion of the immune system. However, the presence of near-identical LEE-encoded proteins in strains that infect different species implies an evolutionary relationship rather than an adaptive one (Zhu *et al.*, 2001). Interestingly, Tir is the most divergent LEE-encoded protein, with less than 60% overall identity between the EHEC O157:H7 and EPEC homologues and only ~40% identity within the C-terminal domain (Kenny, 1999; Paton *et al.*, 1998). Surprisingly, whereas EPEC Tir undergoes tyrosine phosphorylation within host cells, the EHEC O157:H7 Tir molecule does not (DeVinney *et al.*, 1999; Kenny, 1999; Paton *et al.*, 1998). The EPEC Tir tyrosine residue, whose phosphorylation is essential for pedestal formation, is absent in EHEC Tir, where it is substituted for a serine (Kenny, 1999), implying that these molecules trigger pedestal formation by different mechanisms.

This premise has been supported by our recent finding that EPEC Tir is functionally interchangeable for that of EHEC O157:H7, whereas the reverse is not true (Kenny, 2001). Furthermore, the study revealed several differences in the modification pattern of the homologues and linked the observed EHEC Tir dysfunction with an altered modification pattern. The observed difference in

the EHEC Tir modification pattern following delivery into host cells by EPEC, which was linked with actin-nucleating dysfunction, was shown to be due to the presence of the EHEC C-terminal ~170 residues (Kenny, 2001). The linkage of EHEC Tir actin-nucleating activity with a specific phosphorylation-mediated banding pattern supports an important role for non-tyrosine phosphorylation in Tir function. The data also imply that EHEC expresses a factor, absent from EPEC, that facilitates the correct full modification of EHEC Tir within host cells. This conclusion has recently been verified by DeVinney and others, who also provide evidence that the putative accessory factor(s) can be delivered into host cells by EHEC to restore the function of the EPEC delivered EHEC Tir molecule (DeVinney *et al.*, 2001). It is possible that this putative accessory factor is encoded within LEE and has co-diverged with Tir, though the finding that EHEC LEE does not confer the A/E phenotype onto *E. coli* K-12 strains, unlike EPEC LEE (Elliott *et al.*, 1999a; McDaniel & Kaper, 1997), raises the possibility that it lies outside LEE. Screening strategies should rapidly lead to the identifi-

Fig. 1. Proposed mechanism of EPEC Tir insertion into the host plasma membrane. In (1), the bacterial envelope (composed of inner membrane, periplasmic space and outer membrane) spanned by the type III secretion apparatus is depicted. Whereas the LEE-encoded intimin (Int) protein becomes inserted in the outer membrane, the EspA protein is translocated via the type III secretion apparatus across the envelope, where it polymerizes to form an extension enabling the transfer of EspB and EspD directly into the plasma membrane. These three Esp proteins are thought to form a pore at the terminal end of a continuous conduit generated by the type III secretion and Esp translocon to enable the direct delivery of Tir (T⁰) into the host cytoplasm. As Tir possesses two putative hydrophobic transmembrane domains (TM), it is presumed to adopt a conformation that buries these features. Tir is proposed to rapidly associate with the inner face of the plasma membrane, where it is phosphorylated on serine 434 by the cAMP-dependent kinase protein kinase A, PKA (2). This modification is linked to a ~5 kDa increase in apparent molecular mass (T' form) and is proposed to reflect a phosphorylation-induced conformational change in Tir structure (3). This partially modified molecule does not appear to be available for interaction with intimin, indicating that it is not inserted across the plasma membrane (3), and it is possible that the PKA-mediated modification may function to expose another serine/threonine kinase modification site (3). Modification by this putative kinase, probably within the C-terminal domain, appears to require an additional bacterial-encoded factor(s) and results in an additional (~2 kDa; T'') increase in apparent molecular mass (4). As the non-tyrosine-phosphorylated T'' form can interact with intimin (Kenny, 1999), this phosphorylation event is linked with Tir insertion into the plasma membrane. The T'' form now becomes the substrate for an undefined tyrosine kinase (4), leading to the phosphorylation of tyrosine 474 (Kenny, 1999) generating the fully modified T''^{pY} form (5). However, the T''^{pY} form displays no significant actin-nucleating activity (5) unless it interacts with its ligand, intimin (6), which triggers actin nucleation in a manner dependent on the phosphotyrosine-specific recruitment of the Nck adaptor molecule, N-WASP, and the host actin-nucleating machinery (see text). Although the EHEC O157:H7 Tir molecule undergoes numerous phosphorylation events, it is not subjected to tyrosine phosphorylation, implying that it recruits the host's actin-nucleating machinery by an alternative mechanism (see text).

cation of this factor and the examination of its role in EHEC Tir modification and function.

The absence of tyrosine phosphorylation of the EHEC O157:H7 Tir molecule implies that the phosphorylation-related shifts in apparent molecular masses are due to the addition of phosphate groups, again presumably on serine and/or threonine residues. Although EPEC and EHEC Tir only share ~40% identity within their C-terminal domains, both possess the putative PKA recognition motif RRXS, whose modification is linked to the EPEC Tir T⁰ to T' shift. Thus it is likely that this conserved site in EHEC Tir is also a substrate for modification within the host cell, though this remains to be formally tested. However, given the different modification pattern of the homologues within host cells it is also likely that EHEC Tir undergoes additional non-conserved phosphorylation events.

Recent studies have defined the importance of EPEC Tir tyrosine phosphorylation and revealed that it enables the direct binding of the Nck adaptor protein and subsequent recruitment of N-WASP and the host actin-nucleating machinery (Gruenheid *et al.*, 2001; Kalman *et al.*, 1999). As tyrosine phosphorylation is not a feature of EHEC O157:H7 Tir, this reinforces the concept that this pathogen has evolved an alternative mechanism to trigger pedestal formation. It is possible that some of the EHEC Tir non-tyrosine modifications served to facilitate this process by, for example, triggering a conformational change to expose a docking site (perhaps explaining the divergence between the EPEC and EHEC C-terminal domains) for a host or bacterial-encoded adaptor molecule. It is intriguing to speculate that the evolution of this tyrosine-phosphorylation-independent actin-nucleating mechanism has provided EHEC O157:H7 with a selective advantage that has contributed to it being most commonly associated with EHEC disease. Studies are currently under way to identify the EHEC Tir modifications so that their role in EHEC Tir function can be determined and compared with those of EPEC Tir.

Composition of Tir-intimin pedestals

A recent study into the composition of the pedestals generated by EPEC and EHEC (O157:H7 strain) has revealed few differences (Goosney *et al.*, 2001) despite these processes being triggered by alternative mechanisms. Pedestals generated by both strains contain a range of actin-associating proteins, including those also found in focal adhesion sites. Focal adhesions mediate host cell attachment and signalling from the extracellular matrix to the cell via transmembrane integrin molecules. However, pedestals do not appear to possess integrin molecules but contain other proteins, such as ezrin, which is found in structures including microspikes and microvilli and link the cytoplasmic membrane to the cytoskeleton, indicating a unique nature for the pedestals (Goosney *et al.*, 2001). Other proteins such as calpactin and cortactin can be found at the tip and along both EPEC- and EHEC-induced pedestals, although

they are recruited independently of Tir. Proteins, such as WASP, vinculin and zyxin, depend on EPEC Tir tyrosine phosphorylation for recruitment but are also detected in EHEC-generated pedestals. Alpha-actinin and talin have been shown to bind the N-terminus of EPEC Tir, in a phosphotyrosine-independent manner, and may serve to link Tir directly to the cytoskeleton (Freeman *et al.*, 2000; Goosney *et al.*, 2001, 2000b). Indeed, it has been reported that talin is an essential host component in pedestal formation (Cantarelli *et al.*, 2001). Tropomyosin, gelsolin and cofilin are also recruited into pedestals in a phosphotyrosine-independent manner (Goosney *et al.*, 2001). Although Grb2 was identified as an adaptor molecule that was specifically recruited to EPEC pedestals in this study, it has recently been documented as playing no detectable role in pedestal formation, while another adaptor, Nck, has been shown to be essential for EPEC Tir pedestal formation (Goosney *et al.*, 2001; Gruenheid *et al.*, 2001). The only detected difference in EPEC and EHEC pedestal composition was the absence of the Grb2 and CrkII adaptor molecules from EHEC pedestals, though the significance of this is unknown. The role of most of the recruited host proteins in pedestal formation remains to be tested, but one, the membrane receptor CD44, does not appear to play a role in pedestal formation, leading to the speculation that it may participate in alternative signalling processes. The recent finding that EPEC can trigger additional cytoskeletal rearrangements at the site of infection, in which Tir participates (see below), raises the possibility that some of the identified host proteins may participate in either or both of these processes.

Identification of a second LEE-encoded effector molecule

Although Tir is undoubtedly an essential virulence determinant (Marches *et al.*, 2000), it was unlikely to be the only EPEC effector molecule as (i) other pathogens deliver multiple effector molecules and (ii) several EPEC-mediated cellular responses have been detected *in vitro* that are not dependent on Tir (Goosney *et al.*, 2000a; Hueck, 1998; Kaper, 1998). This hypothesis was confirmed by the demonstration that the gene immediately upstream of *tir*, *orf19*, encoded a type III secreted protein that was transferred into host cells in an Esp-(translocon) dependent manner (Kenny & Jepson, 2000). Epifluorescence microscopy analysis identified Orf19 in punctate accumulations throughout the host cytoplasm, while dual staining revealed an association with mitochondria but not endocytic/phagocytic vesicles, the endoplasmic reticulum, the Golgi apparatus or lysosomes. Given this association, *orf19* was renamed *map* (mitochondrial associated protein), and it encodes 203 amino acids, of which the first 44 are predicted to act as N-terminal mitochondrial-targeting and cleavage sequences. Indeed, these features may be functional as a subpopulation of Map undergoes an N-terminal cleavage within host cells (Kenny & Jepson, 2000). If this were the case, such molecules are probably imported

into the mitochondrial matrix or intermembrane space, as Map is not predicted to possess membrane-spanning domains.

Infection studies in the presence of the fluorescent dye TMRE, which is specifically taken up and retained by mitochondria as long as they maintain their membrane potential, indicate that Map disrupts mitochondrial membrane potential (Kenny & Jepson, 2000). This is supported by the observation that Map-associated mitochondria were poor accumulators of an alternative mitochondrial specific reagent, Mitotracker, which, like TMRE, depends on mitochondrial potential for uptake. Indeed, pre-treatment of cells with Mitotracker, which gradually becomes toxic to mitochondria, prior to Map delivery reduces the level of Map associating with mitochondria, indicating that Map also requires a membrane potential to associate with these organelles (Kenny & Jepson, 2000). Pathogen-induced mitochondrial damage is often associated with the triggering of programmed cell death, via the release of pro-apoptotic factors and subsequent activation of proteolytic caspases (Boya *et al.*, 2001). Several reports suggest that EPEC can induce death of tissue-cultured cells by a mechanism displaying features of both apoptosis and necrosis, with a more recent report indicating a link with the expression of the bundle-forming pilus (Abul-Milh *et al.*, 2001; Barnett Foster *et al.*, 2000; Crane *et al.*, 1999). However, one must be cautious with *in vitro* evaluations of the apoptotic nature of pathogens because inappropriate infection conditions and/or the use of immortal cell lines may generate results that do not reflect the *in vivo* situation. Indeed, a recent *in vivo* study using a natural rabbit model infection system did not detect increases in apoptotic rates, but in contrast indicated decreases in normal cellular apoptotic rates (Heczko *et al.*, 2001). This raises the alternative possibility that, like other described pathogenic factors (Boya *et al.*, 2001), Map may have an anti-apoptotic role. Other possible roles for Map-mitochondrial interaction could be to modulate the level of mitochondrial-mediated ATP production to alter the activity of ATP-sensitive enzymes or perhaps to mediate other as yet undetermined mitochondrial controlled functions. Such possibilities are the subject of current studies, which will also address the location of Map within mitochondria, its mechanism of action and role in EPEC pathogenesis.

Multifunctional nature of the EPEC Map and Tir effector molecules

An additional degree of complexity in EPEC-mediated subversion of host cellular processes was recently unveiled by the finding that EPEC can trigger cytoskeletal rearrangements that are distinct from Tir-intimin-triggered pedestal formation (Kenny *et al.*, 2002). This new activity was revealed by the early and transient appearance of unfocused polymerized actin together with filopodia-like extensions at the site of infection (see Fig. 2). Unexpectedly, this activity was shown to be due to the Map effector molecule, with the underlying host process apparently sensitive to Map dosage as indicated

by the fact that increased production of Map by expression from a plasmid (Kenny & Jepson, 2000) stimulated filopodia formation (Kenny *et al.*, 2002). This activity is independent of Map-mitochondria interaction, as formation of filopodia was still mediated by a Map molecule that was unable to target mitochondria, revealing that Map possesses at least two distinct functions. This new Map-driven activity was shown to be dependent on the host GTPase, Cdc42 (orchestrates such processes in uninfected host cells), which is not required for Tir-intimin-triggered pedestal formation (Kalman *et al.*, 1999; Kenny *et al.*, 2002). It is possible that Map, like the *Salmonella* SopE/E2 type III effector proteins, acts as a GEF (guanosine nucleotide exchange factor) to stimulate Cdc42 conversion to its GTP-bound active form (Bishop & Hall, 2000; Friebe *et al.*, 2001; Scheffzek *et al.*, 1998; Stender *et al.*, 2000). However, such an activity has not been demonstrable in *in vitro* assays, though this may be due to the insoluble nature of purified Map. Alternatively, Map may activate Cdc42 by non-conventional mechanisms, as suggested for the *Shigella* IpaC type III effector protein (Tran Van Nhieu *et al.*, 1999), but additional work is required to resolve these issues.

The finding that filopodia formation is a transient event suggested the possible involvement of a GTPase-activating protein (GAP) in down-regulating Cdc42 activity. GAPs preferentially bind the active GTP-bound GTPase, and carry a so-called arginine finger motif (GXL_R; X is any residue), with the invariant arginine residue essential for stimulating the intrinsic GTPase activity of the small GTPase to generate the GDP-bound inactive form (Hall, 1998; Scheffzek *et al.*, 1998). Down-regulation of filopodia was unexpectedly shown to be dependent on both the Tir and intimin molecules. Moreover, Tir carries a putative arginine finger motif, conserved among the majority of Tir homologues, within its most divergent (C-terminal) domain. This region is undoubtedly available for interaction with host proteins, such as Cdc42, as it lies just upstream of the Tir tyrosine kinase substrate site. More compellingly, substitution of the arginine of this motif inhibited EPEC from down-regulating formation of filopodia but not pedestal formation. The dependence of down-regulation on intimin is suggestive of a mechanism whereby the putative Tir GAP-like activity is only unleashed following Tir-intimin interaction, providing EPEC with a simple mechanism to allow sufficient Map-mediated Cdc42-dependent signalling to occur prior to down-regulating as a consequence of Tir-intimin interaction.

So why might Tir and intimin participate in both down-regulating the Map-mediated Cdc42 activity and triggering pedestal formation? The answer may lie in the observation that the Map-mediated Cdc42-dependent signalling is inhibitory to virulence-associated pedestal formation (Kenny *et al.*, 2002). This finding also argues that Map plays an important function within host cells, as otherwise this inhibitory function would have been abolished. Similarly, EPEC's adaptation of Tir and intimin to down-regulate this Map-induced activity

Fig. 2. Map-induced filopodia formation. The EPEC Δmap mutant carrying *map* on a multicopy plasmid was pre-grown in tissue culture media, to accelerate adherence to host cells, and allowed to infect HeLa cells for 30 min prior to fixing and processing for scanning electron microscopy analysis (a) and epifluorescence microscopy (b). Scanning electron microscopy reveals the adherence of EPEC microcolonies to the cell surface and the presence of distinct filopodia-like extensions at the site of infection. Staining of similarly infected cells for the location of bacteria (DNA; also revealing location of host nuclei), polymerized actin and Map for detection by epifluorescence microscopy reveals the presence of actin-rich extensions at the site of adherence, while Map accumulates in punctate structures, previously identified as mitochondria (Kenny & Jepson, 2000), beneath the site of infection. Fig. 2(b) reproduced from Kenny *et al.* (2002) with permission from Blackwell Publishing.

further supports an important role for this process in pathogenicity and suggests that the resulting activity must be tightly regulated. Although Map and Tir appear to be delivered into the host cells in the same time frame, Map-induced formation of filopodia can be followed for ~15 min prior to down-regulation (Kenny & Jepson, 2000; Kenny *et al.*, 2002). This lends itself to a mechanism whereby the duration of Cdc42-mediated Map signalling is dictated by the time taken to modify and insert Tir into the membrane for interaction with intimin, which then triggers both filopodia down-regulation and pedestal formation. An outline of the proposed steps of Map–Tir–intimin-regulated signalling is depicted in Fig. 3.

We currently do not understand the role of the Map-induced Cdc42-mediated activity in EPEC pathogenesis, although, as stated above, the data are consistent with an important role for this process. It has been reported that Cdc42 can modulate the activity of many cellular

processes (such as cell–cell interactions, protein/cytokine expression, endocytosis, phagocytosis and apoptosis) by direct or indirect mechanisms (Bishop & Hall, 2000; Erickson & Cerione, 2001), with some of these processes known to be altered during EPEC infection (Frankel *et al.*, 1998; Kaper, 1998; Vallance & Finlay, 2000). Further studies are now required to identify which host processes are modulated and their role in pathogenesis, though such analysis will undoubtedly be complicated by the multifunctional nature of Map.

Given the recent identification of a multitude of putative virulence genes within the EHEC EDL933 O157:H7 genome (Perna *et al.*, 2001), it is likely that additional non-LEE, and probably LEE-encoded effector molecules will be identified. This will almost certainly lead to the discovery of additional A/E specific and conserved pathogenic mechanisms to aid the elucidation of the complex interactions between host and pathogens. Given the non-invasive lifestyle of A/E pathogens, it is

Fig. 3. Proposed mechanism of EPEC modulation of Tir and Map effector function. In (a), as per Fig. 1, the type III and Esp translocon systems enable the direct delivery of Tir and Map into the host cell. Map, by direct or indirect mechanisms, activates the small GTPase protein Cdc42, converting it to its GTP-bound membrane-associating form that drives actin polymerization and filopodia formation. It is postulated that Cdc42 activation also leads to the modulation of other undefined cellular processes, again by direct and/or indirect mechanisms. Concurrently, Tir is proposed to undergo host- and bacteria-dependent modification, leading to its insertion into the plasma membrane, as described in Fig. 1. In (b), the fully modified membrane-inserted Tir molecule interacts with intimin to unleash two distinct functions. Firstly, this interaction is proposed to release a C-terminally encoded GAP-like activity to down-regulate Cdc42 function by stimulating its conversion to the GDP-bound inactive form and thus down-regulation of filopodia formation. It appears that this mechanism evolved because Map-mediated Cdc42 signalling inhibits Tir-intimin-triggered pedestal formation. This also provides EPEC with a simple mechanism to regulate Map activity and ensure appropriate levels of Cdc42-mediated signalling prior to Tir-intimin-triggered down-regulation. Inactivation of Cdc42 may increase the propensity for Map to target mitochondria, via a putative N-terminally located signal sequence, to induce dysfunction with unknown consequences. Simultaneously, Tir-intimin interaction also triggers signalling events, leading to pedestal formation and other signalling events such as phosphorylation of PLC- γ 1. It is presumed that EPEC continues to deliver Tir, Map and other effector molecules into the cell to subvert a variety of diverse cellular processes that together allow EPEC to generate an outcome favouring its survival, replication and dissemination.

likely that they evolved mechanisms to subvert pathways not targeted by invasive pathogens or manipulate such processes by an alternative mechanism or for different purposes. Thus the study of EPEC should continue to provide unique tools for understanding the pathogenic nature of bacteria and the host cellular processes that they subvert.

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References

- Abe, A., de Grado, M., Pfuetzner, R. A., Sánchez-SanMartín, C., DeVinney, R., Puente, J. L., Strynadka, N. C. J. & Finlay, B. B. (1999). Enteropathogenic *Escherichia coli* translocated intimin receptor, Tir, requires a specific chaperone for stable secretion. *Mol Microbiol* 33, 1162–1175.
- Abul-Milh, M., Wu, Y., Lau, B., Lingwood, C. A. & Foster, D. B. (2001). Induction of epithelial cell death including apoptosis by enteropathogenic *Escherichia coli* expressing bundle-forming pili. *Infect Immun* 69, 7356–7364.
- Barnett Foster, D., Abul-Milh, M., Huesca, M. & Lingwood, C. A. (2000). Enterohemorrhagic *Escherichia coli* induces apoptosis which augments bacterial binding and phosphatidylethanolamine exposure on the plasma membrane outer leaflet. *Infect Immun* 68, 3108–3115.
- Batchelor, M., Prasannan, S., Daniell, S., Reece, S., Connerton, I., Bloomberg, G., Dougan, G., Frankel, G. & Matthews, S. (2000). Structural basis for recognition of the translocated intimin receptor (Tir) by intimin from enteropathogenic *Escherichia coli*. *EMBO J* 19, 2452–2464.
- Bishop, A. L. & Hall, A. (2000). Rho GTPases and their effector proteins. *Biochem J* 348, 241–255.
- Boya, P., Roques, B. & Kroemer, G. (2001). New EMBO members' review: viral and bacterial proteins regulating apoptosis at the mitochondrial level. *EMBO J* 20, 4325–4331.
- Cantarelli, V. V., Takahashi, A., Yanagihara, I., Akeda, Y., Imura, K., Kodama, T., Kono, G., Sato, Y. & Honda, T. (2001). Talin, a host cell protein, interacts directly with the translocated intimin receptor, Tir, of enteropathogenic *Escherichia coli*, and is essential for pedestal formation. *Cell Microbiol* 3, 745–751.
- Crane, J. K., Majumdar, S. & Pickhardt, D. F., 3rd (1999). Host cell death due to enteropathogenic *Escherichia coli* has features of apoptosis. *Infect Immun* 67, 2575–2584.
- Daniell, S. J., Delahay, R. M., Shaw, R. K., Hartland, E. L., Pallen, M. J., Booy, F., Ebel, F., Knutton, S. & Frankel, G. (2001). Coiled-coil domain of enteropathogenic *Escherichia coli* type III secreted protein EspD is involved in EspA filament-mediated cell attachment and hemolysis. *Infect Immun* 69, 4055–4064.
- de Grado, M., Abe, A., Gauthier, A., Steele-Mortimer, O., DeVinney, R. & Finlay, B. B. (1999). Identification of the intimin-binding domain of Tir of enteropathogenic *Escherichia coli*. *Cell Microbiol* 1, 7–17.
- DeVinney, R., Stein, M., Reinscheid, D., Abe, A., Ruschkowski, S. & Finlay, B. B. (1999). Enterohemorrhagic *Escherichia coli* O157:H7 produces Tir, which is translocated to the host cell membrane but is not tyrosine phosphorylated. *Infect Immun* 67, 2389–2398.
- DeVinney, R., Nisan, I., Ruschkowski, S., Rosenshine, I. & Finlay, B. B. (2001). Tir tyrosine phosphorylation and pedestal formation are delayed in enteropathogenic *Escherichia coli* *sepZ::TnpH*A mutant 30-5-1(3). *Infect Immun* 69, 559–563.
- Donnenberg, M. S., Calderwood, S. B., Donohue-Rolfe, A., Keusch, G. T. & Kaper, J. B. (1990). Construction and analysis of TnpH mutants of enteropathogenic *Escherichia coli* unable to invade HEP-2 cells. *Infect Immun* 58, 1565–1571.
- Donnenberg, M. S., Tacket, C. O., James, S. P., Losonsky, G., Nataro, J. P., Wasserman, S. S., Kaper, J. B. & Levine, M. M. (1993). Role of the *eaeA* gene in experimental enteropathogenic *Escherichia coli* infection. *J Clin Invest* 92, 1412–1417.
- Elliott, S. J., Wainwright, L. A., McDaniel, T. K., Jarvis, K. G., Deng, Y. K., Lai, L. C., McNamara, B. P., Donnenberg, M. S. & Kaper, J. B. (1998). The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/69. *Mol Microbiol* 28, 1–4.
- Elliott, S. J., Yu, J. & Kaper, J. B. (1999a). The cloned locus of enterocyte effacement from enterohemorrhagic *Escherichia coli* O157:H7 is unable to confer the attaching and effacing phenotype upon *E. coli* K-12. *Infect Immun* 67, 4260–4263.
- Elliott, S. J., Hutcheson, S. W., Dubois, M. S., Mellies, J. L., Wainwright, L. A., Batchelor, M., Frankel, G., Knutton, S. & Kaper, J. B. (1999b). Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. *Mol Microbiol* 33, 1176–1189.
- Elliott, S. J., Krejany, E. O., Mellies, J. L., Robins-Browne, R. M., Sasakawa, C. & Kaper, J. B. (2001). EspG, a novel type III system-secreted protein from enteropathogenic *Escherichia coli* with similarities to VirA of *Shigella flexneri*. *Infect Immun* 69, 4027–4033.
- Erickson, J. W. & Cerione, R. A. (2001). Multiple roles for Cdc42 in cell regulation. *Curr Opin Cell Biol* 13, 153–157.
- Feliciello, A., Gottesman, M. E. & Avvedimento, E. V. (2001). The biological functions of A-kinase anchor proteins. *J Mol Biol* 308, 99–114.
- Finlay, B. B., Rosenshine, I., Donnenberg, M. S. & Kaper, J. B. (1992). Cytoskeletal composition of attaching and effacing lesions associated with enteropathogenic *Escherichia coli* adherence to HeLa cells. *Infect Immun* 60, 2541–2543.
- Frankel, G., Phillips, A. D., Rosenshine, I., Dougan, G., Kaper, J. B. & Knutton, S. (1998). Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Mol Microbiol* 30, 911–921.
- Freeman, N. L., Zurawski, D. V., Chowrashi, P., Ayoob, J. C., Huang, L., Mittal, B., Sanger, J. M. & Sanger, J. W. (2000). Interaction of the enteropathogenic *Escherichia coli* protein, translocated intimin receptor (Tir), with focal adhesion proteins. *Cell Motil Cytoskeleton* 47, 307–318.
- Friebe, A., Ilchmann, H., Aepfelbacher, M., Ehrbar, K., Machleidt, W. & Hardt, W. D. (2001). SopE and SopE2 from *Salmonella typhimurium* activate different sets of RhoGTPases of the host cell. *J Biol Chem* 276, 34035–34040.
- Friedberg, D., Umanski, T., Fang, Y. & Rosenshine, I. (1999). Hierarchy in the expression of the locus of enterocyte effacement genes of enteropathogenic *Escherichia coli*. *Mol Microbiol* 34, 941–952.
- Goosney, D. L., Gruenheid, S. & Finlay, B. B. (2000a). Gut feelings: enteropathogenic *E. coli* (EPEC) interactions with the host. *Annu Rev Cell Dev Biol* 16, 173–189.
- Goosney, D. L., DeVinney, R., Pfuetzner, R. A., Frey, E. A., Strynadka, N. C. & Finlay, B. B. (2000b). Enteropathogenic *E. coli* translocated intimin receptor, Tir, interacts directly with alpha-actinin. *Curr Biol* 10, 735–738.
- Goosney, D. L., DeVinney, R. & Finlay, B. B. (2001). Recruitment of cytoskeletal and signaling proteins to enteropathogenic and enterohemorrhagic *Escherichia coli* pedestals. *Infect Immun* 69, 3315–3322.
- Gruenheid, S., DeVinney, R., Bladt, F., Goosney, D., Gelkop, S., Gish, G. D., Pawson, T. & Finlay, B. B. (2001). Enteropathogenic *E. coli* Tir binds Nck to initiate actin pedestal formation in host cells. *Nat Cell Biol* 3, 856–859.

- Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* 279, 509–514.
- Hartland, E. L., Batchelor, M., Delahay, R. M., Hale, C., Matthews, S., Dougan, G., Knutton, S., Connerton, I. & Frankel, G. (1999). Binding of intimin from enteropathogenic *Escherichia coli* to Tir and to host cells. *Mol Microbiol* 32, 151–158.
- Heczko, U., Carthy, C. M., O'Brien, B. A. & Finlay, B. B. (2001). Decreased apoptosis in the ileum and ileal Peyer's patches: a feature after infection with rabbit enteropathogenic *Escherichia coli* O103. *Infect Immun* 69, 4580–4589.
- Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* 62, 379–433.
- Ide, T., Laarmann, S., Greune, L., Schillers, H., Oberleithner, H. & Schmidt, M. A. (2001). Characterization of translocation pores inserted into plasma membranes by type III-secreted Esp proteins of enteropathogenic *Escherichia coli*. *Cell Microbiol* 3, 669–679.
- Jarvis, K. G., Giron, J. A., Jerse, A. E., McDaniel, T. K., Donnenberg, M. S. & Kaper, J. B. (1995). Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proc Natl Acad Sci USA* 92, 7996–8000.
- Jerse, A. E., Yu, J., Tall, B. D. & Kaper, J. B. (1990). A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proc Natl Acad Sci USA* 87, 7839–7843.
- Kalman, D., Weiner, O. D., Goosney, D. L., Sedat, J. W., Finlay, B. B., Abo, A. & Bishop, J. M. (1999). Enteropathogenic *E. coli* acts through WASP and Arp2/3 complex to form actin pedestals. *Nat Cell Biol* 1, 389–391.
- Kaper, J. B. (1998). EPEC delivers the goods. *Trends Microbiol* 6, 169–172.
- Kenny, B. (1999). Phosphorylation of tyrosine 474 of the enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule is essential for actin nucleating activity and is preceded by additional host modifications. *Mol Microbiol* 31, 1229–1241.
- Kenny, B. (2001). The enterohaemorrhagic *Escherichia coli* (serotype O157:H7) Tir molecule is not functionally interchangeable for its enteropathogenic *E. coli* (serotype O127:H6) homologue. *Cell Microbiol* 3, 499–510.
- Kenny, B. & Finlay, B. B. (1995). Protein secretion by enteropathogenic *Escherichia coli* is essential for transducing signals to epithelial cells. *Proc Natl Acad Sci USA* 92, 7991–7995.
- Kenny, B. & Finlay, B. B. (1997). Intimin-dependent binding of enteropathogenic *Escherichia coli* to host cells triggers novel signaling events, including tyrosine phosphorylation of phospholipase C gamma. *Infect Immun* 65, 2528–2536.
- Kenny, B. & Jepson, M. (2000). Targeting of an enteropathogenic *E. coli* (EPEC) effector protein to host mitochondria. *Cell Microbiol* 2, 579–590.
- Kenny, B. & Warawa, J. (2001). Enteropathogenic *Escherichia coli* (EPEC) Tir receptor molecule does not undergo full modification when introduced into host cells by EPEC-independent mechanisms. *Infect Immun* 69, 1444–1453.
- Kenny, B., Lai, L. C., Finlay, B. B. & Donnenberg, M. S. (1996). EspA, a protein secreted by enteropathogenic *Escherichia coli*, is required to induce signals in epithelial cells. *Mol Microbiol* 20, 313–323.
- Kenny, B., DeVinney, R., Stein, M., Reinscheid, D. J., Frey, E. A. & Finlay, B. B. (1997). Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91, 511–520.
- Kenny, B., Ellis, S., Leard, A., Warawa, J., Mellor, H. & Jepson, M. A. (2002). Co-ordinate regulation of distinct host cell signalling pathways by multifunctional enteropathogenic *E. coli* (EPEC) effector molecules. *Mol Microbiol* 44, 1095–1107.
- Knutton, S., Lloyd, D. R. & McNeish, A. S. (1987). Adhesion of enteropathogenic *Escherichia coli* to human intestinal enterocytes and cultured human intestinal mucosa. *Infect Immun* 55, 69–77.
- Knutton, S., Baldwin, T., Williams, P. H. & McNeish, A. S. (1989). Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohemorrhagic *Escherichia coli*. *Infect Immun* 57, 1290–1298.
- Knutton, S., Rosenshine, I., Pallen, M. J., Nisan, I., Neves, B. C., Bain, C., Wolff, C., Dougan, G. & Frankel, G. (1998). A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J* 17, 2166–2176.
- Kubori, T., Matsushima, Y., Nakamura, D., Uralil, J., Lara-Tejero, M., Sukhan, A., Galan, J. E. & Aizawa, S. I. (1998). Supramolecular structure of the *Salmonella typhimurium* type III protein secretion system. *Science* 280, 602–605.
- Luo, Y., Frey, E. A., Pfuetzner, R. A., Creagh, A. L., Knoechel, D. G., Haynes, C. A., Finlay, B. B. & Strynadka, N. C. (2000). Crystal structure of enteropathogenic *Escherichia coli* intimin-receptor complex. *Nature* 405, 1073–1077.
- Marches, O., Nougayrede, J. P., Boullier, S. & 8 other authors (2000). Role of tir and intimin in the virulence of rabbit enteropathogenic *Escherichia coli* serotype O103:H2. *Infect Immun* 68, 2171–2182.
- McDaniel, T. K. & Kaper, J. B. (1997). A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. *Mol Microbiol* 23, 399–407.
- McDaniel, T. K., Jarvis, K. G., Donnenberg, M. S. & Kaper, J. B. (1995). A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proc Natl Acad Sci USA* 92, 1664–1668.
- McNamara, B. P., Koutsouris, A., O'Connell, C. B., Nougayrede, J. P., Donnenberg, M. S. & Hecht, G. (2001). Translocated EspF protein from enteropathogenic *Escherichia coli* disrupts host intestinal barrier function. *J Clin Invest* 107, 621–629.
- Moon, H. W., Whipp, S. C., Argenzio, R. A., Levine, M. M. & Giannella, R. A. (1983). Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. *Infect Immun* 41, 1340–1351.
- Nataro, J. P. & Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 11, 142–201.
- Paton, A. W., Manning, P. A., Woodrow, M. C. & Paton, J. C. (1998). Translocated intimin receptors (Tir) of Shiga-toxicogenic *Escherichia coli* isolates belonging to serogroups O26, O111, and O157 react with sera from patients with hemolytic-uremic syndrome and exhibit marked sequence heterogeneity. *Infect Immun* 66, 5580–5586.
- Perna, N. T., Mayhew, G. F., Posfai, G., Elliott, S., Donnenberg, M. S., Kaper, J. B. & Blattner, F. R. (1998). Molecular evolution of a pathogenicity island from enterohemorrhagic *Escherichia coli* O157:H7. *Infect Immun* 66, 3810–3817.
- Perna, N. T., Plunkett, G., 3rd, Burland, V. & 25 other authors (2001). Genome sequence of enterohaemorrhagic *Escherichia coli* O157:H7. *Nature* 409, 529–533.
- Rosenshine, I., Donnenberg, M. S., Kaper, J. B. & Finlay, B. B. (1992). Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phos-

phorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. *EMBO J* **11**, 3551–3560.

Rosenshine, I., Ruschkowski, S., Stein, M., Reinscheid, D. J., Mills, S. D. & Finlay, B. B. (1996). A pathogenic bacterium triggers epithelial signals to form a functional bacterial receptor that mediates actin pseudopod formation. *EMBO J* **15**, 2613–2624.

Scheffzek, K., Ahmadian, M. R. & Wittinghofer, A. (1998). GTPase-activating proteins: helping hands to complement an active site. *Trends Biochem Sci* **23**, 257–262.

Sekiya, K., Ohishi, M., Ogino, T., Tamano, K., Sasakawa, C. & Abe, A. (2001). Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. *Proc Natl Acad Sci U S A* **98**, 11638–11643.

Shaw, R. K., Daniell, S., Ebel, F., Frankel, G. & Knutton, S. (2001). EspA filament-mediated protein translocation into red blood cells. *Cell Microbiol* **3**, 213–222.

Stender, S., Friebe, A., Linder, S., Rohde, M., Miold, S. & Hardt, W. D. (2000). Identification of SopE2 from *Salmonella typhimurium*, a conserved guanine nucleotide exchange factor for Cdc42 of the host cell. *Mol Microbiol* **36**, 1206–1221.

Taylor, K. A., O'Connell, C. B., Luther, P. W. & Donnenberg, M. S. (1998). The EspB protein of enteropathogenic *Escherichia coli* is targeted to the cytoplasm of infected HeLa cells. *Infect Immun* **66**, 5501–5507.

Taylor, K. A., Luther, P. W. & Donnenberg, M. S. (1999). Expression of the EspB protein of enteropathogenic *Escherichia coli* within HeLa cells affects stress fibers and cellular morphology. *Infect Immun* **67**, 120–125.

Tran Van Nhieu, G., Caron, E., Hall, A. & Sansonetti, P. J. (1999). IpaC induces actin polymerization and filopodia formation during *Shigella* entry into epithelial cells. *EMBO J* **18**, 3249–3262.

Vallance, B. A. & Finlay, B. B. (2000). Exploitation of host cells by enteropathogenic *Escherichia coli*. *Proc Natl Acad Sci U S A* **97**, 8799–8806.

Wachter, C., Beinke, C., Mattes, M. & Schmidt, M. A. (1999). Insertion of EspD into epithelial target cell membranes by infecting enteropathogenic *Escherichia coli*. *Mol Microbiol* **31**, 1695–1707.

Warawa, J. & Kenny, B. (2001). Phosphoserine modification of the enteropathogenic *E. coli* Tir molecule is required to trigger conformational changes and efficient pedestal elongation. *Mol Microbiol* **42**, 1269–1280.

Warawa, J., Finlay, B. B. & Kenny, B. (1999). Type III secretion-dependent hemolytic activity of enteropathogenic *Escherichia coli*. *Infect Immun* **67**, 5538–5540.

Wilson, R. K., Shaw, R. K., Daniell, S., Knutton, S. & Frankel, G. (2001). Role of EscF, a putative needle complex protein, in the type III protein translocation system of enteropathogenic *Escherichia coli*. *Cell Microbiol* **3**, 753–762.

Wolff, C., Nisan, I., Hanski, E., Frankel, G. & Rosenshine, I. (1998). Protein translocation into host epithelial cells by infecting enteropathogenic *Escherichia coli*. *Mol Microbiol* **28**, 143–155.

Zhu, C., Agin, T. S., Elliott, S. J., Johnson, L. A., Thate, T. E., Kaper, J. B. & Boedeker, E. C. (2001). Complete nucleotide sequence and analysis of the locus of enterocyte effacement from rabbit diarrheagenic *Escherichia coli* RDEC-1. *Infect Immun* **69**, 2107–2115.