Targeting of an enteropathogenic *Escherichia coli* (EPEC) effector protein to host mitochondria

Brendan Kenny1* and Mark Jepson2
1Department of Pathology and Microbiology, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK. 2Cell Imaging Facility and Department of Biochemistry, University of Bristol, Bristol BS8 1TD, UK.

Summary

Many Gram-negative pathogens use a type III secretion apparatus to deliver effector molecules into host cells to subvert cellular processes in favour of the pathogen. Enteropathogenic *Escherichia coli* (EPEC) uses such a system to deliver the Tir effector molecule into host cells. In this paper, we show that the gene upstream of *tir*, orf19, encodes an additional type III secreted effector protein. Orf19 is delivered into host cells by a mechanism independent of endocytosis, but dependent on EspB. Orf19 is targeted to host mitochondria, where it appears to interfere with the ability to maintain membrane potential. Although the precise role of Orf19 remains to be elucidated, its interaction with mitochondria suggests a possible role in the subversion of key functions of these organelles, such as energy production or control of cell death. This is the first example of a type III secreted protein targeted to mitochondria; it is probable that homologues (present in EPEC and *Shigella* species) and other bacterial effectors will also target this organelle.

Introduction

Many Gram-negative pathogens encode a type III secretion apparatus enabling the release of virulence-associated proteins across the bacterial cell envelope. Some of these secreted proteins function to enable the delivery of other proteins into the host cell where they can interact with host components to subvert cellular processes (for review, see Hueck, 1998). It has become clear that different pathogens can use alternative strategies to subvert the same cellular processes. For example, *Shigella* and *Salmonella* species deliver IpaB and SipB, respectively, which activate interleukin-1β-converting enzyme triggering apoptosis, whereas other pathogens use a more indirect mechanism leading to programmed cell death, such as the hypersensitive response in higher plants (reviewed by Weinrauch and Zychlinsky, 1999) that is associated with altered mitochondrial function (Xie and Chen, 2000).

Enteropathogenic *E. coli* (EPEC) are responsible for causing severe diarrhoea in humans, leading to the death of up to a million infants per year in developing countries. The major phenotype of EPEC is its ability to bind intestinal epithelial cells, efface absorptive microvilli and form cytoskeletal-rich pedestal-like structures beneath the adherent bacteria (for reviews, see Donnenberg *et al*., 1997; Frankel *et al*., 1998). This requires the delivery of the Tir effector molecule into the host plasma membrane in which, upon interaction with intimin (a bacterial outer membrane protein), it triggers cytoskeletal rearrangements (Rosenshine *et al*., 1992; 1996; Kenny *et al*., 1997a) similar to those observed *in vivo*. Only minor levels of Tir are secreted in contrast to the EspA, EspB and EspD proteins, whose primary function is to deliver Tir into the host cell (Kenny *et al*., 1997a; Knutton *et al*., 1998; Wolff *et al*., 1998; Warawa *et al*., 1999). The ability of EPEC to induce other responses in host cells that are independent of Tir or intimin (reviewed by Donnenberg *et al*., 1997; Frankel *et al*., 1998) suggests the existence of other effector molecules.

EPEC type III secreted/effector proteins are encoded on the ~35 kb chromosomally located locus for enterocyte effacement (LEE) region. This region also encodes the type III secretion apparatus, intimin and several chaperone molecules (CesD for EspB/EspD and CesT for Tir) (Jarvis *et al*., 1995; Elliott *et al*., 1998; 1999; Wainwright and Kaper, 1998; Abe *et al*., 1999). In addition to the LEE, EPEC carries a large plasmid encoding at least two virulence-associated loci. One of these loci is involved in the generation and assembly of the type IV bundle-forming pili (BFP) (Sohel *et al*., 1996; Stone *et al*., 1996). BFP are required for efficient bacterial adherence to cultured cells, but their role *in vivo* is in question (Stone *et al*., 1996; Hicks *et al*., 1998). The second plasmid-encoded locus is involved in the positive regulation of LEE proteins (Gomez-Duarte and Kaper, 1995; Tobe *et al*., 1996; Kenny *et al*., 1997b; Mellies *et al*., 1999). The LEE-encoded Orf19 gene product has several homologues (EPEC TrcA/TrcP and *Shigella* IpgB proteins; Tobe *et al*.,...
In this paper, we demonstrate that Orf19 is a type III secreted protein that is delivered into host cells in an espB-dependent manner. Epifluorescence microscopy co-localization studies reveal that Orf19 targets mitochondria, whereas live imaging studies identified a putative mitochondrial membrane potential disrupting activity. Although Orf19 was shown to have different characteristics from its EPEC homologue TrcA, our data support the possibility that other homologues, such as the Shigella IpgB protein, or other pathogen proteins may also be targeted to mitochondria.

Results

Orf19 is a type III secreted protein

Given the location of orf19 immediately upstream of tir, we investigated whether orf19 also encodes a secreted effector molecule. Polymerase chain reaction (PCR) technology was used to generate plasmids encoding an Orf19HSV gene fusion (pSK–orf19HSV), enabling its detection with anti-HSV antibodies, and an Orf19HSVHis fusion to purify the protein for ‘far’ Western protocols and antibody generation (see Experimental procedures).

The EPEC orf19 LEE gene was deleted (see Experimental procedures), and the resulting mutant (orf19) was transformed with pSK–orf19HSV or vector (pSK) alone. These strains, together with EPEC and escN (a type III secretion mutant), were grown in Dulbecco’s modified Eagle medium (DMEM), and cell and supernatant samples were isolated for Western analysis (see Experimental procedures). Type III-dependent secretion of Orf19 was demonstrated by the presence of a band of the predicted molecular mass of Orf19 (<20 kDa) in the supernatants of EPEC, but not the orf19 or escN mutant strains (Fig. 1, lanes 5–7, top) with the anti-Orf19 antibodies. These antibodies detected low levels of this band in the EPEC cellular extract, whereas reduced levels were apparent with the escN mutant (Fig. 1, lanes 1 and 2, top). A minor band was detected in the orf19 cellular extract, probably as a result of cross-reactivity with an underlying band, possibly the Orf19 homologue TrcA (Fig. 1, lane 3, top). An additional non-specifically recognized cellular band (indicated by an arrowhead in Fig. 1) was absent from the supernatant samples, indicating specific release of Orf19 (Fig. 1, top).

Introduction of pSK–orf19HSV into orf19 resulted in the detection of a band of higher molecular mass (owing to the presence of the HSV tag) in both the cell and the supernatant samples (Fig. 1, lanes 4 and 8, top and middle). Plasmid expression of Orf19HSV led to increased cellular levels of Orf19, but without an equal increase in secreted levels. The deletion of orf19 had no detectable effect on EspB secreted levels, whereas expression of Orf19HSV inhibited EspB secretion (Fig. 1, lanes 5–8, bottom). Orf19HSV was not secreted by the escN mutant, confirming its dependence on the type III-dependent translocon for secretion (data not shown).

Orf19 specifically interacts with host proteins

The finding that Orf19 was secreted was surprising in view of the report showing that its homologue, TrcA, was a chaperone-like molecule (Tobe et al., 1999b). Several TrcA–EPEC protein interactions were revealed by ‘far’ Western assays (Tobe et al., 1999b), similar to those used to define Tir–intimin interactions (Kenny et al., 1997a; Kenny, 1999). To reassess this, TrcA, Orf19 and Tir were purified for comparative ‘far’ Western analyses (see Experimental procedures).

Figure 2 shows the purified proteins (top left) and the profiles of the EPEC and host protein extracts (top right) to be probed in the ‘far’ Western analyses. The bottom set shows that, in contrast to TrcA, which bound to a large number of EPEC and host protein bands, Orf19 interacted...
with only host proteins (Fig. 2, bottom). Some of these host, but not EPEC, bands emerged in a control blot (incubated in the absence of purified proteins; data not shown), indicating that Orf19 interacts fairly specifically with two host protein bands (Fig. 2, indicated by arrowheads). The ‘far’ Western protocol was validated by demonstrating specific interaction of Tir with intimin, present in EPEC, but not eae (intimin minus) extracts (Fig. 2, bottom right). Reduced binding on blots co-incubated with Tir and TrcA is suggestive of Tir±TrcA interaction (Fig. 2, bottom right). These data indicate different binding specificities for these homologues.

Detection of Orf19 in host cellular fractions

Given the secreted nature of Orf19 and its specific interaction with host proteins, we tested whether, as with Tir, it could be delivered into host cells. Thus, HeLa cells were infected with the EPEC orf19 strain carrying pSK or pSK–orf19HSV, and the host cells were then fractionated into saponin-released ‘cytoplasmic’, Triton X-100-soluble ‘membrane’ and ‘insoluble’ (contains adherent EPEC, host nuclei and cytoskeleton) fractions (see Experimental procedures). Samples were resolved for Western analysis and probed with anti-HSV antibodies. HSV-related bands were detected in cellular fractions obtained from orf19/pSK–orf19HSV (Fig. 3, bottom), but not from the orf19/pSK infected cells (data not shown).

The bacterial form of Orf19HSV was evident in the insoluble fraction (contains adherent EPEC), with a corresponding band in the cytoplasmic and membrane fractions (Fig. 3, bottom). In contrast to the minor single Orf19HSV cytoplasmic band, the membrane fraction has increased levels plus a second faster migrating form (Orf19HSV*; Fig. 3, lanes 5–7, bottom). This is presumably an N-terminally cleaved form, as it was detected via its C-terminal tag. These cytoplasmic and membrane bands were resistant to trypsin digestion of intact, but not saponin-permeabilized, infected cells, indicating an intracellular or membrane-protected location (Fig. 3, bottom; lanes 1–3 versus 4 and 5–7 versus 8). Orf19HSV, in the insoluble fraction, resisted such treatment as it is present within the bacteria.

Incubation of saponin-permeabilized cells with a high salt wash (2 M NaCl final concentration) failed to extract the membrane-associated HSV bands into the cytoplasmic fraction (Fig. 3, lanes 3 and 7), which is indicative of integral membrane proteins. The detection of Orf19HSV does not require the presence of the standard inhibitor cocktail [phenylmethylsulphonyl fluoride (PMSF), sodium vanadate and sodium fluoride; lanes 2 and 6], which was
absent from the trypsin susceptibility and salt extraction experiments.

The correct assignment of Orf19HSV species to host cellular compartments was verified by probing the blot for Tir, as the compartmentalization of different Tir species within host cells has been defined (Rosenshine et al., 1996; Kenny, 1999). Figure 3 (top) revealed the documented distribution of two (T\(8\)/T\(0\)), three (T\(8\)/T\(0\)/T\(00\)) and two distinct (T\(8\)/T\'(\(0\))) Tir forms in the cytoplasm, membrane and insoluble fractions respectively (Kenny, 1999). This blot also validated the trypsin susceptibility protocol, as the Tir-modified species were resistant to trypsin digestion of intact (Fig. 3, lanes 1–3 and 5–7), but not saponin-treated (Fig. 3, lanes 4 and 8), host cells, as reported previously (Kenny, 1999).

**Detection of Orf19 in punctate structures within the host cytoplasm**

The location of Orf19 within the host cell was examined by immunofluorescence microscopy (see Experimental procedures). Analysis with anti-Orf19 antibodies revealed punctate structures in cells colonized with orf\(19/pSK\)–orf\(19\)HSV, but not orf\(19/pSK\) (data not shown). Similar structures were observed in cells infected with orf\(19\) carrying a plasmid-encoding native Orf19 (Fig. 4A, right), discounting the possibility that the HSV tag affects Orf19 localization. Staining for polymerized actin shows the location of the actin-nucleating bacteria (Fig. 4A, left) and indicates distinct bacterial and Orf19 staining patterns. This was confirmed using laser confocal microscopy to obtain a series of images through orf\(19/pSK\)–orf\(19\)-infected cells (Fig. 4B). The figure (left) reveals the location of actin-nucleating adherent bacteria on the surface (0 \(\mu\)m) and sides (–2 \(\mu\)m) of the host cells, which were virtually absent from the basal region (–4 \(\mu\)m) where host stress fibres are evident. The figure (right) shows the corresponding Orf19 labelling pattern and clearly demonstrates that Orf19 is within the cell and not associated with the surface-located bacteria. Indeed, the majority of the Orf19 is located in the basal (–4 \(\mu\)m) section throughout the cell, but is absent from the nucleus. In some cells, Orf19 labelling was confined to regions close to the adherent bacteria (data not shown), suggesting initial delivery at the site of infection and subsequent diffusion throughout the cell. Similar patterns of Orf19 labelling were apparent in some cells infected with wild-type EPEC E2348/69, but the labelling was less consistent and less intense than that after infection with orf\(19/pSK\)–orf\(19\).

**EspB-dependent translocation of Orf19 into host cells**

Western analysis (Fig. 3) had indicated that Orf19 was associated with the membrane fraction. However, its absence from the plasma membrane, as revealed by confocal microscopy examination (Fig. 4), suggested that it might associate with intracellular membrane compartments. Owing to Orf19’s secreted nature, it was possible that it could have been endocytosed into such membranous compartments. This was not the case, however, as Orf19 was not detected (data not shown) in transferrin-labelled endocytosed compartments or vesicular compartments identified with antibodies to EEA1 (early endosomal), M6P-CI (mannose 6-phosphate-cationic independent; early/late endosomal) or Lgp120 (lysosomal glycoprotein 120; lysosomal).

In contrast, the direct delivery of Orf19 into host cells...
was demonstrated by the dependence of Orf19 detection within host cells on a functional EPEC EspB protein. Thus, although EPEC orf19 and espB strains, both carrying pSK-orf19HSV, secreted similar levels of Orf19HSV into the host extracellular milieu (Western analysis; data not shown), only cells infected with the orf19/pSK-orf19HSV contained Orf19-labelled structures (epifluorescence microscopy; data not shown).

**Orf19 is targeted to host mitochondria**

As Orf19 was predicted to encode a putative mitochondrial presequence cleavage site and it appears to be N-terminally processed within host cells (Fig. 3), we investigated whether it might be targeted to mitochondria. The mitochondrion-selective probe, Mitotracker, revealed both punctate (arrowhead) and stringy (arrows) structures, characteristic of mitochondria (Fig. 5, top). Mitochondrial-specific staining was confirmed with antibodies against a mitochondrial matrix protein, pyruvate dehydrogenase (PDH), which produced an identical staining pattern (Fig. 5, top).

After HeLa infection with orf19/pSK-orf19, host cells were co-incubated with Mitotracker for 30 min before probing for Orf19 and mitochondria location. Figure 5 shows that the Orf19 co-localizes with a subpopulation of the Mitotracker- and PDH-labelled mitochondria. Less Orf19 label was observed in Mitotracker-labelled cells than in anti-PDH-probed cells, suggesting that Mitotracker may interfere with Orf19 recruitment into mitochondria.

---

**Fig. 4.** Detection of Orf19 in host cells by indirect immunofluorescence microscopy. HeLa cells were infected with orf19 carrying pSK or pSK-orf19 for 3 h. Cells were fixed, permeabilized and stained for polymerized actin (phalloidin–TRITC) and Orf19 (FITC) before examination by epifluorescent/confocal microscopy. A series of serial X–Y images of infected cells was captured at 1 μm steps.

A. A composite projection of the top (apical) three sections.

B. Single sections from the pSK-orf19-infected HeLa cells are shown corresponding to the top (apical) surface (0 μM), 2 and 4 μM into the infected cell. Right, the Orf19–FITC staining pattern. Left, the phalloidin–TRITC (polymerized actin) staining.

---

**Fig. 5.** Localization of Orf19 to host mitochondria. Host cells were infected in FCS-free DMEM media with orf19/pSK–orf19 for 2 h and, where indicated, the mitochondrial-selective fixable probe, Mitotracker, was added for the final 30 min of the infection period. Cells were then fixed and stained for Orf19 (FITC) and/or the mitochondrial matrix protein, pyruvate dehydrogenase (PDH). Whereas the PDH (Alexa) staining pattern was coincident with that of Mitotracker, Orf19 labelling was coincident with only a subpopulation of Mitotracker- and PDH-labelled compartments. Both Mitotracker and PDH resulted in a characteristic mitochondrial staining pattern revealing both punctate (arrowhead) and stringy (arrows) structures.
Conversely, mitochondria intensely labelled with anti-Orf19 antibodies often exhibited decreased levels of Mitotracker, but not anti-PDH labelling (data not shown), indicating that Orf19 may itself interfere with the ability of mitochondria to take up and retain Mitotracker.

Mitochondrial membrane disrupting activity of Orf19

Given the localization of Orf19 to the mitochondrion and the above observations, we tested whether Orf19 interferes with mitochondrial function. HeLa cells were prelabelled with the cationic mitochondrial-specific fluorophore, tetramethylrhodamine ethyl ester (TMRE), whose sequestration depends on mitochondrial membrane potential, and infected with various EPEC strains (see Experimental procedures). Figure 6 reveals that TMRE produced a typical mitochondrial staining pattern in orf19/pSK or cfm-14, type III secretion-defective, infected cells. In contrast, infection with orf19/pSK–orf19 produced distinct TMRE-negative zones beneath many bacterial microcolonies (Fig. 6), consistent with loss of TMRE from underlying mitochondria. These TMRE-negative zones were frequently surrounded by a normal TMRE labelling pattern, indicating that this phenomenon was not a result of the relocalization of the organelles away from the site of infection. Furthermore, immunolabelling of PDH rarely indicated any disappearance of this mitochondrial marker from the area beneath microcolonies, indicating that the frequent loss of TMRE from these regions did not result from gross disruption of mitochondrial integrity. Similar TMRE-negative zones were apparent beneath a smaller proportion of bacterial microcolonies after infection with wild-type EPEC E2348/69, indicating that this phenomenon is related to a genuine EPEC phenotype that may be amplified when Orf19 is delivered in increasing amounts after expression from a plasmid.

Delivery of low levels of Orf19 into host cells

To investigate the host cellular level of Orf19, a strain was generated in which the orf19 and tir genes were exchanged for HSV-tagged versions (see Experimental procedures). After various infection periods, non-adherent EPEC and host fractions (Triton X-100-soluble and -insoluble) were isolated for Western analysis with anti-HSV antibodies. Figure 7 reveals that, in contrast to TirHSV, only minor levels of Orf19HSV are detected in the non-adherent bacteria. This ratio appears to be maintained throughout the infection period.

Similar analysis of the host fractions revealed TirHSV in the host insoluble fraction by 1 h and the soluble fraction by 2 h after infection (Fig. 7), with the modified forms evident by 2 h. The cellular levels of these bands appear

![Fig. 6. Orf19 disrupts mitochondrial membrane potential. Host cells were prelabelled with the non-fixable mitochondrial-selective probe, TMRE, for 1 h before adding preinduced EPEC strains. When microcolonies were evident on cells, live cells were examined by phase-contrast/epifluorescence microscopy for the position of the adherent bacteria (phase-contrast; left) and for TMRE (right) within the infected cells. Black arrows indicate the position of adherent bacterial microcolonies, and white arrows indicate the TMRE-negative regions within the cytoplasm. Strains used were wild-type EPEC, the type III secretion-defective mutant cfm-14 and the orf19 mutant carrying pSK or pSK–orf19.](image-url)
Evidence for Orf19 as an injected EPEC effector molecule initially came from Western analysis revealing a subpopulation of Orf19HSV in the host membrane fraction with a small amount in the cytoplasmic fraction. This study also indicated that a subpopulation of membrane-associated Orf19 underwent N-terminal cleavage. These Orf19 species were resistant to trypsin treatment of intact but not saponin-permeabilized cells, supporting an intracellular or membrane-protected location. Corresponding Orf19 bands were not detected in uninfected or control orf19/pSK-infected cells.

The resistance of the Orf19HSV membrane-associated bands to extraction by high salt solutions suggested that these forms might be integrally inserted in the membrane. However, the absence of predicted membrane-spanning domains argues against this, raising the possibility that Orf19 may be anchored in the membrane or be part of a complex/structure too large to exit through the saponin-generated pores. Correct fractionation and trypsin susceptibility protocols were verified by probing the same blots for Tir, in which the reported cellular distribution and trypsin susceptibility profiles of Tir species were reproduced (Rosenshine et al., 1996; Kenny, 1999) (Fig. 3).

Epifluorescence microscopy was used to confirm the presence of Orf19 within orf19/pSK–orf19, but not control, infected host cells. Here, it was observed in punctate accumulations in the cytoplasm and absent from the plasma membrane (the site of bacterial adherence). The possibility that this staining pattern resulted from Orf19 endocytosis into membrane-enclosed compartments was discounted by the inability to co-localize Orf19 to endosomal or lysosomal compartments (data not shown). Direct injection of Orf19 into host cells was implied by the dependence of its delivery on a functional EspB protein. EspB is a component of a translocon required for Tir delivery into host cells (Kenny et al., 1997a; Knutton et al., 1998; Wolff et al., 1998).

A clue to the cellular compartment targeted by Orf19 came from computer analysis (http://psort.nibb.ac.jp), which highlighted a putative mitochondrial presequence
cleavage site, consistent with our observation that a subpopulation of Orf19HSV was N-terminally cleaved in host cells. Targeting of Orf19 to mitochondria was demonstrated by its co-localization with structures labelled with mitochondrial-specific markers (Mitotracker and anti-PDH antibodies). In support of this, the N-terminal portion of Orf19 has features reminiscent of mitochondrial import proteins (Roise and Schatz, 1988), such as (i) positive charges spread along its length; (ii) strong bias against negative charges; (iii) high content of hydrophobic residues; and (iv) the putative cleavage motif. A gradient of Orf19 staining was often apparent in infected cells, indicative of its delivery at the site of infection and targeting to underlying mitochondria. Targeting and import of mitochondrial proteins is dependent on mitochondrial membrane potential (Roise and Schatz, 1988). Orf19 targeting to mitochondria may also be dependent on mitochondrial membrane potential, as less Orf19 staining was detected in cells that had been incubated during the final stages of the infection with Mitotracker. Recent studies indicate that Mitotracker causes mitochondrial depolarization (Scorrano et al., 1999) and, thus, may explain the reduced Orf19 labelling. In addition, the observation that strongly labelled Orf19 structures often had a weak Mitotracker signal (much weaker than Orf19-free mitochondria) suggested that Orf19 itself may have a membrane potential disrupting activity reducing the ability of such mitochondria to recruit Mitotracker.

A mitochondrial membrane potential disrupting activity for Orf19 was supported by live cell imaging of infected cells that had been prelabelled with a mitochondrial-specific dye, TMRE, which depends on mitochondrial membrane potential for its retention. Characteristic mitochondrial staining patterns were obtained in uninfected or control, orf19/pSK or type III secretion-defective, infected cells, whereas orf19/pSK–orf19-infected cells exhibited discrete zones beneath adherent colonies, which were devoid of TMRE stain (Fig. 6). The presence of some of these zones in areas surrounded by TMRE-labelled mitochondria was indicative of the loss of TMRE from underlying mitochondria and not their relocation. Similar PDH-negative zones were not apparent after immunolabelling of these infected cells, supporting the fact that the mitochondria in the TMRE-negative zones were neither relocated nor grossly disrupted. However, decreased PDH labelling was observed beneath a small proportion of orf19/pSK–orf19 microcolonies (data not shown) suggesting that Orf19 may, with time, also induce more profound effects on mitochondrial integrity.

The detection of similar, although less frequent, TMRE-negative zones under wild-type EPEC microcolonies demonstrated that this is a genuine EPEC phenotype and not an artifact related to increased Orf19 delivery into host cells after its expression from a plasmid. Our frequent inability to detect Orf19 in EPEC-infected host cells indicates that relatively low levels of this protein are delivered into host cells. This was verified using a strain in which the LEE orf19 and tir genes were replaced with HSV-tagged versions, resulting in barely detectable levels of Orf19HSV in host cells by Western analysis compared with TirHSV (Fig. 7).

Together, our data suggests that EPEC deliver Orf19 at the site of infection directly into the host cytoplasm in which it is targeted to mitochondria (probably via its putative N-terminal signal sequence and mitochondrial membrane potential). Orf19 interaction with mitochondria appears to lead to N-terminal processing of a subpopulation of Orf19, possibly indicative of import into these organelles. Orf19 delivery appears to lead to the loss of membrane potential of the mitochondria underlying the site of infection leading, perhaps, to the loss of mitochondrial integrity at later stages.

What might be the in vivo function of Orf19? As a major role for mitochondria (for a review, see Bernardi et al., 1999) is the synthesis of ATP, which is compromised after membrane depolarization, it is possible that Orf19 results in a localized alteration of ATP/ADP concentrations in order to affect the function of ATP-sensitive ion channels or other processes. Another possibility concerns the central role of mitochondria in the release of proapoptotic factors (Bernardi et al., 1999). Many type III-containing pathogens modulate cell death in a direct or indirect manner, but no such pathogens have been reported to target effector molecules to these crucial organelles (for a review, see Weinrauch and Zychlinsky, 1999). However, there are few data indicating that EPEC induce apoptosis after infection, although it is possible that EPEC infection increases the cells’ responsiveness to apoptotic signals (Malstrom and James, 1998; Crane et al., 1999). It is also possible that Orf19 could have an anti-apoptotic function, a process apparently induced after Chlamydia infection, an organism recently reported to encode a type III secretion system. This appears to involve preventing the release of proapoptotic factor from mitochondria (for a review, see Weinrauch and Zychlinsky, 1999), which could involve targeting an effector molecule to mitochondria. A role for Orf19 in these processes remains to be tested, whereas its in vivo function may only become fully apparent using a suitable animal infection model system. Such studies are currently under way. As Orf19 is targeted to host mitochondria we propose that it be renamed mitochondrial associated protein (MAP) and the encoding LEE gene, map.

Map (Orf19) is one of a growing number of homologues (EPEC TrcA/TrcP and Shigella IpgB proteins) (Tobe et al., 1999a; b), of which only one other, TrcA, has been studied in any detail and has reportedly a chaperone-like
function. TrcA interacted with several EPEC proteins including a 39 kDa secreted protein putatively identified as a glyceraldehyde 3-phosphate homologue (Kenny and Finlay, 1995). This protein is now known (unpublished data) to be encoded by espD (Lai et al., 1997). In our hands, TrcA interacted somewhat non-specifically with a large number of EPEC and host proteins in contrast to Map (Orf19), which interacted only with host proteins (Fig. 2). It is still a formal possibility that one or more of these Map (Orf19) homologues is perhaps targeted into host cells to interact with mitochondria. Given this report, this question will probably be quickly addressed.

In summary, we have shown that the map (orf19) gene product is a type III secreted protein that is injected directly into host cells in an Esp translocon-dependent manner. Map (Orf19) was located in punctate structures within the host cytoplasm identified as mitochondria, in which it appears to interfere with mitochondrial membrane potential. Although the precise function of Orf19 in host cells is as yet undefined, its host location argues that it may participate in regulating cell death or perhaps some as yet poorly understood mitochondrial-regulated process, which is subverted to enable full EPEC virulence. This study reveals a new strategy used by type III-containing pathogens, opening the possibility that other effector molecules, such as the Orf19 homologues (TrcA and lpgB), or other proteins, are similarly targeted to mitochondria to disrupt mitochondrial function as part of the infection process.

Experimental procedures

For bacterial strains, plasmids and oligonucleotides, see Table 1.

Cell lines

HeLa (ATCC #CCL2, human adenocarcinoma cervical) cells were grown at 37°C in 5% CO₂ in DMEM supplemented with 10% (v/v) fetal calf serum (FGS; Gibco-BRL).

Deletion of orf19 gene sequence

The LEE region encompassing orf19, tir, cesT and 5′ eae has been cloned previously into pSK, creating pSK-tir (Kenny, 1999). The pSK-specific T3 oligonucleotide, together with a synthesized oligonucleotide, XNSNCO, was used to amplify a 0.7 kb fragment encompassing the first 126 bases (42 residues) of orf19, followed by a unique NcoI site. The sequence encompassing the final 141 bases (47 residues) of orf19, tir, cesT and 5′ eae was amplified on a 2.8 kb fragment (including the 5′-located Ncol site) using the synthetic oligonucleotide XPSNCO, together with the pSK-specific T7 oligonucleotide (see Fig. 1). Both fragments were cloned into pSK, generating pSK-orf19 (partial). The pSK-orf19 (partial) BstEI–SalI (~2.4 kb) fragment (tir minus 5′ 10 bp, cesT and 5′ eae) was exchanged for the same fragment from pSK-tir to remove any PCR-generated mutations in the tir–cesT sequence.

Generation of orf19 EPEC strain

The LEE DNA fragment carrying the deleted orf19 gene was cloned into the suicide vector pCVD442 and introduced into EPEC E2348/69 by allelic exchange as described previously (Stein et al., 1996). The wild-type orf19 and tir chromosomal sequences were exchanged for those of orf19HSV and tirHSV by an identical approach in a sequential manner. PCR and Western blot analyses confirmed the generation of the required strains.

Generation of pSK–or19HSV, pSK–or19 and pET–HisT7Orf19 plasmids

A 1.2 kb fragment carrying the orf19 gene sequence was PCR amplified using pSK–tir as a template and the pSK T3-PgutNheNS oligonucleotide set; the latter deletes the Nhel restriction site. This fragment was digested EcoRI–Nhel and cloned into the same sites of pSK–tirHSV (Kenny, 1999) at which a Nhel site is present at the 5′ end of the HSV gene sequence. The resulting plasmid carried the orf19HSV and cesT sequence. The cesT sequence was deleted by SalI digestion, dropping out the 0.7 kb cesT fragment, generating pSK–or19HSV. pSK–or19 was constructed by cloning the 1.7 kb EcoRI–PstI fragment of pSK–tir, carrying orf19 and 5′ 220 bp of tir, into the same sites of pSK. The gene sequence encoding orf19HSV was isolated from pSK–or19HSV on a BamHI–Nhel fragment and cloned into the same sites of pET27b (Novagen) to generate pET–HisT7orf19.

Generation of pSK–tirACHSV, pSK–tirCA and pET–tirACHSVHis plasmids

Two oligonucleotides, tirABCamPS (unique BamHI ~ 50 bp upstream of tirA) and tirANCamNS (Nhel site at stop codon), were used to PCR clone tirAC from EPEC B17 into pSK. The BamHI–Nhel tirAC-containing fragment was cloned into pET28a to generate pET–tirACHSVHis.

Purification of Orf19 and TrcA fusion proteins for antibody production and ‘far’ Westerns

E. coli BL21 (DE3) carrying pET constructs were grown to an optical density (A₆₀₀) of ~0.6 before the addition of IPTG (1 mM final concentration) for 1–3 h. Overexpression led to the formation of inclusion bodies. The cellular pellets were lysed in 50 mM Tris-HCl (pH 8), 1 mM EDTA, 100 mM NaCl containing lysozyme (Sigma; 0.8 mg g⁻¹ wet pellet), deoxycholic acid (Sigma: 4 mg g⁻¹ wet pellet), 1 mM AEBSF protease inhibitor (Melford), and DNase I (20 μg g⁻¹ wet pellet; ICN). The solution was centrifuged (12K RCF) for 15 min and the cytoplasmic supernatant discarded. The insoluble pellet was solubilized in phosphate buffer (PBS; 10 mM phosphate, pH 7.4, 0.5 M NaCl) containing 8 M
urea—10 mM imidazole (BDH) and centrifuged (12K RCF, 15min). A portion of the partially purified Orf19HSVHs protein was resolved by SDS–PAGE (12%), transferred to unsupported nitrocellulose (Millipore Immobilon NC pure) and used to immunize mice.

Tagged proteins were purified further by binding to nickel beads (Pharmacia) and eluting from columns with PBS containing 8 M urea and 225 mM imidazole. ‘Far’ Western protocol was carried out as previously described (Kenny et al., 1997a) diluting the purified protein solutions at least 160-fold. This concentration of urea did not inhibit Tir–intimin interaction (Fig. 2).

Isolation of secreted proteins

EPEC strains were grown overnight in Luria–Bertani (LB) (37°C without shaking) and diluted 1:50 into DMEM (Gibco-BRL). Supernatants were concentrated by the addition of trichloroacetic acid (TCA; 10% v/v; BDH) as described previously (Kenny et al., 1997b).

Cellular fractionation and protein extraction

The procedure used was similar to that described previously (Kenny, 1999). Briefly, HeLa monolayers were infected with EPEC strains (MOI = 1:100) for 3–5 h. The monolayers were washed two or three times in cold PBS, and the HeLa cells were permeabilized by the addition of saponin (Calbiochem) lysis buffer [PBS containing 0.4 mM NaVO₄ (ICN), 1 mM NaF (ICN) and 0.1 mM PMSF; Sigma]. After 5 min incubation on ice, samples were centrifuged (12 K RCF, 5 min, 4°C) and the soluble ‘cytoplasmic’ fraction removed. The insoluble pellet was rinsed in PBS and the membrane proteins separated from the insoluble components by the addition of Triton X-100 lysis buffer (the same as saponin buffer above with the addition of 1% Triton v/v), as described before. For experiments treating host cells with trypsin–EDTA, infected cells were washed twice with PBS and incubated with a trypsin–EDTA solution (0.5–0.2% final concentration; Boehringer Mannheim) until cells detached. Cells were centrifuged (3 K RCF for 30 s at room temperature) and washed trice in PBS. Cells were permeabilized with 0.2% saponin in PBS or trypsin–EDTA for 5 min at room temperature and the cytoplasmic fraction isolated. Membrane and insoluble fractions were then isolated in the presence of inhibitors, as above. In some experiments, host cells were permeabilized in saponin lysis buffer before being extracted with PBS containing 1.5 M NaCl (2 M final concentration) and fractionating cells as above.

Western immunoblot analysis

Protein samples were resolved by SDS–PAGE (Laemmli, 1970) and the proteins transferred to nitrocellulose for Western analysis as described elsewhere (Rosenshine et al., 1992). Blots were blocked in 5% Marvel dried skimmed milk powder. Bands bound by these antibodies were detected by alkaline phosphatase-conjugated secondary antibodies (Jackson Laboratories), as described previously (Rosenshine et al., 1992).

Immunofluorescence microscopy

HeLa cells seeded on glass coverslips were infected with various EPEC strains for up to 5 h. Monolayers were washed three times in PBS and fixed in 2.5% paraformaldehyde (BDH). Cells were permeabilized with 0.1% Triton in PBS and incubated with anti-HSV (Novagen), anti-PDH or anti-Orf19 antibodies (preabsorbed against LB-grown orf19 bacteria), together with an appropriate secondary fluorescein-conjugated antibody, as described previously (Rosenshine et al., 1992). In some experiments, polymerized actin was visualized using tetramethylrhodamine isothiocyanate (TRITC)-conjugated phallolidin (Sigma). Mitotracker CMX-Ros (100 nM final concentration; Molecular Probes) was added 30 min before the end of FCS minus infection cultures. Fluorescent labelling patterns were assessed with a Zeiss Axioskop phase-contrast/eplifluorescence microscope and confocal images obtained using a Leica TCS SP (spectrophotometer) confocal microscopy system attached to a Leica DM IRBE microscope with Ar laser (488 nm) and Kr laser (568 nm) excitation (Leica Microsystems). An oil-immersion objective lens 100×, NA 1.4, was used, and imaging parameters were selected to optimize confocal resolution.
Cytotoxic staining for comparative studies was performed in the same experimental session and images obtained using identical imaging parameters.

**Live imaging of mitochondrial membrane potential**

Mitochondrial membrane potential was assessed after infection of HeLa cells with EPEC strains using the ethyl ester of TMRE (Molecular Probes), whose accumulation in mitochondria depends on its membrane potential. HeLa cells, in the absence of FCS, were preincubated with TMRE (20 nM final concentration) for 1 h and infected with EPEC strains pregrown in DMEM for 2 h. Cells were examined after the microcolony adherence with TMRE fluorescence detected with a 515–560 nm excitation filter and 590 nm longpass emission filter. Fluorescence images were acquired along with phase-contrast images of adherent EPEC microcolonies, with a Hamamatsu C4742–95 12-bit digital CCD camera attached to a Leica DM-IRBE microscope with an oil immersion 40× PL Fluotar lens (NA 1.0). Image capture was controlled using OPENLAB software (Improvision).

**Data imaging**

Raw data for the figures were imported into Adobe Photoshop directly or scanned using a UMAX (Astra 1220S) scanner for import, where they were labelled before printing on a Hewlett Packard LaserJet 6P or Epson stylus 740 colour printer.

**Acknowledgements**

We would like to thank Professor P. H. Williams (Leicester University) for providing EPEC B171, and David Copland and Jonathon Warawa (Bristol) for generating purified proteins and Orf19 polyclonal antibodies. Thanks also go to many people in the Bristol Biochemistry Department for providing advice and reagents (Dr George Banting, anti-endosomal–lysosomal antibodies; Professor Richard Denton, anti-PDH antibodies; and Dr Guy Rutter for TMRE) and to Professor Tim Hirst for critical reading of the manuscript. This work was funded by the Wellcome Trust under a career development fellowship to B.K. Additional support was provided by Royal Society research grants to B.K. and M.A.J. and a Medical Research Council Infrastructure Award supporting the School of Medical Sciences Cell Imaging Facility. Confocal imaging used equipment provided by a Medical Research Council Joint Research Equipment Initiative grant.

**References**

Abe, A., de Grado, M., Pfuetzner, R.A., Sanchez-SanMartin, C., DeVinney, R., Puente, J.L., et al. (1999) Enteropathogenic *Escherichia coli* translocated intimin receptor, Tir, requires a specific chaperone for stable secretion. *Mol Microbiol* 33: 1162–1175.

Bernardi, P., Scorrano, L., Colonna, R., Petronilli, V., and Di Lisa, F. (1999) Mitochondria and cell death. Mechanistic aspects and methodological issues. *Eur J Biochem* 264: 687–701.

Crane, J.K., Majumdar, S., and Pickhardt, D.F., III (1999) Host cell death due to enteropathogenic *Escherichia coli* has features of apoptosis. *Infect Immun* 67: 2575–2584.

Donnenberg, M.S., and Kaper, J.B. (1991) Construction of an eae deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect Immun* 59: 4310–4317.

Donnenberg, M.S., Calderwood, S.B., Donohue-Rolfe, A., Keusch, G.T., and Kaper, J.B. (1990) Construction and analysis of TnphoA mutants of enteropathogenic *Escherichia coli* unable to invade HEP-2 cells. *Infect Immun* 58: 1565–1571.

Donnenberg, M.S., Yu, J., and Kaper, J.B. (1993) A second chromosomal gene necessary for intimate attachment of enteropathogenic *Escherichia coli* to epithelial cells. *J Bacteriol* 175: 4670–4680.

Donnenberg, M.S., Kaper, J.B., and Finlay, B.B. (1997) Interactions between enteropathogenic *Escherichia coli* and host epithelial cells. *Trends Microbiol* 5: 109–114.

Elliott, S.J., Wainwright, L.A., McDaniel, T.K., Jarvis, K.G., Deng, Y.K., Lai, L.C., et al. (1998) The complete sequence of the locus of enterocyte effacement (LEE) from enteropathogenic *Escherichia coli* E2348/91. *Mol Microbiol* 28: 1–4.

Elliott, S.J., Hutcheson, S.W., Dubois, M.S., Millies, J.L., Wainwright, L.A., Batchelor, M., et al. (1999) Identification of CesT, a chaperone for the type III secretion of Tir in enteropathogenic *Escherichia coli*. *Mol Microbiol* 33: 1176–1189.

Frankel, G., Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J.B., and Knutton, S. (1998) Enteropathogenic and enterohemorrhagic *Escherichia coli*: more subversive elements. *Mol Microbiol* 30: 911–921.

Gomez-Duarte, O.G., and Kaper, J.B. (1995) A plasmid-encoded regulatory region activates chromosomal eaeA expression in enteropathogenic *Escherichia coli*. *Infect Immun* 63: 1767–1776.

Goosney, D.L., Celli, J., Kenny, B., and Finlay, B.B. (1999) Enteropathogenic *Escherichia coli* inhibits phagocytosis. *Infect Immun* 67: 490–495.

Hicks, S., Frankel, G., Kaper, J.B., Dougan, G., and Phillips, A.D. (1998) Role of intimin and bundle-forming pilus in enteropathogenic *Escherichia coli* adhesion to pediatric intestinal tissue in vitro. *Infect Immun* 66: 1570–1578.

Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* 62: 379–433.

Jarvis, K.G., Giron, J.A., Jerse, A.E., McDaniel, T.K., Donnenberg, M.S., and 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.

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., and 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., DeVinney, R., Stein, M., Reinscheid, D.J., Frey, E.A., and Finlay, B.B. (1997a) Enteropathogenic *E. coli* (EPEC) transfers its receptor for intimate adherence into mammalian cells. *Cell* 91: 511–520.
Enteropathogenic *E. coli* (EPEC) protein secretion is induced in response to factors similar to those of the gastrointestinal tract. *Infect Immun* **65**: 2606–2612.

Knutton, S., Rosenshine, I., Pallen, M.J., Nisan, I., Neves, B.C., Bain, C., et al. (1998) A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells. *EMBO J* **17**: 2166–2176.

Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.

Lai, L.C., Lai, L.C., Waterman, D.H., Hornick, R.B., Young, C.R., and Sotman, S. (1978) *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *Lancet* **1**: 1119–1122.

Malstrom, C., and James, S. (1998) Inhibition of murine splenic and mucosal lymphocyte function by enteric bacterial products. *Infect Immun* **66**: 3120–3127.

Mellies, J.L., Elliott, S.J., Sperandio, V., Donnenberg, M.S., and Kaper, J.B. (1999) The Per regulon of enteropathogenic *Escherichia coli*: identification of a regulatory cascade and a novel transcriptional activator, the locus of enterocyte effacement (LEE)-encoded regulator (Ler). *Mol Microbiol* **33**: 296–306.

Puente, J.L., Bieber, D., Ramer, S.W., Murray, W., and Schoolnik, G.K. (1996) The bundle-forming pilus of enteropathogenic *Escherichia coli*: transcriptional regulation by environmental signals. *Mol Microbiol* **20**: 87–100.

Roise, D., and Schatz, G. (1988) Mitochondrial presequences. *J Biol Chem* **263**: 4509–4511.

Rosenshine, I., Doonenberg, M.S., Kaper, J.B., and Finlay, B.B. (1992) Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation 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., and 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.

Scorrano, L., Petronilli, V., Colonna, R., Di Lisa, F., and Bernardi, P. (1999) Chloromethyltetramethylrosamine (Mitotracker Orange) induces the mitochondrial permeability transition and inhibits respiratory complex I. Implications for the mechanism of cytochrome c release. *J Biol Chem* **274**: 24657–24663.

Soehl, I., Puente, J.L., Ramer, S.W., Bieber, D., Wu, C.Y., and Schoolnik, G.K. (1996) Enteropathogenic *Escherichia coli*: identification of a gene cluster coding for bundle-forming pilus morphogenesis. *J Bacteriol* **178**: 2613–2628.

Stein, M., Kenny, B., Stein, M.A., and Finlay, B.B. (1996) Characterization of EspC, a 110-kilodalton protein secreted by enteropathogenic *Escherichia coli* which is homologous to members of the immunoglobulin A protease-like family of secreted proteins. *J Bacteriol* **178**: 6546–6554.

Stone, K.D., Zhang, H.Z., Carlson, L.K., and Donnenberg, M.S. (1996) A cluster of fourteen genes from enteropathogenic *Escherichia coli* is sufficient for the biogenesis of a type IV pilus. *Mol Microbiol* **20**: 325–337.

Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**: 60–89.

Tobe, T., Schoolnik, G.K., Soehl, I., Bustamante, V.H., and Puente, J.L. (1996) Cloning and characterization of bfpTVW, genes required for the transcriptional activation of bfpA in enteropathogenic *Escherichia coli*. *Mol Microbiol* **21**: 963–975.

Tobe, T., Hayashi, T., Han, C.G., Schoolnik, G.K., Ohitsu, E., and Sasakawa, C. (1999a) Complete DNA sequence and structural analysis of the enteropathogenic *Escherichia coli* adherence factor plasmid. *Infect Immun* **67**: 5455–5462.

Tobe, T., Tatsuno, I., Katayama, E., Wu, C.Y., Schoolnik, G.K., and Sasakawa, C. (1999b) A novel chromosomal locus of enteropathogenic *Escherichia coli* (EPEC), which encodes a bfp7-regulated chaperone-like protein, TrcA, involved in microcolony formation by EPEC. *Mol Microbiol* **33**: 741–752.

Uchiya, K., Barbieri, M.A., Funato, K., Shah, A.H., Stahl, P.D., and Groisman, E.A. (1999) A *Salmonella* virulence protein that inhibits cellular trafficking. *EMBO J* **18**: 3924–3933.

Wainwright, L.A., and Kaper, J.B. (1998) EspB and EspD require a specific chaperone for proper secretion from enteropathogenic *Escherichia coli*. *Mol Microbiol* **27**: 1247–1260.

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

Weinrauch, Y., and Zychlinsky, A. (1999) The induction of apoptosis by bacterial pathogens. *Annu Rev Microbiol* **53**: 155–187.

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

Xie, Z., and Chen, Z. (2000) Harpin-induced hypersensitive cell death is associated with altered mitochondrial functions in tobacco cells. *Mol Plant–Microbe Interact* **13**: 183–190.