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A novel EspA-associated surface organelle of enteropathogenic *Escherichia coli* involved in protein translocation into epithelial cells

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Enteropathogenic *Escherichia coli* (EPEC), like many bacterial pathogens, employ a type III secretion system to deliver effector proteins across the bacterial cell. In EPEC, four proteins are known to be exported by a type III secretion system—EspA, EspB and EspD required for subversion of host cell signal transduction pathways and a translocated intimin receptor (Tir) protein (formerly Hp90) which is tyrosine-phosphorylated following transfer to the host cell to become a receptor for intimin-mediated intimate attachment and 'attaching and effacing' (A/E) lesion formation. The structural basis for protein translocation has yet to be fully elucidated for any type III secretion system. Here, we describe a novel EspA-containing filamentous organelle that is present on the bacterial surface during the early stage of A/E lesion formation, forms a physical bridge between the bacterium and the infected eukaryotic cell surface and is required for the translocation of EspB into infected epithelial cells.
Keywords: EPEC/EspA/EspB/protein translocation

Introduction

Enteropathogenic *Escherichia coli* (EPEC), an important cause of severe infantile diarrhoeal disease in many parts of the developing world, colonizes the small intestinal mucosa and, by subverting intestinal epithelial cell function, produces a characteristic histopathological feature known as the 'attaching and effacing' (A/E) lesion (Moon *et al.*, 1983) and diarrhoea. The A/E lesion is characterized by localized destruction (effacement) of brush border microvilli, intimate attachment of the bacillus to the host cell membrane and the formation of an underlying pedestal-like structure in the host cell consisting of polymerized actin, α-actinin, ezrin, talin and myosin (Ulshen and Rollo, 1980; Knutton *et al.*, 1987, 1989; Finlay *et al.*, 1992); similar A/E lesions are produced by EPEC in a variety of tissue culture cell lines (Knutton *et al.*, 1989). *In vitro* studies employing cultured epithelial cells and defined EPEC mutants support a three-stage model of A/E lesion formation: (i) initial non-intimate attachment; (ii) signal transduction and cytoskeletal rearrangements in host cells and (iii) intimate bacterial adhesion, actin accumulation and pedestal formation (Donnenberg *et al.*, 1997).

An emerging theme in the pathogenesis of bacterial infections is subversion by bacterial pathogens of host cell functions including signal transduction pathways and cytoskeletal organization (Cornelis and Wolf-Watz, 1997; Donnenberg *et al.*, 1997; Finlay and Cossart, 1997; Finlay and Falkow, 1997). Like many other Gram-negative pathogens of animals (e.g. *Yersinia*, *Salmonella* and *Shigella*) and plants (e.g. *Pseudomonas syringae*, *Erwinia amylovora*, *Ralstonia solanaceum* and *Xanthomonas campestris*), EPEC also relies for its exploitation of host cell machinery on a complex specialized secretion system—a type III secretion system (Jarvis *et al.*, 1995; Lee, 1997). The EPEC type III secretion system is part of a chromosomal pathogenicity island, designated the LEE (for locus for enterocyte effacement) which contains all the genes required to produce A/E lesions (McDaniel *et al.*, 1995, 1997). The LEE also encodes intimin, an outer membrane protein adhesin, required for the intimate attachment of EPEC to host cells (Jerse *et al.*, 1990; Frankel *et al.*, 1994) and for the organization of polymerized actin into a cup-like pedestal beneath each attached bacterium (Donnenberg *et al.*, 1997).

Type III secretion systems have been shown to deliver effector proteins across the bacterial cell envelope and eukaryotic cell membrane to the host cell cytosol (Mecsas and Strauss, 1996; Lee, 1997). The components responsible for the secretion of proteins across the bacterial cell envelope are broadly conserved in all type III secretion systems, so that one type III secretion system can export proteins usually secreted by another type III system (Rosqvist *et al.*, 1995). However, the proteins secreted across the bacterial cell wall vary from system to system: in *Yersinia* they are termed Yops (*Yersinia* outer-membrane proteins; Cornelis and Wolf-Watz, 1997), in *Shigella flexneri* Ipas (Invasion plasmid antigens) (Menard *et al.*, 1996) and in *Salmonella* Sips and Sops (*Salmonella* inner- and outer- membrane proteins, respectively; Wood *et al.*, 1996; Collazo and Galan, 1997). In EPEC, four proteins are known to be exported by a type III secretion system. Three EPEC-secreted proteins (or Esps), EspA, EspB and EspD (Donnenberg *et al.*, 1993; Kenny *et al.*, 1996; Lai *et al.*, 1997), all of which are required for signal transduction in host cells, and a translocated intimin receptor

Table I. List of strains and plasmids

	Description	Reference
Strain		
E2348/69	Wild-type	Levine *et al.* (1985)
JPN15	EAF-plasmid cured	Jerse *et al.* (1990)
UMD872	*espA*$^-$	Kenny *et al.* (1996)
UMD870	*espD*$^-$	Lai *et al.* (1997)
UMD864	*espB*$^-$	Donnenberg *et al.* (1993)
CVD452	*sepB*$^-$	Jarvis *et al.* (1995)
CVD463	*tir*$^-$	S.Elliott and J.B.Kaper (unpublished)
Plasmid		
pMSD2	cloned *espA*	Kenny *et al.* (1996)
pMSD3	cloned *espB*	Donnenberg *et al.* (1993)
pLCL123	cloned *espD*	Lai *et al.* (1997)

(Tir) (formerly Hp90) which is tyrosine-phosphorylated following transfer to the host cell membrane to become the receptor for intimin-mediated intimate attachment and A/E lesion formation (Kenny *et al.*, 1997).

In this paper, we now show that one of the Esps, EspA, is a major component of a large extracellular filamentous appendage which appears on the bacterial surface prior to intimate EPEC attachment, forms a direct link between the bacterium and the host cell surface and is required for the translocation of another Esp, EspB, into infected host cells.

Results

Anti-EspA labels EPEC surface filamentous structures

We used the His-tag system to produce recombinant EspA. The purified protein was used to raise a rabbit polyclonal EspA antiserum which was then used in immunofluorescence studies to stain the EPEC wild-type strain E2348/69 and its derivatives (Table I). We examined bacteria grown in broth- and tissue-culture media since expression of EPEC-associated virulence factors has been shown to be highest in mid-log growth phase in Dulbecco's modified Eagle's medium (DMEM) (Gomez-Duarte and Kaper, 1995; Tobe *et al.*, 1996; Knutton *et al.*, 1997). No labelling with the anti-EspA antibody was seen in overnight bacterial cultures grown in L-broth from any of the strains (data not shown). However, when the antiserum was applied to bacterial cells of the wild-type strain grown to mid-log phase in DMEM, numerous filamentous structures, ~50 nm in diameter and up to 2μm long, were seen on the bacterial surface (Figure 1a). These structures were also produced by plasmid-cured E2348/69 strain JPN15 (Figure 1b), by an *espB*$^-$ strain UMD864 (Figure 1c) and by a *tir*$^-$ strain CVD463 (data not shown) but they were not detected on cells of the *espA*$^-$ strain UMD872 (Figure 1d). They were seen, however, when *espA* was re-introduced into UMD872 on a plasmid, to produce strain UMD872(pMSD2) (data not shown). The filamentous structures were also absent from the *sepB*$^-$ strain CVD452 (Figure 1f), which is deficient in the secretion of Esps (Jarvis *et al.*, 1995). Although of uniform diameter, there was some variability in the length of filaments present on individual bacteria and among different strains. Compared with wild-type E2348/69, plasmid-cured JPN15 and *espB*$^-$ strain UMD864, an *espD*$^-$ strain, UMD870, produced structures that were very short (Figure 1e) whereas strain UMD870(pLCL123), where the *espD* gene is supplied *in trans*, produced filaments longer than those of the wild-type (data not shown). When observed in suspension culture by immunofluorescence, the filaments appeared to be rigid, but hinged at the bacterial surface, and were seen to wave about in the medium, thus making it difficult to obtain sharp fluorescence micrographs of these structures (Figure 1).

The presence of EspA filaments correlated with levels of EspA detected in supernatants of mid-log phase culture from various EPEC-derived strains using the polyclonal antiserum and Western blots (Figure 1g). Levels of EspA similar to those produced by the wild-type were detected in supernatants from the plasmid-cured derivative JPN15, *espB*$^-$ strain, UMD864 and from the mutant strains complemented *in trans* with the relevant genes on recombinant plasmids (Table I); no EspA was detected in the supernatant from the *espA*$^-$ strain UMD872, and only low levels of EspA were secreted by the *espD*$^-$ strain, UMD870, which is in accordance with a previous report (Lai *et al.*, 1997).

Ultrastructure of EspA filaments

To investigate the structure of the EspA filaments we examined bacteria grown to the mid-log phase in DMEM using negative staining and immunogold labelling electron microscopy. Anti-EspA-labelled filaments were seen when wild-type E2348/69 and plasmid-cured derivative JPN15 were examined by immunogold labelling (Figure 2a,b) but no such structures were seen on the *espA*$^-$ strain UMD872 (Figure 2c). Somewhat high background levels of gold labelling were seen routinely with strains secreting EspA but not with strains lacking EspA, suggesting that the antiserum is also staining unpolymerized EspA present in the bacterial suspension. In formalin-fixed preparations (Figure 2b) the filaments appeared as rigid cylindrical rods similar to those seen by immunofluorescence, whereas in unfixed preparations the filaments appeared somewhat collapsed and less rigid (Figure 2a). When combined with negative staining, the EspA filaments appeared to have a substructure and to be composed of smaller structures. This was most clearly seen in filaments present in culture supernatants (Figure 2d and e). The EspA antiserum stained aggregates of smaller, ~7–8 nm diameter, pilus-like structures; filaments with three pilus structures typical of those illustrated in Figure 2d and e were the most common. EspA-associated filaments were not seen when cells of wild-type E2348/69 or other strains were examined by negative staining without immunolabelling, suggesting that these structures are stabilized by the antiserum.

Negative staining electron microscopy also revealed the structurally and antigenically distinct bundle-forming pili (bfp) produced by wild-type strain E2348/69 (not shown) and other rod-like fimbriae, ~7 nm in diameter, produced by some E2348/69 and JPN15 bacteria that also did not stain with the EspA antiserum (Figure 2b, arrow); mannose-sensitive haemagglutination of bacterial cultures suggests that these are probably somatic type 1 fimbriae.

EspA filaments interact with target cells

To avoid complications due to bfp expression by wild-type E2348/69 and the formation of large three-dimensional

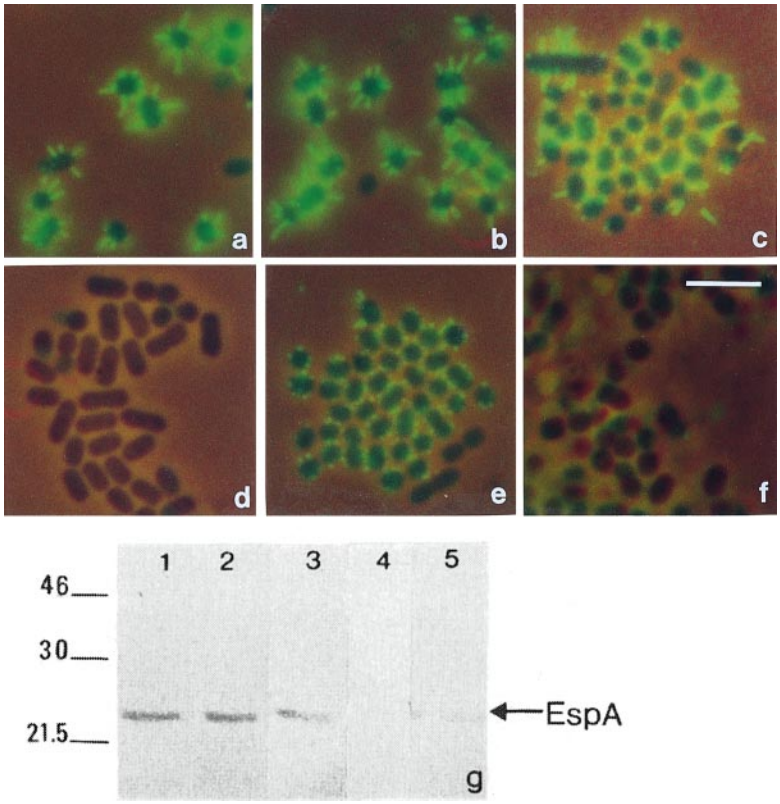

Fig. 1. Combined fluorescence/phase contrast micrographs showing EPEC strains stained with the EspA antiserum. The antiserum stained filamentous surface structures on wild-type strain E2348/69 (**a**), plasmid-cured strain, JPN15 (**b**) and the *espB*$^-$ strain, UMD864 (**c**), and very short filaments on the *espD*$^-$ strain, UMD870 (**e**). There was no staining of the *espA*$^-$ strain, UMD872 (**d**) or the *sepB*$^-$ strain, CVD452 (**f**). (Scale bar, 5 μm). Corresponding Western blots (**g**) showed similar levels of EspA in culture supernatants of strains E2348/69 (lane 1), JPN15 (lane 2), UMD864 (lane 3), reduced levels of EspA in culture supernatants of strain UMD870 (lane 5) and no EspA in culture supernatants of strain UMD872 (lane 4). Molecular weight markers are shown in thousands on the left.

bacterial microcolonies which obscure interaction of bacteria with the cell surface, we used the plasmid-cured derivative, JPN15, to investigate the possible interaction of the EspA-associated organelles with target cells. In tissue-culture cell-adhesion assays we routinely examined EPEC adhesion after 3 and 6 h incubation periods. Since the plasmid-cured derivative, JPN15, adheres less efficiently than the wild-type, we initially examined HEL cell adhesion assays after 6 h by immunofluorescence using the EspA antiserum. At this stage, we observed that most bacteria had formed A/E lesions (assessed by fluorescence actin staining; Knutton *et al.*, 1989) but showed no or scant staining with the antibody. Since bacteria grown in tissue-culture medium express EspA filaments, we speculated that, in the absence of adhesins such as bfp, initial attachment of strain JPN15 might be weak, and that weakly adherent bacteria might be removed in the vigorous washing procedure of the assay. We therefore modified our adhesion assay to include a much gentler washing procedure. Immunofluorescence studies now revealed a large population of cell-adherent bacteria that had not formed A/E lesions, and these bacteria were covered with EspA filaments (Figure 3a); at higher magnification the filaments were seen to form a direct link between the bacterium and the HEL cell surface (Figure 3b). Similarly, when Hep-2 cell preparations infected with JPN15 were pre-incubated with the EspA antiserum and then examined by transmission electron microscopy, anti-EspA labelled filaments were seen forming a bridge between the bacteria and the eukaryotic cell surface (Figure 4a). Structures morphologically similar to EspA filaments produced by JPN15 were also seen by scanning electron microscopy, and in cell adhesion assays such structures also formed a bridge between bacteria and the eukaryotic cell surface (Figure 5a); no such structures were seen by scanning electron microscopy with the EspA deletion mutant (data not shown).

In a similar manner, using short (<1 h) incubation times, EspA filaments linking bacteria and the HEL cell surface were also demonstrated for wild-type strain E2348/69 (data not shown).

EspA filament expression during A/E lesion formation

After a 6 h incubation of cells with plasmid-cured strain JPN15 and thorough washing, the lack of EspA staining indicated that the EspA filaments were absent from mature A/E lesions. To characterize EspA filament expression by wild-type EPEC strain E2348/69 and its plasmid-cured derivative, JPN15, during A/E lesion formation we performed adhesion assays at 1 h intervals for 6 h and stained cells for EspA and for cellular actin (to assess A/E lesion formation). Several stages of EspA filament expression were identified although these were difficult to quantitate because of the dynamic nature of the adhesion process.

Stage 1. EspA filaments were first expressed (in the

absence of cells) when bacteria were grown in tissue culture medium (Figure 1a and b).

Stage 2. Prior to A/E lesion formation, bacteria formed a non-intimate cell attachment, with EspA filaments forming a bridge between the bacterium and eukaryotic cell surface (Figures 3b, 4a, 5a and 6a).

Stage 3. Most wild-type E2348/69 and some plasmid-cured JPN15 bacteria progressed to form intimate cell attachment with associated actin accumulation. At this stage, EspA filaments were excluded from the site of intimate attachment but were present on the remainder of the bacterial surface (Figures 4b, 5b and 6b).

Stage 4. Progressive loss of the remainder of the EspA filaments from the bacterial surface (Figure 6c) produced fully developed A/E lesions (often with extended pedestal structures) devoid of EspA filaments (Figure 6d).

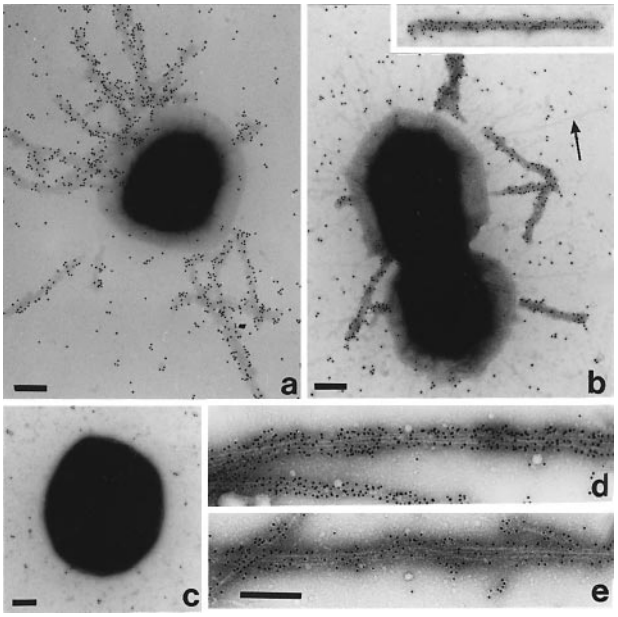

Fig. 2. Electron micrographs showing EPEC strains immunogold labelled with the EspA antiserum. The antiserum stained filamentous surface structures on strains JPN15 (**a**) and E2348/69 (**b**) but there was no staining of the *espA*$^{-}$ strain, UMD872 (**c**). Negative staining revealed a substructure in these filaments (b) and, at higher magnification, filaments present in culture supernatants were seen to be composed of small bundles of thinner ~7–8 nm diameter pilus-like structures (**d** and **e**). Many bacteria produced other ~7 nm diameter fimbriae which did not stain with the antiserum (b, arrow). (Scale bars, 200 nm).

EspA is required for translocation of EspB into host cells

Previous studies showed that both *espA* and *espB* mutants share a common phenotype, i.e. deficiency in subverting host cell signal transduction pathways and A/E lesion formation (Donnenberg *et al.*, 1993; Kenny *et al.*, 1996). Moreover, Lai *et al.* (1997) showed that secretion of EspB is not affected by an *espA* mutation. In this study, we have shown that EspA filaments did not stain with an EspB antiserum (data not shown). However, building upon our observation that the EspA filaments form a bridge between the incoming EPEC and the infected eukaryotic cells and a report by Wolff *et al.* (1998) that EspB is translocated into the host cell, we hypothesized that EspA filaments play a direct role in the translocation process. In order to test this, HeLa cells were infected with wild-type E2348/69, with the *espA*$^{-}$ strain UMD872, and with strain UMD872(pMSD2) where the *espA* gene is supplied *in trans*, and translocation of EspB was tested by immunofluorescence microscopy using the EspB antiserum. Zones of accumulated EspB were detected following permeabilization of E2348/69-infected HeLa cells (Figure 7C and D) but were not detected in non-permeabilized cells (Figure 7A and B); no EspB staining could be detected in the *espA*$^{-}$ strain UMD872-infected HeLa cells with or without permeabilization (Figure 7E and F) but staining was restored to permeabilized cells when *espA* was supplied *in trans* [strain UMD872(pMSD2)] (Figure 7G and H). Therefore, EspA filaments not only connect EPEC with the eukaryotic cell membrane, but are also required for translocation of EspB.

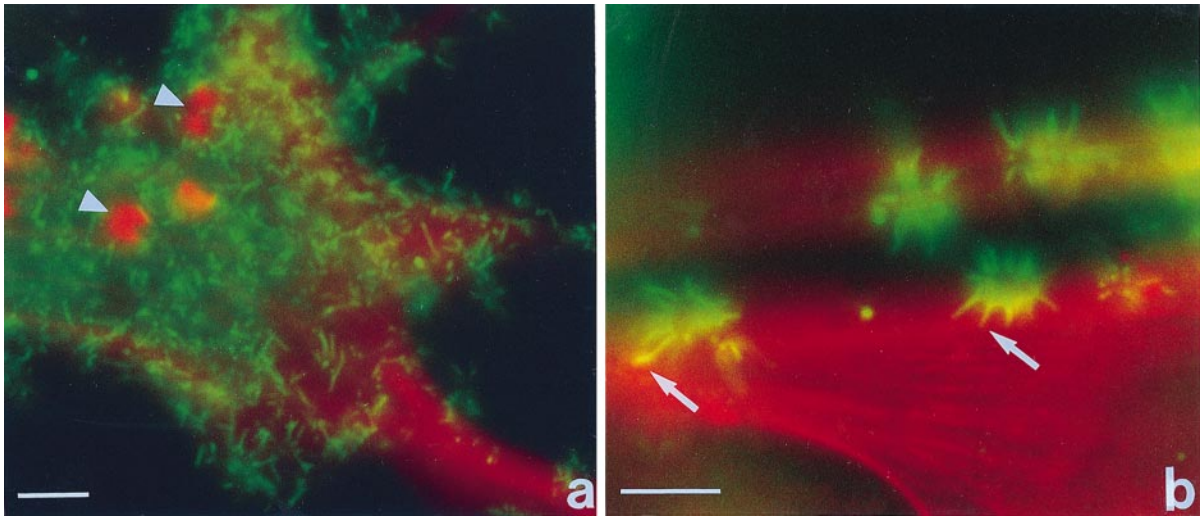

Fig. 3. Fluorescence micrographs showing adhesion of EPEC strain, JPN15, to cultured HEL cells. Bacteria were double stained for EspA (green) and for cellular actin (red). After a 5 h incubation and gentle washing, some bacteria had formed A/E lesions indicated by actin accumulation (**a**, arrowheads); the remainder of the cell surface was covered with bacteria that had not formed A/E lesions but were covered with EspA staining filaments. At higher magnification EspA staining filaments could be seen to bridge bacteria and the cell surface (**b**, arrows). (Scale bar, 5 μm).

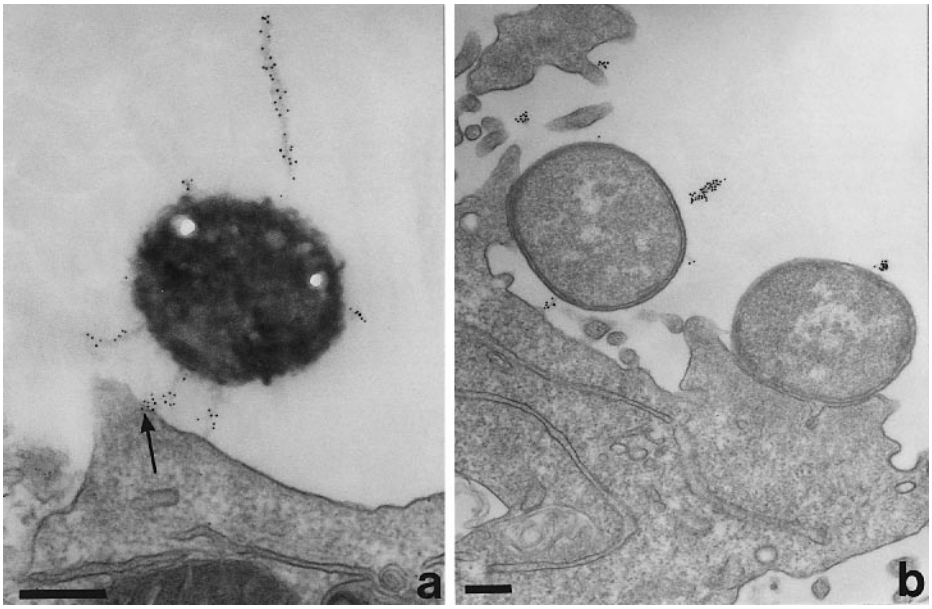

Fig. 4. Transmission electron micrographs showing JPN15 immunogold-labelled with the EspA antiserum after a 3 h incubation with Hep-2 cells. Prior to A/E lesion formation, labelled filaments were seen to bridge bacteria and the Hep-2 cell surface (**a**, arrow). EspA staining filaments were eliminated from the site of intimate bacterial attachment and reduced EspA staining was seen following A/E lesion formation (**b**). (Scale bar, 200 nm).

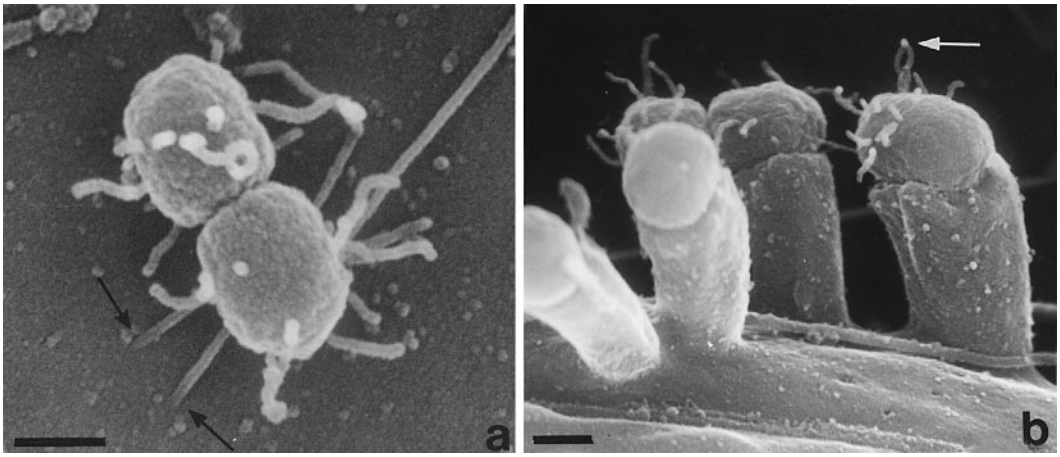

Fig. 5. Scanning electron micrographs showing strain JPN15 adhering to HEL cells. Prior to A/E lesion formation, filamentous surface structures similar to those stained by the EspA antiserum can be seen on the bacterial surface and forming a bridge between bacteria and the HEL cell surface (**a**). Note that filaments which interact with the cell surface appear very rigid and to be embedded in the HEL cell membrane (a, arrows). Filaments can also be seen on the exposed surface of bacteria that have formed A/E lesions (**b**, arrow). (Scale bar, 0.25 μm).

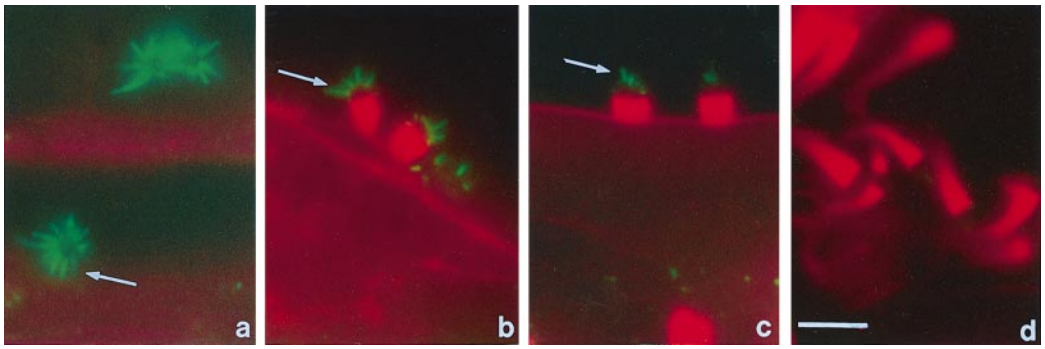

Fig. 6. Fluorescence micrographs showing different stages of EspA filament expression during adhesion of strain JPN15 to cultured HEL cells. Bacteria were double stained for EspA (green) and for cellular actin (red). Initially bacteria formed a non-intimate attachment with EspA filaments covering the bacterial surface and bridging bacteria and the cell surface (**a**). When bacteria formed A/E lesions, EspA filaments were excluded from the region of intimate contact but were still present on the remainder of the bacterial surface (**b**, arrow). Staining of EspA filaments reduced gradually (**c**, arrow) until none was detectable in mature A/E lesions (**d**). (Scale bar, 5μm).

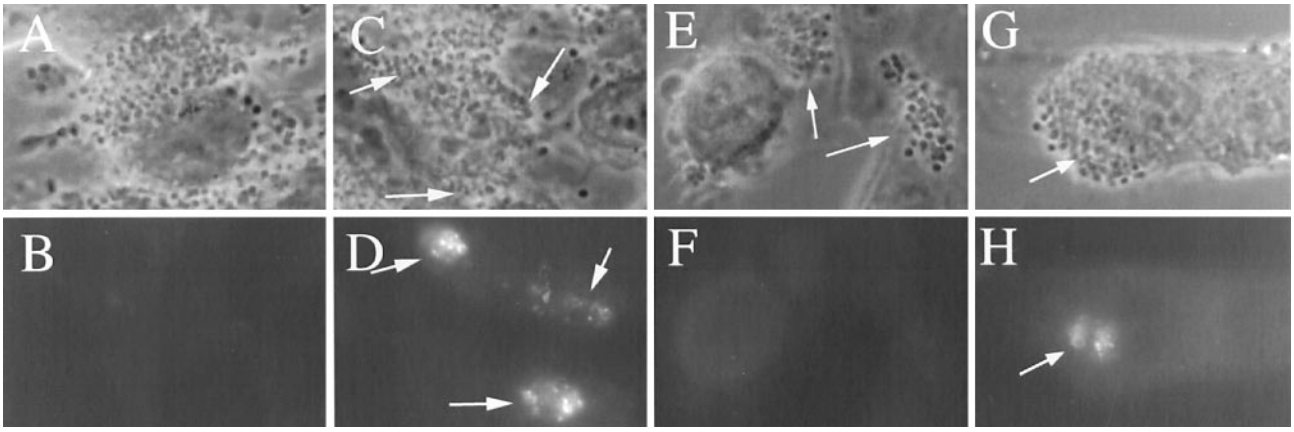

Fig. 7. EspA is required for EspB translocation. HeLa cells were infected for 3.5 h with E2348/69 and UMD872(pMSD2) or for 5 h with UMD872. The infected cells were fixed, permeabilized or not permeabilized and stained with the EspB antiserum. Zones of EspB accumulation were not detected in non-permeabilized HeLa cells infected with wildtype E2348/69 (**A** and **B**) but are evident in permeabilised HeLa cells infected with E2348/69 (**C** and **D**, arrows); zones of EspB accumulation were not detected in HeLa cells infected with the *espA*$^-$ strain UMD872 (**E** and **F**) but they were evident when *espA* was supplied *in trans* [strain UMD872(pMSD2)] (**G** and **H**) (×2000).

Wolff *et al.* (1998) showed that translocated EspB can be found in association with the eukaryotic cell membrane and in the cytosolic fraction. In this study, we tested whether EspB could be detected in either fraction following infection with *espA*$^-$ strain, UMD872. Cytosolic and membrane (Triton X-100 soluble) fractions of E2348/69, as a control, and UMD872-infected HeLa cells were analysed for the presence of EspB by immunoblotting using the EspB antiserum. In addition, these fractions were examined for the presence of tyrosine-phosphorylated Tir using anti-phosphotyrosine antibody. Tyrosine-phosphorylated Tir is membrane-associated (Rosenshine *et al.*, 1996a; Kenny *et al.*, 1997), and thus served as an internal control for assessing the efficiency of the fractionation procedures. As reported by Wolff *et al.* (1998), in HeLa cells infected with E2348/69, Tir was found only in the membrane fraction, whereas EspB was found in both membrane and cytosolic fractions (Figure 8A). In contrast, neither Tir nor EspB were detected in either fraction of HeLa cells infected with *espA*$^-$ strain UMD872 (Figure 8A).

In order to quantify the relative levels of translocated EspB, Wolff *et al.* (1998) used the adenylate cyclase (AC) reporter system (Sory and Cornelis, 1994) and constructed a fusion between the *espB* gene and a DNA fragment encoding the catalytic domain of CyaA (EspB–CyaA fusion protein encoded by plasmid pEspB–CyaA). To further determine the importance of EspA filaments in the translocation of EspB, we compared the ability of 'activated' E2348/69 and *espA*$^-$ strain UMD872 to translocate the EspB–CyaA fusion protein into infected HeLa cells by measuring the level of intracellular [^{3}H]cAMP. Compared with HeLa cells infected with E2348/69 as a control, infection for 1.5 h with E2348/69(pEspB–CyaA) led to a ~143-fold increase in intracellular [^{3}H]cAMP. In contrast, no increase in the level of [^{3}H]cAMP was detected even after prolonged infection (3 h) of HeLa cells with *espA*$^-$ strain UMD872(pEspB–CyaA) (Figure 9A). In order to rule out the possibility that the absence of intracellular EspB is not due to reduced secretion of the fusion protein in the *espA*$^-$ strain background, we compared levels of extracellular EspB and EspB–CyaA

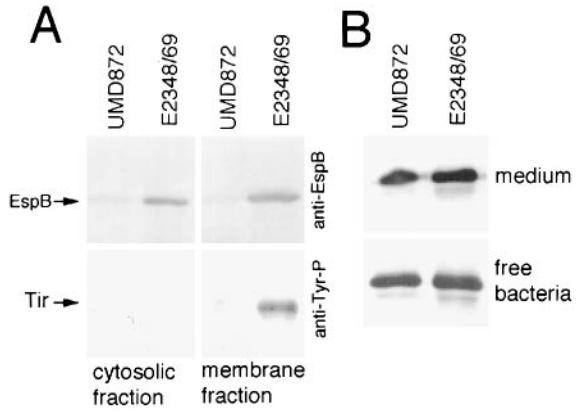

Fig. 8. EspA is required for EspB translocation. (**A**) HeLa cells were infected for 3.5 h with either E2348/69 or UMD872. The infected cells were fractionated into membrane and cytosolic fractions, and the fractions analysed by immunoblotting with the EspB antiserum or with anti-phosphotyrosine antibody. With E2348/69, EspB was localized to both membrane and cytosolic fractions, but EspB was not detected in either fraction of cells infected with the *espA*$^-$ strain UMD872; tyrosine phosphorylated Tir was localized solely in the membrane fraction. (**B**) Lack of translocation of EspB by UMD872 was not due to lack of secretion since comparable levels of EspB were found both in the medium and in free infecting bacteria.

produced by E2348/69 and UMD872. In agreement with a previous report (Lai *et al.*, 1997), we found, using immunoblotting, similar levels of EspB between the two strains both in the growth medium and in free bacteria (Figure 8B). Secretion of EspB–CyaA by *espA*$^-$ strain UMD872 was reduced 7.6-fold compared with that of E2348/69(pEspB–CyaA), as determined by measuring AC enzymatic activity in the medium (Figure 9B). However, this reduced level of activity would not account for the absolute lack of cAMP production in UMD872(pEspB–CyaA)-infected HeLa cells.

Discussion

EPEC exert their pathogenic effects by binding intimately to host intestinal epithelial cells and producing A/E lesions. Three EPEC-secreted proteins, EspA, EspB and EspD,

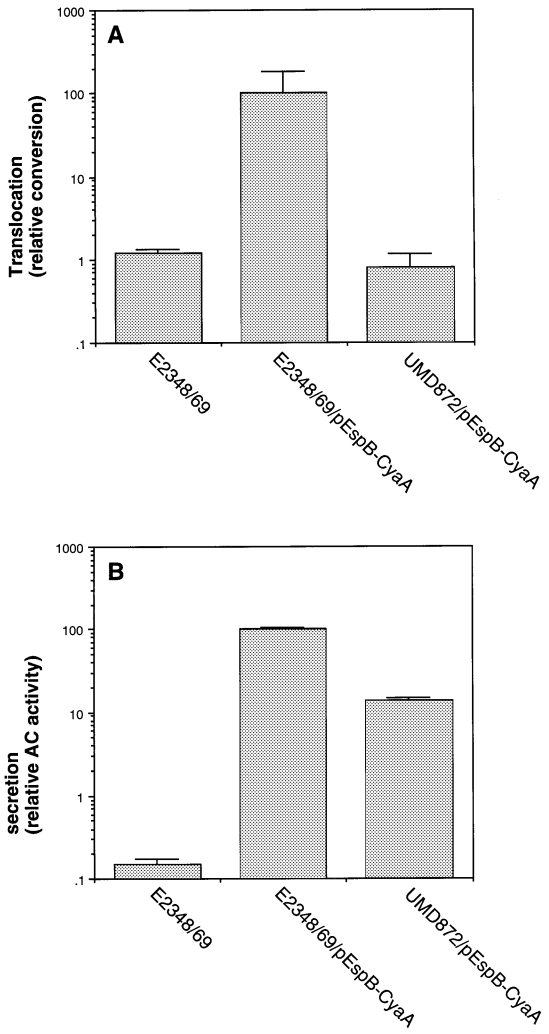

Fig. 9. EspA is required for translocation of EspB–CyaA. HeLa cells were infected either for 1 h with an activated culture of E2348/69/pEspB–CyaA (positive control), E2348/69 (negative control) or for 2.5 h with an activated culture of UMD872 containing pEspB–CyaA. IPTG was then added, the infections continued for a further 30 min and the levels of translocation (% conversion) (**A**) and secreted AC activity (**B**) determined. Infection with E2348/69(pEspB–CyaA) lead to an ~143-fold increase in intracellular [^{3}H]cAMP compared with cells infected with E2348/69 or with UMD872(pEspB–CyaA) (A). However, comparable levels of secreted AC activity were detected when pEspB–CyaA was expressed either in the E2348/69 or UMD872 background (B). Standard deviation values are indicated by bars.

have been shown to be required for the subversion of host-cell signal transduction events required for A/E lesion formation (Donnenberg *et al.*, 1997). However, the mechanisms by which these proteins subvert host cell signal transduction pathways remain unknown. Here, for the first time we have shown that one EPEC-secreted protein, EspA, is a major component of a bacterial surface organelle which is produced during A/E lesion formation, forms a direct link between the bacterium and the host cell and is required for the translocation of another secreted protein, EspB, into host cells. We have incorporated these new features of A/E lesion formation into a model of EPEC interaction with cultured epithelial cells (Figure 10). While this study has not provided any direct evidence as to the mechanism of EspB translocation into host cells, it seems likely that these EspA filaments would play a direct role in the translocation process and it is tempting to speculate that they may act as 'molecular go-betweens', transporting proteins from the bacterium to the host cell. This conclusion is further strengthened by the recent observation that the intimin receptor is, in fact, a LEE-encoded bacterial protein, Tir, whose translocation to the host cell membrane is also dependent upon EspA (Kenny *et al.*, 1997). Furthermore, disappearance of the EspA filaments once A/E lesions have formed would suggest that their expression is regulated, possibly in response to host cell factors, although we cannot rule out the alternative possibility that EspA filament elimination is simply growth-phase regulated, for example as bacteria enter the stationary phase. The structural basis for cell–cell contact and protein translocation has yet to be fully elucidated for any type III secretion system. The observations reported here provide an important new level of detail in the case of EPEC protein translocation and suggest that EspA filaments provide an essential first step in the molecular cross-talk between the bacterium and the host cell. However, it remains to be seen whether similar structures and mechanisms responsible for protein translocation are common to all type III secretion systems.

Although we have not shown directly that the appendages we see by scanning electron microscopy contain EspA, this supposition is strongly supported by indirect evidence—fibrillar structures similar in size to the EspA filaments are seen by transmission electron microscopy after immunogold labelling with anti-EspA and they are not produced by an *espA*$^-$ mutant. Appendages (invasomes), resembling the EspA filaments and visible by scanning electron microscopy on the surface of *Salmonella typhimurium* cells have been described by Ginocchio *et al.* (1994). The invasome and EspA filaments are similar in size and shape, both covering the entire bacterial cell surface and shed after triggering changes in the host cells (A/E lesion formation in the case of EPEC, plasma membrane ruffling in the case of *S.typhimurium*). Both depend on a type III secretion system for their secretion and both types of appendages are absent from bacteria grown in L-broth. However, although Ginocchio *et al.* (1994) reported that invasomes appeared only after exposure to host cells, the presence of the EspA filaments on EPEC grown in DMEM suggests that some constituent(s) of the growth medium trigger the formation of the appendages, rather than signals released by host cells. Another important difference is in the time course of the interaction with host cells—invasomes appear and are shed within 30 min of infection, whereas the appearance and disappearance of EspA filaments occurs over a longer period (several hours). We speculate that *Salmonella* invasomes, like EspA filaments, are involved in mediating protein translocation although, at the present time, no gene products from *Salmonella* have been linked to invasome structures or have been demonstrated to be translocated by them.

The immunogold labelling results are consistent with the EspA filaments being constructed from bundles of smaller fibrils which resemble pili, although the precise number and arrangement of such pili in the intact organelle have yet to be defined. Studies are in progress to determine the detailed structure of these multi-stranded filaments. Roine and colleagues (1997) recently described an extra-

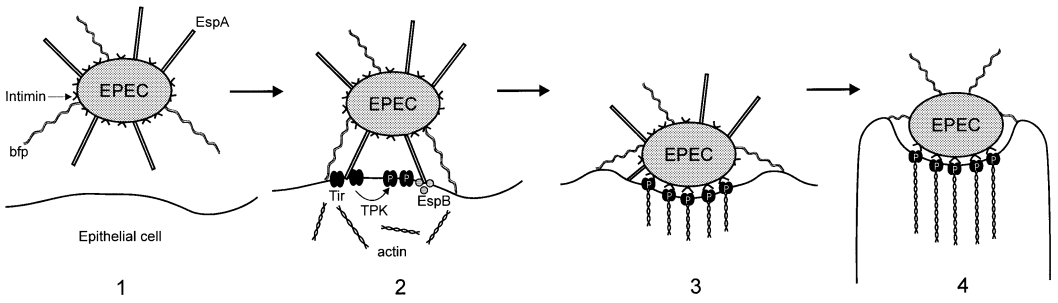

Fig. 10. A model of EPEC interaction with epithelial cells. Growth of bacteria in tissue culture media results in expression of adhesins [bundle forming pili (bfp) and intimin] and production of EspA filaments (stage 1). Initial attachment of EPEC via bfp and EspA filaments stimulates EspB and Tir translocation, and probably translocation of other, as yet to be identified, effector proteins into the host cell. This in turn leads to tyrosine protein kinase (TPK) activation and formation of the intimin receptor (tyrosine phosphorylated Tir) and to actin rearrangements (stage 2). Intimin binds to phosphorylated Tir and polymerized actin accumulates beneath intimately attached bacteria; for this to happen EspA filaments and other surface structures are eliminated from the region of intimate attachment (stage 3). Further actin polymerisation produces the mature A/E lesion in which all EspA filaments and intimin have been eliminated from the bacterial surface (stage 4).

cellular appendage, the Hrp pilus, produced by the plant pathogen *Pseudomonas syringae*. Like EspA, the Hrp pilus consists of a protein exported by a type III secretion system—in this case the protein HrpA. HrpA is essential for the pathogenesis of plant disease, although Roine and co-workers have not presented any evidence to show that the Hrp pilus contacts infected cells. In EPEC, we do not know whether EspA itself mediates the contact of the EspA filament with the eukaryotic cell membrane or whether contact is mediated by another protein located at the tip of the appendage. In agreement with a previous report (Lai *et al.*, 1997), we found that an *espD*$^{-}$ strain secreted only low levels of EspA. We also detected shorter filaments on the *espD*$^{-}$ strain and found that it showed decreased adhesion to target cells relative to the wild-type. This suggests that secretion of EspA and EspD are linked, perhaps because EspD is also a constituent of the filament. EspA filaments are produced by an *espB*$^{-}$ strain, and the anti-EspB antiserum did not stain the filaments, indicating that EspB is not a component of the structure. The identity of the EspA filament receptor on the cell surface is similarly unknown.

Many important species of pathogenic bacteria employ one or more type III secretion systems to deliver subversive effector proteins to the cytosol of eukaryotic cells (Lee, 1997). The characteristic features of the diseases caused by these bacteria vary because of differences in the effector proteins, in the effects of other virulence factors (e.g. intimin in the case of EPEC), or in the properties of the target eukaryotic cells. Despite these differences, the translocation of effector proteins from bacterium to target cell by the type III systems represents a common starting point in the exploitation of host cell functions. Furthermore, the first stage in translocation, movement through the bacterial cell envelope, appears to rely on identical mechanisms in all systems, as several type III systems have been shown mediate secretion of proteins usually exported by other systems (Rosqvist *et al.*, 1995). However, it is often unclear whether a secreted protein is an effector protein, part of the translocation apparatus or both (e.g. SipB of *Salmonella*; Collazo and Galan, 1997). In this study, we have shown a role for the EspA translocase in linking bacterial and host cells; an essential first step in the molecular cross-talk between bacterium and host cell. Wolff *et al.* (1998) provided direct evidence that EspB is translocated into infected HeLa cells. In addition we have shown that while specific staining of EspB was observed in wild-type E2348/69-infected HeLa cells, no such staining was seen in association with cells infected with the *espA*$^{-}$ strain, UMD872. Moreover, EspB, like Tir, was found in neither the cytosolic nor the membrane fraction after infection with the *espA*$^{-}$ strain. We also demonstrated that, when expressed in E2348/69, EspB–CyaA fusion protein is translocated upon infection, while no elevation of relative conversion of [^{3}H]ATP into [^{3}H]cAMP above the control infection was observed when the EspB–CyaA was expressed from *espA*$^{-}$ strain, UMD872. The absence of any increase in the relative conversion is a strong indication that the fusion protein is not translocated, even though *espA*$^{-}$ strain, UMD872, released a lower level of extracellular AC activity. Together these results show that EspA plays a direct role in the translocation of EspB. At present we are testing the possibility that, like EspB (Wolff *et al.*, 1998) and SipB (Collazo and Galan, 1997), EspA might not only be a translocase but may also be an effector/translocated protein itself. In addition, EspA filaments, through their interaction with a cell-surface receptor, might also be able to function as adhesive structures, albeit providing weak adhesion. This could explain the weak initial attachment of plasmid-cured strain, JPN15, which lacks bfp.

The EspA filaments are superficially similar to other bacterial extracellular appendages, such as flagella and pili. We have recently reported that EspA, in common with many other proteins secreted by type III systems, is predicted to contain a coiled-coil domain (Pallen *et al.*, 1997). This not only hints at a common mechanisms for protein translocation in all type III secretion systems, but also suggests that assembly of the EspA filament might be a similar mechanism to that governing the assembly of flagella and pili. In support of this idea, we have found that BLASTP searches report similarities, albeit with low significance (*P* values 0.016–0.999), between EspA and flagellins from a variety of bacterial species (data not shown). In particular EspA shows 33% identity over a 45-residue span with a flagellin from *Yersinia enterocolitica* (Entrez UID 496298) in the region predicted to form a coiled-coil (data not shown). As flagellin monomers are transported through a pore in the flagellar filament (Emerson *et al.*, 1970), it is tempting to speculate that

proteins and/or signals could be carried from EPEC to the host cell through a pore in a hollow EspA filament.

The fact that the EspA filaments (this study) and intimin (Knutton *et al.*, 1997) are eliminated after A/E lesion formation implies that the bacterial cells may have sensed some change in the host cell. Contact-dependent control of bacterial gene expression has recently been described in two settings, synthesis of Yops after *Yersinia*–host-cell contact (Pettersson *et al.*, 1996) and up-regulation of gene expression after binding of P-pili of uropathogenic *E.coli* to erythrocytes (Zhang and Normak, 1996). Based on these precedents, we speculate that the EspA filaments might also, on contacting host cells, transmit a signal from the host cell to the bacterium, altering bacterial gene expression.

Two factors accounted for our discovery of the EspA filaments and their interaction with the eukaryotic cell surface, possession of an antibody against one of the secreted proteins and the use of a more gentle washing procedure in our adherence assays. We suspect that if similar measures were applied to other pathogens with type III secretion systems, structures similar to the EspA filaments and invasome might be found. Two distantly related bacteria, *Rhizobium* and *Chlamydia*, have recently been shown to possess type III secretion systems, and many more are likely to be discovered as we enter the age of bacterial genomics. This means that it is more important than ever that the structures and mechanisms employed by these systems be characterized by the approaches that we have described.

Materials and methods

Bacterial strains, plasmids, tissue culture and cell infection conditions

The bacterial strains used in this study are listed in Table I. A non-polar mutation was introduced into each of the *espA*, *espB* and *espD* genes (Donnenberg *et al.*, 1993; Kenny *et al.*, 1996; Lai *et al.*, 1997). All the E2348/69 derivatives and plasmids were kindly provided by Drs J.Kaper and M.Donnenberg, University of Maryland. Bacterial strains were grown in L-broth, DMEM or L-agar. Media were supplemented with 50 μg/ml kanamycin, 100 μg/ml ampicillin or 30 μg/ml chloramphenicol where appropriate. Hep-2, HeLa and human embryonic lung (HEL) cells were cultured either on glass coverslips (for immunofluorescence staining and scanning electron microscopy) or in 30 mm diameter Petri dishes (for transmission electron microscopy) in DMEM/5% CO_2 supplemented with 10% fetal calf serum (FCS) at 37°C. Bacterial infections were carried out according to the method of Cravioto *et al.* (1979). Subconfluent cell cultures were washed and incubated with bacteria (10 μl bacterial broth culture/ml tissue culture medium) for up to 6 h at 37°C. Cells were washed to remove non-adherent bacteria and fixed in either 4% formalin (for immunofluorescence), 0.1% glutaraldehyde (for immunogold labelling) or 3% glutaraldehyde (for scanning electron microscopy). In some experiments, a gentle (no vigorous agitation) washing procedure was used. EPEC adhere identically to each cell type, but HEL cells were found to be more appropriate for immunofluorescence studies because, being very thin, it was easier to record more in focus micrographs. In some experiments EPEC cultures were activated prior to infection. This was achieved after overnight cultures, grown without shaking in LB broth at 37°C, were diluted 1:50 in DMEM and grown for 3 h without shaking at 37°C and 5% CO_2. The activated EPEC culture, which usually reached OD_{600nm} of 0.1, reacted with cultured cells immediately upon contact (Rosenshine *et al.*, 1996b). When different strains were compared, all cultures were adjusted in DMEM to the same OD_{600nm}, prior to infection. Isopropyl-β-D-thiogalactoside (IPTG) (0.1 mM), was added as needed.

Preparation of His-EspA

A DNA segment encoding EspA (amino acids 14–198) (Kenny *et al.*, 1996) (forward primer *Eco*RI, 5′-GCGAGTACTTCGACATC; reverse primer *Hin*dIII, 5′-TTATTTACCAAGGGATAT) was amplified using E2348/69 as template and cloned into pET28a in *E.coli* BL21 as described (Frankel *et al.*, 1996). The His–EspA polypeptide was purified as suggested by the manufacturer. Briefly, 1 ml of overnight culture of BL21 containing the recombinant pET28a plasmid was inoculated into 100 ml L-broth supplemented with 0.2% glucose and 30 mg/ml kanamycin. The culture was incubated for 2 h at 37°C with shaking and expression of His–EspA was induced by addition of 24 mg IPTG. After an additional 4 h incubation at 30°C the cells were harvested by centrifugation, the supernatant discarded and the pellet resuspended in 8 ml binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) and frozen overnight. The culture was then sonicated at maximum intensity in 10 s bursts for a total of 3 min, with 1 min intervals. The lysate was centrifuged at 4000 r.p.m. for 30 min and the supernatant was loaded onto a 2.5 ml bed volume pre-washed nickel column. After loading of the cell extract the column was washed with the following solutions: 25 ml binding buffer, 7.5 ml wash buffer 1 (30 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9) and 7.5 ml wash buffer 2 (60 mM imidazole, 0.5 M NaCl, 20 mM Tris–HCl, pH 7.9). The bound protein was eluted with 15 ml elute buffer (500 mM imidazole). The fractions were analysed on a 12% SDS–polyacrylamide gel electrophoresis (SDS–PAGE).

Preparation of polyclonal sera

His-tagged EspB (His-EspB) was constructed and purified as described (Frankel *et al.*, 1966). Female Sandy half lop rabbits were immunized subcutaneously with 50–100 μg of His-EspA or His-EspB in complete Freund's adjuvant as described (Knutton *et al*., 1997). The animals were boosted twice with the same antigen in incomplete Freund's adjuvant with 3-week intervals before exsanguination. The antisera were tested positive against the purified EspA or EspB using Western blots and ELISA (data not shown).

Preparation of EPEC-secreted proteins

The analysis of culture supernatants for EPEC-secreted proteins was performed as described previously (Jarvis *et al.*, 1995), with modifications. Bacteria were grown in 10 ml of Eagle's minimal medium at 37°C with shaking to an OD_{600} of 1.0, and aliquots taken to quantify colony forming units (CFU). The bacterial cells were pelleted by centrifugation at 10 000 r.p.m. for 10 min and supernatants passed through filters with a pore size of 0.45 μm. Phenylmethylsulfonyl fluoride (50 μg/ml, Sigma), aprotinin (0.5 μg/ml, Sigma) and EDTA (0.5 mM) were added (all final concentrations), and the Esps concentrated 100-fold with centrifugal filter devices (Millipore Corporation, Bedford, MA). A volume of concentrated samples corresponding to 4×10^6 CFU was loaded and separated by 12% SDS–PAGE. Separated proteins were transferred to nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) as described previously (Knutton *et al.*, 1997) and blocked with 2% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) containing 0.05% Tween 20 for 2 h and probed with anti-EspA antiserum at a dilution of 1:1000 in PBS–0.05% Tween 20. Subsequently, goat anti-rabbit secondary antibody, horseradish peroxidase-conjugate, was used at a concentration of 1:2000 in PBS–0.05% Tween for 1 h, and 4-chloro-1-naphtol (Sigma) used to develop the immunoblots.

Immunofluorescence

To detect intracellular EspB, fixed cells were permeabilized for 5 min with 0.1% Triton X-100 in PBS prior to immune staining. All antibody dilutions and immune reactions were carried out in PBS–0.2% bovine serum albumin (PBS–BSA). Hep-2/HeLa/HEL cell monolayers were incubated with suitable dilutions of EspA or EspB antiserum (generally 1:80) in PBS–BSA for 45 min at room temperature. After three washes in PBS samples were stained with FITC conjugated goat anti-rabbit IgG (Sigma) diluted 1:20 in PBS–BSA for 45 min. In most cases, cells were also labelled for cellular actin by simultaneously staining coverslips with a 5 μg/ml solution of TRITC-phalloidin (Sigma) (Knutton *et al.*, 1989). Preparations were washed a further three times in PBS and mounted in glycerol–PBS. Specimens were examined by incident light fluorescence and phase contrast using either a Leitz Dialux or Zeiss Axiolab microscope.

Electron microscopy

For immunolabelling of bacteria, 10 μl samples of washed bacterial suspensions were applied to carbon coated grids for 5 min, excess liquid removed and the grids immediately placed face down on drops of EspA antiserum (1:80 dilution) for 30 min. After washing, the grids were placed on drops of 10 nm gold-labelled goat anti-rabbit sera (1:20

dilution) (British BioCell International) for 30 min. After further washing in PBS and distilled water, the grids were either air dried or negatively stained with 1% ammonium molybdate and dried. For immunogold labelling of cell-associated bacteria, Hep-2 cell monolayers were briefly fixed for 10 min in 0.1% glutaraldehyde, washed and incubated with EspA antiserum for 2 h at room temperature. Cells were washed and incubated with gold-labelled goat anti-rabbit serum for 12 h at 4°C. After further thorough washing, cells were fixed in 3% buffered glutaraldehyde and processed for thin-section electron microscopy using standard procedures (Knutton, 1995). Samples were examined in a Jeol 1200EX electron microscope operated at 80 kV.

For scanning electron microscopy, HEL cell monolayers were briefly fixed for 10 min in 0.1% glutaraldehyde, washed, incubated with EspA antiserum for 2 h, washed again and fixed in 3% buffered glutaraldehyde. Monolayers were post-fixed in 1% osmium tetroxide, dehydrated through graded acetone solutions and critical point dried. Mounted specimens were sputter coated with gold (Polaron Ltd) and examined in a Jeol 1200EX Scanning Transmission EM operated at 40 kV.

Relative adenylate cyclase (AC) activity and formation of intracellular cAMP

We used the *EspB–CyaA* gene fusion constructed by Wolff *et al.* (1998). In this plasmid the *EspB–CyaA* gene is expressed from the tac promoter (Ptac), enabling IPTG-regulated expression. Relative AC activity in the tissue culture medium obtained from E2348/69(pEspB–CyaA)- and UMD872(pEspB–CyaA)-infected HeLa cells was measured by quantifying the formation of [^{32}P]cAMP from [α-^{32}P]ATP as described (Hanski and Farfel, 1995; Wolff *et al.*, 1998). The [^{32}P]cAMP formed was isolated according to the procedure of Jhonson and Salomon (1991). The results are means of duplicate measurements. Relative per cent enzymatic activity was determined as AC activity from UMD872(pEspB–CyaA) divided by the value obtained with E2348/69(pEspB–CyaA). Measurement of intracellular conversion of [^{3}H]ATP into [^{3}H]cAMP was performed according to Salomon (1991) and Wolff *et al.* (1998). Briefly, HeLa cells (4×10^{6} HeLa cells/100 mm Petri dish) were washed with PBS and incubated with 5 ml of DMEM supplement with [^{3}H]adenine (Amersham, 3 μCi/ml) for 3 h under culturing conditions. Extracellular [^{3}H]adenine was removed by washing, the monolayers infected in the presence of 0.1 mM 1-methyl-3-isobutylxanthine (IBMX, Sigma), and the HeLa cell monolayers washed before lysis with 1 ml of ice cold solution of 2.5% perchloric acid, 0.1 mM cAMP. The lysates were transferred into tubes and incubated for 45 min on ice with occasional shaking. The level of [^{3}H]cAMP formed and the total [^{3}H] uptake were determined as described (Salomon, 1991). These values were used to calculate the percent conversion which reflects the amount of intracellular cAMP formed (Salomon, 1991). The results are expressed as relative conversion, dividing the values obtained from UMD872(pEspB–CyaA)-infected HeLa with those obtained form HeLa cell infected with E2348/69(pEspB–CyaA).

Cellular localization of translocated EspB

In order to localize translocated EspB, infected HeLa cell monolayers were fractionated into five fractions containing: incubation medium, unattached (free) bacteria, the membrane-soluble fraction of cells, the cytosolic fraction of cells and the insoluble fraction containing nuclei, cytoskeletal proteins and proteins of attached bacteria. HeLa cells were seeded at a density of 4×10^{6}/Petri dish. After 16 h, cells were infected for 3.5 h with 5 ml DMEM inoculated with 0.1 ml of overnight EPEC cultures. To acquire the incubation medium fraction and the fraction containing free bacteria, 1 ml of the infection medium was removed and bacteria were isolated by centrifugation. The cleared DMEM medium (50 μl) was mixed with 2.5× loading buffer (50 μl), and the washed bacterial pellet was resuspended in 100 μl of 2.5× loading buffer. Samples containing these fractions were boiled for 7 min, spun (5 min, 23 000 *g*) and the supernatants subjected to SDS–PAGE. To isolate the other fractions, HeLa cells were washed twice with 5 ml cold PBS, scraped into 1 ml of PBS and spun (2 min, 1600 *g*). The pellets were suspended in 1 ml of sonication buffer (50 mM Tris—HCl, pH 7.6, 0.4 mM $NaVO_4$, 0.1 mg/ml PMSF and 10 mg/ml leupeptin) and sonicated for 1 s. This ruptured HeLa cell plasma membranes leaving out intact nuclei and EPEC. The sonicated extracts were spun (15 min, 100 000 *g*), 0.8 ml of the supernatant was removed and precipitated with 10% trichloroacetic acid (TCA). Finally, the pellets were dissolved in 50 ml 1× SDS loading buffer. The pellet of the sonicated fraction (containing plasma membranes, bacteria and nuclei) was suspended in 150 ml of Triton/PBS lysis buffer (1% Triton X-100, 0.4 mM $NaVO_4$, 0.1 mg/ml PMSF and 10 mg/ml leupeptin, in PBS), the lysate was spun (5 min, 23 000 *g*), and the supernatant containing membrane proteins isolated. To obtain the insoluble fraction the remaining pellet was dissolved in 100 ml of 2.5× loading buffer. The lysates were boiled for 7 min, spun (5 min 23 000 *g*) and supernatants subjected to SDS–PAGE. The protein content of each fraction was subjected to SDS–PAGE and Western blotting, using either monoclonal anti-phosphotyrosine (PT66, Sigma) or polyclonal anti-EspB antibodies.

Sequence analysis

BlastP 1.0 was used via a web-server (http://www.ncbi.nlm.nih.gov/BLAST/) to search the NCBI's non-redundant protein database. BlastP 2.0, which produces gapped alignments, was used to search the OWL protein sequence database on a server at Oxford University (http://www.molbiol.ox.ac.uk). For both Blast searches the default options were adopted. PropSearch was used to search the Swissprot protein sequence database on the EMBL-Hedielberg server (http://www.embl-heidelberg.de/prs.html).

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