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A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12

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Summary

Attaching and effacing (AE) bacteria are a diverse group of gastrointestinal pathogens, comprising members of four genera, that cause the intestinal epithelial microvilli to be replaced with raised clusters of filamentous actin that conform to the surface of attached bacteria. We have cloned a 35.4 kb 'pathogenicity island' from the prototype AE bacterium, enteropathogenic *Escherichia coli*, containing all previously described AE genes. Transfer of this pathogenicity island to avirulent *E. coli* converts the recipients into strains that secrete virulence proteins, induce host signal-transduction pathways, and cause AE lesions on cultured epithelial cells. These results demonstrate that this pathogenicity island contains all pathogen-specific genes necessary for inducing AE lesions, and that the defining feature of this class of pathogens can be acquired by an avirulent bacterium in a single genetic step.

Introduction

Diverse bacterial pathogens of the gastrointestinal tract remodel the actin cytoskeleton of infected host cells in a common manner, inducing what are known as attaching and effacing (AE) lesions (Moon *et al*., 1983; Ulshen and Rollo, 1980; Staley *et al*., 1969). In AE lesions microvilli are replaced by compact microfilamentous structures known as 'pedestals' that protrude from the cell surface and cup individual bacteria (Moon *et al*., 1983). Although the bacteria remain outside the cell, their association with the host pedestal is intimate: the pedestal surface conforms to the curvature of the bacterium and maintains a distance of less than 10 nm across much of the bacterial surface (Moon *et al*., 1983). AE lesions are caused by a variety of proved or suspected pathogens of the gastrointestinal tract, including enteropathogenic *Escherichia coli* (EPEC), a potentially fatal diarrhoeal pathogen that strikes infants in developing countries, enterohaemorrhagic *E. coli* O157:H7, the emerging food-borne pathogen that causes bloody diarrhoeae and haemolytic uremic syndrome in the developed world (Tzipori *et al*., 1986), *Hafnia alvei* strains isolated from children with diarrhoeae (Albert *et al*., 1992), and *Helicobacter pylori*, the leading cause of peptic ulcers (Segal *et al*., 1996). AE lesions are also caused by a variety of animal pathogens, including *E. coli*, that cause diarrhoeae in dogs, pigs and rabbits (Cantey and Blake, 1977; Drolet *et al*., 1994; Zhu *et al*., 1994), and *Citrobacter rodentium*, the cause of transmissible colonic hyperplasia in mice (Schauer and Falkow, 1993). The organism in which the AE phenotype was first described, and remains best studied, is EPEC.

Coincident with AE lesion formation, EPEC trigger host signal-transduction pathways (Baldwin *et al*., 1990; 1993; Foubister *et al*., 1994; Rosenshine *et al*., 1992). These pathways are manifest by a number of biochemical hallmarks, including rises in intracellular levels of calcium and inositol triphosphate and by the phosphorylation of several host proteins. The signalling events are linked to AE, as chelating calcium or preventing its release from intracellular stores inhibits lesion formation (Baldwin *et al*., 1993). This link is also supported by genetic studies, as all defined mutations that eliminate signalling also eliminate the AE phenotype (Jarvis *et al*., 1995; Rosenshine *et al*., 1992; Foubister *et al*., 1994a,b; Kenny *et al*., 1996).

AE lesion formation is a complex process, and at least 12 genes are implicated by mutation or by similarity to genes of other pathogens as being necessary for the phenotype (Kenny *et al*., 1996; Jarvis *et al*., 1995; Donnenberg *et al*., 1993b; Jerse *et al*., 1990). Analysis of EPEC mutants has enabled the sorting of these genes into three groups based on the role they play in the AE process. (i) Two genes, *espA* and *espB*, encode proteins that become secreted from the bacterium and associate with the host cell (Kenny and Finlay, 1995; Kenny *et al*., 1996; Donnenberg *et al*., 1993b). Distinct roles for each

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esp gene have not been established, but mutation shows that both are essential for host signalling. (ii) Nine secretory genes, *sepA* to *J* are believed necessary for the secretion of the *esp* signalling proteins (Jarvis *et al*., 1995). All *sep* genes are similar at the amino acid level to genes that encode components of type III secretory systems that secrete virulence factors of other bacterial pathogens. (iii) One adhesin gene, *eae*, encodes the 94 kDa outer membrane protein, intimin, which directly binds the host cell and facilitates the tight association of the bacterial and host membranes (Frankel *et al*., 1994; Jerse *et al*., 1990). This gene (and, by association, the AE phenotype) is necessary for full virulence in humans, as an *eae* mutant shows a significantly diminished diarrhoeal attack rate in volunteers relative to the wild-type parent strain (Donnenberg *et al*., 1993a). Genes homologous to *eae* have been found in all AE bacteria with the exception of *H. pylori*, implying a common mechanism of AE lesion formation among these pathogens (Yu *et al*., 1992; Schauer and Falkow, 1993; Frankel *et al*., 1994).

In recent years it has emerged as a common theme among bacterial pathogens that virulence genes are clustered in distinct stretches of the bacterial chromosome. These clusters are known as pathogenicity islands (Lee, 1996; Knapp *et al*., 1986). They often exceed 30 kb in length and can contain multiple operons encoding several distinct virulence phenotypes (Lee, 1996). We recently described a 35.4 kb pathogenicity island of EPEC termed the LEE, for locus of enterocyte effacement, which contains all known genes necessary for the AE phenotype (McDaniel *et al*., 1995). Probes derived from sequences throughout the LEE's length hybridized to all AE pathogens known to contain an *eae* gene, but not to avirulent relatives of the same genera or species (McDaniel *et al*., 1995). Because of these properties it seemed probable, but was unproved, that the LEE is a functional cassette encoding the AE phenotype. In the present study we attempted to define all sequences necessary for the phenotype. A cosmid clone containing the LEE and little flanking DNA conferred on several laboratory *E. coli* strains the ability to secrete the EspB virulence protein and to induce host signalling, actin rearrangements, and pedestal formation on cultured epithelial cells. These data show that the LEE is a functional unit containing all pathogen-specific genetic information necessary to enable the bacterium to effect the profound changes in the eukaryotic cytoskeleton that define the AE lesion.

Results

Cloning the LEE

The nucleotide content of the published sequences from the LEE is poor in GC bp (Jerse *et al*., 1990; Donnenberg *et al*., 1993b; Jarvis *et al*., 1995; McDaniel *et al*., 1995). Therefore a restriction enzyme with a GC-rich recognition site would be expected to cleave far less frequently in the LEE than in the *E. coli* chromosome as a whole. It therefore seemed possible that an enzyme might exist that does not cut within the LEE but cuts in the nearby flanking DNA of the EPEC chromosome, which would enable the isolation of the LEE on a single DNA fragment containing little flanking DNA. A computerized restriction analysis of the existing LEE and flanking *E. coli* sequences revealed that a candidate for such an enzyme was *Sma*I (recognition site, CCCGGG): no *Sma*I recognition site was present in the sequenced portions of the LEE, but sites were present approx. 720 bp to the left of the LEE and precisely 46 bp to the right. Restriction mapping of the unsequenced regions of the LEE confirmed that *Sma*I did not cut within the LEE and, therefore, that the LEE could be cloned on a single fragment containing only approx. 770 bp of flanking DNA (data not shown).

The LEE was therefore cloned from the chromosome of the prototypic EPEC strain E2348/69 as a single *Sma*I fragment into the cosmid vector pCVD551. This generated the recombinant clone pCVD462. Southern hybridization using previously cloned LEE probes verified that the pCVD462 clone contained the entire, intact LEE (data not shown). This clone was transferred into a variety of avirulent laboratory *E. coli* K-12 strains, including DH5α, HB101 and W3110, and tested for phenotypes associated with AE lesion formation.

Phenotypic testing of the cloned LEE

The earliest known event in AE lesion formation is the secretion of Esp proteins via the *sep* type III secretory system. If the cloned LEE encodes AE lesion formation, one would expect it to encode functional type III protein secretion as well. To assay this phenotype, we tested for the presence of one of the *sep* system's secreted targets, the *espB* gene product, in supernatants of bacterial cultures using a Western blot-based assay. The primary antiserum used in this assay (a gift of Michael Donnenberg) was a rabbit polyclonal antiserum raised against a synthetic peptide corresponding to amino acids 279–298 of the E2348/69 EspB protein. Concentrated supernatants of EPEC E2348/69, HB101(pCVD462), HB101(pCVD462, pMAR7), HB101(pCVD551), and HB101(pMAR7) were probed. Plasmid pMAR7 is an ampicillin-resistant derivative of a naturally occurring 90 kb adherence plasmid found in EPEC E2348/69. This plasmid contains a region that regulates mRNA levels of several genes of the LEE (Gómez-Duarte and Kaper, 1995), so we thought it possible that the presence of the plasmid would be necessary for the expression of LEE-encoded phenotypes. In the supernatants of EPEC E2348/69 and the two HB101 clones

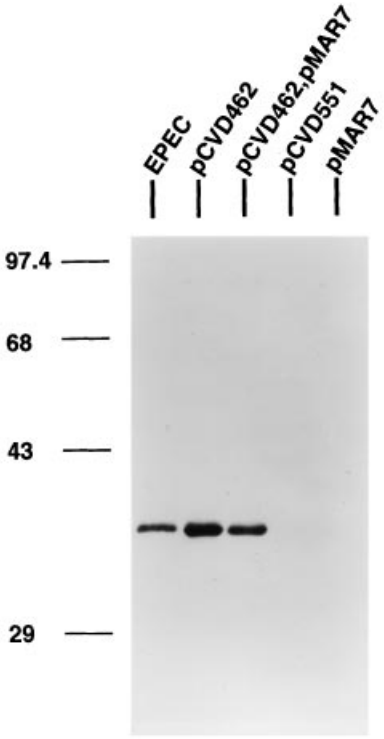

Fig. 1. Anti-EspB Western blot of supernatants from EPEC and recombinant clones. EPEC E2348/69, HB101(pCVD462), HB101(pCVD462, pMAR7), HB101(pCVD551) and HB101(pMAR7) were grown with shaking at 37°C in LB broth until an OD_{600} value of 1.0. After centrifugation, supernatants were filtered and concentrated 100-fold, separated by SDS–PAGE, and transferred to a PVDF membrane. The membrane was incubated with anti-EspB polyclonal rabbit antiserum, then with horseradish peroxidase-coupled antisera against the primary antibody. A band with an apparent molecular mass of 37 kDa corresponding to the *espB* product is seen in lanes containing supernatants of EPEC E2348/69, HB101(pCVD462), and HB101(pCVD462, pMAR7). No such product is seen in the supernatants of the HB101(pCVD551) vector control or HB101(pMAR7). Strains are indicated above their lanes. All plasmids are in HB101. The size scale to the left of the autoradiograph is in kDa.

containing the pCVD462 LEE cosmid, the anti-EspB antiserum detected a single band with an apparent molecular mass (37 kDa) previously reported for the secreted EspB product (Fig. 1). No EspB was detected in the supernatants of HB101 containing the pCVD551 cloning vector, or the pMAR7 adherence plasmid in the absence of the LEE (Fig. 1). The ability of pCVD462 to confer EspB secretion was confirmed using a different background strain: the protein was detected in supernatants of DH5α(pCVD462), but not the DH5α(pCVD551) vector control (data not shown). These results show that the cloned LEE is sufficient to produce and secrete the EspB protein, implying a functional type III secretory system. They also indicate that the regulatory region of the pMAR7 plasmid is unnecessary for this secretion, although the possibility that the region participates in regulation of the phenotype *in vivo* is not ruled out.

Secretion of EspB is essential, but not sufficient, for EPEC-induced host signalling (Foubister *et al*., 1994a; Kenny and Finlay, 1995; Jarvis *et al*., 1995). To determine whether the cloned LEE encodes all additional factors necessary to induce host signalling, we tested the signalling phenotype by probing for the phosphorylation of HP-90, the major substrate of EPEC-induced host protein tyrosine phosphorylation (Rosenshine *et al*., 1992). After 4 h bacterial infections of Int-407 cells, Triton X-100 fractionated host proteins were blotted to a membrane and probed with anti-phosphotyrosine monoclonal antibody 4G10. HB101(pCVD462), but not the vector control, induced phosphorylation of a 90 kDa host protein indistinguishable in size from the major protein phosphorylated during EPEC infection (Fig. 2). Paralleling previously reported results with EPEC, the presence of the pMAR7 adherence plasmid in the LEE clone enhanced, but was not necessary for, the appearance of phosphorylated HP-90. Neither

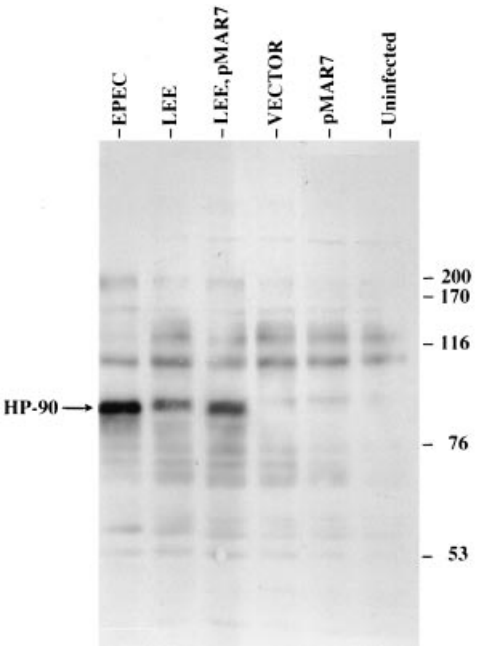

Fig. 2. Anti-phosphotyrosine Western blot of proteins from Int-407 cells infected with EPEC or recombinant clones. Semiconfluent Int-407 cell monolayers were infected for 4 h with EPEC, HB101(pCVD462), HB101(pCVD462, pMAR7), HB101 (pCVD551), or HB101(pMAR7). Cells were solubilized in Triton X-100, and the Triton-soluble fraction was run on an SDS–PAGE gel. After blotting onto a nitrocellulose membrane, proteins were probed with a mAb to phosphotyrosine. Phosphotyrosine-containing proteins were visualized by ECL autoradiography after cross-staining with a horseradish peroxidase-coupled antibody to the primary antibody. Tyrosine phosphorylation of a 90 kDa protein (indicated by the arrow) is induced by infection with EPEC and recombinant strains containing the cloned LEE, but not negative controls containing no bacteria or recombinants without the cloned LEE. Lanes: EPEC, strain E2348/69; LEE, HB101(pCVD462); LEE, pMAR7, HB101(pCVD462, pMAR7); Vector, pCVD551; pMAR7, HB101(pMAR7).

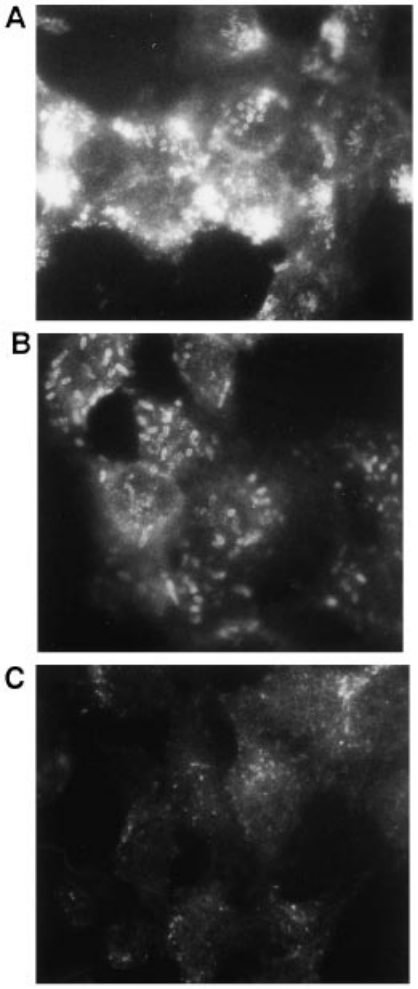

Fig. 3. Anti-phosphotyrosine immunofluorescence staining of Int-407 cells infected with EPEC or recombinant clones. Semiconfluent Int-407 cell monolayers were infected for 3 h with EPEC E2348/69, W3110(pCVD462) or W3110(pCVD551). Fixed, permeabilized cells were then incubated with a mAb against phosphotyrosine, cross-stained with a FITC-conjugated antiserum against the primary antibody, then viewed under fluorescence microscopy. A peri-bacterial pattern of staining is seen in cells infected with EPEC E2348/69 (A), the LEE clone W3110(pCVD462) (B), but not in the vector control W3110(pCVD551) (C).

pMAR7 alone nor the pCVD551 vector control induced a signal that could be distinguished from an uninfected control, indicating a requirement for the LEE for this effect.

Rosenshine *et al*. (1992) have shown that the tyrosine phosphorylation induced by EPEC is a phenomenon that co-localizes with sites of bacterial attachment. Therefore, we tested the ability of the pCVD462 LEE clone to induce tyrosine phosphorylation. Infected epithelial cells were stained with antiphosphotyrosine monoclonal antibodies and observed under immunofluorescence microscopy. In separate infections, Int-407 embryonic intestinal cells were incubated for 3 h with EPEC E2348/69, W3110, W3110(pCVD462), or W3110(pCVD551). In concurrence with the previous findings of Rosenshine *et al*. (1992), EPEC strain E2348/69 induced focal staining that mimicked its localized adherence pattern (Fig. 3A). Similarly, the W3110(pCVD462) induced a pattern of staining that paralleled bacterial adherence (Fig. 3B). The vector control produced no signal that could be distinguished from uninfected cells (Fig. 3C). Thus, the phosphorylation of host proteins conferred by the cloned LEE mimics that of wild-type EPEC not only in the major substrate of phosphorylation, but also in the localized nature of the effect.

Next, the pCVD462 clone was tested for the ability to induce the focal actin accumulation associated with the AE phenotype by staining infected cells with fluorescein isothiocyanate and observing under fluorescence microscopy. EPEC strain E2348/69 and *E. coli* DH5α (pCVD462) showed bright rings of staining associated with adherent bacteria, characteristic of AE lesion formation (Fig. 4). In parallel infections no actin rearrangements were seen after 3 h or 6 h with DH5α alone or DH5α containing the pCVD551 cloning vector (Fig. 4). Giemsa staining revealed that all of these strains adhere to HEp-2 cells (data not shown). Double staining of infected cells with fluorescein isothiocyanate (FITC)–phalloidin and Giemsa confirmed that, as with EPEC strain E2348/69, the actin rearrangements induced by the pCVD462 clone co-localize with adherent bacteria (data not shown). These results indicate that the cloned LEE contains all genes necessary to induce focal actin rearrangements similar to those induced by EPEC. The ability of the pCVD462 clone to confer the induction of actin rearrangements was confirmed by placing the clone in three other avirulent *E. coli* strain backgrounds: the pCVD462 clone, but not the pCVD551 vector control, conferred the ability to induce actin rearrangements in W3110 and HB101.

Because the cloned LEE confers multiple phenotypes associated with AE lesion formation, we finally tested whether it confers AE lesion formation *per se*. Blinded electron microscopic observation of Caco-2 cells infected for 3 h with EPEC E2348/69, HB101(pMAR7) or HB101(pMAR7, pCVD462) confirmed and extended the results obtained from HEp-2 cell infections. The pMAR7 adherence plasmid was included in all strains, as in the absence of the plasmid bacterial adherence was too infrequent to observe reliably. E2348/69 and HB101(pMAR7, pCVD462) exhibited the hallmarks of the AE lesion, including absence of microvilli at sites of attachment, intimate association of bacterial and host cell membranes, and raised pedestal structures (Fig. 5, A and B). Pedestals were characterized by an absence of organelles, ribosomes, or other cytoplasmic granules, consistent with a compact microfilamentous structure (Fig. 5, A and B). With both strains occasional bacteria were seen that appeared to be internalized. These features of these infections sharply contrasted to those of Caco-2 cells infected with HB101(pMAR7).

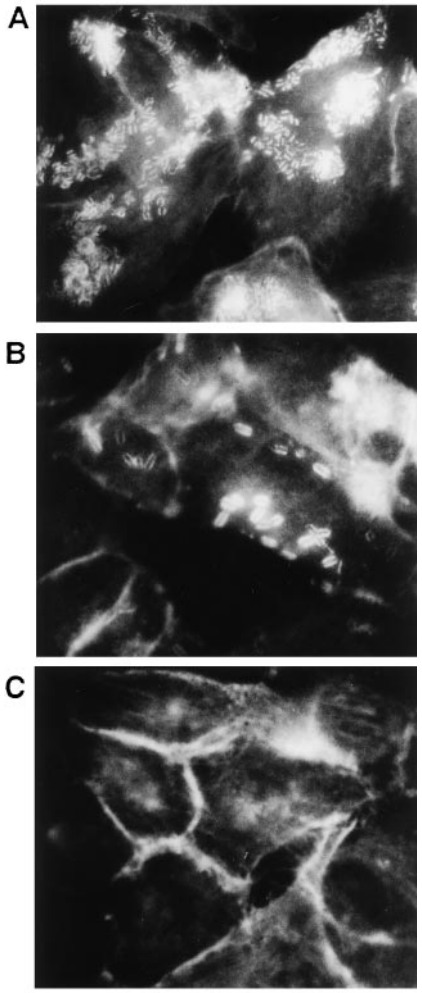

Fig. 4. Fluorescence actin staining of HEp-2 cells infected with EPEC or recombinant clones. Semiconfluent HEp-2 cell monolayers were infected for 3 h with EPEC E2348/69, DH5α(pCVD462), or DH5α(pCVD551), stained for filamentous actin with FITC-phalloidin, then viewed by fluorescence microscopy. Intense actin fluorescence can be seen surrounding adherent bacteria in samples infected with EPEC E2348/69 (A), DH5α(pCVD462) (B), but not in the vector control, DH5α(pCVD551) (C).

Although adherence of bacteria to the monolayer was abundant, this adherence was, without exception, to intact microvilli (Fig. 5C); no observations were made of intimate contact between bacteria and host cell membranes, of cytoskeletal alterations associated with bacteria, or of internalized bacteria (Fig. 5C). Thus, the presence of the LEE profoundly affected the interaction of bacteria with this model of infection. The LEE conferred the attaching and effacing phenotype.

Discussion

The bacterium-induced rearrangement of the intestinal brush-border microvilli known as the AE lesion was first observed by Staley *et al*. (1969) during electron microscopic studies of rabbits experimentally infected with EPEC (Staley *et al*., 1969). The first observations of AE lesions in humans were made in 1980, in biopsies of intestinal tissue from EPEC-infected children (Ulshen and Rollo, 1980). Since this discovery, the phenotype has been subject to ever-increasing investigation, and interest has accelerated in recent years for two reasons. First, investigators have found that the AE phenotype is not just an oddity of EPEC, but is shared by an expanding class of pathogens comprising bacteria of four genera (Schauer

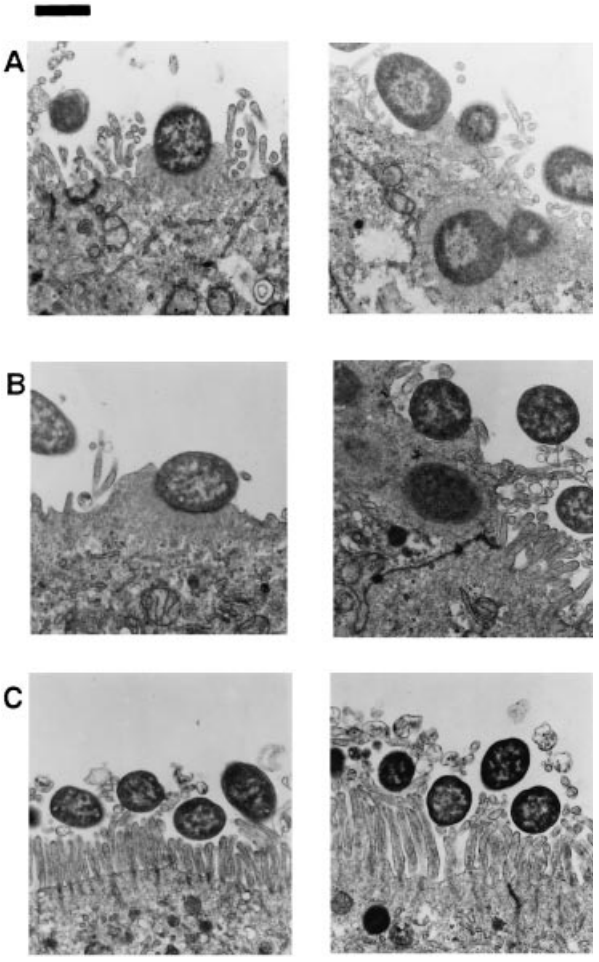

Fig. 5. Transmission electron microscopy of Caco-2 cells infected with EPEC E2348/69 or recombinant clones. Polarized Caco-2 monolayers were infected for 3 h with E2348/69, HB101(pMAR7, pCVD462), or HB101(pMAR7). Specimens were washed, fixed, thin sectioned, stained, then viewed under transmission electron microscopy. Intimate attachment and pedestal formation, characteristic of AE lesions, are evident in specimens infected with EPEC E2348/69 (A), HB101(pMAR7, pCVD462) (B), but not HB101(pMAR7) (C). Note the internalized bacteria in EPEC and HB101(pMAR7, pCVD462) specimens. HB101 (pMAR7) adhere well, but do not attach intimately, efface microvilli, or cause other noticeable cytoskeletal disruptions. The scale bar represents 1 μm. Scale is the same in all photos.

and Falkow, 1993; Tzipori *et al*., 1986; Segal *et al*., 1996; Albert *et al*., 1992). Included in this list are two important and recently identified pathogens of the developed world: *E. coli* O157:H7 and *H. pylori*, the most common agents of haemolytic uremic syndrome and peptic ulcers, respectively (Segal *et al*., 1996; Tzipori *et al*., 1986). The second reason for increased interest in the AE lesion is the growing awareness that bacterial pathogens and, more specifically, the pathways by which they alter the cells of their hosts, provide definable and genetically tractable probes for studying the workings of eukaryotic cells. Because AE bacteria appear to alter the host cytoskeleton in a manner distinct from other actin-rearranging bacteria, an understanding of the AE phenotype should yield new insights into native host pathways for cytoskeleton remodelling.

A necessary step in dissecting the mechanism of AE lesion formation is to delimit the genes responsible for the phenotype. In the present study, we address this issue by cloning a 35.4 kb pathogenicity island of EPEC known as the LEE. Addition of the cloned LEE to avirulent laboratory *E. coli* strains conferred the ability to induce pedestal formation and intimate bacterial attachment indistinguishable from those seen in infections with wild-type EPEC. Furthermore, two pathogenic events associated with EPEC AE lesion formation – the secretion of the EspB virulence protein and the induction of host protein tyrosine kinases – were also conferred by the cloned LEE. These events confirm that strains containing the cloned LEE induce AE lesion formation by the same mechanism used by wild-type EPEC. The results of these studies show that the LEE contains all pathogen-specific genes required to produce the AE phenotype.

While this last statement is true, the present study does not exclude the possibility that some genes within the LEE play no role in AE lesion formation. However, previous work from our laboratory and from others would suggest that the majority of the LEE's genes are necessary for the phenotype. Mutations in 10 different genes, distributed among 29 kb of the LEE, inactivate AE lesion formation (Donnenberg *et al*., 1990; 1993b; Jerse *et al*., 1990; Kenny *et al*., 1996). Moreover, sequences throughout the LEE's length are highly conserved among phylogenetically distant AE pathogens, as would be predicted for sequences which are essential for the phenotype (McDaniel *et al*., 1995). The nucleotide sequence of the entire LEE has recently been completed in our and Michael Donnenberg's laboratories (T. K. McDaniel, L.-C.. Lai, K. Jarvis, Y.-K. Deng, L. Wainwright, M. S. Donnenberg and J. B. Kaper, unpublished data); therefore the roles of the LEE's individual genes in AE lesion formation can be tested by systematic mutation.

The AE phenotype results from a complex process involving the interaction of many bacterial genes. Steps of this process include: (i) the assembly of a type III secretary apparatus composed of at least nine gene products; (ii) the production and secretion of two or more proteins that interact with the host cell and initiate the rearrangement of the actin cytoskeleton; and (iii) the expression of an outer membrane adhesin that mediates intimate binding of the bacterium to the host cell and formation of the pedestal. The clustering of all known genes necessary for AE lesion formation within a pathogenicity island would imply that the LEE is a functional cassette conferring this complex phenotype. Several previously described features of the LEE suggest that it has been acquired by EPEC (and other AE bacteria) during recent evolution. These features include: (i) a G + C nucleotide content (38.4%) that is strikingly lower than that of the *E. coli* chromosome as a whole (51%); (ii) the stringent conservation of LEE sequences within AE pathogens of three genera, including the distantly related *H. alvei*; and (iii) the LEE's insertion in the *E. coli* chromosome within the *selC* tRNA locus, which is the target of insertion of two known mobile DNA elements: pai I, a distinct pathogenicity island found in pathogenic *E. coli* strains that cause urinary tract infections (Knapp *et al*., 1986); and a bacteriophage, ϕR73. If the LEE is a unit that has recently spread among, and provided the defining feature of, AE bacteria, a necessary prediction is that its transmission would convert an avirulent recipient strain into one capable of causing AE lesions. The functionality of the cloned LEE within otherwise avirulent *E. coli*, as described in this study, fulfills this prediction, and demonstrates that a single genetic step can transform the bacterium–host interaction from a simple interaction of benign adherence to a multistep process that damages the host.

Experimental procedures

Maintenance and growth of bacterial strains

Antibiotic selection was applied where appropriate at the following concentrations: ampicillin, 200 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; nalidixic acid, 100 μg ml^{-1}; streptomycin, 100 μg ml^{-1}; tetracycline, 30 μg ml^{-1}.

General recombinant DNA techniques

DNA precipitation, agarose gel electrophoresis, ethidium bromide staining, DNA quantification, alkaline-lysis plasmid extraction, restriction digestion, and DNA ligation were performed by standard protocols as described by Sambrook *et al*. (Sambrook *et al*., 1989). DNA-modifying enzymes and restriction enzymes were from Gibco BRL or New England Biolabs, except *Psp*AI, which came from Stratagene. Chromosomal DNA was purified by extraction with phenol and chloroform as described (Bastin *et al*., 1991). Southern blots were incubated with ^{32}P-radiolabelled DNA probes overnight in hybridization solution (5× standard saline citrate (SSC; 1× SSC = 150 mM sodium chloride, 15 mM sodium citrate,

pH 7.0), 0.1% SDS, 1 mM EDTA, 1× Denhardt's solution (0.02% BSA, 0.02% polyvinylpyrollidone, 0.02% Ficoll 400)) at 65°C. Blots were washed briefly in ≈50 ml of wash solution (2× SSC, 0.1% SDS) at room temperature, then for 1 h in ≈500 ml wash solution at 65°C, then again briefly at room temperature in ≈50 ml of wash solution, then dried and imaged on X-ray film.

Fluorescence actin staining (FAS)

Actin rearrangements were detected by fluorescence microscopy of fixed cells after staining with fluorescein isothiocyanate-coupled phalloidin, as described by Knutton *et al*. (1989) Specimens were observed under a Zeiss Axioskop routine microscope connected to a Zeiss HBO 100 W Attoarc arc lamp, using an FITC filter.

Detection of tyrosine phosphorylated proteins

Phosphotyrosine was detected by immunofluorescence microscopy of fixed cells or by Western blots of Triton X-100 fractions of host cell proteins. The assays were performed essentially as described by Rosenshine *et al*. (1992), except that the primary antibody used in each assay was antiphosphotyrosine mAb 4G10 (UBI). Dilutions of the 4G10 antibody were 1:50 for immunofluorescence microscopy and 1:2000 for Western blotting.

Concentration of bacterial supernatant proteins

Single bacterial colonies were picked from agar plates and seeded into 100 ml Luria–Bertani (LB) broth. Cells were grown shaking with antibiotic selection, as appropriate, at 37°C. Bacteria were grown until their OD_{600} value reached 1.0, and pelleted by centrifuging for 10 min at 15 600 × *g* in a Sorvall RC5B Plus centrifuge (DuPont Medical Products). Cultures were filtered through a 0.45 μm Nalgene filter (Nalge) to remove all remaining bacteria. Protease inhibitors were added to supernatants (0.5 ml of 10 mg ml^{-1} PMSF, 25 μl of 2 mg ml^{-1} aprotonin, and 1 ml of 0.5 M EDTA), and supernatants were concentrated to a volume of 1 ml in a 150 ml Omegacell 10 000-molecular-weight-cutoff filter spin-cell unit (Filtron Technology) under 379.5 kPa nitrogen at 4°C.

Detection of EspB protein from bacterial supernatants

Concentrated bacterial supernatants (15 μl each) were boiled in 5% β-mercaptoethanol sample-loading buffer, separated on a 15% SDS–PAGE gel, then transferred to an Immobilon P polyvinylidene fluoride membrane (Millipore) in a Bio-Rad Trans-Blot electrophoretic chamber. Membranes were blocked in PBS/BSA/Tween for 1 h, and incubated for 1 h in a 1:5000 titre (in dilute PBS/BSA/Tween) of rabbit polyclonal antisera that had been raised against a synthetic peptide corresponding to amino acids 279–298 of the EPEC E2348/69 EspB protein (a gift of Michael Donnenberg). The membrane was washed three times for 5 min in PBS/Tween, incubated for 1 h in a 1:40000 titre of goat anti-rabbit IgG-HRP conjugate in dilute PBS/BSA/Tween, then washed three times for 10 min in PBS/Tween. Reactive proteins were detected by ECL (Amersham) and autoradiography.

Transmission electron microscopy

Caco-2 cell monolayers grown for 18 d (a gift of Sergio Uzzau), were used for infections. Resistance values of all monolayers exceeded 160 Ω cm^{-2} (range, 160–169 Ω cm^{-2}) at the time of infection. Before addition of bacteria, monolayers were washed three times with PBS then incubated for 30 min at 37°C in 5% CO_2 in fresh media lacking serum or antibiotics. Static overnight broth cultures of EPEC (30 μl) and HB101 clones (90 μl) grown in appropriate antibiotics were used to infect separate monolayers. Infection lasted for 3 h at 37°C in 5% CO_2. Monolayers were washed, fixed and post-fixed, then stained *en bloc* in 2% uranyl acetate, dehydrated through graded ethanol solutions, embedded in polybed/propylene oxide and thin sectioned. Thin sections were stained with 2% uranyl acetate and 0.2% lead citrate, then observed using a JEOL 1200EX electron microscope.

Cloning the LEE

The LEE was initially cloned into pCVD550, an *ori* p15a-based (copy number approx. 20) cosmid cloning vector (McDaniel, 1996). Ten micrograms of EPEC E2348/69 genomic DNA and approximately equimolar amounts of pCVD550 were digested with the enzyme *Psp*AI, an isoschizomer of *Sma*I that generates cohesive termini. The two products were ligated with T4 nucleotide ligase and packaged into lambda phage particles. Packaged recombinants were used to transduce *E. coli* DH5α and selected on ampicillin plates. Several-thousand recombinants were screened for the presence of the LEE by hybridizing colony lifts of the recombinants with radiolabelled LEE-specific probe, derived from the *eae* gene (Jerse *et al*., 1990). One well-isolated positive colony was saved for analysis. Extraction and restriction mapping of this clone's cosmid, designated pLEE1, confirmed that it contained the entire 35.4 kb LEE insert (data not shown), but also indicated that it contained two copies of the cloning vector ligated in tandem. Because large tandem DNA repeats can destabilize cosmid inserts (Sambrook *et al*., 1989), it was decided to clone the insert again. For this second cloning it was desirable to use a different vector, as the ampicillin resistance of the pCVD550 vector would be incompatible with the ampicillin-resistant pMAR7 plasmid used in subsequent experiments; cosmid vector pCVD551 (McDaniel, 1996), which also contains a pl5a origin of replication, but has chloramphenicol as a selectable marker, was used for this purpose. To clone the LEE into pCVD551, the insert of the pLEE1 clone was excised from the pCVD551 vector by digestion with *Psp*AI. Without purification of the excised insert from the pCVD550 vector, the digestion product was ligated to *Psp*AI-digested pCVD551. The ligation mixture was packaged into phage and used to transduce DH5α. Transductants were plated on chloramphenicol agar plates to select for those containing the pCVD551 cloning vector. To ensure that recombinants did not additionally contain the pCVD550 vector, which was present in the ligation mixture, chloramphenicol-resistant clones were tested for sensitivity to ampicillin.

Plasmid DNA from 10 chloramphenicol-resistant, ampicillin-sensitive clones was analysed by restriction mapping. One of the 10 clones, designated pCVD462, contained all appropriate LEE fragments and only one copy of the vector.

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