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A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells

(bacterial adhesion/bacterial pathogenesis/diarrhea)

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ABSTRACT **The ability of enteropathogenic *Escherichia coli* (EPEC) to form attaching and effacing intestinal lesions is a major characteristic of EPEC pathogenesis. Using Tn*phoA* mutagenesis we have identified a chromosomal gene (*eae*, for *E. coli* attaching and effacing) that is necessary for this activity. A DNA probe derived from this gene hybridizes to 100% of *E. coli* of EPEC serogroups that demonstrate attaching and effacing activity on tissue culture cells as well as other pathogenic *E. coli* that produce attaching and effacing intestinal lesions, such as RDEC-1 (an EPEC of weanling rabbits) and enterohemorrhagic *E. coli*. The predicted amino acid sequence derived from the nucleotide sequence of *eae* shows significant homology to that of the invasin of *Yersinia pseudotuberculosis*.**

Enteropathogenic *Escherichia coli* (EPEC) are an important cause of infant diarrhea in the developing world (1–5). Diarrhea caused by EPEC can be severe, as evidenced by a 30% fatality rate in a recent nursery outbreak (5). Once a serious cause of "summer diarrhea" and nursery outbreaks in industrialized countries, diarrhea due to EPEC now occurs less frequently in these areas, although outbreaks in nurseries and day-care centers are reported occasionally (6, 7).

Although EPEC were the first *E. coli* to be recognized as a diarrheal pathogen, the elucidation of EPEC virulence factors has lagged behind that of enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC). Unlike that of ETEC and EHEC, the pathogenesis of EPEC does not appear to involve a toxin and no fimbrial colonization factors have been described. A major advance in the understanding of EPEC pathogenesis was the demonstration that EPEC possess a high molecular weight plasmid which is required for full virulence in volunteers (8) and is associated with the ability to adhere to HEp-2 epidermoid carcinoma cells in a pattern described as localized adherence (9, 10). This adherence phenotype is a characteristic of *E. coli* of the major EPEC serotypes (11) and is detectable with a DNA probe derived from one such plasmid called the EAF probe (*E*PEC *a*dherence *f*actor).

Perhaps the most important feature of EPEC pathogenesis is the ability of EPEC to produce characteristic histopathological intestinal lesions in humans or experimental animal models. This lesion has been described by Moon *et al.* (12) as an "attaching and effacing" (A/E) lesion and is characterized by the intimate adherence of bacteria to the enterocyte, dissolution of the brush border at the site of bacterial attachment, and disruption of the cellular cytoskeleton. Within the enterocyte, high concentrations of filamentous actin are present at the site of bacterial attachment and the enterocyte membrane is frequently seen cupping the bacteria, often forming a pedestal-like structure. The production of this lesion can occur in the absence of the EAF plasmid, as evidenced by the observation that A/E lesions are produced by EAF plasmid-cured derivatives of EPEC isolates in experimental animals (13) and on cultured human intestinal mucosa (14) but not by *E. coli* K-12 strains containing an EAF plasmid, although such strains do adhere to HEp-2 cells (15). The detection of the A/E lesion on tissue culture cells has recently been facilitated by Knutton *et al.* (16) through the development of the fluorescence actin staining (FAS) assay. This assay utilizes fluorescein isothiocyanate-labeled phalloidin to detect the polymerized actin filaments that are concentrated at the site of bacterial attachment in the EPEC lesion.

While the mechanism by which EPEC cause diarrhea is not clear, this characteristic lesion is believed to be a crucial component of EPEC pathogenesis. We have used a genetic approach to investigate this critical aspect of EPEC pathogenesis by isolating chromosomal Tn*phoA* mutants of an EPEC strain that do not produce histopathological lesions on the human intestinal Caco-2 cell line and that demonstrate reduced fluorescence on HEp-2 cells in the FAS assay. Analysis of these mutants has yielded significant insights into the fundamental nature of EPEC pathogenesis.¶

MATERIAL AND METHODS

Bacterial Strains. One hundred ninety-three *E. coli* strains of the EPEC serogroups O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (6, 7) were tested for hybridization with the *eae* probe. The above strains, as well as clinical isolates of ETEC, EHEC, and EIEC and *E. coli* strains isolated from healthy adults, were from the culture collection of the Center for Vaccine Development, University of Maryland (Baltimore).

Genetic Techniques. Conjugation was performed by standard methods (17). The transposon Tn*phoA* was introduced into the JPN15 chromosome from the suicide vector pRT733 (18). Electroporation was performed with a Bio-Rad Gene Pulser according to the manufacturer's instructions. DNA probes were ^{32}P-labeled by random priming (19). A 2.8-kilobase (kb) *Bgl* II fragment containing the kanamycin-resistance gene (*kan*) of Tn*phoA* was used as a probe. *In vitro*

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Abbreviations: A/E, attaching and effacing; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; EIEC, enteroinvasive *E. coli*; EHEC, enterohemorrhagic *E. coli*; EAF, EPEC adherence factor; FAS, fluorescence actin staining.

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¶The sequence of the *eae* gene discussed in this paper has been deposited in the GenBank data base (accession no. M34051).

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transcription and translation were performed with a prokaryotic DNA-directed translation system from Amersham according to the manufacturer's instructions. The nucleotide sequence adjacent to the Tn*phoA* fusion points of each mutant was determined by the method of Chen and Seeburg (20), using an 18-base oligonucleotide primer based on nucleotides 24–41 of the IS*50* insertion sequence of Tn*phoA*.The 1.2-kb *Bgl* II–*Sal* I and 2.0-kb *Sal* I–*Eco*RV fragments of pCVD437 were cloned into M13mp18/M13mp19 vectors and nested deletions were generated using the Erase-a-Base system (Promega). The nucleotide sequence was determined by the dideoxy chain-termination method (21) using a Sequenase kit (United States Biochemical). For homology studies, nucleotide sequences were aligned using the GAP program from the Genetics Computer Group, University of Wisconsin, and relationships between the amino acids were assessed as described by Gribskov and Burgess (22).

Tissue Culture Cell Assays. The ability to adhere to HEp-2 cells in a localized manner was determined using the HEp-2 cell adherence assay (23) and the FAS assay was performed using HEp-2 cells as described (16). Caco-2 cells were cultivated by standard methods (24). The monolayers were grown in 35 × 10-mm Petri dishes for 11–13 days to allow differentiation of the brush border, and bacteria were incubated with the cells as done in the HEp-2 cell adherence assay except the incubation period was extended for 8 hr. After incubation, the monolayer was washed with phosphate-buffered saline to remove unattached bacteria and the cells plus adherent bacteria were fixed with 4% paraformaldehyde and 1% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4). The cells were pelleted and then embedded in paraffin, and thin sections were stained with hematoxylin and eosin for light microscopy. Samples for transmission electron microscopy were postfixed with 1% osmium tetroxide and thin sections were prepared by standard methods (25).

Membrane Extraction. Cultures were grown in 200 ml of L broth at 37°C with aeration for 12 hr. The bacteria were harvested by centrifugation (10 min, 4°C, 10,000 × *g*) and then passed through a French pressure cell (138 MPa) three times. Unbroken cells were removed by centrifugation (10 min, 4°C, 10,000 × *g*). The supernatant was incubated with Triton X-100 [1% (vol/vol) final concentration] for 30 min at 37°C and then centrifuged in a Beckman ultracentrifuge (60 min, 4°C, 100,000 × *g*). Pellets were resuspended in 10 mM Tris (pH 8.0) and boiled in SDS/PAGE sample buffer for 5 min before loading onto gels.

SDS/PAGE and Immunoblotting. Outer membrane proteins were fractioned by SDS/7% PAGE (26) and electrophoretically transferred to nitrocellulose sheets. Immunoblotting was done by standard methods (27) using rabbit anti-bacterial alkaline phosphatase (a gift of David Lowe, University of Utah) as the primary antibody followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma).

RESULTS

Isolation of Mutants Deficient in Attaching Activity. Based on the hypothesis that bacterial factors involved in A/E activity would be excreted or located on the bacterial cell surface, we selected Tn*phoA* mutagenesis (28) as a practical way of creating mutants deficient in this activity. The Tn*phoA* system follows the rapid isolation of mutants [detectable as blue colonies on agar containing 5-bromo-4-chloro-3-indolyl phosphate (XP agar)] that contain an in-frame insertion of Tn*phoA* in a gene encoding secreted products.

We chose to mutate strain JPN15, a spontaneously EAF plasmid-cured derivative of EPEC strain E2348/69 that was isolated from a volunteer fed E2348/69 (8). JPN15 produces positive FAS activity on HEp-2 cells after 6 hr of incubation and produces A/E lesions on Caco-2 cells, a human intestinal cell line. After mutagenesis of JPN15 with Tn*phoA*, 96 kanamycin-resistant colonies that were blue on XP agar were isolated and screened by the FAS assay. The occurrence of intense spots of fluorescence on the HEp-2 cells corresponding to the presence of attached bacteria as determined by phase-contrast microscopy was considered to be a positive result. The parent JPN15 and 93 out of 96 JPN15::Tn*phoA* mutants demonstrated the positive FAS phenotype (Fig. 1*A*). No fluorescence other than the normal background fluorescence produced by actin filaments in HEp-2 cells was seen in three of the mutants (JPN15.20, JPN15.36, and JPN15.96) after 6 hr of incubation (Fig. 1*B*). Mutant JPN15.36, unlike JPN15.20 and JPN15.96, was ampicillin-resistant and hybridized with pBR322 on colony blots, suggesting that the entire pRT733 plasmid (a derivative of pBR322) had integrated into the chromosome of this mutant.

To provide the mutants with a greater opportunity to come into contact with the HEp-2 cells, and therefore give factors that mediate the attaching and/or effacing activity a greater chance to act, the EAF plasmid pMAR7 (9) was introduced into the two ampicillin-sensitive mutants by conjugation. As expected, the acquisition of pMAR7 by the mutants resulted in their ability to adhere to HEp-2 cells in the localized adherence pattern. These strains, JPN15.20(pMAR7) and JPN15.96(pMAR7), and strain JPN15(pMAR7) were then tested in the FAS assay after 3- and 6-hr incubations. Strain JPN15(pMAR7), also created by introducing pMAR7 into JPN15 by conjugation, gave strong fluorescence at 3 hr, but

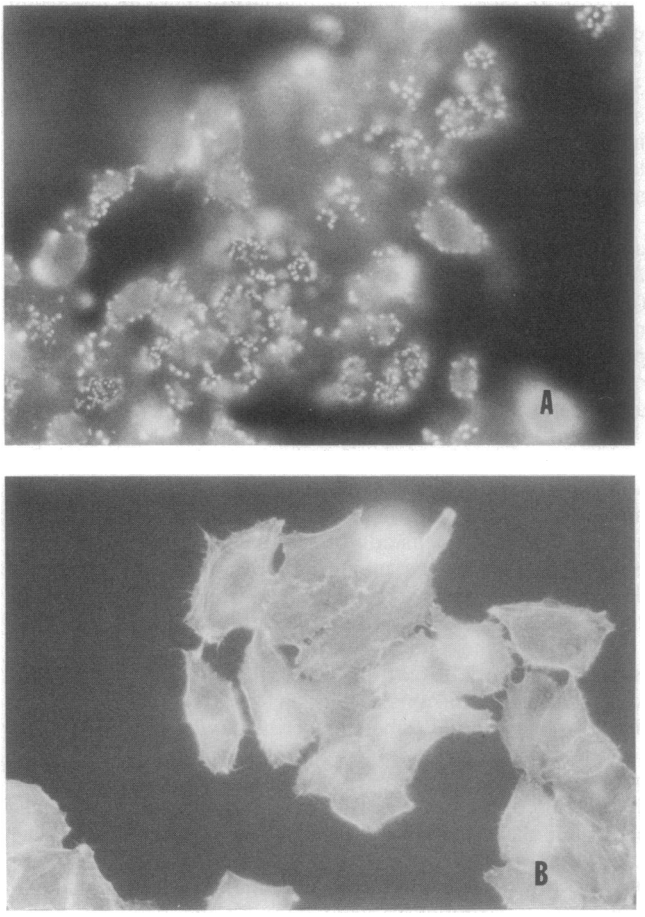

FIG. 1. Fluorescein-phalloidin staining of HEp-2 cells after 6 hr of incubation with JPN15 (*A*) or JPN15.96 (*B*). Samples were examined using a dual phase and fluorescence objective. (×240 and ×250, respectively.)

mutants JPN15.20(pMAR7) and JPN15.96(pMAR7) demonstrated an altered FAS pattern in which a dim fluorescence was seen where microcolonies appeared (data not shown). This pattern has been described as a "shadow" phenotype by Donnenberg *et al.* (29). *E. coli* strain HB101(pMAR7) exhibited localized adherence to the HEp-2 cells but did not produce any fluorescence at the site of microcolony attachment.

The ability of these mutants to adhere to and produce A/E lesions on Caco-2 cells was examined. Although Caco-2 cells are colonic in origin, they spontaneously differentiate into cells that produce a brush border and other characteristics of small intestinal enterocytes (30). EAF plasmid-containing strains E2348/69, JPN15.20(pMAR7), and JPN15.96-(pMAR7) demonstrated marked adherence to the cells as determined by light microscopy of hematoxylin- and eosin-stained sections. Strain JPN15, which lacks an EAF plasmid, showed only moderate levels of adherence to the cells. Mutants JPN15.20, JPN15.36, and JPN15.96, which also lack an EAF plasmid, did not exhibit detectable adherence.

When sections were viewed by transmission electron microscopy, the classic A/E lesions were demonstrated by strains E2348/69 and JPN15 (Fig. 2*A*). As has been observed in other models, the frequency of lesions produced by JPN15 was much less than that of E2348/69. Bacteria were rarely seen in sections from mutants JPN15.20, JPN15.36, and JPN15.96, and none were associated with the cells. Strains JPN15.20(pMAR7) and JPN15.96(pMAR7) (Fig. 2*B*) exhibited brush-border adherence to the cells, in a manner identical to that seen with HB101(pMAR7) (data not shown). No other evidence of EPEC histopathology, such as cytoskeletal damage or high concentrations of polymerized actin, was seen in Caco-2 cells that were incubated with the mutants.

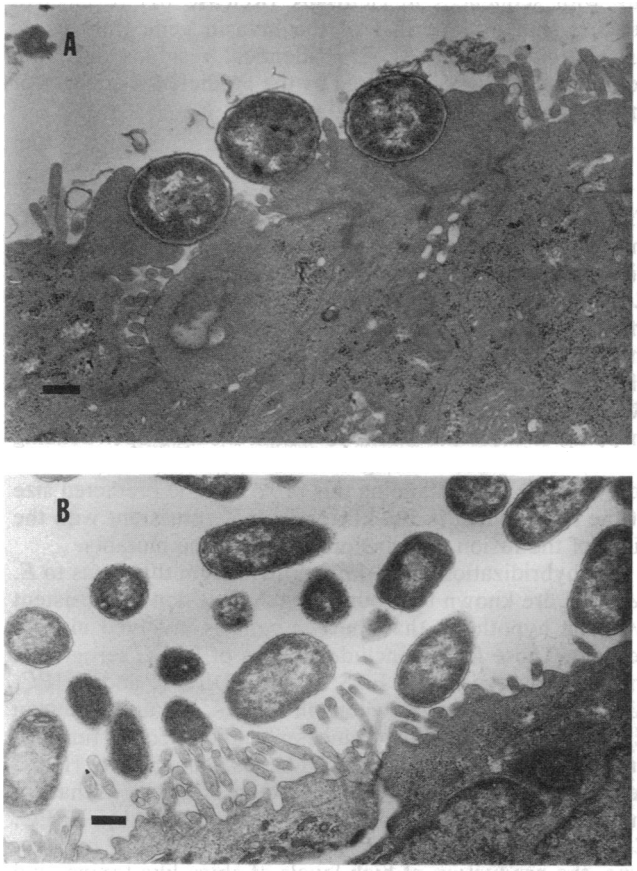

FIG. 2. Transmission electron micrographs of Caco-2 cells incubated with JPN15 (*A*) or JPN15.96(pMAR7) (*B*). Thin sections were examined using a Siemans IA or a JEOL 100B transmission electron microscope at 60 kV. (Bar = 0.5 μm.)

Mapping of the Mutations. The presence of a single Tn*phoA* insertion in mutants JPN15.36 and JPN15.96 was confirmed by Southern hybridization. A single *Mlu* I fragment from chromosomal digests of each of these mutants hybridized with the *kan* probe. In contrast, two fragments from mutant JPN15.20 hybridized with this probe, indicating that double insertions had occurred in this mutant. It was later discovered that a large deletion had occurred in mutant JPN15.20, and so this mutant was not analyzed further.

Chromosomal DNA from JPN15.36 and JPN15.96 was digested with *Sal* I, which cuts 3′ of the *kan* gene of Tn*phoA*, and probed with the *kan* probe. An 11-kb *Sal* I fragment from mutant JPN15.36 and a 5.5-kb *Sal* I fragment from mutant JPN15.96 hybridized with the probe. These fragments were subsequently cloned to create pJY3 and pJY4, respectively, by ligating *Sal* I-digested chromosomal DNA into pBR322 and selecting for kanamycin-resistant transformants of *E. coli* DH5α. Approximately 200 base pairs of DNA adjacent to the Tn*phoA* fusion point of pJY3 and pJY4 were sequenced and oligonucleotide probes of 20 and 18 bases that corresponded to the respective sequences were made. These oligonucleotide probes were hybridized to cosmids from a previously constructed gene bank of E2348/69. Nine cosmids hybridized with the oligonucleotide probes from both clones, but none conferred a positive fluorescence staining pattern in the FAS assay or adherence to HEp-2 cells in *E. coli* HB101.

One of these cosmids, pCVD436, was selected for further study. A 7.0-kb *Bgl* II fragment was cloned from pCVD436 into the *Bam*HI site of vector pTTQ181 (31) to create pCVD437. The location of the Tn*phoA* insertions in each mutant was determined by restriction endonuclease mapping of pCVD437, pJY3, and pJY4 (Fig. 3).

Analysis of Alkaline Phosphatase Fusion Proteins. Preliminary data suggested that the fusion proteins were located in the membrane fraction of the mutants. Separation of the Triton X-100-insoluble membrane proteins by SDS/PAGE followed by immunoblotting with rabbit anti-bacterial alkaline phosphatase revealed alkaline phosphatase fusion proteins of 96 kDa and 128 kDa in mutants JPN15.36 and JPN15.96, respectively (Fig. 4). No band was recognized by the antiserum in the membrane preparation of the parent JPN15.

The mapping data presented in Fig. 3 and the molecular sizes of the fusion proteins are consistent with the insertions in JPN15.36 and JPN15.96 being in the same gene and with the direction of transcription as indicated in Fig. 3. Nucleotide sequence determination of the 3.2-kb *Bgl* II–*Eco*RV fragment of pCVD437 confirmed that the Tn*phoA* insertion

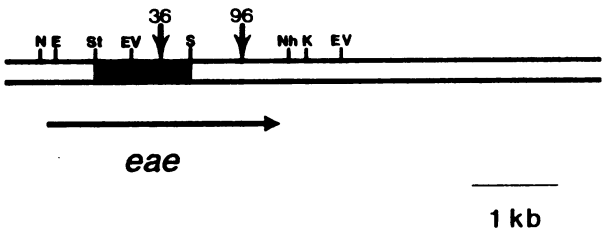

FIG. 3. Restriction enzyme map of the 7.0-kb *Bgl* II fragment of pCVD437. DNA to the right of the *Eco*RV site shown downstream of *eae* has not been mapped. The locations of the Tn*phoA* insertions in mutants JPN15.36 and JPN15.96 are indicated by vertical arrows. The 2817-base open reading frame is indicated by the horizontal arrow, which is placed at the approximate start site of the gene as estimated from the size of the alkaline phosphatase fusion proteins produced by the mutants. The *Sal* I–*Stu* I fragment used as the EAE probe is shown in black. Restriction endonuclease sites: E, *Eco*RI; EV, *Eco*RV; K, *Kpn* I; N, *Nde* I; Nh, *Nhe* I; S, *Sal* I; St, *Stu* I.

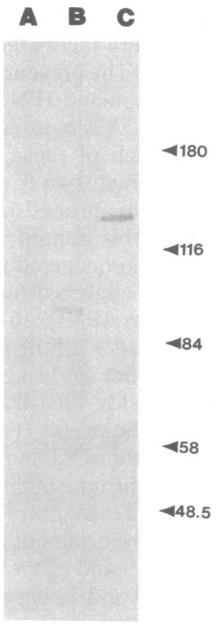

FIG. 4. Immunoblot of Triton X-100-insoluble membrane proteins of JPN15 (lane A), JPN15.36 (lane B), and JPN15.96 (lane C). After SDS/7% PAGE, proteins were transferred to nitrocellulose and probed with anti-bacterial alkaline phosphatase as described in the text. Molecular mass markers are in kilodaltons.

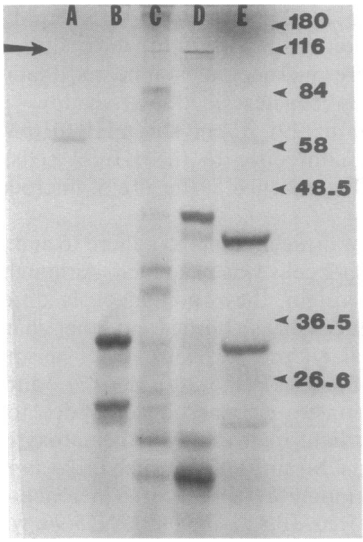

FIG. 5. *In vitro* transcription/translation products of cosmid pCVD436, the *Bgl* II subclone pCVD437, and their respective cloning vectors separated by SDS/10% PAGE. Molecular masses (kDa) were determined from 7%, 10%, and 12% gels. The 107-kDa protein produced by pCVD436 and pCVD437 is indicated by the arrow. Lanes: A, no-DNA control; B, pHC79; C, pCVD436; D, pCVD437; E, pTTQ181.

sites of JPN15.36 and JPN15.96 are within the same 2817-base-pair open reading frame. This gene, which we have designated *eae* (*E. coli* *a*ttaching and *e*ffacing), could encode a 102-kDa protein.

Expression of pCVD437. Although neither pCVD437 nor the cosmid pCVD436 demonstrates positive FAS activity or adherence to HEp-2 cells in *E. coli* HB101 or DH5α backgrounds, the introduction of pCVD437 (but not the cloning vector pTTQ181) into JPN15.96 by electroporation restored full FAS activity on HEp-2 cells. (We are unable to introduce pCVD437 into mutant JPN15.36, as both pCVD437 and JPN15.36 encode resistance to ampicillin.) *In vitro* transcription/translation analysis of pCVD436 and pCVD437 was performed to examine the peptides encoded by these plasmids (Fig. 5). pCVD437 produces peptides of 107, 44, 39, 21, and 19 kDa that are not encoded by the vector pTTQ181. pCVD436 encodes peptides of the same molecular mass plus peptides of 80, 79, 73, 71, 43, 38, and 25 kDa that are not encoded by the vector pHC79. The 107-kDa protein is most likely the product of *eae*, as it is most consistent with the size of the protein predicted by the nucleotide sequence of *eae*.

Construction and Evaluation of the *eae* Probe. A probe spanning the location of the Tn*phoA* insertion in mutant JPN15.36 was constructed by cloning the 1-kb *Sal* I–*Stu* I fragment of pCVD437 into *Sal* I/*Sma* I-digested pUC19 to create pCVD434. The fragment was removed by digestion with *Sal* I and *Kpn* I (which produces the original *Sal* I–*Stu* I fragment plus 3 bases of pUC19 DNA) and evaluated for its ability to hybridize with EPEC and other bacteria by colony blot hybridization.

One hundred ninety-three *E. coli* isolates representing 11 EPEC serogroups were tested in the FAS assay after 6 hr of incubation with HEp-2 cells and for their ability to hybridize with this 1-kb fragment. Ninety-nine isolates were positive in the FAS assay and all 99 hybridized with the DNA fragment. Conversely, all but 2 isolates (an O114 and an O111) that hybridized with the DNA fragment demonstrated positive FAS activity. None of 25 normal flora *E. coli*, 25 ETEC isolates, or 11 EIEC isolates hybridized with the probe. Interestingly, RDEC-1, an EPEC of weanling rabbits (32), and 29 out of 30 EHEC isolates of serogroups O157:H7 and O26:H11 hybridized with the probe.

Homology with the *Yersinia pseudotuberculosis* Invasin Gene. Comparison of the predicted amino acid sequence of *eae* with sequences in GenBank (Release 61) revealed a striking similarity to that of the invasin gene (*inv*) of *Y. pseudotuberculosis* (33): 31% identical residues and 50% conserved residues are shared by the predicted sequences of invasin and the *eae* gene product.

DISCUSSION

The first description of the A/E lesion produced in EPEC infection was in 1969, by Staley *et al.* (34). Although this histopathology has since been described in infants with EPEC diarrhea (35–37) and in various animal models (12, 38), there has been no progress in identifying the bacterial factors that mediate this aspect of EPEC pathogenesis. The isolation of Tn*phoA* mutants of an EPEC strain that have lost their ability to produce A/E lesions on tissue culture cells and the mapping of these mutations to within a single open reading frame, which we have designated *eae*, constitute a significant step towards understanding this process. The predicted size of the *eae* product is 102 kDa, which is consistent with the sizes of the fusion proteins produced by the mutants.

The hybridization of a 1-kb fragment from this locus to *E. coli* that are known to produce the A/E lesion is consistent with the hypothesis that the *eae* gene is involved in A/E activity. These *E. coli* include isolates of EPEC serogroups that are positive in the FAS assay, RDEC-1, and EHEC. RDEC-1 produces A/E lesions in rabbits, but unlike human EPEC strains, RDEC-1 produces plasmid-encoded fimbriae known as AF/R1 fimbriae (39) and does not adhere to HEp-2 cells (unpublished data). EHEC produce A/E lesions that are morphologically identical to those produced by EPEC. EHEC, however, can be distinguished from EPEC by serotype, the production of high levels of shiga-like toxins, and the possession of a distinct (*ca.* 60 MDa) plasmid that encodes fimbriae (40). We consider the detection of sequences homologous to *eae* in RDEC-1 and EHEC of great interest, since it suggests that the mechanism by which these patho-

gens produce the A/E lesion may be the same and raises questions of their evolutionary relatedness. One intriguing possibility is that these pathogens share a common progenitor but diverged with the acquisition of mobile elements such as the EAF plasmid by human EPEC strains, the AF/R1 plasmid by RDEC-1, and the EHEC plasmid and phage-encoded shiga-like toxins by EHEC.

Although the homology between the predicted amino acid sequence of the *eae* gene and that of the invasin gene (*inv*) of *Y. pseudotuberculosis* is limited, it is interesting in light of the recent reports of EPEC invasion of tissue culture cells (41–43). Isberg and Leong (44) have elegantly shown that the invasin protein of *Y. pseudotuberculosis* is an adhesin that binds to HEp-2 cells, and have proposed that invasion of HEp-2 cells by *Y. pseudotuberculosis* occurs by endocytosis of the adherent bacteria. The similarity between the product of *eae* and the invasin of *Y. pseudotuberculosis* is further supported by the work of Donnenberg *et al.* (29), which reports the isolation of Tn*phoA* mutants of E2348/69 that have lost the ability to invade HEp-2 cells and demonstrate a shadow pattern of fluorescence identical to that produced by JPN15.96(pMAR7) in the FAS assay. These mutants map within the *eae* gene, suggesting the *eae* locus is necessary for invasion (29).

Although the *eae* locus is necessary for producing the A/E histopathology that is characteristic of EPEC pathogenesis, the role of the *eae* gene product is not clear. The *eae* locus is necessary but not sufficient for this activity as evidenced by the inability to demonstrate positive FAS activity in *E. coli* K-12 strains carrying *eae*. The fact that none of the Tn*phoA* mutants of the *eae* locus adhered to Caco-2 cells, unlike the parent JPN15, suggests that these mutants lack an adhesin. The involvement of other loci in the production of the A/E lesion requires further investigation.

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