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Enterohemorrhagic *Escherichia coli* O157:H7 Requires Intimin To Colonize the Gnotobiotic Pig Intestine and To Adhere to HEp-2 Cells†

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In a previous study, enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 with a deletion and insertion in the eaeA gene encoding intimin was used to establish that intimin is required for the organism to attach to and efface microvilli in the piglet intestine (M. S. Donnenberg, S. Tzipori, M. L. McKee, A. D. O’Brien, J. Alroy, and J. B. Kaper, J. Clin. Invest. 92:1418–1424, 1993). However, in the same investigation, a role for intimin in EHEC adherence to HEp-2 cells could not be definitively demonstrated. To analyze the basis for this discrepancy, we constructed an in-frame deletion of eaeA and compared the adherence capacity of this mutant with that of the wild-type strain in vitro and in vivo. We observed a direct correlation between the requisite for intimin in EHEC O157:H7 colonization of the gnotobiotic piglet intestine and adherence of the bacterium to HEp-2 cells. The in vitro-in vivo correlation lends credence to the use of the HEp-2 cell adherence model for further study of the intimin protein.

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Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is the leading cause of bloody diarrhea in the United States (5). EHEC colonizes the large intestine of humans and produces Shiga-like toxins (SLTs) that are considered to be essential for EHEC virulence (reviewed in references 27 and 36). In animal models (3, 11, 29, 30, 37), EHEC causes attaching and effacing (A/E) intestinal lesions similar to those caused by enteropathogenic *E. coli* (EPEC) in humans (32) and experimental animals (26, 34). In EPEC, the eaeA (E. coli attaching and effacing) locus, which encodes the protein intimin, has been shown to be necessary, but not sufficient, to cause the A/E lesion in vitro (6, 17). EHEC also carries an eaeA homolog (17), and Donnenberg et al. (7) sought to define the role of the EHEC eaeA gene in A/E lesion formation. These investigators constructed an insertion-deletion mutant of EHEC O157:H7 strain 86-24, called UMD619, that was unable either to adhere to HEp-2 cells in vitro or to colonize the piglet intestine. Plasmids encoding eaeA conferred in vivo adherence and A/E lesion formation to UMD619, but the mutant carrying either EHEC or EPEC eaeA remained unable to adhere to HEp-2 cells in vitro, possibly as suggested by the authors, because the mutation was polar (7).

In the present study, we sought to resolve the contradiction between the in vitro and in vivo data of the earlier investigation by constructing an in-frame deletion in the eaeA gene of EHEC 86-24 to obviate any potential polar effects. We then compared this mutant with its isogenic wild-type partner in the HEp-2 cell adherence assay and in the gnotobiotic piglet infection model.

The eaeA locus from O157:H7 strain 86-24 (13) was cloned by PCR amplification with the GeneAmp PCR kit (Perkin-Elmer Cetus, Norwalk, Conn.) according to the manufacturer’s specifications. The gene fragment was amplified directly from the wild-type 86-24 chromosome with primers derived from previously published EHEC eaeA sequences (Fig. 1) (2, 39). The amplification resulted in a 3,144-bp fragment that encoded the entire eaeA open reading frame and included 186 bp upstream of the coding sequence. The PCR product was treated with T4 DNA polynucleotide kinase and DNA polymerase I (Bethesda Research Laboratories, Gaithersburg, Md.) according to the manufacturer’s protocol to create blunt ends. The treated PCR product was ligated into the EcoRV site of the low-copy-number vector pBRKS (33). The eaeA gene was cloned in both orientations to allow transcription from either P\_\mu_ or P\_T7, and the constructs were designated pEB311 and pEB310, respectively. The recombinants were maintained under the constitutive control of the lac repressor in host strain XL1BlueF (Stratagene Cloning Systems, La Jolla, Calif.). Expression of the insert in pEB310 from the T7 RNA polymerase-dependent promoter (P\_T7 [according to the method described in reference 35]) resulted in an approximately 97-kDa protein as the major product, with a minor product around 80 kDa. The size of the larger expressed protein is consistent with the molecular mass observed for intimin by others (16, 20).

Plasmid pEB290 (Fig. 1) was used to generate the deletion mutant. This plasmid was constructed from a PCR product amplified from the 86-24 chromosome with primer MM1 (starts at the second codon of the eaeA structural gene and includes an ScaI restriction site [Fig. 1 legend]) in combination with primer MM2. The resultant 2,953-bp fragment derived by PCR was digested with ScaI and XbaI and ligated into pBlue-scriptSK* (Stratagene) that had been restricted with SmaI and XbaI. As determined by DNA sequencing of the ends of the pEB290 insert, the 3’ 250 bp had been lost from pEB290. Therefore, we concluded that pEB290 carries a truncated eaeA locus.
We then tested our isogenic strains, 86-24, 86-24[eaeΔ10], and 86-24[eaeΔ10] carrying pEB310, for adherence to HEp-2 cells as described previously (7, 25). We used microscopic evaluation as our primary criterion for scoring a strain as adherent or nonadherent, because quantitative data sometimes gave false-positive findings (24). In confirmation of our previous report (7, 25), wild-type 86-24 formed microcolonies when the bacteria interacted with HEp-2 (human laryngeal epithelial) or HCT-8 (human ileocecal epithelial) cells. This localized adherence (LA) was fluorescence actin staining (FAS) positive, which indicates the polymerization of F-actin at the site of bacterial attachment (Fig. 2A and B). The mutant 86-24[eaeΔ10] was unable to adhere to HEp-2 cells (Fig. 2C). When eae4 was introduced into 86-24[eaeΔ10] on either pEB310 or pEB311, the LA/FAS phenotype was fully restored (Fig. 2D), an observation which demonstrated that intimin alone complements the mutation. Since both of the clones permitted complementation of 86-24[eaeΔ10], the native promoter for eae4 is probably present in the PCR-amplified sequences. B2F1, a naturally eae4-negative O91:H21 EHEC strain isolated from a patient with hemolytic uremic syndrome (15, 28), was also tested with the in vitro adherence assay. The few B2F1 bacteria that bound to HEp-2 cells did so in a diffuse pattern. In contrast, B2F1 transformed with pEB310 exhibited a LA pattern and produced a weakly positive FAS phenotype (data not shown). We also tested 86-24[eaeΔ10] carrying pCVD444 or pCVD436 with the adherence assay. Plasmid pCVD444 contains the eae4 locus from EHEC EDL933 (39) and was previously shown not to complement UMD619 in vitro (7). Cosmid pCVD436 contains the entire eae4 gene cluster from EPEC E2348/69 (17) and also did not complement UMD619 (7). However, both of these constructs complemented the in-frame eae4 mutation in 86-24. Conversely, pEB310 was unable to render UMD619 adherent to the HEp-2 cells (data not shown). We conclude that pEB310, pEB311, pCVD436, and pCVD444 produce a functional intimin product, but only 86-24[eaeΔ10] is able to express intimin or the additional factor or factors required for the full LA/FAS phenotype on HEp-2 cells. These data indicate that a gene downstream of eae4 and in the same operon is required.

To create the in-frame deletion in the chromosomal copy of 86-24 eae4, the wild-type copy of the gene was replaced by double homologous recombination with an internally deleted copy. Plasmid pEB290 was transformed into GM119 (dam-6 dcm-3 [1]) to obtain unmethylated DNA, which was sensitive to the restriction endonuclease BclI. Plasmid DNA was isolated and restricted with BclI to remove an internal 1,125-bp fragment from the gene (Fig. 1). The resulting sticky ends were ligated to create pEB300. The deleted eae4 gene was excised by digestion of pEB300 with XhoI and HindIII, and the fragment containing the eae4 gene was ligated into the BamHII site of a suicide vector, pAM450. Plasmid pAM450 is a derivative of pMAK705 (14) that has a temperature-sensitive origin of replication, carries the sacB/R locus from Bacillus subtilis, which renders the host strain sensitive to sucrose (12, 19), and encodes resistance to ampicillin. These features allow homologous recombination and positive selection for a second recombination event, resulting in resolution and loss of vector sequences. The suicide:eae4 construct, pEB305, was transformed into the wild type, 86-24, by electroporation. Double recombinants that had been cured of the vector sequences were selected by growth on medium containing sucrose and then were screened for ampicillin sensitivity (4, 22). The chromosomal deletion was confirmed by (i) the reduced size of the eae4 fragment after PCR amplification with primers MM1 and MM2, (ii) Southern blot analysis of the mutated chromosomal DNA, (iii) loss of restriction sites within the eae4 gene, and (iv) the failure of an internal probe to recognize the mutated chromosome (data not shown). The resulting strain was designated 86-24[eaeΔ10]. The mutation was confirmed to be in frame by in vitro transcription and translation analysis of the PCR-derived product from 86-24[eaeΔ10]. A truncated protein product of the predicted size (about 68,000 Da) was identified by [35S]methionine labeling of the translation product with the E. coli S30 extract prokaryotic translation kit for linear DNA (Promega, Madison, Wis.) (data not shown). The mutant was otherwise identical to wild-type 86-24 in all characteristics tested, including growth in Luria broth (10 g of tryptone, 5 g of yeast extract, and 5 g of sodium chloride), agglutination with O157 and H7 antisera, inability to ferment sorbitol, and growth on MacConkey agar at 37°C.

![FIG. 1. Plasmid constructs of the wild-type 86-24 eae4 gene (large dark bar) were derived from the sn20MM2 PCR product as described in the text. The primers used for amplification reactions are indicated by the small arrows. The direction of transcription is indicated by the large arrow. Plasmid pEB310 includes the entire 3,144-bp ampiclon cloned into the EcoRV site of pBRKS in the orientation of the T7 RNA polymerase promoter (P₆). Plasmid pEB290 is a clone of the fragment from a reaction with primers MM1 and MM2 in vector pHuescriptSK⁺. Plasmid pEB300 is pEB290 with the internal BclI fragment deleted. The XbaI-HindIII fragment of pEB300, which includes the deleted eae4 gene, was ligated into the suicide vector pAM450 to create construct pEB305. The following primers were used: MM1, ATAACATGACTCATGGTGG; Sn20, CGTGTGATGCTCAATGGAAAC; and MM2, TCTAGAGAAACCGTGAAATGTTGCTCT. RI, EcoRI; B, BclI; RV, EcoRV.](image-url)
for in vitro attachment of EHEC to HeP-2 cells. The hypothesized additional factors may not be directly involved in the adherence of EHEC in vitro but rather may aid in the localization or presentation of intimin by the bacterium. Neither plasmid pEB310 nor pEB311 was able to confer HeP-2 cell adherence to a K-12 host strain (DH5α or XL1-Blue). These data are consistent with the finding that EPEC eaeA is not sufficient to confer adherence to K-12 strains (17) and with a similar observation made by Dytoc et al. (10) with EHEC eaeA cloned from strain CL8.

Next, we evaluated the role of intimin in intestinal colonization, A/E lesion formation, and EHEC-mediated colitis and diarrhea in the gnotobiotic piglet by the method of Francis et al. (11). Five pairs of colostrum-deprived, 24-h-old piglets from the same litter were fed ~10⁷ organisms of either 86-24, 86-24eaeΔ10, 86-24eaeΔ10(pEB310), B2F1, or EDL933 (31) (as the O157:H7, eaeA-positive control). The piglets were euthanized 48 h after challenge (when 72 h old). Animals fed 86-24eaeΔ10(pEB310) were treated with ampicillin at a dose of 250 mg per os per day to ensure maintenance of the recombinant plasmid. During the 48-h period between challenge and euthanasia and prior to necropsy, all piglets were examined for evidence of diarrhea and other signs of disease. After gross examination at necropsy, tissue specimens were fixed in 10% neutral buffered formalin, processed by routine methods, sectioned, and stained with hematoxylin and eosin for histologic examination. Specimens from the duodenum, jejunum, proximal ileum, terminal ileum, cecum, spiral colon (two areas), and the rectum also were fixed in 3% glutaraldehyde (in 0.1 M NaCaC buffer [pH 7.4] with 5% sucrose) for transmission electron microscopic (EM) examination. Glutaraldehyde-fixed tissues were dehydrated by routine methods and embedded in Epon 812 (Ernest Fullam, Latham, N.Y.). Ultrathin sections (70 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate, and photographed with a JEOL 100CX microscope at 80 kV. No bacterial contamination of the pigs was observed from anaerobic and aerobic cultures of the colonic and cecal contents of the animals prior to experimental infection. Recombinant plasmids were maintained in the EHEC strains tested in the piglets, as confirmed by extraction and analysis of plasmid DNA (21) from the bacteria recovered (data not shown).

The in vivo challenge results are summarized in Table 1. Both pairs of piglets inoculated with the wild-type parent
strain, 86-24, and the eae-positive control strain, EDL933, developed diarrhea and had edema in the mesentery of the spiral colon at necropsy. Histologically, strains 86-24 and EDL933 primarily colonized the cecum and spiral colon. Minimal multifocal bacterial adherence was also seen in the terminal ileum of one of two piglets inoculated with EDL933. Histologically and by culture, no evidence of bacterial dissemination to the liver, kidneys, lungs, or brain was detected with either strain. Intimate bacterial adherence and A/E lesion, as described by Staley (34) and Moon (26) for EPEC, were evident by both light microscopy and EM examination of cecum and colon sections of piglets infected with either EDL933 or 86-24 (Fig.

FIG. 3. Electron micrographs of enterocytes in the spiral colon from piglets infected with wild-type 86-24 (A, B, and C), the in-frame deletion mutant 86-24 eaeΔ10 (D), or 86-24 eaeΔ10 carrying pEB310 (E and F). Newborn gnotobiotic piglets were infected for 48 h before euthanasia and necropsy as described in the text. Tissue sections were stained with uranyl acetate and lead citrate for EM analysis. Bacteria were observed intimately associated with the epithelial cells on which microvilli had been effaced (A, ×3,500; B, ×13,800). In some instances, microvilli had sloughed into the gut lumen with bacteria still attached (C). All regions along the spiral colon appeared normal in piglets infected with the mutant strain (D), but when the same strain carried pEB310, wild-type activity was restored (E, ×3,450; F, ×14,200). Intestinal sections from piglets infected with B2F1 resembled those from piglets infected with 86-24 eaeΔ10.
abolished FAS activity but not cytoadherence by the bacte-
rophage phenotype of the CL8-KO1 mutant. This ob-
observation that could confound interpretation of the adher-
ance to enterocyte glycocalyx.

Both piglets inoculated with the mutant strain, 86-24eaeAΔ10, had
formed feces at necropsy. Histologically and by EM ex-
amination, there was no evidence that strain 86-24eaeAΔ10 was able
to colonize piglet intestine and cause the A/E lesion (Fig. 3D).
The few bacteria seen by light microscopy and EM ex-
amination were in the mucus overlying the mucosal epithelium
of the cecum and spiral colon. One of two piglets inoculated with
86-24eaeAΔ10 had slight mesocolonic edema, but no other
gross or microscopic lesions were seen in either piglet. Piglets
inoculated with 86-24eaeAΔ10 (pEB310) had pasty feces and me-
socolonic edema at necrosis. Strain 86-24eaeAΔ10 (pEB310) in-
timately adhered to mucosal enterocytes and caused A/E les-
sions in the cecum and spiral colon (Fig. 3E and F). Histologically, perivesical lymphohistiocytic typhlocolitis sim-
ilar to that caused by wild-type 86-24 and EDL933 was also seen.
One of two piglets inoculated with strain B2F1 had pasty feces and mesocolonic edema at necropsy; the other piglet had
formed stool and no gross lesions. Neither piglet had micro-
scope colitis, and in both piglets, strain B2F1 rods in the
intestines were rare, nonadherent to mucosal enterocytes, and
mainly seen in the gut lumen by histologic and EM examina-
tion (data not shown).

The B2F1 and eaeA Δ10 mutant data indicate that adherence to
mucosal enterocytes is critical for EHEC to cause the A/E
lesion. Complementation of this effect in 86-24eaeAΔ10 by
pEB310 indicates the pivotal role of intimin in intimate adher-
ance of EHEC to the intestinal epithelium. Intimate adherence
in the gnotobiotic piglet model also appears necessary for
certain other lesions, such as enterocyte sloughing and inflam-
mation, to develop. Mesocolonic edema in the absence of
bacterial adherence may be evidence of the effects of SLT
absorbed directly from the gut lumen, since SLT-II (at least
subtype SLT-IIc) can bind to pig intestinal epithelium (38),
and SLT-I (9) and SLT-II (21) injected parenterally induce this
lesion in pigs. In this report, we have shown an absolute re-
quirement for the eae locus both in vitro (for LA and FAS)
and in vivo (for A/E lesion formation) through the use of a
mutant with an in-frame deletion in EHEC O157:H7 eaeA.
The in vivo data confirm the previous finding of Donnenberg
et al. (7) that the eae gene product, intimin, is required for A/E
lesion formation in vivo. We have extended the previous find-
ing by the use of gnotobiotic pigs, which were shown to be free
of contaminating microflora, rather than conventional pigs.
Therefore, the lesions observed in the intestinal tissues are
ascribable only to the challenge strains in the current inves-
tigation. Our in vitro results differ from those of Donnenberg
et al. (7), who found that an insertion-deletion mutation in eaeA
was not complemented for the capacity to adhere to HEP-2 cells
by plasmids encoding intimin. Our in vivo data are also
different from those of Louie et al. (20), who reported that an
insertional inactivation of the eaeA locus in EHEC strain CL8
abolished FAS activity but not cytadherence by the bacte-
rium. The CL8 strain has been found to bind to HEP-2 cells in
a pattern similar to that of enteropathogenic E. coli (32a), an
observation that could confound interpretation of the adher-
ence phenotype of the CL8-KO1 mutant.

The most probable explanation for these discrepant results

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