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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 requirement 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.

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 up-stream 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 specifications. The gene fragment was amplified directly from the 86-24 chromosome with primer MM1 at -250 bp had been lost from pEB290.

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 plBluecriptSK+ (Strategene) 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.

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To create the in-frame deletion in the chromosomal copy of 86-24 eaeA, 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 eaeA gene was excised by digestion of pEB300 with XbaI and HindIII, and the fragment containing the eaeA sequence was ligated into the BamHI site of a suicide vector, pAM450. Plasmid pAM450 is a derivative of pMAK705 (14) that has a temperature-sensitive aderivative of pMAK705 (14) that has a temperature-sensitive

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We then tested our isogenic strains, 86-24, 86-24ΔeaeA, and 86-24ΔeaeA 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 86-24ΔeaeA was unable to adhere to HEp-2 cells (Fig. 2C). When eaeA was introduced into 86-24ΔeaeA 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 86-24ΔeaeA, the native promoter for eaeA is probably present in the PCR-amplified sequences. B2F1, a naturally eaeA-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 86-24ΔeaeA carrying pCV3444 or pCV3463 with the adherence assay. Plasmid pCV3444 contains the eaeA locus from EHEC EDL933 (39) and was previously shown not to complement UM619 in vitro (7). Cosmid pCV3463 contains the entire eae gene cluster from EPEC E2348/69 (17) and also did not complement UM619 (7). However, both of these constructs complemented the in-frame eaeA mutation in 86-24. Conversely, pEB310 was unable to render UM619 adherent to the HEp-2 cells (data not shown). We conclude that pEB310, pEB311, pCV3463, and pCV3444 produce a functional intimin product, but only 86-24ΔeaeA 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 eaeA and in the same operon is required.
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 \(10^8\) organisms of either 86-24, 86-24\(\triangle eae\), 86-24\(\triangle eae\) (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 86-24\(\triangle eae\) (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

FIG. 2. Phase-contrast (A, C, and D) and fluorescent (B) micrographs of EHEC adherence to HEp-2 cells in vitro. HEp-2 cell monolayers were infected for 6 h with either wild-type EHEC O157:H7 86-24 (A and B), 86-24\(\triangle eae\) (C), or 86-24\(\triangle eae\) (pEB310) (D) and then were stained with fluorescein-conjugated phalloidin to visualize F-actin as described previously (23). The wild-type strain, 86-24, forms microcolonies on the HEp-2 cell surface (two representative microcolonies at arrowheads [A]), which results in an FAS-positive phenotype (B). All of the HEp-2 cells in A and B have adherent microcolonies, but not all of the bacterial clusters are in the same plane of focus. The eaeA mutant 86-24\(\triangle eae\) is unable to adhere to the HEp-2 cells (C). Adherence (arrowhead [D]) of the mutant was restored by plasmid pEB310 carrying the wild-type eaeA locus from 86-24. Magnification, \(\times\) ×345.
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. 3).
3A). A/E lesions included the accumulation of electron-dense material at the site of attachment (Fig. 3B). In some areas, sloughed enterocyte fragments and microvilli with attached bacteria were noted in the gut lumen (Fig. 3C). In histologic sections of cecum and spiral colon tissue of piglets infected with 86-24 or EDL933, an inflammatory infiltrate was seen. Inflammation was characterized by scattered neutrophils in the lamina propria and mild diffuse accumulation of serous fluid and perivascular lymphocytes and macrophages in the submucosa.

Both piglets inoculated with the mutant strain, 86-24 eaeAΔ10, had formed feces at necropsy. Histologically and by EM examination, there was no evidence that strain 86-24 eaeAΔ10 was able to colonize piglet intestine and cause the A/E lesion (Fig. 3D). The few bacteria seen by light microscopy and EM examination were in the mucus overlaying the mucosal epithelium of the cecum and spiral colon. One of two piglets inoculated with 86-24 eaeAΔ10 had slight mesocolonic edema, but no other gross or microscopic lesions were seen in either piglet. Piglets inoculated with 86-24 eaeAΔ10 (pEB310) had pasty feces and mesocolonic edema at necropsy. Strain 86-24 eaeAΔ10 (pEB310) intimately adhered to mucosal enterocytes and caused A/E lesions in the cecum and spiral colon (Fig. 3E and F). Histologically, perivascular lymphohistiocytic typhlitis similar 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 microscopic 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 examination (data not shown).

The B2F1 and eaeAΔ10 mutant data indicate that adherence to mucosal enterocytes is critical for EHEC to cause the A/E lesion. Compensation of this effect in 86-24 eaeAΔ10 by pEB310 indicates the pivotal role of intimin in intimate adherence 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 inflammation, 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 for type-1 adherent cells) may cause polar effects on downstream of intimin.

In the gnotobiotic model, the intimin required for intimin function is that the eaeA mutations in both UMD619 and CL8-KO1 have had polar effects on genes downstream of eaeA. The in-frame mutant 86-24 eaeAΔ10 described here appeared to have no such polar effects, a hypothesis supported by the fact that the mutation was complemented by intimin alone. The apparently polar nature of the previous eaeA mutants suggested that multiple gene products are involved in EHEC adherence to epithelial cells. Indeed, McDaniel et al. have reported that a 35-kb chromosomal region called the locus of enterocyte effacement (LEE) contains the genes necessary to cause the A/E lesion and is shared among A/E bacteria (23). More recently, Lai and Donnenberg discovered three open reading frames (10) that are associated with intimin and are involved in A/E lesion formation that are interrupted in EHEC 86-24 (18). A detailed molecular analysis of the region between eaeA and eaeB (8) in EHEC 86-24 will ultimately reveal the loci in this region that are interrupted and not targeted in UMD619. Finally, our results support the relevance of the HEP-2 (or HCT-8) cell assay as a model to study structure and function of EHEC intimin. However, it remains unclear whether this in vitro model or the in vivo gnotobiotic pig model is more reflective of EHEC infection in humans. Thus, the data from both systems should be taken together when dissecting the mechanism by which EHEC O157:H7 cells adhere to the gut epithelium and cause disease.

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