Attaching and Effacing Locus of a *Citrobacter freundii* Biotype That Causes Transmissible Murine Colonic Hyperplasia

DAVID B. SCHAUER[1]†* AND STANLEY FALKOW[1,2]

Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, California 94305-5402,[1] and Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840[2]

Citrobacter freundii biotype 4280 produces attaching and effacing (AE) lesions in the large intestine of laboratory mice and is the causative agent of transmissible murine colonic hyperplasia. AE lesions are also produced by enteropathogenic *Escherichia coli* in humans. Southern analysis revealed that biotype 4280, but not 20 other strains of *C. freundii*, contained DNA homologous to the *eae* (*E. coli* attaching and effacing) gene which is necessary for AE activity by enteropathogenic *E. coli* in vitro. We have cloned and determined the nucleotide sequence of the *C. freundii eae* homolog. Our findings suggest that the *eae* locus of *C. freundii* biotype 4280 is necessary for AE activity and has a role in the pathogenesis of transmissible murine colonic hyperplasia.

A specific variant of *Citrobacter freundii*, designated *C. freundii* biotype 4280, is the etiologic agent of transmissible murine colonic hyperplasia (5), a naturally occurring disease of laboratory mice characterized by epithelial cell proliferation in the mucosa of the descending colon (6). In experimentally infected mice, mucosal epithelial hyperplasia is detectable as early as 4 days after oral inoculation with *C. freundii* biotype 4280 (6). Maximal hyperplasia develops 2 to 3 weeks after infection, and in adult mice the hyperplasia slowly regresses, with the gut returning to normal 6 to 8 weeks after infection (6). Adult mice infected with *C. freundii* biotype 4280 develop colonic hyperplasia but exhibit little morbidity or mortality (6). In contrast, suckling mice develop secondary inflammation in the colon, which is associated with retarded growth, soft feces, and a greater than 50% mortality rate (6). Between 4 and 10 days after oral inoculation of either adult or suckling mice, large numbers of bacteria are found to be intimately associated with the epithelial cell surface in the descending colon. At the site of bacterial attachment, there is dissolution of the brush border and cupping of adherent bacteria by the epithelial cell plasma membrane, often forming raised plaques or pedestals. Cytoskeletal rearrangements occur in the underlying cytoplasm, and there is disruption of the terminal web (17). These histopathologic changes are indistinguishable from those produced by enteropathogenic *Escherichia coli* (EPEC) and termed attaching and effacing (AE) lesions (21).

AE lesions are produced in rabbits by the rabbit EPEC strain RDEC-1 (31) and are produced in humans by human EPEC strains (25). Human isolates of enterohemorrhagic *E. coli* (EHEC) (23) and some human isolates of *Hafnia alvei* (1) also produce AE lesions in experimentally infected animals. A chromosomal locus of a human EPEC isolate that is necessary for the formation of AE lesions in vitro has been identified (16). An internal fragment of this gene, named *eae*, for *E. coli* attaching and effacing, hybridizes with DNA from bacteria that produce AE lesions in vivo, including human

EPEC strains, RDEC-1, most EHEC strains (16), and some *H. alvei* strains (2). The EPEC (16) and EHEC (9, 32) *eae* genes have been cloned and sequenced and are highly homologous throughout most of their open reading frames, with the exception of the more divergent 3' ends (9, 32). It has been suggested that Eae is a bacterial adhesin that is involved in producing the intimate adherence to epithelial cells characteristic of AE lesions (15, 24).

In this study, we report the cloning, nucleotide sequence determination, and expression of a locus from *C. freundii* biotype 4280 that is homologous to *eae*.

(Results from this study were presented in part at the 91st General Meeting of the American Society for Microbiology, Dallas, Tex., May 1991.)

MATERIALS AND METHODS

Media, bacterial strains, and plasmids. Lennox L (LB) broth and LB agar (GIBCO Laboratories, Gaithersburg, Md.) were used for routine cultivation of bacteria. MacConkey agar (Difco Laboratories, Detroit, Mich.) was prepared according to the directions of the manufacturer. For metabolic labeling with [^{35}S]methionine, M9 minimal medium (27) was supplemented with 1.0% (wt/vol) methionine assay medium (Difco), 0.004% threonine and leucine, 0.2% glucose, and 0.0005% thiamine. Ampicillin was added at 100 µg/ml for the culture of strains containing plasmids. The *eae*-positive human EPEC strain JPN15(pMAR7) was kindly provided by J. Kaper (University of Maryland School of Medicine). *C. freundii* biotype 4280 was provided by S. Barthold (Yale University School of Medicine). The additional strains of *C. freundii* that were examined for *eae* and EPEC adherence factor (EAF) homology included 18 clinical isolates from various animal species obtained from the University of California Davis School of Veterinary Medicine; ATCC 29219, an H$_2$S-negative strain isolated from a human kidney; and ATCC 8090, the *C. freundii* type strain. The *E. coli* laboratory strains DH5α (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and XL1-Blue (Stratagene, La Jolla, Calif.) were used as hosts for recombinant

* Corresponding author.
† Present address: Division of Toxicology, Massachusetts Institute of Technology, Cambridge, MA 02139.

TABLE 1. Plasmids used in this study

Plasmid	Description
pDBS2	pREG153 cosmid clone with *eae* homology from a genomic library of *C. freundii* biotype 4280; Ap^r (this report)
pDBS4	Subclone of pDBS2 in which the 4.7-kb *Bam*HI-*Hin*dIII fragment was cloned into pBluescript KS−; Ap^r (this report)
pDBS5	Subclone of pDBS2 in which the 4.7-kb *Bam*HI-*Hin*dIII fragment was cloned into pT7-5; Ap^r (this report)
pDBS6	Subclone of pDBS2 in which the 4.7-kb *Bam*HI-*Hin*dIII fragment was cloned into pT7-6; Ap^r (this report)
pCVD434	*eae* probe containing a 1-kb *Sal*I-*Stu*I fragment of the EPEC *eae* gene cloned into pUC19; Ap^r (16)
pJPN16	EAF probe containing a 1-kb *Bam*HI-*Sal*I fragment of the EPEC EAF plasmid pMAR2 cloned into pCVD315; Ap^r (22)
pREG153	Cosmid cloning vector derived from plasmid pR388; Ap^r (12)
pT7-5,-6	Expression vectors containing the bacteriophage T7 φ10 promoter, multiple cloning site, and ColE1 origin of replication; Ap^r (30)
pBluescript KS−	High-copy cloning vector; Ap^r (Stratagene)

plasmids. The plasmids used in this study are listed in Table 1.

Animal infections. Three-week-old female Swiss Webster mice (Charles River Laboratories, Wilmington, Mass.) were orally inoculated with 10 μl of an overnight culture of *C. freundii* biotype 4280 or sterile broth. Six days after infection, animals were sacrificed and were necropsied. The most distal 5 mm of the descending colon was removed and processed for transmission electron microscopy. The remainder of the descending colon was aseptically collected, weighed, and homogenized in a Stomacher Lab-Blender 80 (Tekmar, Cincinnati, Ohio). Serial dilutions of the tissue homogenate were plated on MacConkey agar to determine the CFU per gram of stool-filled colon of *C. freundii* biotype 4280. *C. freundii* biotype 4280 has a distinct colony morphology on this medium; the center of each colony is pink, while the periphery is white (10).

Microscopy. Animal tissue was fixed in buffered 2% glutaraldehyde for 2 h at 4°C. The tissue was then rinsed twice in buffer (0.1 M sodium phosphate buffer, pH 6.3) and postfixed in 1% OsO$_4$ for 1 h at room temperature. After being rinsed twice with distilled water, the tissue was stained en bloc with 2.5% aqueous uranyl acetate for 1 h in the dark. The tissue was again rinsed twice with distilled water and then was dehydrated through a graded series of ethanol solutions followed by propylene oxide and was embedded in a firm Spurr resin (Polysciences, Inc., Warrington, Pa.). Samples were sectioned, stained with uranyl acetate and lead citrate, and examined on a Philips 201c electron microscope.

Recombinant DNA methods. Total cell DNA was isolated as described previously (20). Plasmid DNA was isolated by one of two methods. pDBS2 was isolated by Triton X-100 lysis as previously described (11). Other plasmids were isolated by alkaline lysis (27). DNA ligations, restriction endonuclease digestions, and gel electrophoresis were performed as described previously (27). Plasmid DNA was introduced into *E. coli* by electroporation with a Gene Pulser

and Pulse Controller (Bio-Rad Laboratories, Richmond, Calif.). DNA sequence analysis was performed by the dideoxy-chain termination method with Sequenase (U.S. Biochemical Corp., Cleveland, Ohio) according to the procedure recommended by the manufacturer. Enzymes were purchased from New England BioLabs, Inc., Beverly, Mass., and Bethesda Research Laboratories. For homology studies, sequences were aligned by the FastDB program of the IntelliGenetics Suite, release 5.4 (IntelliGenetics, Inc., Mountain View, Calif.).

DNA hybridization. The 1-kb *Sal*I-*Stu*I *eae* gene probe and the 1-kb *Bam*HI-*Sal*I EAF probe were isolated from low-melting-point agarose gels after digestion with appropriate endonucleases and were labeled with [α-^{32}P]dCTP (Amersham Corp., Arlington Heights, Ill.) by nick translation (27). Total cell DNA and purified cosmid DNA were digested with endonucleases, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose filters (0.45-μm pore size; Schleicher and Schuell, Inc., Keene, N.H.) by the capillary method and were hybridized as described previously (27). After hybridization, the nitrocellulose filters were washed at 65°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.5% sodium dodecyl sulfate (SDS) for high stringency or 2× SSC with 0.5% SDS for low stringency. Hybridization was detected by autoradiography with XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) in the presence of an intensifying screen. Colony hybridization was performed as described previously (27).

Construction of a *C. freundii* biotype 4280 genomic library. Total cell DNA from *C. freundii* biotype 4280 was partially digested with *Sau*3AI and was ligated to *Bam*HI-digested, phosphatase-treated pREG153 (12). The DNA was packaged (Gigapack II Gold; Stratagene) and the recombinant phage pool was transduced into DH5α. The EPEC *eae* gene probe was used to screen 870 transductants by colony hybridization.

Overexpression of the cloned gene. Metabolic labeling and autoradiography of polypeptides encoded by genes cloned into vectors containing the bacteriophage T7 φ10 promoter (gift of S. Tabor, Harvard Medical School) were performed by a modification of a previously described technique (30). Briefly, XL1-Blue cells, containing T7 RNA polymerase-dependent expression vectors, were grown to an optical density of 0.5 at 600 nm. After the bacteria were washed and resuspended in labeling medium, transcription was induced by infecting the bacteria with the recombinant M13 phage mGP1-2 and adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. Where indicated, rifampin (200 μg/ml) was added to inhibit bacterial transcription. The bacteria were pulsed for 5 min with [^{35}S]methionine (2 μCi/ml) (TRAN^{35}S-LABEL; ICN Biomedicals, Inc., Costa Mesa, Calif.), harvested by centrifugation, and resuspended in SDS loading buffer (27). The labeled bacterial proteins were separated on a 10% gel by SDS-polyacrylamide gel electrophoresis (PAGE) (27). The gel was stained with Coomassie brilliant blue and dried, and the signal was detected by autoradiography with XAR-5 film.

Nucleotide sequence accession number. The nucleotide sequence of the *C. freundii eae* gene has been submitted to GenBank, EMBL, and DDJB and has been assigned the accession number L11691.

RESULTS

AE activity of *C. freundii* biotype 4280. Mice were experimentally infected to confirm the ability of *C. freundii* biotype
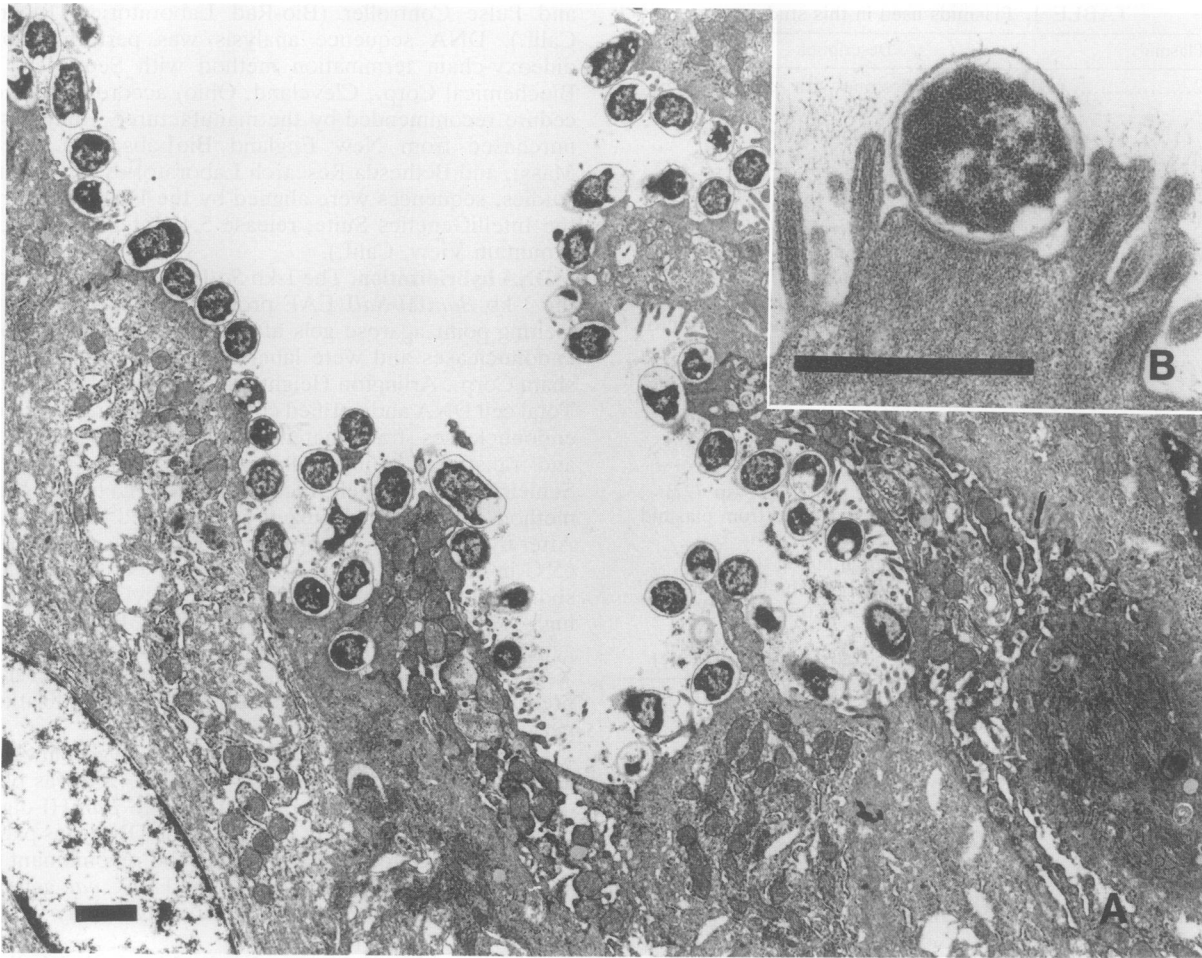
FIG. 1. Transmission electron micrograph of AE lesions in the descending colon of a mouse 6 days after oral inoculation with *C. freundii* biotype 4280 (A) and a single bacterium eliciting cytoskeletal rearrangements and destruction of the terminal web (B). Each bar equals 1 μm.

4280 to produce AE lesions. Animals orally inoculated with this bacterium developed typical AE lesions in the descending colon that were evident 6 days postinoculation (Fig. 1). Quantitative microbiology of tissues taken from these animals indicated that *C. freundii* biotype 4280 had colonized the descending colon to a density of 10^8 to 10^{10} CFU/g of stool-filled colon. Animals inoculated with sterile broth did not develop AE lesions and were not colonized with *C. freundii* biotype 4280.

Hybridization of the *eae* probe with *C. freundii* DNA. A 1-kb internal restriction fragment of the EPEC *eae* gene has been previously reported to hybridize to DNA from EHEC, RDEC-1 (16), and *H. alvei* (2). Total cell DNA from 21 *C. freundii* isolates was probed for *eae* homology by Southern analysis. Under conditions of high stringency, the *eae* gene probe hybridized to a single 18-kb *Eco*RI fragment of DNA from *C. freundii* biotype 4280 but did not hybridize to DNA from the other *C. freundii* strains (Fig. 2 and data not shown). Even under conditions of low stringency, no hybridization of the *eae* probe to DNA from the other *C. freundii* strains was detected (data not shown). A recombinant cosmid, designated pDBS2, was identified from a genomic library of *C. freundii* biotype 4280 by colony hybridization with the *eae* probe and was confirmed by Southern analysis

to contain the entire 18-kb *Eco*RI fragment of *C. freundii* biotype 4280 DNA (data not shown).

DNA from the 21 *C. freundii* isolates were also examined for EAF homology. Human EPEC strains contain related, high-molecular-weight EAF plasmids which are required for full virulence in vivo (19) and mediate localized adherence to mammalian cells in vitro (3). Homology to the EAF probe, a 1-kb fragment of the EAF plasmid pMAR2, correlates with localized adherence in vitro (22). Even under conditions of low stringency, no hybridization of the EAF probe with *C. freundii* DNA was detected (data not shown).

Nucleotide sequence of the *C. freundii* biotype 4280 *eae* locus. A 4.7-kb fragment of DNA from the recombinant cosmid pDBS2 (Fig. 3) was subcloned into pBluescript KS− to produce the plasmid pDBS4. The nucleotide sequence of the *C. freundii* DNA insert in pDBS4 was then determined. Nucleotide sequence analysis revealed several open reading frames, one of which was 2,808 bp in length and could potentially encode a protein of 936 amino acids. It has been reported previously that the EPEC and EHEC *eae* sequences, with open reading frames of 2,817 and 2,802 bp, respectively, are conserved but have significant sequence divergence in the 3′-most 700 bp (9, 32). The greatest homology between the *eae* genes of *C. freundii* and *E. coli*
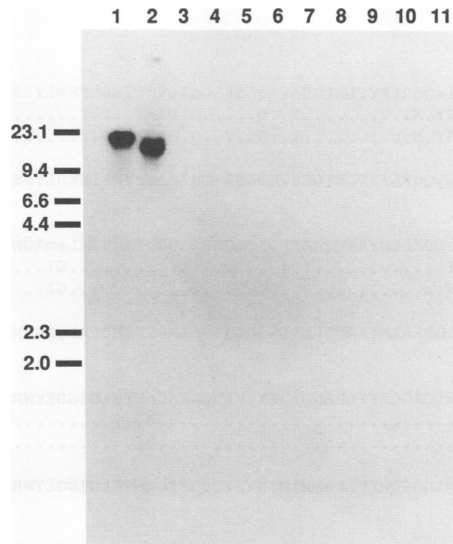
FIG. 2. Southern blot of EcoRI-digested total cell DNA hybridized with the EPEC eae gene probe and washed under conditions of high stringency. Lanes 1 and 2 contain DNA from the human EPEC strain JPN15(pMAR7) and DNA from C. freundii biotype 4280, respectively. Lanes 3 through 11 show DNA from C. freundii strains isolated from the following: 3, rabbit gallbladder; 4, finch lung; 5, feline pleura; 6, equine abscess; 7, fowl abscess; 8, equine lung; 9, canine muscle; 10, python gastrointestinal tract; and 11, tortoise kidney.

was also found in the 5' end of the sequences. There was 87% nucleotide identity in the first 2,106 bp of the C. freundii eae gene to the EPEC eae gene and 84% nucleotide identity to the EHEC eae gene. The remaining 3' 702 bp of the C. freundii gene had only 61% nucleotide identity to the EPEC gene and only 57% identity to the EHEC gene. The 5' homology between C. freundii and EPEC was found to extend upstream of the coding region of eae. Immediately upstream of the EPEC eae gene is an open reading frame of 468 bp, the function of which is unknown. This open reading frame, which has been called ORFU, was also found to be present in C. freundii biotype 4280. Downstream of the C. freundii eae gene, beginning 14 bp 3' of the stop codon, a series of no less than 35 direct repeats of the heptameric sequence CCCGGCA was identified. The actual number of repeats and the complete nucleotide sequence 3' of these repeats were not determined.

An alignment of the predicted Eae proteins of C. freundii, EPEC, and EHEC is shown in Fig. 4. The predicted C. freundii Eae protein has 78% amino acid identity and 82% similarity to the EPEC Eae and 76% identity and 80% similarity to the EHEC Eae.
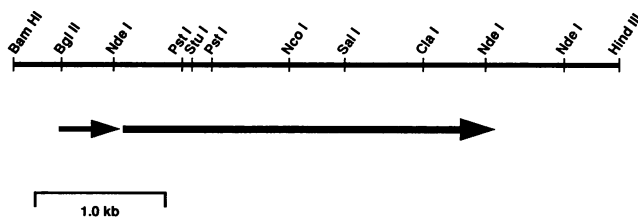

FIG. 3. Physical map of the 4.7-kb BamHI-HindIII fragment of C. freundii biotype 4280 DNA that contains eae homology. Arrows indicate the open reading frames with homology to ORFU and eae.

Expression of the C. freundii biotype 4280 eae homolog. While the predicted molecular mass of the EPEC eae gene product is 102 kDa, the molecular mass of the Eae protein estimated by mobility with one-dimensional SDS-PAGE is 94 kDa (15). There is also some evidence that the EHEC eae gene product is a protein of ca. 94 kDa (29). We determined the relative mobility of the protein encoded by the C. freundii eae locus in E. coli. For this purpose, the 4.7-kb BamHI-HindIII fragment of pDBS4 was subcloned into the expression vectors pT7-5 and pT7-6 to produce pDBS5 and pDBS6. These two plasmids differ only in the orientation of the cloned C. freundii DNA fragment with respect to the T7 phage φ10 promoter. After inducing transcription from the φ10 promoter, in the presence of rifampin, the major translational product encoded by pDBS5 was found to be ca. 94 kDa (Fig. 5). No polypeptides were detected when pDBS6 was induced in the presence of rifampin.

DISCUSSION

We have identified a candidate virulence determinant of C. freundii biotype 4280 that is not present in other strains of C. freundii. Typical biotypes of C. freundii are commonly isolated from the environment, cause opportunistic infection in many animal species and in humans (26), and fail to cause colonic hyperplasia when orally inoculated into laboratory mice (8). In contrast, C. freundii biotype 4280 does not infect rats or hamsters (8) and, in mice, produces disease that is limited to the gastrointestinal tract (5). The C. freundii biotype 4280 eae locus has a high degree of nucleotide homology to the EPEC eae gene, which is necessary for the formation of AE lesions by EPEC on mammalian cells in vitro (16). We are analyzing eae mutants of C. freundii biotype 4280 to confirm the role of this locus in the formation of AE lesions in vivo and in the induction of colonic hyperplasia. We are also expressing the C. freundii biotype 4280 eae homolog in typical biotypes of C. freundii and testing these constructs for the ability to colonize and produce disease in mice.

The function of the eae gene product in EPEC and EHEC pathogenesis is not completely understood. EPEC mutants that do not express eae do not produce AE lesions and, in the absence of the EAF plasmid, do not adhere to mammalian cells in vitro. However, when expressed in a K-12 strain of E. coli, the EPEC eae gene is not sufficient to promote AE lesion formation or adherence to mammalian cells. The eae loci of EPEC (16) and EHEC (9, 32) share sequence similarity with the inv (invasin) gene of Yersinia pseudotuberculosis and Yersinia enterocolitica. The inv gene product is an outer membrane protein that mediates adherence to and entry into mammalian cells in vitro through interactions with β_1 integrins on the eukaryotic cell surface (13, 14). The C. freundii biotype 4280 locus was also found to have homology with inv. Our sequence data suggest that the C. freundii eae locus encodes a protein that is a member of a family of related bacterial virulence determinants that can function as adhesins.

Examination of the predicted amino acid sequence of the C. freundii eae locus revealed greater homology to the Eae proteins of EPEC and EHEC in the amino-terminal region than in the carboxy-terminal region. The carboxy-terminal 236 residues of the predicted C. freundii polypeptide exhibited about the same degree of homology to EPEC Eae and EHEC Eae as the E. coli proteins do to each other (between 56 and 62% amino acid similarity). It has been suggested that the carboxy-terminal divergence between the Eae proteins

```
C.f.    1 MIiHGFctgTRHKHKLrKTFIMLgAGLGLFFsVNQNSFANGENYFKLraDSKLinnNiaQdRLFYTLKTGEsVAqLSKSQgIsvpvIWSLNKHLYSSESE
EPEC    1 ..T...YaR.......K......S........Y...............GS....LTH.SY.N..........T..D.....D.NLST..............
EHEC    1 ..T..cYtR.......K..l...S........Y...............GS....LTHdSY.N..........T..D.....D.NLST..............

con       MItHGfytrTRHKHKLkKTfIMLsAGLGLFFyVNQNSFANGENYFKLgsDSKLlthnsyQnRLFYTLKTGEtVAdLSKSQdInlstIWSLNKHLYSSESE


C.f.  101 MMKAsPGQQIILPLKKLsaEYStLPiLGtAPvVAAadVAGHT    kKMSqDtTKSNtsDDKALNYAAQQAASLGSQLQSRSLNGDYAKDTALsmAGNQAS
EPEC  101 ....e...........PF...A..L..S..L...GG.....NKLT...P.V....MT.......................................GI......
EHEC  101 ....a...........PF...A..L..S..L...GG.....NKLT...P.V....MT.......................................GI......

con       MMKA-PGQQIILPLKKLpfEYSaLP1LGsAP1VAAggVAGHTnk1tKMSpDvTKSNmtDDKALNYAAQQAASLGSQLQSRSLNGDYAKDTALg1AGNQAS


C.f.  198 SQmQAWLQHYGTAEVNLQSGNNFDGSSLDFLLPFYDtEnMLAFGQVGARYIDSRFTANLGAGQRFFLPENMLGYNVFIDQDFSGnNTRLGIGGEYWRDYF
EPEC  201 ..L.........................S.K.........................................D..............
EHEC  201 ...Q.............d...........S.K.....................a...............D..............

con       SQlQAWLQHYGTAEVNLQSGnNFDGSSLDFLLPFYDsEkMLAFGQVGARYIDSRFTANLGAGQRFFLPeNMLGYNVFIDQDFSGdNTRLGIGGEYWRDYF


C.f.  298 KSSVNGYFRMSGWHESYNKKDYDERPANGFDIRFNGYLPSYPALGgKLMYEQYYGDNVALFNaDKLySNPGAvTVGVNYTPIPLVTMGIDYRHGTGNEND
EPEC  301 ..............................................A.............S...Q.....A...........................
EHEC  301 ...........rr.....h.....................A..iY.............S...Q.....A...........................

con       KSSVNGYFRMsgWHESYnKKDYDERPANGFDIRFNGYLPSYPALGaKLmYEQYYGDNVALFNsDKLqSNPGAaTVGVNYTPIPLVTMGIDYRHGTGNEND


C.f.  398 LLYSMQFhYQFDKPWSQQIEPQYVNELRTLSGSRYDLVQRNNNIILdYKKQDILSmNIPHnINGTEhSThKIQLIVKSKYGLeRIVWDDStLRtQGGQIQ
EPEC  401 .......R..................................E........L....D.....r..Q.............D.......A..S......
EHEC  401 .......R.....s..............................E........L....D........Q.............D.......A..S......

con       LLYSMQFrYQFDKPWSQQIEPQYVNELRTLSGSRYDLVQRNNNIILeYKKQDILSlNIPHdINGTEhSTqKIQLIVKSKYGLdRIVWDDSaLRsQGGQIQ


C.f.  498 HSerkahnDYQAILPAYVQGGSNVYKVTrRAYDRNGNSSNNVqLTITVLSNGQVVDkVGiTnFTADKTSAKADnsdtITYTATVKKNGVAQANVPVSFNI
EPEC  501 ..GSQSAQ...............A............1............Q..V.D..........gtea......................
EHEC  501 ..GSQSAQ...............i....A........................Q..V.D.........a......................

con       HSgsqsaqDYQAILPAYVQGGSNvYKVTaRAYDRNGNSSNNVqLTITVLSNGQVVDqVGvTdFTADKTSAKADn-dtITYTATVKKNGVAQANVPVSFNI


C.f.  598 VSGTAtLSAkSANTNsSGKATVTLKSDKPGQVVVSAKTAEMTSALNANAVIFVDQTKASITEIKvDKTiAtAdnkDtIeYTVKVMKGgnPiSgQkVTF
EPEC  601 .....v...N.....g..............................................A...T.V.NGq.A.t........dk.V.N.e...
EHEC  601 .......g.N....dan.........st.............s.....s....f...........A...T.V.NG..A.k........n.q.VnN.s...st

con       VSGTAtLsAnSAnTn-sGKATVTLKSdkPGQVVVSAKTAEMtSALNAnAVIFvDQTKASITEIKaDKTtAvAngkDaI-YTVKVMKgg-PvsnQ-VTFst


C.f.  696 skdfGtLnkteatTDqNGYAtVkLsSgTPGKaiVSAkVSeVnteVKAatVEFFapLsIDgnkvtviGTGVtGsLPknWLQYGQVkLqAtGGNGKYTWkS
EPEC  699 tttl.k.Snstek..t....k.T.T.t....sl....r.:D.avd...pE....tt.t..dgnieIv....k.k..tv.......n.K.S.........r.
EHEC  701 nfgm.ngkSq.q...gnd.r..iT.T.ssa...t...t..D ga....tE.t..de.k.. ...dI..nn.r.e..ni......fk.K.S..d.t.s.y.

con       n---fg-ls-t-atTd-nGyAtvtLtS-tpGKa-VSA-VSdv--eVKA-eVeFF--L-ID-nkv-iiGtgV-G-LP--WLQYGQvkLkAsGGnGkYtW-S


C.f.  795 sNtkIASVDnSgvitlnekgsaTitvVsgdnqsatytintpdNiilavdkinRmaYseAesrCqaissnLaqSksvLENiyskWGAANKYpYYsSSnslt
EPEC  798 a.pa.....A.sGqVTLkeKGt.tIs.iSsDnQTatYTIatp.sl.vpnmsK.vt.nD.vnt.KNfggk.PS.Qne...vfka.......e..k..qtIi
EHEC  799 e..s..t..A. GkVTLngKGsvvIkatSgDkQTvsYTI kapsymikvdKqay.aD.m.i.KN    1.PStQt..sd..ds.......sh...m..I.

con       -Nt-IAsVDaS-g-vtl--kg-t-i-v-s-d-qt--yti---n--i-----kr--Y-dA-s-Ckn----Lpssq-vLeniy--WGAANKY-yYsSsnsit


C.f.  895 aWikQstsDsaSGVsnTYDLVttNsLtNvKAtdkNAfAvCVK 936
EPEC  898 s.vq.Taq.ak...aS.....kQ.P.n.i..ses..Y.t... 939
EHEC  893 .....Ts.eqr....S..n.i.Q.P.pg.nvntp.vY....e 934

con       aWikQt-sd--SGVssTYdLvtqNpL-nvka---NayAvCVk
```

FIG. 4. Alignment of the predicted amino acid sequences from the *eae* loci of *C. freundii* biotype 4280 (*C.f.* [GenBank accession number L11691]), EPEC (16), and EHEC (32). Sequences were aligned by using the GENALIGN program of the IntelliGenetics Suite, release 5.4. Residues identical with those of *C. freundii* are indicated by dots. In the consensus (con) sequence, residues with identity among all three sequences are in uppercase and those with identity between two sequences are in lowercase. Divergence among all three sequences is represented by dashes.
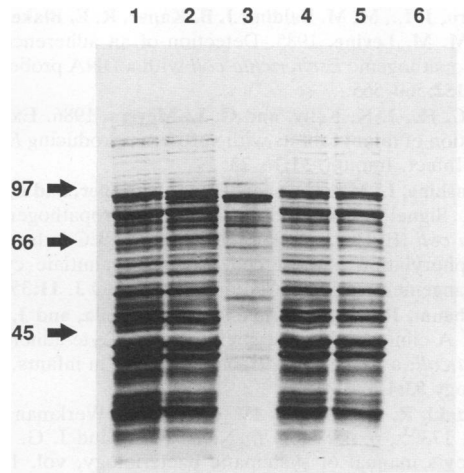
FIG. 5. Incorporation of [³⁵S]methionine into proteins of *E. coli* XL1-Blue with the expression vector pT7-5 (lane 1); pDBS5, which is pT7-5 containing the 4.7-kb DNA fragment from *C. freundii* biotype 4280 DNA (lane 2); pDBS5 in the presence of rifampin (lane 3); the expression vector pT7-6, which has a polylinker in the opposite orientation of pT7-5 (lane 4); pDBS6, which is pT7-6 containing the 4.7-kb DNA fragment from *C. freundii* biotype 4280 DNA (lane 5); and pDBS6 in the presence of rifampin (lane 6).

of EPEC and EHEC is responsible for the antigenic specificity of these proteins and may contribute to differences in the sites of intestinal colonization by EPEC and EHEC (9, 32). It remains to be determined if the carboxy-terminal region of the putative *C. freundii* Eae protein contains antigenic epitopes or a receptor-binding domain distinct from both EPEC Eae and EHEC Eae.

While human and rabbit EPEC strains, EHEC strains, and *C. freundii* biotype 4280 all produce AE lesions, the natural host and resulting disease for each of these organisms are distinct. Virulence factors that are not common to all of the AE *E. coli* and that contribute to the unique pathophysiology of each organism have been identified. EAF plasmids are present in most human EPEC isolates but are not present in RDEC-1 or EHEC strains (22). Southern hybridization revealed no EAF homology in *C. freundii* biotype 4280. EHEC strains produce bacteriophage-encoded Shiga-like toxins (SLTs) (18), and SLT-producing *C. freundii* strains have recently been identified (28). The SLT-producing *C. freundii* strains are distinct from the 4280 biotype of *C. freundii* in that the SLT-producing strains produce H₂S and are motile (28), while *C. freundii* biotype 4280 does not produce H₂S and is nonmotile (5). Consistent with these biochemical differences, *C. freundii* biotype 4280 does not produce hemorrhagic colitis or hemolytic-uremic syndrome in mice (6). The phylogenetic relationship between EPEC, EHEC, SLT-producing *C. freundii*, and *C. freundii* biotype 4280 has not been established. Bacteria that produce AE lesions may acquire plasmid-borne and bacteriophage-encoded determinants that are responsible for the distinct pathophysiology seen in each bacterial disease. Alternatively, there may be horizontal transfer of the *eae* gene, and the direct repeats downstream of the *C. freundii* biotype 4280 locus may indicate recombinational events involving *eae*. Identification and nucleotide sequence determination of an *eae* locus from SLT-producing *C. freundii* should provide some insight into the relationship between these organisms.

C. freundii biotype 4280 induces epithelial proliferation

that leads to mucosal hyperplasia in the descending colon of laboratory mice. The role of AE lesion formation in the induction of colonic hyperplasia remains to be determined. It is possible that mucosal colonization by *C. freundii* biotype 4280 results in the formation of AE lesions, while other bacterial factors induce the proliferative host response. On the other hand, formation of AE lesions in the descending colon may be sufficient to elicit epithelial proliferation. The fact that EPEC and EHEC do not produce pronounced hyperplasia may be due to differences in virulence factors, including the Eae proteins, but could also be a reflection of the site of colonization. The normal turnover rate of enterocytes increases along the proximal-distal axis of the gastrointestinal tract (7), and *C. freundii* biotype 4280 colonizes the most distal portion of the colon (6). AE lesion formation could result in secretory diarrhea in the small bowel but lead to epithelial proliferation in the descending colon. Finally, while terminally differentiated colonic enterocytes are the primary targets for AE lesion formation by *C. freundii* biotype 4280, colonic hyperplasia is a result of increased proliferation by stem cells migrating from the colonic crypts (4). Identification of the factors that trigger the proliferative response in transmissible murine colonic hyperplasia is likely to provide insight into the biology of enterocyte turnover.

REFERENCES

1. **Albert, M. J., K. Alam, M. Islam, J. Montanaro, A. S. M. Hamidur Rahman, K. Haider, M. A. Hossain, A. K. M. G. Kibriya, and S. Tzipori.** 1991. *Hafnia alvei*, a probable cause of diarrhea in humans. Infect. Immun. **59:**1507–1513.
2. **Albert, M. J., S. M. Faruque, M. Ansaruzzaman, M. M. Islam, K. Haider, K. Alam, I. Kabir, and R. Robins-Browne.** 1992. Sharing of virulence-associated properties at the phenotypic and genetic levels between enteropathogenic *Escherichia coli* and *Hafnia alvei*. J. Med. Microbiol. **37:**310–314.
3. **Baldini, M. M., J. B. Kaper, M. M. Levine, D. C. A. Candy, and H. Moon.** 1986. Localization of a determinant for HEp-2 adherence by enteropathogenic *Escherichia coli*. Infect. Immun. **52:**334–336.
4. **Barthold, S. W.** 1979. Autoradiographic cytokinetics of colonic mucosal hyperplasia in mice. Cancer Res. **39:**24–29.
5. **Barthold, S. W., G. L. Coleman, P. N. Bhatt, G. W. Osbaldiston, and A. M. Jonas.** 1976. The etiology of transmissible murine colonic hyperplasia. Lab. Anim. Sci. **26:**889–894.
6. **Barthold, S. W., G. L. Coleman, R. O. Jacoby, E. M. Livstone, and A. M. Jonas.** 1978. Transmissible murine colonic hyperplasia. Vet. Pathol. **15:**223–236.
7. **Barthold, S. W., and A. M. Jonas.** 1977. Morphogenesis of early 1,2-dimethylhydrazine-induced lesions and latent period reduction of colon carcinogenesis in mice by a variant of *Citrobacter freundii*. Cancer Res. **37:**4352–4360.
8. **Barthold, S. W., G. W. Osbaldiston, and A. M. Jonas.** 1977. Dietary, bacterial, and host genetic interactions in the pathogenesis of transmissible murine colonic hyperplasia. Lab. Anim. Sci. **27:**938–945.
9. **Beebakhee, G., M. Louie, J. De Azavedo, and J. Brunton.** 1992. Cloning and nucleotide sequence of the *eae* gene homologue

from enterohemorrhagic *Escherichia coli* serotype O157:H7. FEMS Microbiol. Lett. **91**:63–68.

10. **Brennan, P. C., T. E. Fritz, R. J. Flynn, and C. M. Poole.** 1965. *Citrobacter freundii* associated with diarrhea in laboratory mice. Lab. Anim. Care **15**:266–275.

11. **Collins, C. M., and S. Falkow.** 1990. Genetic analysis of *Escherichia coli* urease genes: evidence for two distinct loci. J. Bacteriol. **172**:7138–7144.

12. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature (London) **317**:262–264.

13. **Isberg, R. R., and J. M. Leong.** 1990. Multiple β_1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell **60**:861–871.

14. **Isberg, R. R., D. L. Voorhis, and S. Falkow.** 1987. Identification of invasin: a protein that allows enteric bacteria to penetrate cultured mammalian cells. Cell **50**:769–778.

15. **Jerse, A. E., and J. B. Kaper.** 1991. The *eae* gene of enteropathogenic *Escherichia coli* encodes a 94-kilodalton membrane protein, the expression of which is influenced by the EAF plasmid. Infect. Immun. **59**:4302–4309.

16. **Jerse, A. E., J. Yu, B. D. Tall, and J. B. Kaper.** 1990. A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. Proc. Natl. Acad. Sci. USA **87**:7839–7843.

17. **Johnson, E., and S. W. Barthold.** 1979. The ultrastructure of transmissible murine colonic hyperplasia. Am. J. Pathol. **97**:291–301.

18. **Levine, M. M.** 1987. *Escherichia coli* that cause diarrhea: enterotoxigenic, enteropathogenic, enteroinvasive, enterohemorrhagic, and enteroadherent. J. Infect. Dis. **155**:377–389.

19. **Levine, M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrheal response of man to some classical enteropathogenic *Escherichia coli* is dependent upon a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. **152**:550–559.

20. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35**:253–263.

21. **Moon, H. W., S. C. Whipp, R. A. Argenzio, M. M. Levine, and R. A. Giannella.** 1983. Attaching and effacing activities of rabbit and human enteropathogenic *Escherichia coli* in pig and rabbit intestines. Infect. Immun. **41**:1340–1351.

22. **Nataro, J. L., M. M. Baldini, J. B. Kaper, R. E. Blake, N. Bravo, and M. M. Levine.** 1985. Detection of an adherence factor of enteropathogenic *Escherichia coli* with a DNA probe. J. Infect. Dis. **152**:560–565.

23. **Pai, C. H., J. K. Kelly, and G. L. Meyers.** 1986. Experimental infection of infant rabbits with verotoxin-producing *Escherichia coli*. Infect. Immun. **51**:16–23.

24. **Rosenshine, I., M. S. Donnenberg, J. B. Kaper, and B. B. Finlay.** 1992. Signal transduction between enteropathogenic *Escherichia coli* (EPEC) and epithelial cells: EPEC induces tyrosine phosphorylation of host cell proteins to initiate cytoskeletal rearrangement and bacterial uptake. EMBO J. **11**:3551–3560.

25. **Rothbaum, R., A. J. McAdams, R. Giannella, and J. C. Partin.** 1982. A clinicopathologic study of enterocyte-adherent *Escherichia coli*: a cause of protracted diarrhea in infants. Gastroenterology **83**:441–454.

26. **Sakazaki, R.** 1984. Genus IV. *Citrobacter* Werkman and Gillen 1932, 173^{AL}, p. 458–461. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams and Wilkins, Baltimore.

27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

28. **Schmidt, H., M. Montag, J. Bockemühl, J. Heesemann, and H. Karch.** 1993. Shiga-like toxin II-related cytotoxins in *Citrobacter freundii* strains from human and beef samples. Infect. Immun. **61**:534–543.

29. **Sherman, P., F. Cockerill, R. Soni, and J. Brunton.** 1991. Outer membranes are competitive inhibitors of *Escherichia coli* of serotype O157:H7 adherence to epithelial cells. Infect. Immun. **59**:890–899.

30. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82**:1074–1078.

31. **Takeuchi, A., L. R. Inman, P. D. O'Hanley, J. R. Cantey, and W. B. Lushbaugh.** 1978. Scanning and transmission electron microscopic study of *Escherichia coli* O15 (RDEC-1) enteric infection in rabbits. Infect. Immun. **19**:686–694.

32. **Yu, J., and J. B. Kaper.** 1992. Cloning and characterization of the *eae* gene of enterohemorrhagic *Escherichia coli* O157:H7. Mol. Microbiol. **6**:411–417.