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A Second Chromosomal Gene Necessary for Intimate Attachment of Enteropathogenic *Escherichia coli* to Epithelial Cells

MICHAEL S. DONNENBERG,[1,2]* JUN YU,[3]† AND JAMES B. KAPER[3]

Medical Service, Department of Veterans Affairs Medical Center,[1] and Division of Infectious Diseases[2] and Center for Vaccine Development,[3] Department of Medicine, University of Maryland School of Medicine, 10 South Pine Street, Baltimore, Maryland 21201

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Enteropathogenic *Escherichia coli* (EPEC) is capable of attaching intimately to epithelial cells and effacing their microvilli. A chromosomal locus, *eaeA* (originally *eae*), is required for the intimate attachment aspect of this effect. We report the mapping of a region of the EPEC chromosome that is located immediately downstream of the *eaeA* gene and that is also necessary for intimate attachment. An isogenic in-frame deletion mutation in one of the open reading frames identified in this region was engineered. Because the resulting mutant, like an *eaeA* deletion mutant, is deficient in the ability to attach intimately to epithelial cells, the mutated gene is designated *eaeB*. Full activity is restored to the *eaeB* mutant when the cloned gene is reintroduced on a plasmid. The *eaeB* mutant remains capable of producing intimin, the product of the *eaeA* gene. No differences in the fractionation properties or electrophoretic mobility of intimin are apparent in the *eaeB* mutant. The product of the *eaeB* locus was identified by in vitro transcription-translation. The nucleotide sequence of the *eaeB* gene predicts a protein that contains a sequence motif common to several aminotransferase enzymes. These results indicate that the attaching and effacing effect is a complex phenotype dependent on a gene cluster present on the EPEC chromosome.

Enteropathogenic *Escherichia coli* (EPEC) strains were the first *E. coli* strains associated with gastroenteritis (6) and continue to be a leading cause of diarrhea among infants in developing countries (10, 17, 20, 28). Recent progress in understanding the pathogenesis of EPEC infections has led to the proposal of a three-stage model (15). According to this model, the initial adherence of the organism to the epithelial cell, recognized in tissue cultures as localized adherence, is characterized by the formation of microcolonies on the cell surface and is dependent on a large plasmid common to EPEC strains (3, 37). Following initial contact, the bacterium transduces a signal to the epithelial cell that results in the activation of host cell tyrosine kinase activity (39) and the elevation of intracellular calcium concentrations (4). The bacterium then becomes intimately attached to the membrane of the epithelial cell, with damage to host cell microvilli and accumulation of cytoskeletal proteins beneath the adherent organism (18, 29, 33). The latter two steps are collectively referred to as attaching and effacing (36). Subsequent to these events, a subset of the bacteria enters the epithelial cells (2, 12, 34).

Since invasion is the last step in this cascade and may depend on prior events, such as initial adherence, intimate attachment, and signal transduction, an analysis of Tn*phoA* mutants of EPEC that are deficient in the ability to invade epithelial cells might lead to insights into the genetic basis of each step (11). Indeed, this has proven to be the case. Tn*phoA* mutants deficient in invasion and localized adherence have been isolated. Study of such mutants with Tn*phoA* insertions in the EPEC plasmid has led to the identification of a type IV fimbrial operon in EPEC that is necessary for initial adherence (13). Mutants capable of localized adherence and intimate attachment but deficient in signal transduction have been described (39), and an analysis of the genetic defects in these mutants is in progress. In addition, mutants capable of localized adherence and signal transduction but deficient in intimate attachment have been isolated. Among the seven mutants in this last category are five with insertions in the chromosomal *eaeA* gene and two with insertions that map downstream of this gene but on the same 13.7-kb *Mlu*I fragment (11).

The *eaeA* gene, initially termed *eae*, was isolated as a locus necessary for the attaching and effacing activity of EPEC (27). This gene is found on the chromosome of EPEC and enterohemorrhagic *E. coli* strains (25), and sequences that hybridize with an *eaeA* gene probe have been detected in isolates of *Hafnia alvei* and *Citrobacter freundii* that display attaching and effacing activity (1, 41). The requirement of the *eaeA* gene for intimate attachment to epithelial cells and identification of this gene as a virulence determinant of EPEC were confirmed by construction of an isogenic *eaeA* deletion mutant (14) and subsequent study of this mutant as well as the wild-type strain from which it was derived in a randomized double-blind volunteer trial (16).

The *eaeA* gene encodes a 94-kDa outer membrane protein (OMP), referred to as intimin, that is recognized by sera from volunteers convalescing from experimental EPEC infections (26). The predicted amino acid sequence of intimin is highly similar to those of the invasins of *Yersinia pseudotuberculosis* and *Y. enterocolitica* (45). Invasins bind with a high affinity to members of the β_1 family of integrin receptors to mediate the efficient uptake of bacteria (44). While intimin is necessary for EPEC invasion, unlike invasin, intimin alone does not convey the invasive phenotype to *E. coli* laboratory strains.

Although intimin is necessary for the intimate attachment aspect of the attaching and effacing effect, *eaeA* mutants remain capable of activating host cell tyrosine kinases (39), damaging microvilli (15), and inducing diarrhea in a minority

* Corresponding author.
† Present address: The Biological Laboratory, University of Kent, Canterbury, Kent CT2 7NJ, United Kingdom.

TABLE 1. Strains, plasmids, and phage used in this study

Strain, plasmid, or phage	Genotype or relevant properties	Source or reference
Strains		
E2348/69	Prototypic O127:H6 EPEC strain; nalidixic acid resistant	30, 31
12-4-1(2)	E2348/69 mutant containing two Tn*phoA* insertions, one of which maps 2.4 kb downstream of *eaeA*	11
14-5-1(2)	E2348/69 mutant containing two Tn*phoA* insertions, one of which maps 4.7 kb downstream of *eaeA*	11
CVD206	E2348/69 Δ*eaeA8*	14
UMD864	E2348/69 Δ*eaeB1*	This study
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	40
SY327λ*pir*	Δ(*lac-pro*) *argE*(Am) *rif nalA recA56* (λ*pir*)	35
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r (λ*pir*)	35, 42
Plasmids		
pACYC184	Cloning vector encoding chloramphenicol and tetracycline resistance	7
pBR322	Cloning vector encoding ampicillin and tetracycline resistance	40
pUC19	High-copy-number cloning vector encoding ampicillin resistance	40
pCR1000	Cloning vector for PCR-derived sequences encoding kanamycin resistance	Invitrogen Corp., San Diego, Calif.
pCVD442	π-dependent, *sacB*-containing positive-selection suicide vector	14
pAT153	Vector encoding ampicillin and tetracycline resistance; positive control for in vitro transcription-translation	Amersham
pCVD433	Derived from pACYC184 by insertion of *Mlu*I linkers into an *Eco*RV site	11
pCVD437	7.3-kb *Bgl*II fragment of the *eae* gene cluster cloned into pTTQ181	27
pJY26	10.3-kb *Sal*I-*Mlu*I fragment of the *eae* gene cluster cloned into pCVD433	This study
pMSD1	2.7-kb *Bgl*II fragment of the *eae* gene cluster cloned into the *Bam*HI site of pBR322	This study
pMSD2	pJY26 with a deletion of 2.7- and 2.3-kb *Bgl*II fragments (not shown in Fig. 2)	This study
pMSD3	2.3-kb *Bgl*II fragment of the *eae* gene cluster cloned into the *Bam*HI site of pACYC184	This study
pMSD4	2.3-kb *Bgl*II fragment cloned back into pMSD2 in the proper orientation	This study
pMSD5	6.6-kb *Sal*I-*Nru*I fragment of pMSD4 cloned into *Sal*I-*Sma*I-cut pUC19	This study
pMSD4.22	Derived from pMSD4 by exonuclease digestion (Erase-a-base kit; Promega, Madison, Wis.) following restriction with *Bgl*I and *Sal*I and containing a deletion of approximately 3.1 kb from the 5′ end	This study
pMSD6	PCR product of the 2.3-kb *Bgl*II fragment with a deletion of nucleotides 801 to 1560, cloned into pCR1000	This study
pMSD7	pCVD442 cloned into the *Sal*I site of pMSD6	This study
pMSD8	pMSD7 with a deletion of the *Sph*I fragment containing pCR1000 sequences	This study
Phage M13mp19	Filamentous bacteriophage cloning vector	40

of volunteers (16). In addition, these mutants are capable of inducing the accumulation of filamentous actin in epithelial cells to which they adhere (11, 14, 27). However, the intensity and distribution of the actin signal generated by these mutants are altered in comparison with those of the wild-type strain, such that the signal is less sharply focused beneath the organisms. This reduced ability to stimulate the accumulation of filamentous actin, as detected by fluorescence microscopy, results in a pattern that has been described as an actin "shadow" (11) and is correlated with similar patterns seen when staining is done for α-actinin and phosphotyrosine (39). These findings suggest that intimin is not necessary for signal transduction by EPEC but rather participates in the organization and focusing of the cytoskeletal effects of the signal.

Evidence suggests that the *eaeA* gene does not act alone to mediate intimate attachment. As mentioned above, two mutants that have Tn*phoA* insertions 2.4 and 4.7 kb downstream of *eaeA* were isolated (11). The phenotype of these mutants is similar to that of eaeA mutants; i.e., the former mutants fail to attach intimately to epithelial cells and are deficient in inducing filamentous actin accumulation in the cells to which they adhere. Unfortunately, each of these mutants has two Tn*phoA* insertions. In both cases, the Tn*phoA* insertion that maps near *eaeA* fails to produce an active fusion protein when cloned, while the insertion that maps outside this region produces a fusion protein with alkaline phosphatase activity. The purpose of this study was to determine whether these mutants define a gene(s) in addition to *eaeA* that is necessary for the intimate attachment of EPEC to epithelial cells.

MATERIALS AND METHODS

Bacterial strains, media, and tissue cultures. The strains, plasmids, and phage used in this study are described in Table 1. Bacteria were stored at −70°C in 50% Luria-Bertani (LB) broth–50% (vol/vol) glycerol and grown on LB agar plates or in LB broth with chloramphenicol (20 μg/ml), ampicillin (200 μg/ml), or nalidixic acid (50 μg/ml) added as needed. HEp-2 cells were grown in Eagle minimal essential medium at 37°C with 5% CO_2. Localized adherence assays and fluorescent-actin staining (FAS) were performed as previously described (9, 11, 29). For these assays, each strain was tested in duplicate on at least two occasions. Wild-type EPEC strain E2348/69 was used as the positive control, and mutants 12-4-1(2) and 14-5-1(2) were included in all assays testing the ability of plasmids to complement the mutants. In all cases,

TABLE 2. Oligonucleotides used in this study

Designation	Sequence	Description or purpose
Donne-1	5′-TCG CAG TTA ATT CTG TTG TAG AAA ATC TTC AG-3′	Spans a deletion of nucleotides 48 to 759 of *eaeB*
Donne-2	5′-CTG AAG ATT TTC TAC AAC AGA ATT AAC TGC GA-3′	Complementary to Donne-1
Donne-3	5′-GGG TCG ACG ATC TAC GCG GAT GGA CA-3′	Upstream end of the 2.3-kb *Bgl*II fragment with the *Sal*I site incorporated
Donne-4	5′-GGG CAT GCG ATC TTT TAT CGT TTT CA-3′	Downstream end of the 2.3-kb *Bgl*II fragment with the *Sph*I site incorporated
Donne-7	5′-GGC GAA TTA TAT ACA GAG-3′	Nucleotides −102 to −85 relative to the 5′ end of *eaeB* used for sequencing to confirm the deletion
Donne-13	5′-GCC ACA AAG AAA CTC CTT-3′	Nucleotides 90 to 73 relative to the 3′ end of *eaeB* used for sequencing
Donne-18	5′-CAG AGC GGC TGT CGC A-3′	Nucleotides 303 to 288 of *eaeB* used for sequencing
Donne-19	5′-GTT GAA CCG GAA ATC C-3′	Nucleotides 698 to 683 of *eaeB* used for sequencing

the observer was blinded to the identities of the strains tested.

Molecular genetic techniques. Routine techniques were performed by standard methods (40). Oligonucleotides (Table 2) were constructed at the Biopolymer Laboratory of the University of Maryland at Baltimore. The polymerase chain reaction (PCR) was performed with 50-μl samples and a minicycler (MJ Research, Watertown, Mass.). Electroporation of strains in 10% glycerol was performed with 0.1-cm cuvettes and an *E. coli* pulser (Bio-Rad Laboratories, Richmond, Calif.) set at 1.8 kV and 25 μF.

DNA sequencing. The nucleotide sequence of the 2.3-kb *Bgl*II fragment downstream of the *eaeA* gene was determined by a variety of techniques. The fragment was cloned into M13mp19. A series of nested deletions (22) were made from the end closer to the *eaeA* gene (referred to as the upstream end), and the entire nucleotide sequence of one strand was determined with single-stranded DNA, [α-^{35}S]dATP, and the Sequenase enzyme (United States Biochemicals, Naperville, Ill.) (43). Information generated in this manner was used to construct oligonucleotide primers (Table 2) for double-strand sequencing (8) of pMSD3 to confirm the sequence of one open reading frame (ORF) on the other strand. In some instances, gaps were filled in by automated sequencing performed at the University of Maryland at Baltimore Biopolymer Laboratory with the DyeDeoxy Terminator cycle sequencing kit and a model 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). Data were analyzed with the package developed by the Genetics Computer Group of the University of Wisconsin. The DNA sequence was determined directly from products of the PCR by automated sequencing after purification with GeneClean (Bio 101, Inc., La Jolla, Calif.).

Construction of a deletion mutant. A four-primer PCR technique (23) was used to engineer an in-frame deletion mutation (Fig. 1). Oligonucleotides Donne-1 and Donne-2 are complementary and represent nucleotides 785 through 800 and nucleotides 1561 through 1575 of the 2.3-kb *Bgl*II *eae* fragment, respectively. In separate reactions (30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 5 min), oligonucleotides Donne-1 and Donne-4 and oligonucleotides Donne-2 and Donne-3 were used to amplify, from wild-type genomic DNA, PCR products representing sequences from each end of the fragment, in each case spanning a deletion of nucleotides 801 to 1560 of the fragment. Samples (1 μl) of the products of each reaction were combined and subjected to 30 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 2 min, and extension at 72°C for 3 min in the absence of

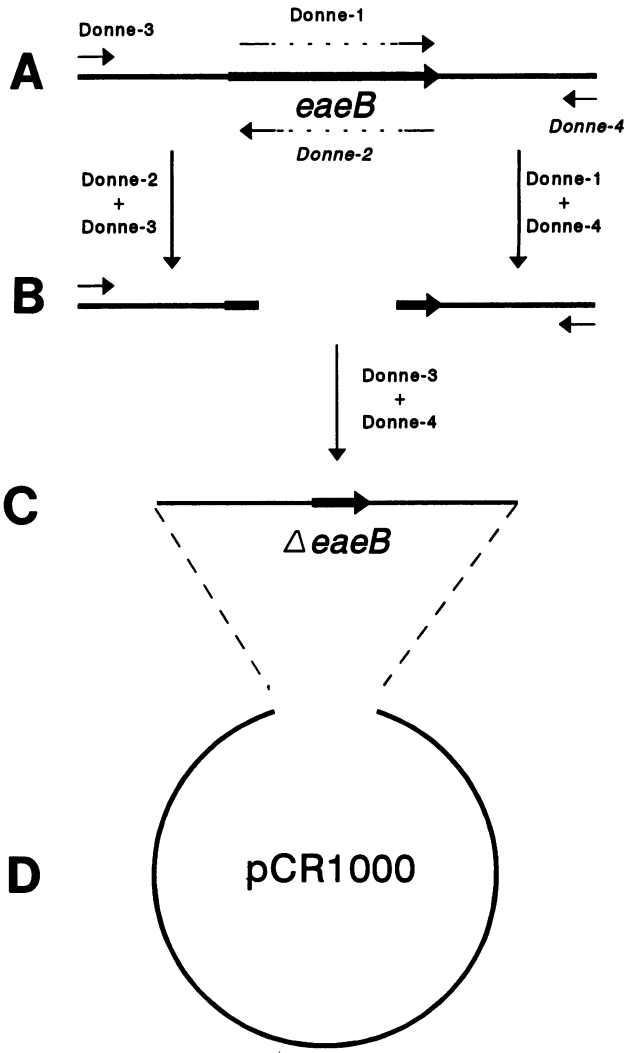


FIG. 1. Strategy for creating a deletion mutation. Oligonucleotide primers are listed in Table 2. (A) Locations of primers (thin arrows) in relation to the 2.3-kb *Bgl*II fragment (solid line) shown in Fig. 2 and to the ORF subsequently designated *eaeB*. Broken lines signify regions of *eaeB* not included in primers Donne-1 and Donne-2. (B) Separate PCRs amplified two fragments from the original. These fragments have overlapping sequences spanning the deletion. (C) The products depicted in panel B were combined and amplified with Donne-3 and Donne-4, the outer pair of primers. These steps resulted in the fragment shown, which contains an internal deletion within the target sequence. (D) The fragment shown in panel C was cloned into vector pCR1000.

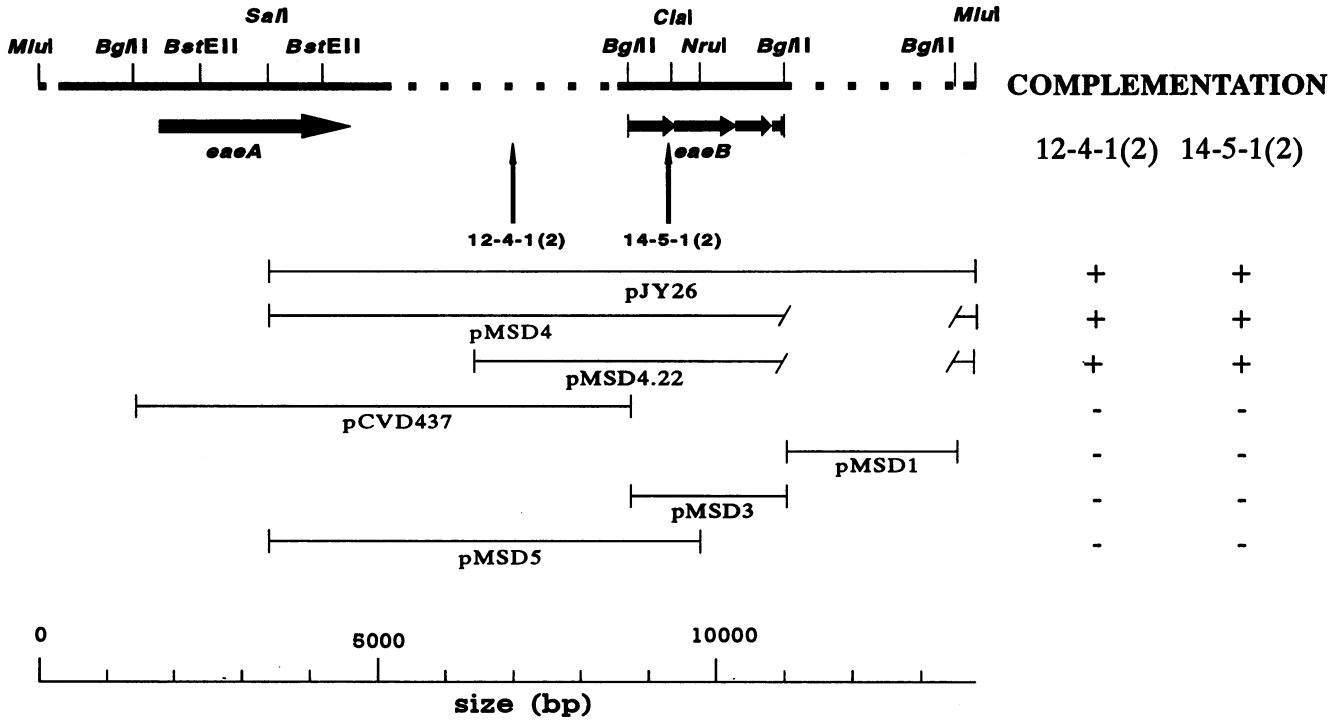
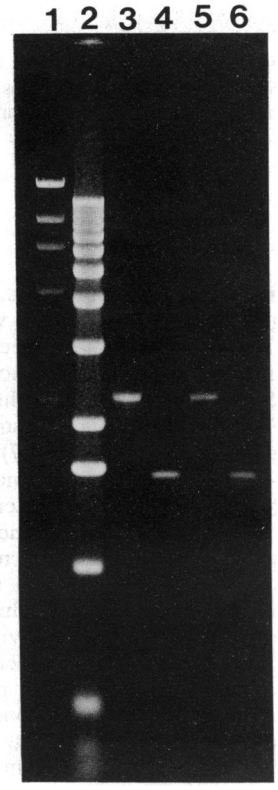


FIG. 2. Map of the *eae* region of EPEC strain E2348/69 indicating the locations of Tn*phoA* insertion mutations and the ability of plasmids containing fragments of the region to complement the mutants and restore activity in the FAS assay. Portions of the region for which there are no nucleotide sequence data are indicated by broken lines. The locations of Tn*phoA* insertions in mutants 12-4-1(2) and 14-5-1(2) are indicated by vertical arrows. Horizontal arrows show genes or ORFs detected by nucleotide sequencing. Fragments cloned to form various plasmids are shown below the map. The ability to complement each mutant is indicated to the right of each plasmid.



added primers and for 30 more cycles after the addition of primers Donne-3 and Donne-4 to amplify a full-length recombinant product representing the initial *Bgl*II fragment with a deletion of nucleotides 801 to 1560. The resulting fragment was cloned into positive-selection suicide vector pCVD442 for introduction into the wild-type EPEC strain by allelic exchange (14).

In vitro transcription-translation. For in vitro transcription-translation, a commercial kit (Amersham, Arlington Heights, Ill.) was used in accordance with the manufacturer's instructions. Controls included a sample containing no DNA, plasmid pAT153 (included with the kit), and plasmid pCVD433 (the vector used for the construction of pMSD3 and pMSD4.22). Samples tested included plasmids pMSD4.22 and pMSD3 and PCR products amplified from bacterial colonies with primers Donne-3 and Donne-4 and purified from an agarose gel with GeneClean. Approximately 2.5 μg of DNA was used in each reaction, except for the pMSD3 sample, which was inadvertently run with approximately 0.5 μg of DNA. Samples were separated by sodium

FIG. 3. PCR products amplified from wild-type EPEC strain E2348/69 (lanes 3 and 5) and the *eaeB* deletion mutant (lanes 4 and 6) with primers Donne-3 and Donne-4 from either end of the 2.3-kb *Bgl*II fragment containing *eaeB*. Lane 1 contains λ DNA digested with *Hin*dIII, and lane 2 contains a 1-kb DNA ladder (Bethesda Research Laboratories, Gaithersburgh, Md.) as size standards. Lanes 3 and 4 contain the products of PCRs, and lanes 5 and 6 contain those products after gel purification for use in an in vitro transcription-translation assay. The product from the *eaeB* mutant is the expected 0.7 kb smaller than that from the wild type.

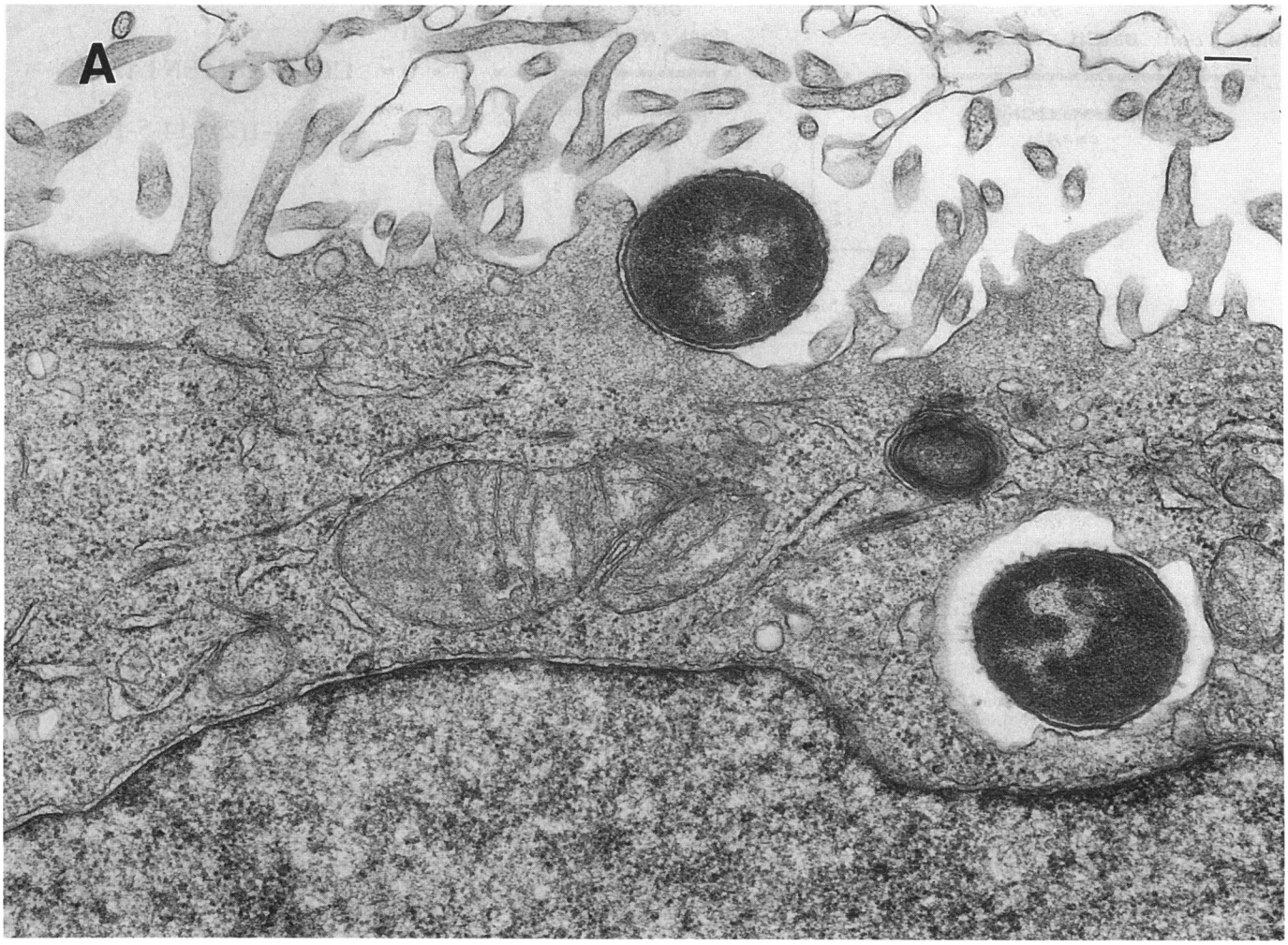


FIG. 4. Electron microscopy of HEp-2 cells infected with wild-type EPEC strain E2348/69 (A), *eaeB* deletion mutant UMD864 (B), and UMD864 containing pMSD3 (C). Typical intimate attachment and alteration of cytoskeletal components in epithelial cells are seen with the wild-type strain and the mutant containing the cloned *eaeB* gene. The mutant not containing this gene displays only nonintimate adherence to the cells. Bars, 0.2 μm.

dodecyl sulfate (SDS)–12% polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography.

OMP preparations. Triton X-100-insoluble proteins were isolated as previously described (14). Intimin was visualized with specific antiserum by Western blotting (immunoblotting) of OMPs separated by SDS–7% PAGE (27).

Electron microscopy. HEp-2 cells grown for 48 h on Transwell filters (Costar, Cambridge, Mass.) were infected with bacteria for 3 h at 37°C with 5% CO_2, washed three times with phosphate-buffered saline, and fixed in 2% formaldehyde–1% glutaraldehyde. Specimens were postfixed in 1% osmium tetroxide. Thin sections were examined with a JEOL JEM 1200-EX transmission electron microscope. The number of bacteria observed and the number of bacteria noted to be intimately attached to epithelial cells were recorded for each specimen without knowledge of strain identity.

Nucleotide sequence accession number. The sequence reported here was deposited in the GenBank-EMBL data base under accession number Z21555.

RESULTS

Complementation of transposon mutants. Plasmids containing cloned fragments of the *eae* region were introduced into mutants 12-4-1(2) and 14-5-1(2) by electroporation and tested for their ability to restore bright fluorescence to the mutants in the FAS assay. The results of these experiments are illustrated in Fig. 2. The ability of plasmid pCVD437 to complement an *eaeA* transposon mutant (27) but not mutants 12-4-1(2) and 14-5-1(2) substantiates the mapping data and clearly indicates that the defect in the latter mutants is not due to mutation of *eaeA*. Furthermore, the ability of fragments from this region to complement both mutants suggests that in both cases, the (inactive) Tn*phoA* insertion in this region, rather than the insertions that produce active fusion proteins, is responsible for the loss of activity. The smallest fragment shown to restore bright fluorescence to both mutants in the FAS assay is represented by pMSD4.22, containing a 4.3-kb fragment of the *eae* region. Note also that pMSD4 complements both mutants, but pMSD5, which lacks a 1.1-kb *Nru*I-*Bgl*II fragment from the 3′ end of

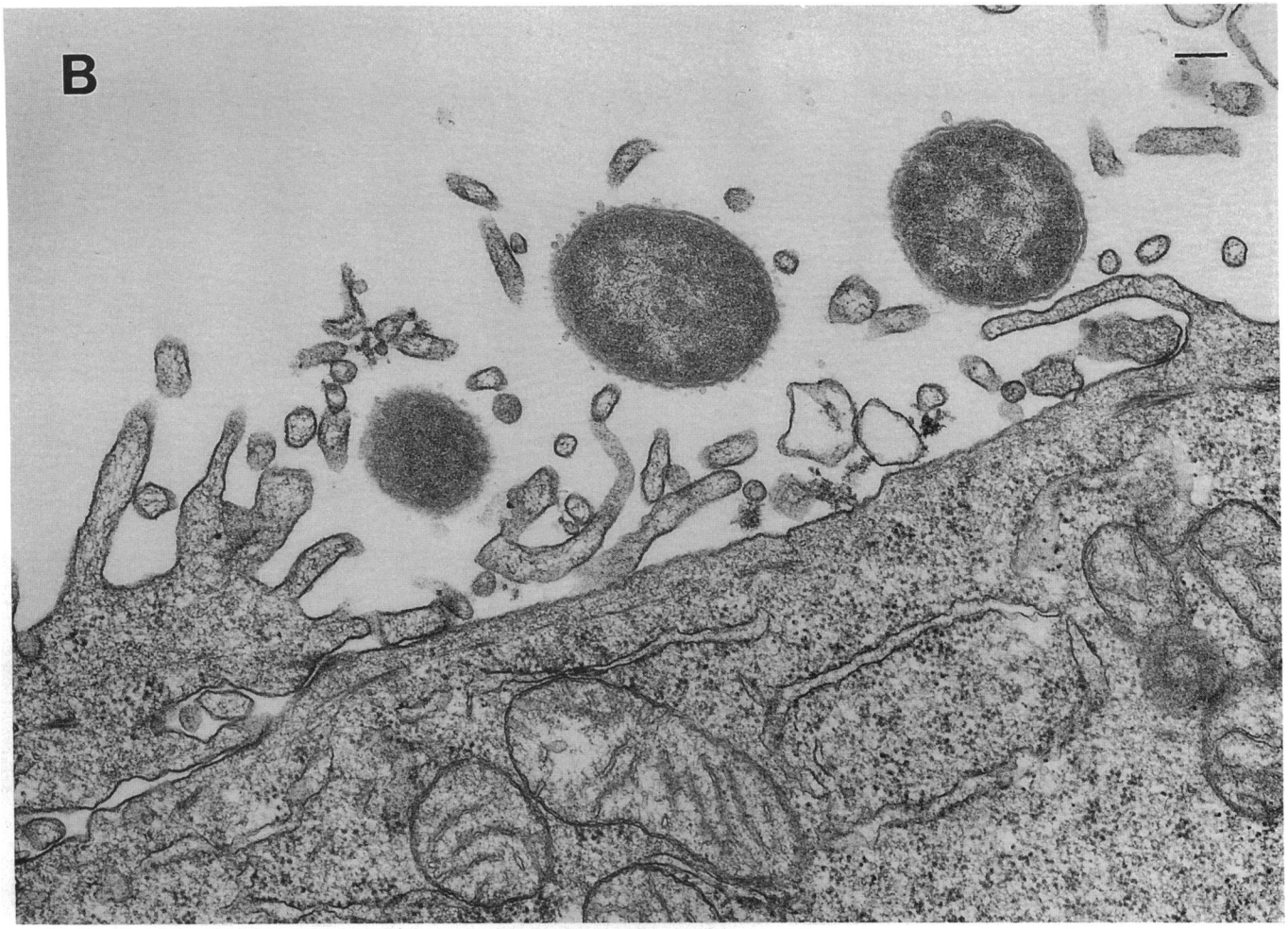


FIG. 4—*Continued.*

pMSD4 (as well as a 0.3-kb *Bgl*II-*Mlu*I fragment) complements neither mutant. In no case did a plasmid containing a fragment of the region complement one but not the other mutant.

Construction of a deletion mutant. Preliminary sequence data generated from one strand of the 2.3-kb *Bgl*II fragment downstream of *eaeA* suggested the presence of four complete or partial ORFs on this fragment (Fig. 2). The complementation data presented above regarding pMSD4 and pMSD5 suggested that one or more of these ORFs might represent a gene necessary for intimate attachment (alternatively, sequences from the 0.3-kb *Bgl*II-*Mlu*I fragment present in pMSD4 but not pMSD5 could be responsible). For testing of the hypothesis that at least one of the ORFs identified is required for intimate attachment and for avoiding the polar effects associated with transposon insertion mutations, an isogenic in-frame deletion mutation was engineered in the second ORF in the wild-type strain. A PCR product that represents the 2.3-kb *Bgl*II fragment with a deletion of 74% of this ORF (nucleotides 48 to 759) was generated. The PCR product was initially cloned in plasmid pCR1000 to create pMSD6. This plasmid was digested with *Sal*I, ligated to SalI-digested pCVD442, and used to transform DH5α to ampicillin resistance. The resulting plasmid was digested with *Sph*I, religated to excise sequences derived from pCR1000, and used to transform SY327λ*pir* to ampicillin resistance. The precise deletion of the predicted nucleotides from plasmid pMSD8 was confirmed by DNA sequence analysis with primer Donne-7 (Table 2).

After introduction of pMSD8 into SM10λ*pir* by electroporation, the plasmid was transferred to E2348/69 by conjugation. Three exconjugants were selected from plates containing ampicillin and nalidixic acid. Since plasmids with the R6K replicon cannot replicate in the absence of the *pir*-encoded π protein (35), ampicillin-resistant exconjugants represented merodiploid strains into which the plasmid had been integrated by homologous recombination. Two colonies derived from each exconjugant were selected at 30°C on modified LB agar plates (without NaCl) containing 5% sucrose (5). Because the *sacB* gene product, also present on the suicide vector, is lethal to gram-negative bacteria in the presence of sucrose (38), sucrose-resistant colonies were likely to have undergone a second recombination event, with loss of the vector and one copy of the target sequences. Sequences of the target region were amplified from each of the six sucrose-resistant, ampicillin-sensitive clones by the PCR with primers Donne-3 and Donne-4 from the ends of the *Bgl*II fragment. The products from the wild-type strain and five of the colonies were 2.3 kb in length, consistent with the wild-type allele. The product from one of the colonies was

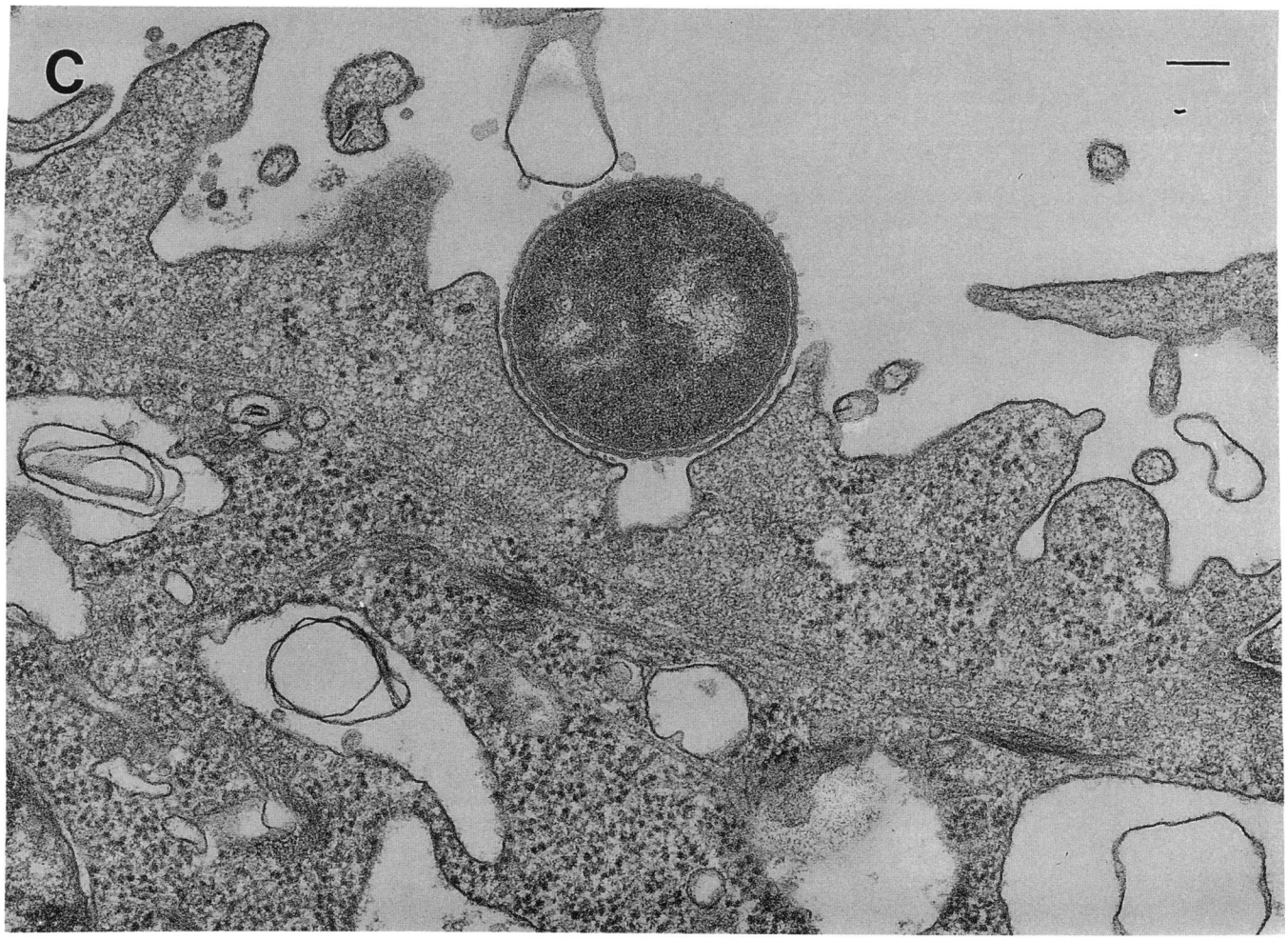


FIG. 4—*Continued.*

1.6 kb in length, consistent with the deletion of approximately 0.7 kb from within the target ORF (Fig. 3). Finally, the DNA sequence of the PCR product obtained from this colony was determined directly by use of primer Donne-7 (Table 2) and found to be identical to the wild-type sequence over 200 nucleotides, except for the deletion of nucleotides 48 to 759 of the target ORF. The strain with the deleted allele was designated UMD864.

Phenotype of the deletion mutant. Mutant UMD864 displayed a colony morphology indistinguishable from that of the wild type. The ability to adhere to HEp-2 epithelial cells in a localized pattern was unimpaired. However, the mutant failed to induce bright fluorescence in HEp-2 cells stained with fluorescein isothiocyanate-phalloidin, indicating that it was deficient in inducing changes in host cell actin. As was the case with the transposon mutants, bright fluorescence in the FAS assay was restored to the deletion mutant by the introduction of plasmid pMSD4.22. However, unlike the situation for the transposon mutants, bright fluorescence in the FAS assay could be restored to the deletion mutant by plasmid pMSD3. This result indicates that sequences upstream of the *Bgl*II fragment cloned in pMSD3 contain an additional locus or loci that is necessary for this phenotype and that is interrupted in the transposon mutants but not in the deletion mutant.

When HEp-2 cells were infected with wild-type strain E2348/69 or with UMD864 containing plasmid pMSD3 and examined by electron microscopy, typical intimate attachment with pedestal formation and deposition of electron-dense material characteristic of the attaching and effacing effect were observed (Fig. 4). Intimate attachment to epithelial cells was noted for 12 of 21 E2348/69 bacteria counted and for 18 of 30 UMD864(pMSD3) bacteria observed ($P = 0.832$, chi-square test). In contrast, *eaeB* deletion mutant UMD864 without the plasmid adhered only at a distance from epithelial cells, in a manner similar to that previously described for *eaeA* mutants (14, 27). None of 51 bacteria observed was intimately attached to epithelial cells ($P < 10^{-6}$ versus the wild type). Therefore, because deletion mutant UMD864 and *eaeA* deletion mutant CVD206 have similar phenotypes, the locus deleted from the former strain is designated *eaeB*.

Effect of the *eaeB* deletion mutation on intimin. The similar phenotypes of mutants with deletions within the *eaeA* and *eaeB* loci suggest that these genes are involved in a common pathway. One hypothesis, given the sequence similarities between intimin, the product of the *eaeA* gene, and invasins from yersiniae is that intimin serves as an intimate adhesin that anchors the bacterium to the epithelial cell and that *eaeB* is necessary for the production or proper cellular

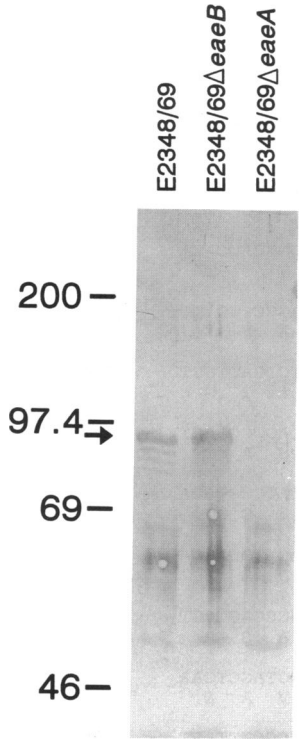


FIG. 5. Immunoblotting of OMPs isolated from wild-type EPEC strain E2348/69, *eaeB* deletion mutant strain UMD864, and *eaeA* deletion mutant strain CVD206. Triton X-100-insoluble proteins were isolated from cell lysates. Samples containing 25 μg of protein were separated by 7% discontinuous PAGE, transferred to polyvinyl difluoride membranes (Millipore Corp., Bedford, Mass.), and stained with antiserum prepared against an intimin-alkaline phosphatase fusion protein (27). The arrow indicates the position of intimin. The mobilities of molecular weight markers (in thousands) are indicated.

localization of intimin. Therefore, immunoblots of OMPs from the *eaeB* mutant were probed with anti-intimin antiserum to determine whether intimin is produced and is fractionated with the outer membrane in the *eaeB* deletion mutant. Wild-type E2348/69 and CVD206, the *eaeA* deletion mutant, were included as controls. An example of one immunoblot obtained is shown in Fig. 5, which demonstrates that intimin indeed is produced and is fractionated into the Triton X-100-insoluble compartment in the *eaeB* mutant. Although this assay is not quantitative, no gross alteration in the amount of intimin produced in the mutant was evident. Nor was there any apparent alteration in the relative mobility of intimin (Fig. 5 and additional data not shown).

Nucleotide sequence of *eaeB*. The nucleotide sequence of *eaeB* is presented in Fig. 6. An ORF of 966 nucleotides is predicted to yield a product with a molecular mass of 33,140 Da. No hydrophobic leader sequence is apparent. The ORF is preceded by excellent matches to consensus sequences for −35 and −10 promoter regions and by a strong Shine-Dalgarno sequence. An unusual sequence of 20 amino acids, 18 of which are either serine or threonine, appears near the amino terminus of the predicted protein. Although the high overall serine and threonine contents of this protein are reminiscent of the internalin protein of *Listeria monocytogenes* (19), the predicted amino acid sequence of the EaeB protein in fact shows no striking similarities to that of internalin or any other protein in the Swiss-Prot data base. When the EaeB sequence was examined for common motifs, however, a highly conserved pyridoxal-phosphate binding site was noted (Table 3). This sequence is found in several aminotransferase enzymes involved in a variety of biosynthetic pathways.

In vitro transcription-translation. Bands with apparent molecular masses corresponding to those of pre-β-lactamase and chloramphenicol acetyltransferase were seen in samples containing plasmids with the genes for these products (Fig. 7). A specific band migrating with an apparent molecular mass of 39 kDa was associated with the *eaeB* gene. This band was present in samples from plasmids pMSD4.22 and pMSD3 but absent from control vector pCVD433. This band was also present in the sample containing the PCR product of E2348/69 but not in that containing the corresponding product of the *eaeB* mutant. In addition, bands with masses of approximately 40, 23, and 21 kDa were seen in the sample from pMSD4.22.

DISCUSSION

We report here the identification and characterization of a second gene required for the intimate attachment of EPEC to epithelial cells. The *eaeB* locus was identified by analysis of two Tn*phoA* mutants of EPEC strain E2348/69 that have insertions located downstream of the *eaeA* gene and phenotypes similar to those of *eaeA* mutants. Complementation assays and preliminary DNA sequencing data led to the implication of one or more ORFs as potentially necessary for intimate attachment. The hypothesis that one ORF represented a gene required for intimate attachment was tested by construction of an isogenic in-frame deletion mutant from the wild-type strain. This construction was accomplished by first engineering the deletion of ca. 74% of the ORF with the aid of the PCR. The mutated allele was then exchanged for the wild-type allele in strain E2348/69 by use of a positive-selection suicide vector (14). The integrity of the deletion mutant was confirmed by the PCR and nucleotide sequence analysis.

The deletion mutant, UMD864, was found to be deficient in the ability to induce the accumulation of filamentous actin in epithelial cells. In addition, no intimate attachment of UMD864 to epithelial cells was detected by electron microscopy. In these respects, the mutant is similar to an *eaeA* deletion mutant. Therefore, the locus identified and mutated in this construct was designated *eaeB*. The defects in the *eaeB* mutant were reversed by the introduction of plasmids containing the cloned *eaeB* gene, confirming that this mutation was responsible for the phenotype observed. In addition, it was noted that plasmid pMSD3, which contained *eaeB* and which was unable to complement the two Tn*phoA* insertion mutants, was capable of restoring activity to the *eaeB* deletion mutant. This result suggests that additional loci (*eaeC*, etc.) necessary for intimate attachment in EPEC may be disrupted in the insertion mutants. Northern (RNA) blot experiments have indicated that *eaeB* and *eaeA* mRNAs are not part of the same transcript (21), suggesting that EPEC possess on their chromosome a gene cluster, rather than a single operon, necessary for intimate attachment.

The *eaeB* gene encodes an unusual protein. No signal sequence for protein export is apparent, suggesting that, unlike intimin, the EaeB protein may be located in the cytoplasm. The predicted size of the EaeB protein is approximately 33 kDa. However, the protein is noted to migrate with a relative mobility of approximately 39 kDa. The

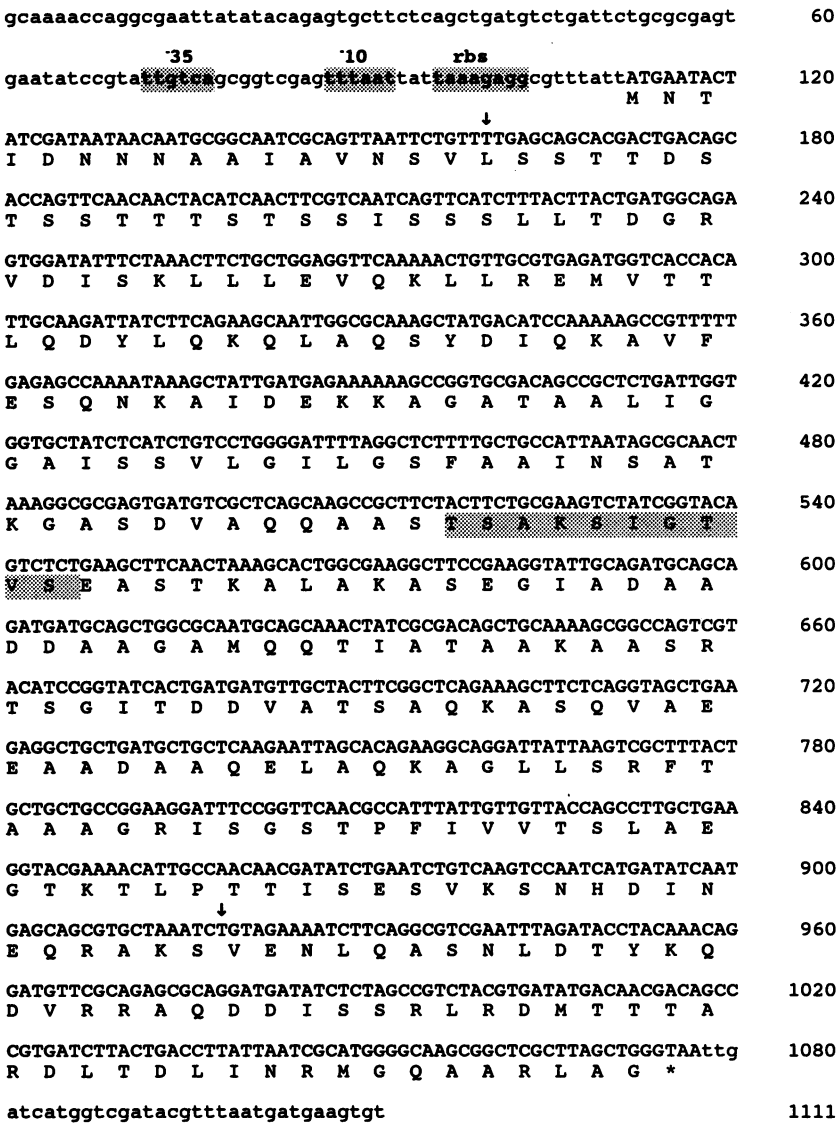


FIG. 6. Nucleotide sequence of the *eaeB* gene. Potential promoters and ribosome binding sites (rbs) are indicated by shaded lowercase letters. Arrows indicate the beginning and end of sequences deleted in the *eaeB* mutant. Amino acids corresponding to a motif common to a subclass of aminotransferases are highlighted.

synthesis of this band directed both by plasmids containing the cloned gene and by a PCR product from the wild-type strain but not by the corresponding fragment from the deletion mutant leaves little doubt as to the origin of the protein. We speculate that the difference between the predicted size and that observed by electrophoresis is due to aberrant migration, as has been described for other proteins, including internalin (19). The predicted sequence of the EaeB protein is noteworthy because of a large stretch of the related amino acids serine and threonine near the amino terminus. No clues as to the function of such a stretch were obtained by a search of computerized data bases. However, the predicted sequence of the EaeB protein includes a motif common to the pyridoxal phosphate binding domain of several aminotransferase enzymes. Many of these enzymes participate in biosynthetic pathways and catalyze the transfer of a small moiety from coenzyme A to an amino acid.

The EaeB protein may exert its effects directly on epithelial cells or indirectly by acting through intimin or another protein. Support for the hypothesis that the EaeB protein acts indirectly includes the absence of an obvious signal sequence for protein export and the identification in the *eaeB* sequence of a motif common to certain aminotransferase enzymes. The latter observation raises the intriguing hypothesis that the EaeB protein covalently modifies another protein necessary for intimate attachment and thereby activates it. One candidate for such a target is intimin which, on the basis of sequence similarities with the invasin proteins of *Yersinia* spp., may be the adhesin responsible for intimate attachment to epithelial cells. However, we were unable to detect any effect of the *eaeB* mutation on intimin expression, cellular localization, or mobility by gel electrophoresis. These results do not rule out such an effect. Indeed, the hemolysin operon of *E. coli* provides a paradigm for the activation of a virulence factor by a subtle modification that cannot be detected by electrophoresis (24, 32). Alterna-

TABLE 3. Comparison of an EaeB sequence identified by the MOTIFS program (Genetics Computer Group) with those of other proteins that share the pyridoxal-phosphate binding motif

Protein	Species	Sequence[a]
EaeB	EPEC	**TSAKSIGTVS**
Glycine acetyltransferase	*E. coli*	**TLGKALGGAS**
8-Amino-7-oxononanoate synthase	*E. coli*	**TFGKGFGVSG**
Histidinol-phosphate aminotransferase	*E. coli*	**TLSKAFALAG**
5-Aminolevulinic acid synthase	*E. coli*	**TFGKGFGVSG**
5-Aminolevulinic acid synthase	*Rhodobacter capsulatus*	**TLAKAYGVFG**
5-Aminolevulinic acid synthase	*Saccharomyces cerevisiae*	**TLGKSFGSVG**
Consensus[b]		**S S S** **TXAKAX$^{A}_{G}$XXA** **G G G**

[a] Boldfacing indicates conserved residues.
[b] X, any amino acid. The consensus is based on sequences shown and additional sequences of 5-aminolevulinic acid synthase from other species (data not shown).

tively, the EaeB protein may be involved either directly or indirectly in the signal transduction arm of the attaching and effacing effect.

In summary, we have identified a second EPEC gene required for intimate attachment to epithelial cells. It is apparent from the results of this study and other studies that the ability of EPEC to attach intimately to epithelial cells, disrupt the cytoskeleton, and efface microvilli is a complex phenotype involving many genes. Much work remains to be done to identify the loci involved and characterize the proteins necessary for producing this effect.

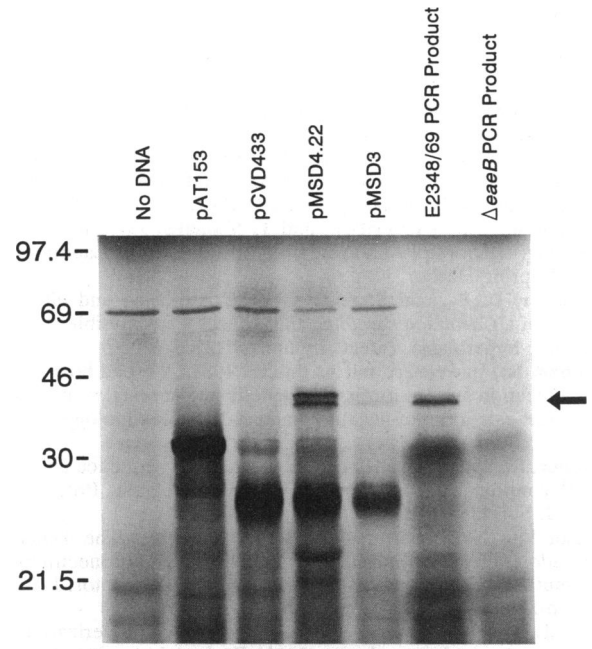


FIG. 7. In vitro transcription-translation of fragments of the *eae* region. Equivalent amounts of DNA were used in each reaction, except for the sample containing pMSD3. Control lanes included a no-DNA sample and samples of plasmid vectors pAT153 and pCVD433 encoding pre-β-lactamase and chloramphenicol acetyltransferase, respectively. A band migrating at approximately 39 kDa is indicated by the arrow.

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