INFECTION AND IMMUNITY, Dec. 1991, p. 4302–4309
0019-9567/91/124302-08$02.00/0
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Vol. 59, No. 12

The *eae* Gene of Enteropathogenic *Escherichia coli* Encodes a 94-Kilodalton Membrane Protein, the Expression of Which Is Influenced by the EAF Plasmid

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The production of a characteristic intestinal histopathology called attaching and effacing (A/E) lesions by enteropathogenic *Escherichia coli* (EPEC) is a major characteristic of EPEC pathogenesis. We previously identified a chromosomal gene (*eae*) of EPEC necessary for the production of A/E lesions on human tissue culture cells. Using antiserum raised to an Eae-PhoA fusion protein, we found that the *eae* gene encodes a 94-kDa membrane protein. This antiserum recognized a 94-kDa membrane protein in parent strain E2348/69 and a protein of similar size in *E. coli* HB101 carrying *eae* on a plasmid but did not recognize any proteins in *E. coli* HB101 carrying a plasmid with an internal deletion in the *eae* gene. Antigenically related proteins of ca. 94 kDa were detected in a collection of EPEC strains representing seven EPEC serogroups and in two EHEC strains of serotype O26:H11. Volunteer sera drawn 28 days after but not before ingestion of strain E2348/69 recognized the 94-kDa Eae protein as well as a 128-kDa Eae-PhoA fusion protein, suggesting that the Eae protein is likely to be a previously reported 94-kDa protein shown to be immunogenic in volunteers. The amount of detectable Eae protein was increased in the presence of a high-molecular-weight plasmid which is associated with the ability to produce localized adherence to tissue culture cells. These data suggest that the virulence plasmid of EPEC strain E2348/69 may have a regulatory role in the production of A/E activity.

Enteropathogenic *Escherichia coli* (EPEC) strains are a significant cause of acute and persistent infant diarrhea in the developing world (1, 3, 12, 40, 49). While diarrhea due to EPEC in industrialized countries is limited to occasional outbreaks in nurseries and day-care centers (6, 37), in developing countries it occurs at a high incidence, especially in infants less than 6 months to 1 year old (13, 18, 19, 30, 32).

Although EPEC strains were the first *E. coli* strains to be recognized as a cause of diarrhea, potential virulence phenotypes characteristic for EPEC have been identified only relatively recently, despite nearly 50 years of study. One of these phenotypes is the ability to adhere in vitro to tissue culture cells in a pattern called localized adherence (36, 43). The localized adherence phenotype is characterized by clusters of bacteria (referred to as microcolonies) adhering to localized regions of tissue culture cells, such as HEp-2 or HeLa cells. The localized adherence phenotype is specific for *E. coli* of enterpathogenic serotypes (43) and is associated with a high-molecular-weight plasmid present in most EPEC strains (4, 36). EPEC strains cured of this plasmid lose the ability to exhibit localized adherence, and the introduction of this plasmid into laboratory *E. coli* host strains results in the acquisition of this phenotype by these strains (4). Although the genes encoding HEp-2 cell adherence have not been identified, a 1-kb DNA fragment, the EAF (EPEC adherence factor) probe, cloned from one such plasmid, is highly sensitive and specific in detecting *E. coli* isolates that demonstrate localized adherence to HEp-2 or HeLa cells (35, 36).

A second phenotype that is recognized as a virulence characteristic of EPEC is the ability to produce a characteristic histopathology known as attaching and effacing (A/E) lesions in the intestines of infected infants (42, 48, 53) and animals (34, 39, 52) and on tissue culture cells (2, 27). The A/E lesion histopathology is characterized by the close adherence of bacteria to the enterocyte and by the effacement of the microvilli and the disruption of the cytoskeleton of the affected cell. The enterocyte membrane is often found "cupping" around the bacteria, and dense concentrations of microfilaments, including filamentous actin (28), are seen in the cell beneath the site of bacterial attachment. Once accomplished only by electron microscopy, detection of A/E lesions has been facilitated by the development of the fluorescence actin staining (FAS) assay, which enables the rapid detection of A/E lesions on tissue culture cells by virtue of the binding of fluorescein isothiocyanate-labeled phalloidin to filamentous actin found in the eukaryotic cell beneath the attached bacteria (28).

A/E lesions are thus far the best clue as to how EPEC cause diarrhea, with some authors proposing that EPEC produce diarrhea via malabsorption resulting from brush border effacement (34) and others reporting a close correlation between A/E activity and increased intracellular calcium and phosphorylation of host proteins potentially leading to intestinal secretion (5). Although studies with cultured intestinal mucosa (29) and gnotobiotic piglets (52) showed that the EAF plasmid is not required for the production of A/E lesions, the importance of the EAF plasmid in virulence should not be underestimated. The EAF plasmid was shown by Levine et al. (31) to be required for full virulence in humans when significantly fewer volunteers developed diarrhea after ingesting an EAF-negative EPEC derivative than after ingesting the wild-type strain. In addition, studies with gnotobiotic piglets (52) and in vitro assays (29) showed that

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TABLE 1. Bacterial strains

Strain	Description
E2348/69	EPEC strain (O127:H7) isolated from an outbreak in Taunton, England (31)
JPN15	Strain E2348/69 spontaneously cured of EAF plasmid pMAR2 after passage through a volunteer given E2348/69 (26)
JPN15(pMAR7)	Strain JPN15 into which EAF plasmid pMAR7 was introduced by conjugation (34a)
JPN15.36	*eae*::Tn*phoA* mutant of strain JPN15; does not produce A/E activity on Caco-2 cells and is negative in the FAS assay; Km^r Ap^r (26)
JPN15.96	*eae*::Tn*phoA* mutant of strain JPN15; does not produce A/E activity on Caco-2 cells and is negative in the FAS assay; Km^r (26)
JPN15.96(pMAR7)	*eae*::Tn*phoA* mutant JPN15.96 into which an EAF plasmid was introduced by conjugation; demonstrates brush border adherence to Caco-2 cells and a dim, reduced fluorescence ("shadow") in the FAS assay compared with the wild-type strain; Ap^r, Km^r (26)

although the EAF plasmid was not required for A/E activity, its presence increased the number of A/E lesions produced by an EPEC strain. The manner in which the EAF plasmid increases A/E activity is not known, although it has been hypothesized that the EAF plasmid enhances A/E activity by mediating the initial adherence to cells (29).

The mechanism behind A/E activity is not yet understood. We recently identified in a wild-type EPEC strain a chromosomal locus, *eae* (*E. coli* attaching and effacing), that is necessary for the production of A/E activity on human intestinal tissue culture cells (26). The *eae* gene consists of a 2,817-bp open reading frame capable of encoding a 102-kDa protein. Sequences homologous to a 1-kb fragment from within the *eae* open reading frame were detected in enterohemorrhagic *E. coli* (EHEC) and RDEC-1, two diarrheal pathogens that also produce A/E lesions in gnotobiotic piglets (51, 52) and weanling rabbits (47), respectively. In our previous study, two *eae*::Tn*phoA* mutants, JPN15.36 and JPN15.96, which produce Eae-PhoA fusion proteins of 96 (JPN15.36) and 128 (JPN15.96) kDa, were isolated (26). Using antiserum raised to an Eae-PhoA fusion protein, we have now identified a 94-kDa membrane protein as the product of the *eae* gene. The antigenic relatedness of Eae proteins in EPEC strains of other serogroups was also investigated, as was the immunogenicity of Eae proteins in humans. In addition, we have demonstrated that the presence of the EAF plasmid increases the expression of both the Eae-PhoA fusion protein of an *eae*::Tn*phoA* mutant and the 94-kDa Eae protein in the parent strain.

MATERIALS AND METHODS

Media, bacterial strains, and plasmids. Bacteria were cultured in L broth (10 g of tryptone, 10 g of NaCl, and 5 g of yeast extract per liter of H_2O [pH 7.4]). The bacterial strains and plasmids used are described in Tables 1 and 2. Additional EPEC and EHEC strains screened for antigenically related Eae proteins were taken from the collection of the Center for Vaccine Development (Baltimore, Md.). These strains were isolated from Peru, Chile, Israel, Canada, the United States, and other sources and were tested previously for hybridization with the 1-kb *eae* probe and for FAS activity on HEp-2 cells (26). The EHEC strains examined hybridized to the EHEC CVD419 probe (33) as well as to probes for Shiga-like toxins I and/or II. Plasmid pCVD438 (Cm^r Tc^s) contains the *eae* open reading frame plus 120 bases downstream of *eae* and 450 bases of a partial open reading frame upstream of *eae*. Plasmid pCVD438 was provided by Jun Yu and was created by ligating the *Sma*I-*Sph*I fragment of pJY21 (a derivative of cosmid pCVD436 [Table 1]) into the *Eco*RV-*Sph*I site of pACYC184 (54). Plasmid pAJ21 (also known as pCVD439 [15]) contains a mutated *eae* gene lacking an internal 1.8-kb *Bst*EII fragment and was constructed by digestion of pCVD438 with *Bst*EII and religation. Plasmids pCVD438, pAJ21, and pACYC184 were electroporated into mutants JPN15.36, JPN15.96, and JPN15.96(pMAR7) as described previously (26).

Bacterial fractionations. Cultures were grown in 200 ml of L broth at 37°C with aeration for 12 h. Osmotic shock fluid was obtained as described by Rosen and Heppel (41). The bacteria were passed through a French pressure cell (20,000 lb/in^2), and unbroken cells were removed by centrifugation (10 min, 4°C, 10,000 × *g*). The supernatant was centrifuged in a Beckman ultracentrifuge (100,000 × *g*, 60 min, 4°C); the resultant supernatant was collected as the cytoplasmic fraction, and the pellet containing the whole-membrane fraction was resuspended in 10 mM Tris (pH 8.0). To obtain the outer membrane fraction, we incubated the supernatant resulting from treatment with the French pressure cell with Triton X-100 (1% final concentration) for 30 min at 37°C and centrifuged it at 100,000 × *g* for 60 min at 4°C. The Triton X-100-insoluble pellet containing the outer membrane proteins was resuspended in 10 mM Tris (pH 8.0). The concentration of protein in each fraction was estimated in microtiter plates with a protein assay kit from Bio-Rad Laboratories, Richmond, Calif., in accordance with the manufacturer's

TABLE 2. Plasmids

Plasmid	Description
pMAR7	Derivative of native EAF plasmid pMAR2 containing Tn*801*; Ap^r (4)
pCVD436	Cosmid clone containing the *eae* gene isolated from a genomic library of E2348/69; Ap^r (26)
pCVD437	Subclone of pCVD436 in which the 7.0-kb *Bgl*II fragment containing the *eae* of pCVD436 was cloned into the *Bam*HI site of pTTQ181; Ap^r (26)
pCVD438	Subclone of pCVD437 carrying the *eae* open reading frame with minimal flanking DNA; construction is described in the text; Cm^r
pAJ21	Subclone of pCVD438 in which the 1.8-kb *Bst*EII fragment internal to the *eae* open reading frame was deleted; also known as pCVD439 (15); Cm^r

instructions. Samples were assayed for enzymatic activity or analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) after being boiled for 5 min in SDS-PAGE loading buffer (2× buffer = 62.5 mM Tris-HCl [pH 6.8], 10% glycerol [vol/vol], 5% β-mercaptoethanol [vol/vol], 2% SDS [wt/vol], 0.01% bromophenol blue [wt/vol]) before being loaded onto gels.

Triton X-100-insoluble membrane extractions. For studies on expression and identification of the Eae protein, Triton X-100-insoluble membrane proteins were extracted from 200-ml L broth cultures as described previously (26). For screening of strains for the presence of the 94-kDa Eae protein, Triton X-100-insoluble membrane proteins were extracted in the same way, except that bacteria were grown to the early stationary phase (A_{600}, 0.8 to 1.2) in 30 ml of L broth (37°C, 200 rpm). The protein concentration of the final preparation was determined as described above. Samples were boiled in SDS-PAGE loading buffer for 5 min before being loaded onto gels.

Quantitative enzymatic assays. The alkaline phosphatase activity of whole bacteria was determined by the method of Brickman and Beckwith (7). For the determination of the alkaline phosphatase activity of purified protein extracts, the same method was used, except that units were calculated per milligram of protein (the protein concentration was determined as described above) instead of per A_{600} unit of the bacterial culture. NADH dehydrogenase activity was determined by monitoring the change in the A_{340} of NADH in 10 mM Tris (pH 8.0) at 25°C. In brief, a standard curve plotting the concentration of NADH against the A_{340} was made, and the number of A_{340} units per micromole of NADH was calculated. Cell fractions were incubated with NADH (final substrate concentration, 250 μM) at room temperature for 5 min, and the change in A_{340} was monitored over a 5-min interval. The change in A_{340} per minute was calculated and compared with the standard curve to determine micromoles of NADH hydrolyzed per minute. β-Lactamase activity was determined by the method of Hirai et al. (22) with cephaloridine (Sigma, St. Louis, Mo.) as the substrate. Units of NADH dehydrogenase and β-lactamase are defined as the amount of enzyme that hydrolyzes 1 μmol of substrate per min per mg of protein at 25°C.

SDS-PAGE and immunoblotting. Triton X-100-insoluble membrane proteins were separated by electrophoresis on 7.0 or 7.5% polyacrylamide gels in the presence of SDS (21) and electrophoretically transferred to nitrocellulose sheets (8). Molecular weight determinations were based on mobilities relative to those of SDS-7B prestained markers (Sigma). Immunoblotting was done by standard methods (50). The primary antibodies used were (i) rabbit anti-bacterial alkaline phosphatase (a gift from David Low; 1:1,000 dilution), (ii) rabbit serum raised to the 128-kDa Eae-PhoA fusion protein (1:750 dilution), (iii) rabbit serum drawn prior to immunization (1:750 dilution), or (iv) human sera (from the volunteer study done by Levine et al. [31]) drawn prior to administration of EPEC strain E2348/69 and 28 days afterwards (1:200 dilution). The secondary antibody was goat anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (Sigma) or goat anti-human immunoglobulin G conjugated to alkaline phosphatase (Sigma).

Rabbit immunizations. Rabbit antisera against the 128-kDa Eae-PhoA fusion protein of mutant JPN15.96 were prepared by first fractionating 1 mg of a Triton X-100-insoluble membrane protein preparation from mutant JPN15.96 on a 7% SDS-polyacrylamide gel. Following electrophoresis, the major portion (ca. 75%) of the gel was stained with Coomassie stain (0.25 g of Coomassie brilliant blue R-250 [Sigma] per liter of 25% isopropanol–10% acetic acid [vol/vol]) and destained (10% 2-isopropanol–10% acetic acid in H_2O). The proteins on the unstained remainder of the gel were transferred to nitrocellulose paper (8), which was subsequently cut into two halves. For definitive identification of the fusion protein, one-half of the nitrocellulose was stained with amido black (0.1% amido black [wt/vol]–45% methanol–10% acetic acid [vol/vol]) and destained (90% methanol–2% acetic acid [vol/vol]). The remainder of the nitrocellulose was immunoblotted with rabbit antisera raised against bacterial alkaline phosphatase as the primary antibody. The band identified by these antisera was distinguished from the other proteins on the gel by aligning the amido black-stained blot with the immunoblot. The 128-kDa fusion protein was readily identified in the Coomassie blue-stained gel by comparison of the stained gel portion with the protein pattern on the amido black-stained nitrocellulose. The 128-kDa protein band was excised from the Coomassie blue-stained gel, homogenized in complete Freund's adjuvant at a final volume of 1 ml, and injected subcutaneously at four sites in a 1.5-kg New Zealand White rabbit. The rabbit was boosted, again in four sites, with 1 mg of excised fusion protein emulsified in Freund's incomplete adjuvant for a total of three boosts until a reaction was seen in immunoblotting against the 128-kDa fusion protein.

RESULTS

Localization of alkaline phosphatase fusion proteins. As previously described (26), *eae*::Tn*phoA* mutants JPN15.36 and JPN15.96 produce blue colonies on XP (5-bromo-3-chloro-indolyl phosphate) indicator plates, suggesting that the fusion proteins produced by these strains are present in the periplasm, membrane fraction, or culture supernatant. The cellular location of the Eae-PhoA fusion proteins produced by mutants JPN15.36 and JPN15.96 was further investigated by measuring the alkaline phosphatase activity in the supernatant, osmotic shock fluid, and cytoplasmic and membrane fractions of each mutant and the parent strain. NADH dehydrogenase and β-lactamase activities were measured in each fraction as markers for the presence of the inner membrane and periplasmic space, respectively. JPN15.36 already expresses β-lactamase (26); to provide β-lactamase to JPN15 and JPN15.96, we introduced plasmid pBR322 into these strains by electroporation. Alkaline phosphatase activity was detected predominantly in the membrane fractions of JPN15.36 and JPN15.96(pBR322) and at levels 100 times higher than that found in the membrane fraction of parent strain JPN15(pBR322) (Table 3). No detectable alkaline phosphatase activity was found in the culture supernatants of these strains. As expected, β-lactamase and NADH dehydrogenase activities were found predominantly in the osmotic shock fluid and membrane fraction, respectively, of all three strains. Treatment of the membrane fraction with 1% Triton X-100 resulted in an approximately 50% loss of alkaline phosphatase activity in the membrane fractions of both mutants. NADH dehydrogenase activity, on the other hand, was almost completely lost in the membrane fractions after treatment with this detergent. These results suggest that the Eae-PhoA fusion proteins are associated with the outer membranes of *E. coli* JPN15.96 and JPN15.36.

Identification of the *eae* gene product in the wild-type parent strain. To create a reagent that would detect the native Eae protein, we raised antiserum to the 128-kDa Eae-PhoA

TABLE 3. Determination of the cellular location of Eae-PhoA fusion proteins by quantitation of alkaline phosphatase, NADH dehydrogenase, and β-lactamase activities in cell fractions of JPN15(pBR322), JPN15.36, and JPN15.96(pBR322)

Strain	Enzyme	Activity[a] in:			
		Osmotic shock fluid	Whole membranes	Cytoplasm	Triton X-100-insoluble membranes
JPN15(pBR322)	Alkaline phosphatase[b]	1,329 (51) [66.5]	564 (15) [28.2]	107 (5) [5.3]	93 (16)
	NADH dehydrogenase[c]	3.1 (0.9) [3.9]	75.4 (5.0) [96.1]	ND	1.5 (0.4)
	β-Lactamase[c]	6,697 (21) [99.9]	7 (1) [0.1]	ND	ND
JPN15.36	Alkaline phosphatase	10,922 (244) [18.2]	47,316 (536) [78.6]	1937 (18) [3.2]	20,249 (290)
	NADH dehydrogenase	2.9 (0.1) [3.3]	86.2 (7.3) [96.6]	ND	2.9 (1.4)
	β-Lactamase	333 (29) [98.9]	4 (1) [1.2]	ND	ND
JPN15.96(pBR322)	Alkaline phosphatase	3,662 (59) [7.6]	43,481 (142) [90.6]	850 (3) [1.8]	23,466 (295)
	NADH dehydrogenase	5.8 (0) [8.5]	62.6 (3.4) [91.5]	ND	2.2 (0.7)
	β-Lactamase	5,374 (35) [99.8]	11 (1) [0.2]	ND	ND

[a] Numbers in parentheses are standard errors. Numbers in brackets are percentages of combined activities in osmotic shock fluid, cytoplasmic fraction, and whole-membrane fraction. ND, not determined.
[b] Units of alkaline phosphatase activity were calculated as described previously (7) and are expressed per milligram of protein.
[c] Units of β-lactamase and NADH dehydrogenase activities are expressed as micromoles of substrate hydrolyzed per minute per milligram of protein at 25°C.

fusion protein of mutant JPN15.96 and used it to probe Triton X-100-insoluble membrane proteins extracted from each Tn*phoA* mutant and from parent strains JPN15 and E2348/69 by immunoblotting. Rabbit serum raised against the 128-kDa fusion protein of mutant JPN15.96 recognized this fusion protein (Fig. 1B, lane 2, faint band) as well as a 94-kDa protein in parent strain JPN15 and EAF plasmid-positive parent strain E2348/69 (Fig. 1B, lanes 3 and 4, respectively). The serum did not recognize the 96-kDa fusion protein of mutant JPN15.36 (Fig. 1B, lane 1), suggesting that fewer or less immunogenic epitopes may be present on the 96-kDa fusion protein of JPN15.36 than on the 128-kDa fusion protein of JPN15.96. The serum also did not react with purified bacterial alkaline phosphatase (data not shown), consistent with the lack of recognition of the alkaline phosphatase portion of the 96-kDa fusion protein of JPN15.36. Figure 1A shows the reaction of rabbit antiserum raised against bacterial alkaline phosphatase with the 96- and 128-kDa Eae-PhoA fusion proteins of mutants JPN15.36 and JPN15.96 (lanes 1 and 2, respectively). No proteins were detected by this serum in strains E2346/69 and JPN15 lacking Tn*phoA* (Fig. 1A, lanes 3 and 4). Preimmunization rabbit serum did not react with any proteins of the four strains (Fig. 1C).

Further evidence that the product of the *eae* gene is the

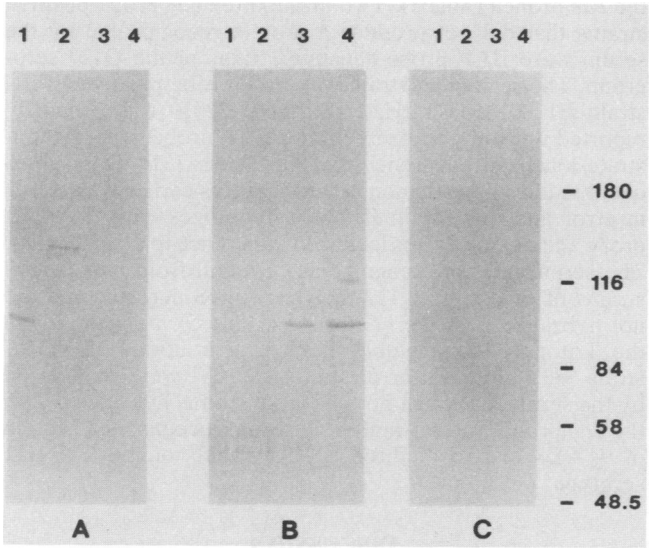

FIG. 1. Immunoblot of Triton X-100-insoluble membrane proteins (50 μg of protein per lane) separated on a 7% denaturing polyacrylamide gel, transferred to nitrocellulose, and probed with anti-bacterial alkaline phosphatase serum (A), serum from a rabbit immunized with the 128-kDa Eae-PhoA fusion protein of mutant JPN15.96 (B), and preimmunization serum from the same rabbit (C). Lanes: 1, JPN15.36; 2, JPN15.96; 3, JPN15; 4, E2348/69. Molecular masses are in kilodaltons.

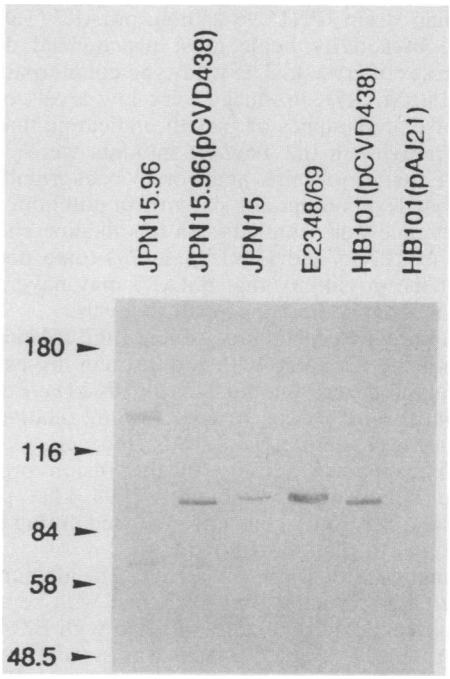

FIG. 2. Immunoblot of Triton X-100-insoluble membrane proteins of wild-type strains JPN15 and E2348/69, mutant JPN15.96, HB101 carrying pCVD438 or pAJ21, and mutant JPN15.96 carrying pCVD438. Proteins were extracted from stationary-phase cultures, separated on a 7.0% denaturing polyacrylamide gel (35 μg of protein per lane), transferred to nitrocellulose, and probed with serum from a rabbit immunized with the 128-kDa Eae-PhoA fusion protein of mutant JPN15.96. Molecular masses are in kilodaltons.

TABLE 4. Alkaline phosphatase activity of *eae*::Tn*phoA* mutant JPN15.96 in the presence and absence of pMAR7 during different stages of growth in L broth

Growth phase (A_{600})	Alkaline phosphatase activity[a] of:		
	JPN15.96	JPN15.96(pMAR7)	Ratio[b]
Early log (0.03)	1.1 (0.05)	34.0 (0.1)	31
Mid log (0.28)	8.4 (0.1)	126.3 (0.5)	15
Late log (0.55)	18.4 (0.2)	172.8 (2.0)	9

[a] Alkaline phosphatase activity was measured with whole bacteria; units are expressed per A_{600} unit as described previously (7). Numbers in parentheses are standard errors.

[b] Ratio of the alkaline phosphatase activity produced by JPN15.96(pMAR7) to that produced by JPN15.96.

94-kDa protein detected by the rabbit serum is given in Fig. 2. Triton X-100-insoluble membranes were extracted from *E. coli* HB101 carrying the cloned *eae* gene in pCVD438 or *eae* deletion plasmid pAJ21 and probed with the antiserum raised to the 128-kDa Eae-PhoA fusion protein. The serum recognized a protein of a similar size (93 kDa) in HB101(pCVD 438) but not in HB101(pAJ21) (Fig. 2). Both a 93-kDa protein and the 128-kDa Eae-PhoA fusion protein were recognized in extracts of mutant JPN15.96 carrying pCVD438, reflecting the presence of both the chromosomal *eae*::Tn*phoA* gene fusion and the cloned, intact *eae* gene (Fig. 2, lane 2).

Influence of the EAF plasmid on the expression of the 128-kDa Eae-PhoA fusion protein in mutant JPN15.96. The alkaline phosphatase activity of JPN15.96 with and without EAF plasmid pMAR7 was determined to examine the influence of this plasmid on the expression of the *eae* gene. Strain JPN15.96(pMAR7) produced higher alkaline phosphatase activity than strain JPN15.96 without pMAR7 (Table 4), the difference in activity being most pronounced during the earlier phases of growth. The wild-type counterparts, JPN15 and JPN15(pMAR7), produced very low levels of alkaline phosphatase at all stages of growth, indicating that the high levels of activity in the Tn*phoA* mutants were due to the Eae-PhoA fusion proteins and not to background alkaline phosphatase levels (data not shown). In addition, no significant difference was seen between the alkaline phosphatase activities of JPN15 and JPN15(pMAR7) (data not shown), ruling out the possibility that pMAR7 may have a positive effect on native alkaline phosphatase levels.

Consistent with the ability of pMAR7 to increase the amount of the Eae-PhoA fusion protein in *eae* mutant JPN15.96, increased amounts of the 94-kDa Eae protein were produced by parent strain E2348/69 relative to EAF plasmid-negative derivative JPN15 (Fig. 2). As with the alkaline phosphatase activity of the fusion protein, the difference between the amounts of 94 kDa protein in E2348/69 and JPN15 was most pronounced during the earlier stages of growth (data not shown).

Immunoblots with volunteer serum. The immunogenicity of the *eae* gene product was examined with serum drawn from volunteers experimentally infected with E2348/69. The 94-kDa protein of strain E2348/69 was strongly recognized by serum drawn from volunteers after infection but not by serum drawn before infection (Fig. 3). Similarly, the 128-kDa Eae-PhoA fusion protein of mutant JPN15.96 (but no protein of 94 kDa) was strongly recognized by post- but not by preinfection volunteer serum (Fig. 3).

Antigenic heterogeneity of other serogroups. To determine whether the antiserum raised against the 128-kDa fusion protein of mutant JPN15.96 recognizes membrane proteins

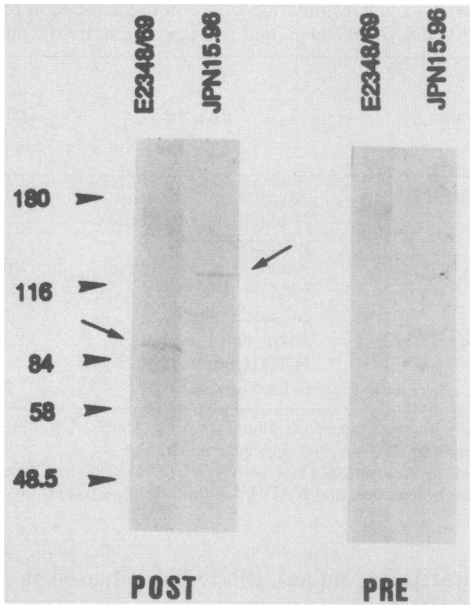

FIG. 3. Immunoblot of Triton X-100-insoluble membrane proteins of strain E2348/69 and mutant JPN15 probed with pre- and postinfection volunteer sera. Proteins were separated on a 7.5% denaturing polyacrylamide gel (35 μg of protein per lane), transferred to nitrocellulose, and probed with volunteer serum drawn before infection with EPEC strain E2348/69 or 28 days after infection. The 94- and 128-kDa proteins are marked by long arrows. Molecular masses are in kilodaltons.

in other EPEC strains that are both FAS positive and *eae* probe positive, we extracted Triton X-100-insoluble membrane proteins from 17 strains of various EPEC serogroups and examined them by immunoblotting. A protein of 94 kDa was identified in 12 of 15 strains positive for both FAS and the *eae* probe (Table 5). Two of the three *eae* probe-positive strains that did not produce a protein recognizable by the serum were EAF probe-negative strains of the O127 serogroup. The remaining strain was EAF probe-positive EPEC strain E128010 (O114:H2). (Strain E128010 was originally reported not to hybridize with the EAF probe or to demonstrate localized adherence to HEp-2 cells [31]. Our subsequent studies have demonstrated that this earlier report was in error and that strain E128010 hybridizes with the EAF probe and demonstrates localized adherence to HEp-2 cells). The serum did not recognize any protein from two *E. coli* strains of serogroup O127 that were FAS negative and did not hybridize with the *eae* probe (Table 5). No proteins in the Triton X-100-insoluble membrane fractions of EHEC strain 933 (O157:H7) or of strain RDEC-1 were recognized by the serum, although both of these strains hybridized with the *eae* probe. Interestingly, the serum recognized a protein of 94 kDa in two of three EHEC strains of the O26:H11 serotype.

DISCUSSION

Using Tn*phoA* mutagenesis of EPEC strain E2348/69, we previously identified a locus (*eae*) that is necessary for A/E activity on human tissue culture cells (26). In this study, we identified a 94-kDa protein as the product of *eae* through the use of antiserum raised to an Eae-PhoA fusion protein. This serum recognized a 94-kDa protein in wild-type parent strain E2348/69 and its EAF plasmid-cured derivative JPN15 as

TABLE 5. Screening of EPEC strains for the presence of antigenically related Eae proteins with antiserum raised to the 128-kDa Eae-PhoA fusion protein of mutant JPN15.96[a]

Strain (no tested)	EAF	*eae*	FAS	94-kDa protein
E2348/69	+	+	+	+
JPN15	−	+	+	+
RDEC-1	−	+	−	−
EPEC strains of serogroup[b]:				
O127 (2)	−	−	−	−
O127 (2)	−	+	+	−
O127 (2)	+	+	+	+
O55 (2)	+	+	+	+
O86 (1)	+	+	+	+
O111 (2)	+	+	+	+
O114 (1)	+	+	+	−
O114 (1)	+	+	+	+
O119 (2)	+	+	+	+
O142 (2)	+	+	+	+
EHEC strains of serotype:				
O26:H11 (2)	−	+	ND	+
O26:H11 (1)	−	+	ND	−
O157:H7, strain 933	−	+	ND	−

[a] EAF and *eae*, hybridization (+) or no hybridizaton (−) with the EAF probe and the *eae* probe, respectively. FAS, staining (+) or no staining (−). ND, not determined. 94-kDa protein, recognition (+) or no recognition (−) of a 94-kDa protein by antiserum raised to the 128-kDa Eae-PhoA fusion protein.

[b] H types were not determined for all strains but included two O127:H− (both EAF plasmid positive) and one each of O55:H6, O111a,b:H2, O111a,b:H−, O119:H6, and O142:H6.

well as a protein of a similar size in *E. coli* HB101 carrying pCVD438, a plasmid containing the cloned *eae* gene. In contrast, the serum did not recognize any proteins extracted from *E. coli* HB101 carrying pAJ21, a derivative of pCVD 438 in which an internal deletion was made in the *eae* gene. Fractionation experiments revealed that the Eae-PhoA fusion proteins produced by mutants JPN15.36 and JPN15.96 are located in the membrane fraction. Treatment of the membrane fraction with the detergent Triton X-100 resulted in the loss of about one-half of the alkaline phosphatase activity, suggesting that the fusion proteins are associated with both the outer and the inner membranes. However, as this detergent has been shown to partially solubilize the outer membrane as well as the inner membrane at the concentrations used in this study (16), the Eae protein is likely to be associated with the outer membrane fraction.

The slight difference between the size of the Eae protein based on the predicted amino acid sequence of *eae* (102 kDa) and that of the protein detected by the above-described serum in the wild-type strains (94 kDa) may be explained by posttranslational processing or by the limited resolution of the SDS-PAGE technique, particularly in light of the fact that the Eae protein is very basic, with a predicted pI of 9.5 (54). A slight, but reproducible, difference in size was noted between the 94-kDa protein produced by the wild-type strains and the 93-kDa protein produced by HB101 or JPN15.96 carrying *eae* on a multicopy plasmid.

The finding that a 94-kDa protein is the product of the *eae* locus is particularly intriguing in light of volunteer challenge studies that identified an EPEC protein of this size as being immunogenic in humans. In the study by Levine et al. (31), 9 of 10 volunteers given strain E2348/69 developed an immune response to a 94-kDa outer membrane protein. The remaining volunteer possessed an antibody against this protein in both pre- and postchallenge sera. Interestingly, this volunteer was the only one of the 10 who did not develop diarrhea when infected with strain E2348/69. We demonstrated that the 94-kDa Eae protein was recognized by serum drawn 28 days after infection from one of the volunteers who developed a response but not by preinfection serum drawn from this volunteer. The demonstration that the 128-kDa Eae-PhoA fusion protein but not a 94-kDa protein was recognized by this serum in protein preparations from mutant JPN15.96 is further evidence that the 94-kDa Eae protein and the 94-kDa protein identified by Levine et al. (31) are the same.

Levine et al. (31) and Chart et al. (11) independently showed that a 94-kDa membrane protein was detected in wild-type EPEC strains but not in EAF plasmid-cured derivatives of these strains. These data initially suggested that the 94-kDa membrane protein of EPEC may be encoded by the EAF plasmid. We now show that the 94-kDa Eae protein is found in both wild-type strain E2348/69 and its EAF plasmid-cured derivative JPN15. This apparent discrepancy may be explained by our evidence that the presence of the EAF plasmid increases the production of the 94-kDa Eae protein. This conclusion is supported by the demonstration of increased alkaline phosphatase activity in *eae*::Tn*phoA* mutant JPN15.96 carrying pMAR7, indicating that there is increased production of the Eae-PhoA fusion protein in JPN15.96 when pMAR7 is present. The difference in expression was greatest in earlier phases of growth, when alkaline phosphatase activity was found to be 31-fold higher in the mutant carrying pMAR7 than in the mutant alone. Consistent with the alkaline phosphatase results was the detection of higher amounts of the 94-kDa Eae protein in E2348/69 than in JPN15.

The demonstration that *eae* expression is positively regulated by the EAF plasmid is potentially significant, as it may help further define the role of the EAF plasmid in virulence as well as explain earlier observations in vivo (52) and in vitro (29) that increased numbers of A/E lesions are produced by EPEC strains that possess this 60-MDa plasmid. Knutton et al. (29) proposed that the EAF plasmid increases the efficiency of A/E activity by providing initial adherence to the cells. This hypothesis can now be modified or expanded to include the possibility that the lower amount and delayed production of A/E activity demonstrated by an EAF plasmid-negative strain (compared with a strain which carries an EAF plasmid) are the result of a lower amount of Eae protein expressed in the absence of the EAF plasmid. Consistent with this hypothesis is the observation that *eae*::Tn*phoA* mutants lacking the EAF plasmid but carrying the *eae* gene on a multicopy plasmid produce a positive FAS result more rapidly than does the EAF plasmid-negative parent strain carrying a single copy of *eae* on the chromosome (25). Although we do not yet know how the EAF plasmid influences the expression of the *eae* gene, we have recently cloned a restriction fragment from pMAR2 that increases the expression of an Eae-PhoA fusion protein in *trans* (20). Interestingly, recent reports concerning colonization factor antigen II of enterotoxigenic *E. coli* describe a plasmid gene (*rns*) which positively regulates the expression of fimbrial structural genes encoded on the chromosome or on a separate plasmid (9, 38). Sequences homologous to *rns* are not present in E2348/69 (10).

When rabbit antiserum that recognizes the 94-kDa Eae protein in EPEC strain E2348/69 (serotype O127:H6) was used, antigenic relatedness was detected among Eae proteins produced by EPEC strains of different serogroups. All strains possessing a protein recognized by this antiserum

hybridized with the *eae* probe and were positive in the FAS assay. Two of the strains that did not produce a protein recognized by the rabbit antiserum hybridized with the *eae* probe but not with the probe for the EAF plasmid. In light of the evidence that the EAF plasmid has an apparent positive effect on the production of the 94-kDa Eae protein in strain E2348/69, these strains may be producing insufficient quantities of the Eae protein to be detected in the absence of the EAF plasmid. No protein was detected by the serum in EPEC strain E128010 (O114:H2), although the serum did recognize a 94-kDa protein in strain 512/69, which is also of the O114 serogroup. This finding is consistent with the hypothesis that the 94-kDa Eae protein is the same 94-kDa protein identified by Levine et al. (31), since these authors also did not detect a 94-kDa protein in strain E128010 using serum from volunteers given EPEC strain E2348/69. The finding that Eae proteins of EPEC strains of different serogroups are antigenically related is inconsistent, however, with the report of Chart et al. (11), who found that only EPEC strains of the O111 serogroup produced a 94-kDa protein that reacted with antiserum raised to another O111 EPEC strain. This discrepancy may be explained by the possibility that the 94-kDa protein studied by these investigators is not the product of the *eae* gene or by the fact that the bacteria used by these investigators to immunize the rabbit were first treated with Formalin, which may alter epitopes that are common among Eae proteins of EPEC strains.

Although EHEC strain 933 (O157:H7) and strain RDEC-1 both hybridized with the *eae* probe, neither produced detectable levels of proteins that were recognized by the serum raised to the 128-kDa Eae-PhoA fusion protein. However, a 94-kDa protein was detected by the serum in two of three *eae* probe-positive EHEC strains of serotype O26:H11. If this finding proves to be a consistent feature of EHEC strains of the O26:H11 serotype, it suggests that EHEC strains of this serotype may be more closely linked to EPEC strains than are EHEC strains of the O157:H7 serotype. Interestingly, Scotland et al. (44) found that 36 of 37 O26:H11 EHEC strains adhered to HEp-2 cells in a localized pattern, a phenotype which is not found in O157:H7 strains but which is characteristic of most EPEC strains.

The function of the 94-kDa Eae protein is unclear at this time. We currently hypothesize that it may serve as an adhesin, on the basis of the ability of strain JPN15, but not *eae*::Tn*phoA* mutants of this strain, to adhere to Caco-2 cells (26) and the similarities found between the Eae protein and the invasin protein of *Yersinia pseudotuberculosis* (23, 24), including (i) homology between the predicted amino acid sequences of the Eae protein and invasin (26), (ii) the inability of *eae* mutants to invade tissue culture cells (14, 17), and (iii) the apparent location of the Eae protein in the outer membrane. Interestingly, a 94-kDa outer membrane protein of EHEC strain CL-56 (O157:H7) was recently implicated by Sherman et al. (45, 46) as a bacterial adhesin involved in A/E activity. This conclusion was based on the demonstration that antisera specific for this protein inhibited both adherence of this strain to and FAS activity of this strain on HEp-2 cells. The similarity in size and cellular location between this protein and the 94-kDa Eae protein of EPEC suggests that the protein described by Sherman et al. may be the Eae protein of that strain. Consistent with our inability to detect a 94-kDa protein in an *eae* probe-positive O157:H7 strain using antiserum specific for the Eae protein of E2348/69, these investigators also reported that antiserum specific for the EHEC 94-kDa protein was unable to inhibit the FAS activity of EPEC strain E2348/69 (45). These data suggest that although these proteins may serve similar functions in the production of A/E lesions, they differ antigenically. We are currently studying the mechanisms responsible for A/E activity and the role of the Eae protein in this bacterium-host cell interaction.

ACKNOWLEDGMENTS

We thank Yu Lim, Rony Adams, Ben Tall, Jeanette Jones, and Wendy Martin for technical assistance, Jun Yu for plasmid pCVD438, and Dave Maneval for technical advice and many helpful discussions.

This work was supported by grant AI21657 from the National Institutes of Health.

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