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A Plasmid-Encoded Regulatory Region Activates Chromosomal *eaeA* Expression in Enteropathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC) organisms produce a characteristic histopathology in intestinal epithelial cells called attaching and effacing lesions. The *eaeA* gene is associated with attaching and effacing lesions and encodes intimin, a 94-kDa outer membrane protein. A 60-MDa plasmid, pMAR2, is essential for full virulence of EPEC strain E2348/69 (O127:H6). We have cloned sequences from pMAR2 that increase expression of the chromosomal *eaeA* gene as shown by increased alkaline phosphatase activity of an *eaeA*::Tn*phoA* gene fusion, increased expression of the intimin protein, and increased production of *eaeA* mRNA. These sequences are called *per* for plasmid-encoded regulator. pMAR2-cured JPN15 containing cloned per sequences adheres to HEp-2 cells in greater numbers than JPN15 carrying the plasmid vector only. The cloned *per* sequences contain four open reading frames (ORFs) which have been designated *perA* through *perD*. Only *perC* can by itself activate expression of *eaeA*::Tn*phoA*, although the levels of alkaline phosphatase activity seen with this ORF alone are considerably lower than those seen when all four ORFs are present. The molecular sizes of polypeptides predicted from *perA*, *perB*, *perC*, and *perD* ORFs are 24, 14.8, 10.5, and 9.4 kDa, respectively. The PerA predicted protein shares homology with members of the AraC family of bacterial regulators, but PerB, PerC, and PerD have no striking homology with previously described prokaryotic proteins. Our studies indicate that plasmid-encoded factors regulate the expression of *eaeA* and possibly genes encoding other outer membrane proteins and may be important for virulence of EPEC.

Enteropathogenic *Escherichia coli* (EPEC) causes acute diarrhea in infants less than 1 year of age (26). The exact mechanism(s) by which EPEC causes diarrhea is unknown, but one important characteristic is the pathognomonic histopathology called attaching and effacing. This phenotype consists of intimate adherence of bacteria to enterocytes, dissolution of the brush border, and disruption of the underlying cytoskeleton (35). Jerse et al. (22) have reported a chromosomal locus, *eaeA*, associated with this pathology. *eaeA* encodes intimin, a 94-kDa outer membrane protein (OMP) which is immunogenic in volunteers challenged with EPEC (21, 27). Mutation of *eaeA* disrupts the ability of EPEC to associate intimately with epithelial cells. Volunteer challenge studies carried out by Donnenberg et al. (11) showed that an *eaeA* deletion mutant strain was less virulent than the wild-type E2348/69 strain, thus identifying intimin as the first proven virulence factor of EPEC.

Jerse and Kaper (21) have reported that the expression of the *eaeA* gene is affected by pMAR2, a 60-MDa plasmid that encodes the bundle-forming pilus (BFP) (17). This plasmid is widely distributed among EPEC strains but is not essential for the attaching and effacing phenotype since strain E2348/69 cured of pMAR2 can still cause attaching and effacing, albeit with less efficiency (24). Expression of intimin is higher in E2348/69 than in its plasmid-cured derivative, JPN15 (21). This result also correlates with the volunteer studies carried out by Levine et al. (27) in which only volunteers fed the wild-type strain E2348/69 mounted an antibody response to the 94-kDa protein intimin, whereas volunteers fed the plasmid-cured derivative did not mount such an antibody response. These observations suggested that plasmid-encoded factors may be involved in in vivo expression of the *eaeA* gene. Furthermore, the alkaline phosphatase activity of JPN15.96 carrying an *eaeA*::*phoA* fusion was higher in the presence of the 60-MDa plasmid (21). These data suggest that there are factors encoded in the EPEC plasmid that control the expression of the chromosomal *eaeA* gene.

In this paper, we report the isolation of a region from the 60-MDa EPEC plasmid that increases the expression of the *eaeA* gene. When cloned in *trans*, this region increases *eaeA* expression as shown by increased alkaline phosphatase activity of an *eaeA*::Tn*phoA* mutant, increased expression of OMPs, and increased production of *eaeA* mRNA.

MATERIALS AND METHODS

Bacterial strains and plasmids. EPEC strain E2348/69 (O127:H6) and its derivatives were used in this study (Table 1). *E. coli* HB101 and DH5α were used as hosts for recombinant DNA cloned into the plasmid vectors pACYC184 and pBluescript SKII. Standard genetic methods (31) were used except as described below. Plasmid DNA was prepared by the method of Birnboim and Doly (3), and transformation of recombinant plasmids was carried out by the $CaCl_2$ method (6).

DNA sequence analysis. The DNA sequence of the cloned *per* genes was determined with the Sequenase Kit, version 2 (U.S. Biochemical Corp., Cleveland, Ohio), with α-^{35}S-ATP (Amersham Corp., Arlington Heights, Ill.) as described in the manufacturer's instructions. A set of nested deletions was obtained from a pBluescript clone carrying a 3.5-kb *Eco*RI fragment (pOG020) by use of exonuclease III. Priming of DNA sequencing reactions utilized the universal forward primer and the M13 reverse primer. The DNA sequence upstream of the *per* region and beyond the *Eco*RI restriction site in pOG020 was determined with pJPN14 as a template and a 16-mer oligonucleotide complementary to nucleotides 216 to 231 of *per* as a primer. The DNA sequence was analyzed by use of the programs from the Genetics Computer Group, University of Wisconsin (16). The FASTA and TFASTA programs were used to search the GenBank database (release 82.0), and the Pileup program was used for alignment of the predicted protein sequences.

Construction of a *perA* mutant of E2348/69 by allelic exchange. pOG082 is a

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TABLE 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description	Reference
E2348/69	EPEC (strain O127:H6) isolated from an outbreak in Taunton, England	25
JPN15	Strain E2348/69 spontaneously cured of pMAR2 plasmid	22
JPN15.96	*eaeA*::Tn*phoA* mutant of strain JPN15	22
pJPN14	15-kb fragment from pMAR2 plasmid cloned into pACYC184	37
OG003	*perA*::*cat* mutant of strain E2348/69 (Cm^r) carrying pOG127	This study
HB101	*supE44 hsdS20*(r^- m^-) *recA13 ara-14 proA2 acY1 galK2 rpsL20 xyl-5 mtl-1*	CVD collection
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*Δ*M15*)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	BRL[a], Gaithersburg, Md.
SY327λ*pir*	Δ(*lac-pro*) *argE*(Am) *rif nalA recA56* (λ*pir*)	34
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λ*pir*	34
pMAR2	60-MDa virulence plasmid from E2348/69	1
pMAR7	pMAR2 plasmid marked with Tn*801* (Ap^r)	1
pACYC184	Cloning vector (Tc^r Cm^r)	CVD[b] collection
pCVD442	Suicide vector (Ap^r Km^r)	9
pBluescriptSKII	Cloning vector (Ap^r)	Stratagene, La Jolla, Calif.
pCVD450	3.5-kb *Eco*RI fragment from pMAR2 plasmid cloned into pACYC184 (Tc^r)	This study
pOG009	31-kb *Eco*RI fragment from pMAR2 plasmid cloned into pACYC184 (Tc^r)	This study
pOG020	3.5-kb *Eco*RI fragment from pCVD450 cloned into pBluescript SK	This study
pOG062	2-kb *Eco*RI-*Eco*RV fragment from pCVD450 cloned into pACYC184 (Cm^r)	This study
pOG082	ExoIII[c] deletion mutant of the 3.5-kb *Eco*RI fragment carrying *perABC* ORFs cloned into pBluescript SKII	This study
pOG110	*perA* interrupted by *cat* and cloned into pCVD442	This study
pOG127	pMAR2 *perA*::*cat*	This study
pOG130	ExoIII deletion mutant of the 3.5-kb *Eco*RI fragment carrying *perBCD* ORFs and most of *perA*, cloned into pACYC184 vector	This study
pOG135	ExoIII deletion of the 3.5-kb *Eco*RI fragment carrying *perBCD* ORFs cloned into pACYC184	This study
pOG136	ExoIII deletion mutant of the 3.5-kb *Eco*RI fragment carrying *perCD* ORFs cloned into pACYC184	This study
pOG138	0.5-kb *Pst*I-*Eco*RV internal fragment of the 3.5-kb *Eco*RI fragment of pCVD450	This study
pOG139	Derivative of pOG136 deleted of 3′ sequences downstream from a *Bgl*II site	This study
pOG131	ExoIII deletion mutant of the 3.5-kb *Eco*RI fragment carrying *perA* ORF cloned into pACYC184 vector	This study
pOG133	Derivative of pJPN14 carrying a 5.5-kb *Pst*I fragment that overlaps with the 5′ portion of the 3.5-kb insert of pCVD450	This study

[a] BRL, Bethesda Research Laboratories.
[b] CVD, Center for Vaccine Development, Baltimore, Md.
[c] ExoIII, exonuclease III.

pBluescript clone derived from pCVD450 that carries a 1.8-kb insert containing the entire *perA* gene. The *perA* gene in pOG082 was interrupted by inserting the chloramphenicol acetyltransferase (*cat*) gene of the pACYC184 plasmid vector in a site 290 bp downstream from the beginning of the *perA* coding region. The *perA* gene interrupted by *cat* was then cloned into pCVD442, a positive-selection suicide vector containing the *sacB* gene (9). The resulting plasmid clone, pOG110 (Ap^r Cm^r), was first isolated after transformation of *E. coli* SY327λ*pir* and used subsequently to transform *E. coli* SM10λ*pir* (Km^r) for further conjugation.

A *perA* mutation in the 60-MDa plasmid of strain E2348/69 was constructed as follows. Plasmid pOG110 (Ap^r Cm^r) present in *E. coli* SM10λ*pir* (Km^r) was transferred to E2348/69 (Nal^r) by plate matings using L agar plates without antibiotics. Transconjugants were isolated by selecting for Ap^r Cm^r Nal^r Km^s colonies that grew on minimal media. Two transconjugants were obtained with the phenotype described above in which pOG110 presumably inserted into the pMAR2 plasmid of E2348/69 by homologous recombination. To select a second recombination event that would lead to the removal of the suicide plasmid with the wild-type *perA* gene and the retention of the pMAR2 plasmid with a *perA*::*cat* mutation, transconjugants were grown on 5.0% sucrose L agar plates as described elsewhere (9). A colony that was resistant to sucrose, chloramphenicol, and nalidixic acid and sensitive to kanamycin and ampicillin and in which the *perA* gene on the pMAR2 plasmid had been interrupted by the *perA*::*cat* insert was obtained. The resulting EPEC mutant strain was named OG003, and its plasmid was designated pOG127. pOG127 was transferred to strain JPN15.96 by conjugation.

Alkaline phosphatase assay. Measurement of *eaeA* expression was determined by calculating the alkaline phosphatase activity of EaeA-PhoA fusions present in JPN15.96 and derivatives. The alkaline phosphatase activity of whole bacteria was determined by the method of Brickman and Beckwith (4).

OMP analysis. Bacteria grown in L broth for 3 h at 37°C were pelleted, resuspended in 10 mM Tris-HCl (pH 8.0)–10 mM EDTA buffer (Tris-EDTA buffer), and passed through a French press at 20,000 lb/in^2. Unbroken cells were removed by centrifugation. The supernatant was then incubated in 1% Triton X-100 for 30 min and centrifuged at 100,000 × *g* for 60 min at 4°C. Pellets containing OMPs were resuspended in Tris-EDTA buffer, and 25 μg of OMPs was loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Gels were either stained with Coomassie blue or further processed for immunoblotting. Rabbit serum raised against the EaeA-PhoA fusion (22) was used as the primary antibody, and conjugated goat anti-rabbit immunoglobulin was used as the secondary antibody. The color reaction was developed with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

Northern (RNA) blot analysis. Total RNA was extracted from bacteria with RNAzol B (Biotecx Laboratories, Inc., Houston, Tex.) as described in the manufacturer's instructions. Since *eaeA* expression is higher at earlier phases of growth (21), RNA was isolated from bacteria in the early log phase (A_{600} = 0.2). Twenty micrograms of RNA samples was denatured at 70°C for 10 min in 50% formamide–2.2 M formaldehyde–1× MOPS buffer (MOPS buffer is 40 mM morpholinepropanesulfonic acid, 10 mM sodium acetate-3H_2O, and 1 mM EDTA [pH 7.2]) in a total volume of 25 μl. Samples were placed on ice and mixed with 4 μl of loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cynol) before separation in a 1% agarose–0.38 M formaldehyde gel in the presence of 1× MOPS buffer. The RNA was then transferred to a GeneScreen Plus nylon membrane (E. I. du Pont de Nemours, Boston, Mass.). The blots were hybridized with a 1-kb *eaeA* probe described previously (21) that was labelled with [α-^{32}P]dATP by random priming. Transfer, prehybridization, hybridization, and washing of the blots were performed as recommended by the manufacturers of the GeneScreen Plus membrane. Dried blots were exposed to X-ray film for autoradiography.

Reverse transcriptase mapping. Reverse transcriptase mapping was performed by the method of Débarboullé and Raibaud (8), except as noted below. A synthetic DNA oligonucleotide, 5′-AAACAATCCTAAA-CCAGCACTAAG CAT-3′, located 64 bp downstream from the *eaeA* ATG initiation codon and complementary to the coding strand, was annealed to 20 μg of total bacterial RNA isolated as described above in a total volume of 7 μl. The annealing reaction mixture was incubated for 1 min at 94°C and then for 1 h at 45°C. The sample was mixed with 10 μl of the 2× extension buffer (0.2 M Tris [pH 8.3], 0.1

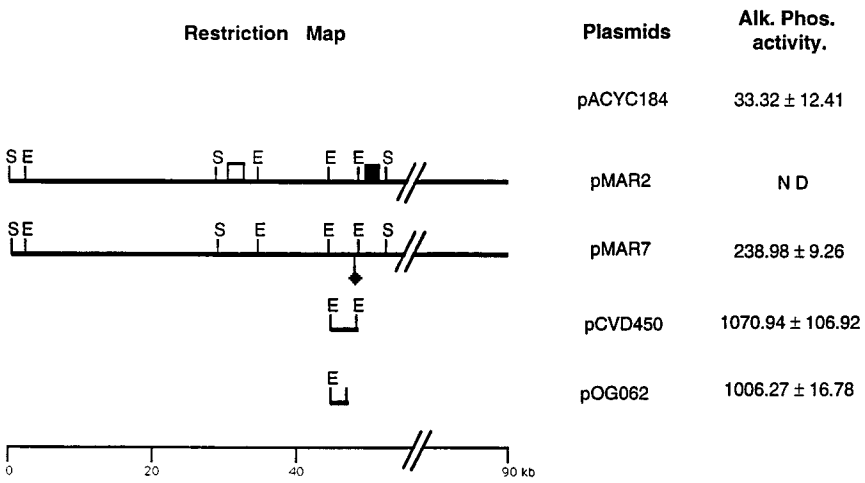

FIG. 1. Alkaline phosphatase activity of JPN15.96 and derivatives carrying the pACYC184 plasmid vector or recombinant plasmids derived from pMAR2. The map coordinates in kilobases are based on the map of pMAR2 described by Baldini et al. (2). *Sal*I (S) and relevant *Eco*RI (E) restriction sites are indicated. ND, not determined. The open box on the pMAR2 map shows the location of the *bfpA* gene; the closed box indicates the location of the EAF probe. The closed diamond on pMAR7 shows the location of the Tn*801* insertion.

M KCl, 8 mM dithiothreitol, 16 mM $MgCl_2$, 50 mM NaCl, 0.16 mg of bovine serum albumin per ml, 400 μM dCTP, 400 μM dGTP, 400 μM dTTP, 10 μM dATP), 20 U of RNase inhibitor (Promega, Madison, Wis.), 20 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., Gaithersburg, Md.), and 10 μCi of [α-^{32}P]dATP. The extension reaction was carried out at 45°C for 1 h, at which time 5 μl of a 10 mM dNTP chase was added and the reaction mixture was incubated at 45°C for an additional 15 min. Primer extension products were separated on standard sequencing gels of 6% polyacrylamide. Gels were dried and exposed to an X-ray film for autoradiography.

HEp-2 cell adherence assay. Testing for adherence of EPEC strains to HEp-2 cells was carried out as described by Cravioto et al. (7). For quantitation of adherence of EPEC to HEp-2 cells, the following modifications were applied. HEp-2 cells were grown at 37°C with 5% CO_2 in Eagle's modified minimal essential medium (EMEM; Applied Scientific, San Francisco, Calif.) supplemented with 100 mM L-glutamine and 10% fetal calf serum. Fresh EMEM supplemented with 0.1% mannose was added to monolayers of HEp-2 cells grown in 24-well tissue culture plates (Becton Dickinson, Oxnard, Calif.), and ~10^5 bacterial cells per ml were added. After 3 h of incubation at 37°C, the medium was replaced with fresh EMEM, and after 6 h of total incubation, unbound bacteria were removed by washing four times with phosphate-buffered saline (PBS). HEp-2 cells were lysed with 1% Triton X-100 for 15 min, and lysates were diluted and plated onto L-agar plates to determine the number of CFU per monolayer.

Colony blotting. The bacterial strains used for colony hybridization were obtained from the culture collections of the Center for Vaccine Development, University of Maryland School of Medicine, Baltimore. The *E. coli* strains included EPEC strains from the major serogroups (O55, O86, O111, O114, O119, O125, O126, O127, O128, and O142), enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, enteroinvasive *E. coli*, enteroaggregative *E. coli*, uropathogenic *E. coli*, and normal flora *E. coli*. The non-*E. coli* strains included *Hafnia alvei*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, and *Citrobacter freundii*. Colony blots were prepared on Whatman 541 filters (30) and hybridized to the *per* probe under high-stringency conditions (36). The *per* probe, consisting of a 1.2-kb *Eco*RI-*Pst*I DNA fragment carrying *perA*, *perB*, and the 5′ end of *perC*, was labelled with [α-^{32}P]dATP by random priming. A subset of *E. coli* and non-*E. coli* strains was tested with a 0.3-kb *Pst*I-*Bgl*II DNA fragment containing only *perC* DNA, and the results were identical to those obtained with the *perAB* probe.

RESULTS

Cloning of DNA fragments from pMAR2 that increase *eaeA* expression. pMAR2 is the 60-MDa plasmid contained in EPEC strain E2348/69 (Table 1). To identify DNA fragments from pMAR2 capable of modulating the expression of the *eaeA* gene, a plasmid library was made by cloning partially digested *Eco*RI fragments from pMAR2 into the plasmid vector pACYC184. The resultant clones were transferred to EPEC strain JPN15.96, a plasmid-cured derivative of E2348/69 containing an *eaeA*::*phoA* fusion, and their ability to activate *eaeA* was assayed by measuring the activity of the *phoA* reporter gene. Plasmid pMAR7, an ampicillin-resistant derivative of pMAR that was shown previously to increase the alkaline phosphatase activity of the *eaeA*::*phoA* fusion in JPN15.96 (21), was used as the positive control.

One clone derived from pMAR2, pCVD450, contained a 3.5-kb insert and increased the alkaline phosphatase activity of JPN15.96 ca. 30-fold (Fig. 1). When examined in HB101 or JPN15, the plasmid-cured derivative of E2348/69, pCVD450, did not increase alkaline phosphatase activity, indicating that this clone did not have an effect on the native *E. coli* alkaline phosphatase gene (data not shown). A further subclone, pOG062, contained a 2.0-kb *Eco*RI-*Eco*RV insert from pCVD450 and increased the alkaline phosphatase activity of JPN15.96 to the same level as that of pCVD450. The 2.0-kb region cloned into pOG062 that is responsible for the increased expression of *eaeA* has been designated *per* (plasmid-encoded regulatory region).

Characterization of the *per* region. The DNA sequence of the *per* region is shown in Fig. 2. Four open reading frames (ORFs) were identified on one strand of the *per* region and designated *perA*, *perB*, *perC*, and *perD*. No large ORFs were identified in the opposite strand. The predicted molecular sizes of the potential polypeptides encoded in the *per* region were 24, 14.8, 10.5, and 9.4 kDa for PerA through PerD, respectively. A series of deletions in the *per* region was constructed to localize the portion of *per* that is directly involved in regulation of *eaeA*. The only individual ORF that could increase expression of alkaline phosphatase above levels seen with the vector only was *perC* (Fig. 3). Although *perC* was necessary and sufficient to increase expression of *eaeA*::*phoA*, the levels of alkaline phosphatase activity observed with this ORF (ca. 200 U) were considerably lower than those seen when all four ORFs were present (ca. 1,224 U). When *perBCD* and *perCD* were tested, activation of *eaeA* expression was higher than that with *perC* alone but still lower than that seen with the entire *per* region (Fig. 3).

The predicted protein products of the *per* ORFs were analyzed for similarity to other regulatory factors. The predicted product of *perC* is a 10.5-kDa polypeptide with a pI of 10.6 but

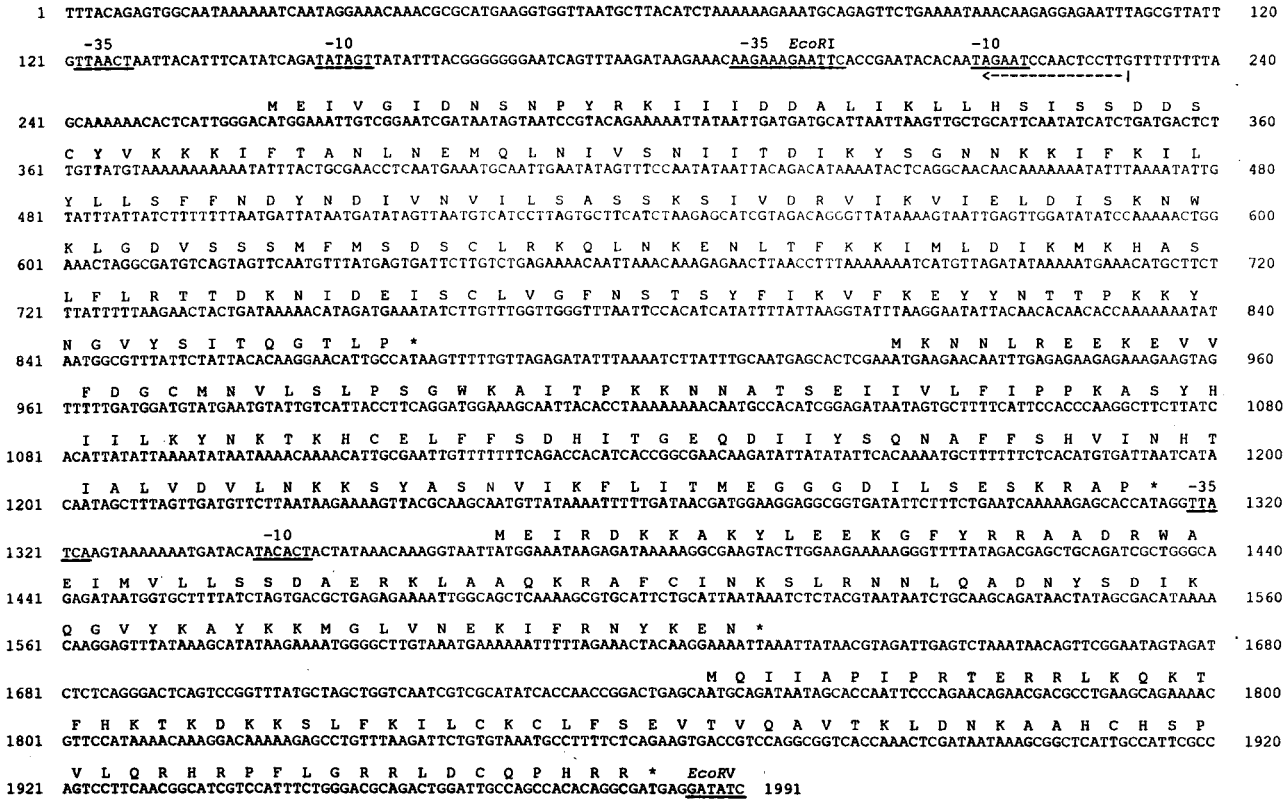

FIG. 2. DNA sequence and predicted protein products of the *per* region. Potential −10 and −35 promoter regions are underlined. The *Eco*RI and *Eco*RV restriction sites are indicated. The 16-mer oligonucleotide 5′CAAGGAGTTGGATTCT 3′ used to sequence upstream of the *Eco*RI site is shown by an arrow. Stop codons are indicated by asterisks. The sequence has been deposited in the GenBank/EMBL database (accession number Z48561).

possessing no compelling similarity to previously described proteins. However, the predicted 24-kDa *perA* gene product shows homology to the AraC family of bacterial regulators, including regulators of virulence genes such as VirF of *Shigella flexneri* (43) and CfaD (44) and Rns (5) of enterotoxigenic *E. coli* (Fig. 4). PerA and VirF share 54% similarity and 31% identity, whereas PerA and CfaD or Rns share 51% similarity and 24% identity. The sequence homology between the PerA

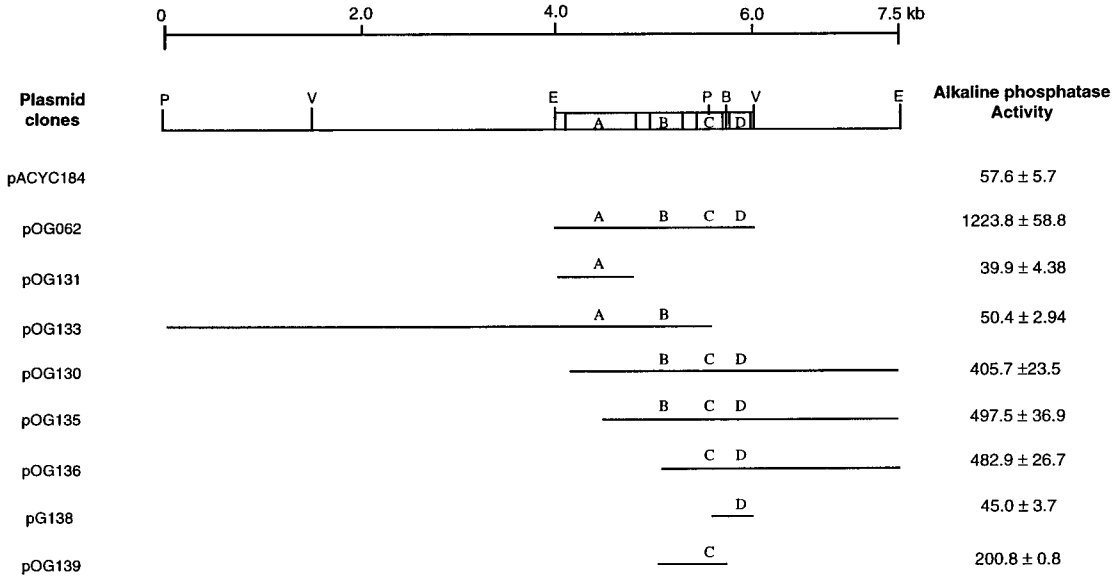

FIG. 3. Deletion analysis of the *per* region. Alkaline phosphatase activity of the *eaeA*::*phoA* fusion was measured with EPEC strain JPN15.96 carrying recombinant plasmids with different portions of the *per* region. Alkaline phosphatase activity is given in units per A_{600} unit ± standard deviation. All plasmids shown in this figure are derivatives of pACYC184. The restriction map of the *per* region and flanking DNA is shown on the top of the figure. Restriction site abbreviations: B, *Bgl*II; E, *Eco*RI; P, *Pst*I; V, *Eco*RV. The ORFs of the *per* region are indicated by capital letters A, B, C, and D.

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Rns   MDFKYTEEKETIKINNIMIHKYTVLYTSNCIMDIYSEEEKITCFSNRLVF
CfaD  MDFKYTEEKEMIKINNIMIHKYTVLYTSNCIMDIYSEEEKITCFSNRLVF
VirF  MMD   GHKNKIDI KVRLHNYIILYAKRCSMTVSSGNETLTIDEGQIAF
PerA  ..............................................

Rns   LERGVNISVRMQKQILSEKPYVAFRLNGDMLRHLKDALMIIYGMSKIDTN
CfaD  LERGVNISVRIQKKILSERPYVAFRLMGDILRHLKNALMIIYGMSKVDTN
VirF  IERNIQINVSI KKSDSINPFEIISLDRNLLLSIIRIMEPIYSFQHSYSE
PerA            MEIVGIDNSNPYRKIIIDDALIKL         LHSISSD

Rns   ACRSMSRKIM   TTEVNKTLLDE LKNINSHDNSAFISSLIYLISKLEN
CfaD  DCRGMSRKIM   TTEVNKTLLDE LKNINSHDDSAFISSLIYLISKIEN
VirF  EKRGLNKKIFLLSEEEVSIDLFKS IKEMPFGKRKIY  SLACLLSAVSD
PerA  DSCYVKKKIFTANLNEMQLNIVSNIITDIKYSGNNKKIFKILYLLSFFND

Rns   NEKIIESIYISSVSFFSDKVRNLIEKDLSRKWTLGIIADAFNASEITIRK
CfaD  NEKIIQSIYISSVSFFSDKVRNVIEKDLSRKWTLGIIADAFNVSEITIRK
VirF  EEALYTSISIASSLSFSDQIRKIVEKNIEKRWRLSDISNNLNLSEIAVRK
PerA  YNDIVNVILSASSKSIVDRVIKVIELDISKVWKLGDVSSSMFMSDSCLRK

Rns   RLESENTNFNQILMQLRMSKAALLLLENSYQISQISNMIGISSASYFIRI
CfaD  RLESENTNFNQILMQLRMSKAALLLLENSYQISQISNMIGISSASYFIRV
VirF  RLESEKLTFQQILLDIRMHHAAKLLLNSQSYINDVSRLIGISSPSYFIRK
PerA  QLNKENLTFKKIMLDIKMKHASLFLRTTDKNIDEISCLVGFNSTSYFIKV

Rns   FNKHYGVTPKQFFTYFKGG
CfaD  FNKHYGVTPKQFFTYFKGG
VirF  FNEYYGITPKKFYLYHKKF
PerA  FKEYYNTTPKKYNGVYSITQGTLP
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FIG. 4. Comparison of the predicted amino acid sequences of the Rns, CfaD, and VirF proteins with that of PerA. Sequences were aligned by the Pileup program. The amino acids in the putative HTH regions are underlined. Identical amino acid residues are shaded. Conserved residues are indicated by small closed squares.

protein and VirF, Rns, and CfaD was more pronounced towards the carboxy-terminal end where two predicted helix-turn-helix (HTH) motifs are found. The second HTH motif of PerA shares homology with the predicted HTH motif of AraC located at residues 235 through 262, which is highly conserved among members of the AraC family of DNA binding proteins (44). In addition to the homology seen with the regulators of virulence factors, similar homology was also found between PerA and regulators of housekeeping genes, including EnvY (regulator of porin gene expression in *E. coli* [29]), AppY (regulator of acid phosphatase gene expression in *E. coli* [46]), RhaS (regulator of the rhamnose operon of *E. coli* [45]), and UreR (regulator of the urease operon of *Proteus mirabilis* [39]) (data not shown).

Because of the striking similarity of PerA to other regulators of enteric virulence factors, a *perA* insertional mutant was constructed in the native pMAR2 by allelic exchange. The mutated plasmid, pOG127, was transferred to strain JPN15.96 to test for expression of the *eaeA*::*phoA* fusion. In parallel experiments, the alkaline phosphatase activity of JPN15.96 (pOG127) was 197.3 ± 4.2 U, whereas the activity of JPN15.96 (pMAR7) was 628.8 ± 18.6 U and that of JPN15.96 was 79.3 ± 1.7 U. The level of alkaline phosphatase activity of JPN15.96 (pOG127) after transformation with pCVD450 was 927.0 ± 1.4 U, indicating restoration of PerA regulatory activity by genetic complementation.

The predicted 14.8-kDa *perB* product showed no striking similarity with any previously reported prokaryotic protein, but similarity was seen with the following eukaryotic DNA binding proteins: Zg53, a zinc finger protein of *Xenopus laevis* (64% similarity and 23.4% identity with PerB between residues 21 and 149 of Zg53) (23); GagJ, a nucleic acid binding protein of *Drosophila melanogaster* (66% similarity and 17.9% identity with PerB between residues 94 and 221 of GagJ) (41); and Ercc3, a DNA excision repair protein of humans (64% similarity and 18.7% identity with PerB between residues 514 and 647 of Erc3) (47).

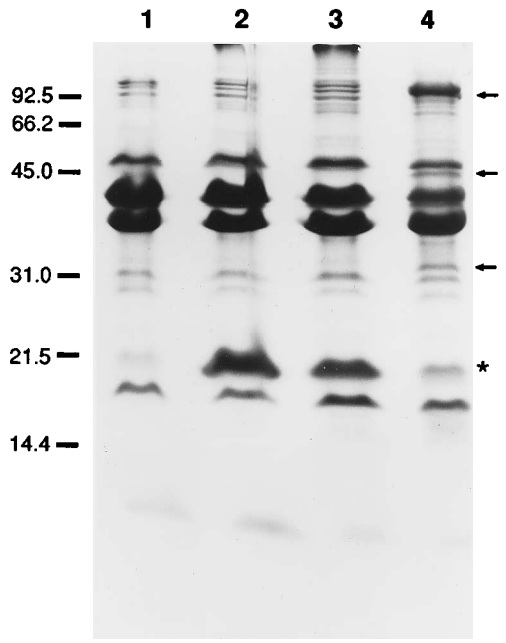

FIG. 5. OMP analysis. Equal amounts of Triton X-100-insoluble OMPs were separated by SDS–7.5% PAGE and stained with Coomassie blue. Lanes: 1, wild-type EPEC strain E2348/69; 2, JPN15; 3, JPN15(pACYC184); 4, JPN15(pCVD450). Arrows indicate increased expression of proteins of 94, 50, and 33 kDa. Asterisk indicates decreased expression of a 20-kDa protein. Molecular size markers in kilodaltons are shown on the left.

perD is a 234-bp-long ORF encoding a predicted polypeptide of 9.4 kDa with a predicted pI of 11.4. No striking similarity was detected at the protein level between this ORF and any previously described prokaryotic or eukaryotic protein or transcriptional factor. However, at the nucleotide level, *perD* shared 77.0% identity over 141 bp with insertion element IS*630* from *Shigella sonnei* and 71.0% identity over 148 bp with the transposon Tn*7*.

OMP analysis. The product of the *eaeA* gene is a 94-kDa protein called intimin (22). Analysis of OMPs isolated from E2348/69, the plasmid-cured JPN15, JPN15(pACYC184), and JPN15(pCVD450) showed that JPN15(pCVD450) had higher levels of intimin than JPN15 alone or JPN15(pACYC184) (Fig. 5), a result consistent with the elevated alkaline phosphatase activity of JPN15.96 carrying pCVD450. Immunoblots with antiserum raised against intimin (21) showed that the increased 94-kDa OMP was intimin (data not shown). In addition to increased production of intimin, increased amounts of two other OMPs with molecular sizes of 50 and 33 kDa were also seen with JPN15(pCVD450) (Fig. 5). These proteins did not react with antisera against intimin (data not shown). Besides the increased expression of the three OMPs, decreased expression of a 20-kDa protein in the presence of *per* was also observed (Fig. 5).

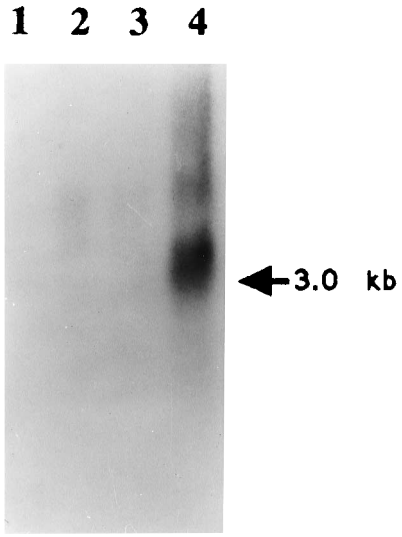

FIG. 6. Northern blot analysis of *eaeA* transcripts after hybridization with an *eaeA*-specific radiolabelled probe. Total RA was isolated from bacteria grown to the early log phase, and 20 μg of denatured RNA was loaded per well. Lanes: 1, HB101(pCVD450); 2, JPN15; 3, JPN15(pACYC184); 4, JPN15(pCVD450).

mRNA analysis. The effect of the *per* region on *eaeA* gene expression is at the level of transcription, as shown by the striking increase of *eaeA* mRNA in JPN15(pCVD450) compared with that in JPN15 or JPN15(pACYC184) (Fig. 6). The major *eaeA* transcript of 3.0 kb made from JPN15(pCVD450) correlates with the size of the 2,820-bp *eaeA* ORF. A minor *eaeA* transcript of 4.2 kb is also seen in this strain. RNA extracted from HB101(pCVD450) showed no transcripts homologous to the *eaeA* probe (Fig. 6).

The site of transcription initiation of the *eaeA* gene was determined by extending a primer hybridized to total RNA from JPN15(pCVD450) by use of reverse transcriptase. A single start site for transcription was identified 130 bp upstream from the ATG start codon of *eaeA* (Fig. 7), a location consistent with the 3.0-kb size of the *eaeA* transcript.

Effect of *per* on EPEC adherence to HEp-2 cells. The addition of *per* to JPN15 increases not only the strain's expression of *eaeA* but also its adherence to HEp-2 tissue culture cells. As shown in Fig. 8, the addition of the cloned *per* region in pCVD450 increased the adherence of JPN15 to HEp-2 cells by ca. 100-fold. The increased adherence induced by *per* is specific for *eaeA* since JPN15.96(pCVD450), containing an inactivated *eaeA* gene, adhered at the same level as that of JPN15. Even with the cloned *per* region in a multicopy plasmid, adherence of JPN15(pCVD450) was still ca. fourfold less than that seen with the wild-type strain E2348/69, presumably because of the absence on this plasmid of the BFP, which is encoded on the wild-type plasmid (17).

As described previously, EPEC wild-type strain E2348/69 adheres to HEp-2 cells in a localized pattern, while strains JPN15 and JPN15.96, lacking pMAR2, adhere poorly to HEp-2 cells and do not show a localized adherence pattern (Fig. 9). Interestingly, the increased adherence of JPN15 (pCVD450) resulted in an adherence pattern resembling localized adherence, although diffuse adherence was also observed (Fig. 9D). The *eaeA* mutant strain JPN15.96(pCVD 450), which also lacks BFP fimbriae, adheres very poorly to HEp-2 cells (Fig. 8 and 9C).

Prevalence of the *per* region. A collection of *E. coli* and non-*E. coli* strains was tested with a *per* gene probe to determine the prevalence of the *per* region among EPEC and other enteric bacteria. Seventy of 78 EPEC strains tested in this study hybridized with the *per* probe (Table 2). All 78 strains were positive for the *eaeA* probe as shown by Jerse et al. (20), and 70 of these strains were positive for the *E. coli* adherence factor (EAF) probe. The EAF probe is a 1-kb DNA fragment from pMAR2 which was found to be associated with the plasmid-encoded localized adherence phenotype of EPEC strains (38). The eight EPEC strains that were negative for the EAF probe and positive for *eaeA* were also negative for *per*. With

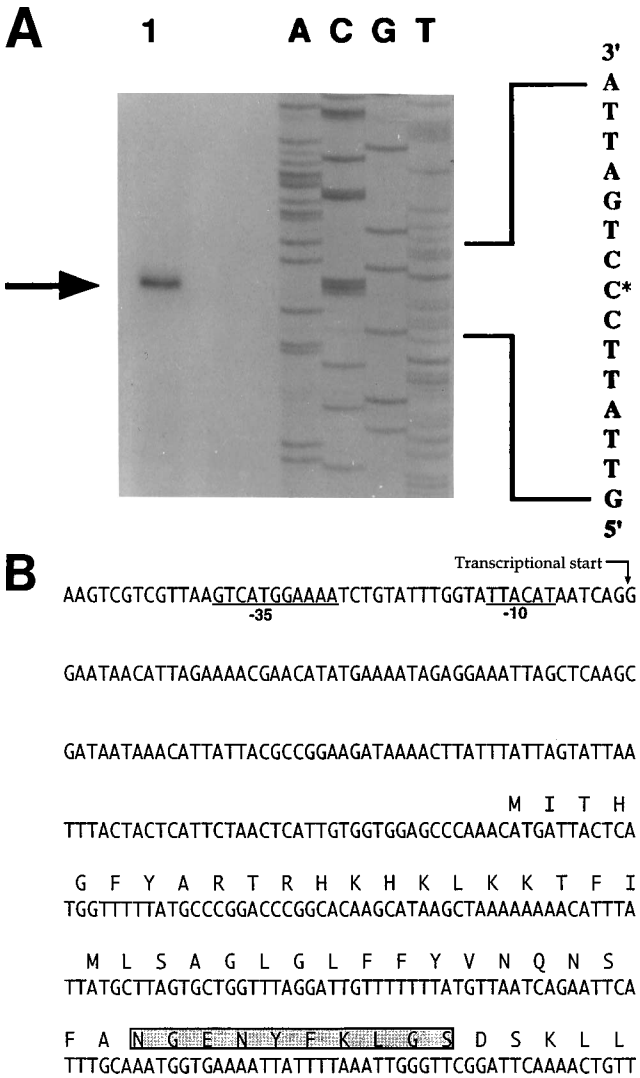

FIG. 7. Transcriptional start site of the *eaeA* transcript. (A) Reverse transcriptase mapping of the *eaeA* transcriptional start. Lanes: 1, primer extension product of JPN15(pCVD450) total RNA after RNA reverse transcription with a 27-mer specific oligonucleotide; A, C, G, and T, lanes corresponding to the bases of a DNA sequencing reaction on a pBR322 recombinant plasmid carrying the upstream portion of the *eaeA* gene. The DNA sequencing reaction used the same 27-mer oligonucleotide used for the RNA priming extension. The arrow indicates the position of the *eaeA* transcript with respect to the *eaeA* gene. *, base at which transcription starts. No *eaeA* mRNA transcript was observed when JPN15 without *per* was similarly analyzed (data not shown). (B) DNA sequence of the 5′ region of *eaeA*. The arrow indicates the *eaeA* transcriptional start site, which is located 130 bp upstream from the first in-frame ATG codon in the *eaeA* ORF. The first predicted methionine residue is 39 residues away from the actual N terminus of the processed intimin protein for which the N-terminal amino acid sequence is indicated by a shaded box (19). Potential −10 and −35 regions are underlined.

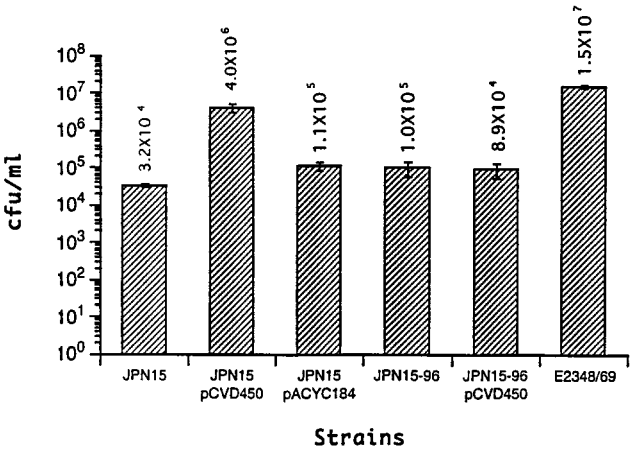

FIG. 8. Quantitative assay of adherence of EPEC strains to HEp-2 cells. The effect of *per* on the adherence of EPEC to HEp-2 cells was evaluated by incubating derivatives of E2348/69 with HEp-2 cells. After removal of unbound bacteria, the CFU of adherent bacteria per milliliter were determined. The actual number of CFU per milliliter ± standard error is given above each column. The names of the strains tested are shown below each bar.

one exception, *per* sequences were not found in any other diarrheagenic *E. coli* strains tested, including enterohemorrhagic *E. coli*, enteroinvasive *E. coli*, enterotoxigenic *E. coli*, enteroaggregative *E. coli*, the rabbit diarrheal pathogen RDEC, uropathogenic *E. coli*, or normal flora *E. coli* (Table 2). The one exceptional non-EPEC strain that possessed *per* sequences was a non-O157 *E. coli* strain which was positive for *eaeA* and the pCVD419 enterohemorrhagic *E. coli*-specific (29) probes but negative for Shiga-like toxins. Non-*E. coli* strains tested, including *C. freundii* biotype 4280 (positive for *eaeA*), *S. typhimurium*, *S. enteritidis*, *S. typhi*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, were also negative for the *per* probe.

DISCUSSION

trans-Acting regulatory factors that modulate expression of genes encoding virulence factors have been found in many bacterial pathogens (33). We now report that enteropathogenic *E. coli* contains a plasmid-encoded regulatory region (*per*) that positively regulates the expression of the chromosomal *eaeA* gene. The *eaeA* gene encodes intimin, a 94-kDa OMP which mediates the intimate adherence of EPEC to epithelial cells (9, 22) and is essential for full virulence of EPEC (10, 11). Previous volunteer challenge experiments demonstrated that loss of the 60-MDa pMAR2 plasmid was associated with decreased virulence and low expression of intimin (27). In this study, we have shown that the low expression of intimin in plasmid-cured strains correlates with the absence of *per* and that addition of the cloned *per* region increases expression of *eaeA*. The 60-MDa plasmid of EPEC also encodes BFP, which mediates localized adherence to HEp-2 cells in a nonintimate fashion (17). Thus, the decreased virulence of the plasmid-cured derivative of E2348/69 in volunteers challenged with this strain could be due to (i) loss of the BFP, (ii) low expression of intimin, and/or (iii) loss of another virulence factor encoded on the 60-MDa plasmid. In all probability, the decreased virulence of plasmid-cured derivatives is a combination of (i) and (ii), although only volunteer trials of isogenic strains specifically mutated in *per* or *bfp* will answer this question.

The *per* region was cloned by screening for recombinant

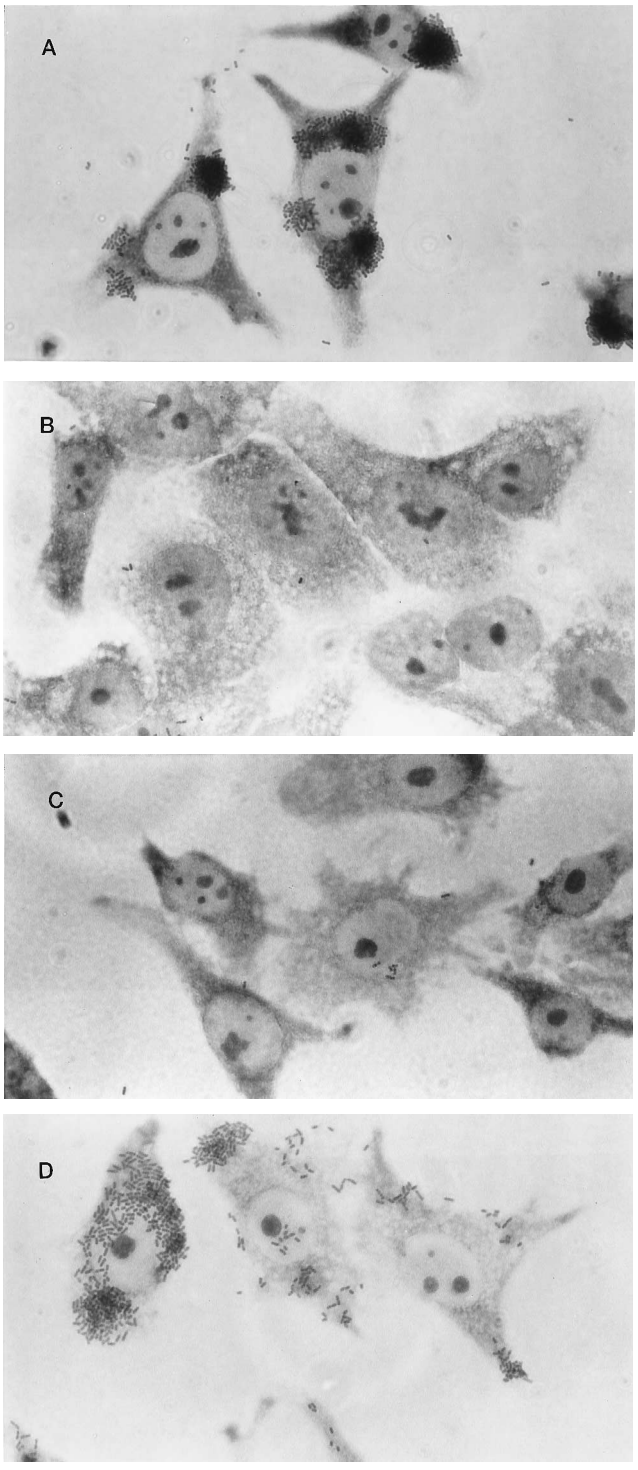

FIG. 9. Assay of localized adherence of EPEC strains to HEp-2 cells. EPEC strains were incubated with HEp-2 cells for 3 h; unbound bacteria were removed by washing. Cells were stained with Giemsa before examination under the light microscope. (A) Wild-type EPEC strain E2348/69; (B) EPEC plasmid-cured strain JPN15; (C) EPEC *eaeA*::Tn*phoA* mutant strain JPN15.96(pCVD450); (D) EPEC strain JPN15(pCVD450). Magnification, ×850.

TABLE 2. Hybridization of the *perAB* probe to *E. coli* isolates[a]

Strain[b]	No. of strains tested	No. of strains positive for *perAB*	Description
EPEC[c]	78	70	EPEC isolates of serogroups O55, O119, O126, O127, O128, and O142
EHEC[d]	65	1[e]	Enterohemorragic *E. coli* isolates positive for the CVD419 EHEC probe (28)
EIEC	4	0	Enteroinvasive *E. coli* isolates of serogroups O28ac, O124, O136, and O143
ETEC	11	0	Enterotoxigenic *E. coli* isolates positive for LT and/or ST toxin DNA probes
EAggEC	4	0	Enteroaggregative *E. coli* isolates
Normal flora strains	5	0	Obtained from normal stools of healthy volunteers

[a] All isolates were obtained from the bacterial strain collection of the Center for Vaccine Development.
[b] EHEC, enterohemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; ETEC, enterotoxigenic *E. coli*; EAggEC, enteroaggregative *E. coli*.
[c] All EPEC isolates were positive for the *eaeA* probe. Seventy isolates were positive for the EAF probe.
[d] Fifty-five of the isolates were positive for the *eaeA* probe.
[e] This strain is *eaeA* positive, O157:H7 negative, and Shiga-like toxin negative.

plasmids that could increase the alkaline phosphatase activity of an *eaeA*::*phoA* fusion. The cloned *per* genes also increased expression of the intimin protein, as shown by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, and increased adherence of EPEC strain JPN15 to HEp-2 cells. Northern blot analysis indicates that the *per* region controls *eaeA* gene expression by increasing transcription of a 3-kb *eaeA* transcript. The increase in expression of *eaeA* by the cloned *per* region contained in pCVD450 is much higher than the increase in expression seen with the wild-type pMAR2 plasmid. It is likely that the higher copy number of the cloning vector accounts for this increase. Alternatively, elements present in the wild-type plasmid and absent in the recombinant clone may negatively control the expression of *per* genes. To rule out the possibility that the regulation of *eaeA* by cloned *per* genes was an artifact resulting from the use of a multicopy plasmid vector, we constructed an insertion mutation in the first ORF of the *per* region in the native pMAR2 plasmid. This mutation greatly diminished expression of *eaeA*.

The four ORFs in the *per* region, designated *perA*, *perB*, *perC*, and *perD*, encode predicted polypeptides of 24, 14.8, 10.5, and 9.4 kDa, respectively. When individual *per* ORFs were tested for the ability to increase the alkaline phosphatase activity of the *eaeA*::*phoA* fusion, only *perC* was able to upregulate expression of the gene fusion by itself. The activation of *eaeA* expression by *perC* was, however, lower than the expression induced by all four ORFs. Various combinations of *perC* and one or two additional *per* ORFs further increased alkaline phosphatase activity but only to levels that were 30 to 40% of the activity seen with all four ORFs. It is possible that the proteins encoded by the *per* region are part of a regulatory cascade in which the initial components are necessary to amplify the regulatory response, whereas the last components may be directly involved with DNA binding and transcription of the target genes. To determine which of the *per* genes are necessary for regulation of *eaeA* in the native plasmid, nonpolar, in-frame deletion mutants must be constructed in each of the ORFs and then introduced into pMAR2 by allelic exchange. It is also possible that *perC* is the only gene whose product acts on *eaeA* and that other parts of the *per* region contain elements that boost *perC* transcription or translation or increase *perC* mRNA stability.

The most compelling sequence similarity found with the predicted *per* gene products was that of PerA with known transcriptional activators of virulence genes such as VirF of *Shigella* spp. and CfaD and Rns of ETEC. The 24-kDa PerA protein has two HTH motifs at the carboxy-terminal end and a predicted pI of 9.8, which are typical features of transcriptional regulators (40). The HTH motifs of PerA share homology with other members of the AraC family of bacterial regulators, some of which activate the expression of virulence genes while others regulate the expression of genes involved in the uptake and metabolism of carbon sources (13, 42). Mutation of the *perA* gene in pMAR2 resulted in diminished regulation of *eaeA*, although whether this effect was due to the specific mutation of *perA* or to polar effects on downstream *per* ORFs is uncertain.

The predicted PerB product shared similarities with several eukaryotic DNA binding proteins, but PerC and PerD showed no compelling similarities to published proteins. None of the predicted Per proteins possess typical leader peptide sequences, and all have high predicted pI values, features of DNA binding proteins. At the nucleotide level, *perD* showed >70% identity over 140 to 150 bases with Tn*7* and IS*630*.

The *per* sequences were found nearly exclusively in EPEC strains and not in other diarrheagenic *E. coli* bacteria or other enteric bacteria. The one exceptional strain in which *per* was found was a non-O157 *E. coli* strain that shared characteristics of both EPEC and enterohemorrhagic *E. coli*. In the 78 EPEC strains examined, there was a perfect correlation between the presence of *per* sequences and sequences hybridizing to the EAF probe. This concurrence is not surprising since the *per* sequences map closely to the 1-kb *Sal*I-*Bam*HI fragment constituting the EAF probe (Fig. 1). Although the EAF fragment and the *per* sequences do not overlap, sequences homologous to IS*630* are found in both *perD* and the EAF fragment (15), suggesting that these sequences may be part of the same transposable element. The function of the EAF probe sequences is not known, but mutation of the *Sal*I site at the end of the EAF fragment leads to inactivation of the localized adherence phenotype (2).

OMP profiles indicate that the *per* region increases not only the expression of intimin, the *eaeA* gene product, but also the expression of two additional OMPs of 50 and 33 kDa, suggesting that the *per* gene products may act as a global regulatory system. In addition, expression of a 20-kDa OMP was decreased in the presence of the *per* region. These OMPs are most likely encoded in the chromosome since they are expressed by an EPEC strain cured of the 60-MDa plasmid but carrying the cloned *per* region. We have also found that *eaeB*, a chromosomal gene which does not encode an OMP but which is essential for intimate adherence and signal transduction in the epithelial cell (12, 14), is also regulated by the *per* region (18). A multicomponent regulatory system regulating the expression of several virulence genes has been found in numerous bacterial pathogens (33), and it is not surprising that EPEC would possess a similar system.

The regulation of a chromosomal gene by a plasmid-en-

coded regulator raises interesting questions about the evolution of EPEC. The situation is not unprecedented since the plasmid-encoded Rns protein of enterotoxigenic *E. coli* regulates expression of chromosomal fimbrial genes (5). Similar to the *rns* gene, the G+C content of *perABC* is 29.6%, which would suggest that *per* originated from a species other than *E. coli*. The striking nucleotide homology found between *perD* and transposable elements such as Tn*7* and IS*630* suggests that the *per* region was introduced into EPEC on a transposable element. We have recently shown that the chromosomal *eaeA* and *eaeB* genes of EPEC are contained on a 35-kb region that is inserted into the exact chromosomal site where a block of virulence genes of uropathogenic *E. coli* is inserted (32). The entire 35-kb region, called LEE for locus of enterocyte effacement, is highly conserved among other attaching and effacing pathogens, such as *E. coli* O157:H7, *H. alvei*, RDEC-1, and *C. freundii* biotype 4280, but is absent from bacteria that do not produce attaching and effacing lesions. It is conceivable but unproven that both the *per* and the LEE region were introduced into EPEC by mobile genetic elements.

In summary, we have cloned a region of the 60-MDa EAF plasmid which controls expression of multiple chromosomal genes in EPEC. It is unknown at the moment if the Per proteins directly activate transcription of *eaeA* and other genes or if the Per proteins are only part of a regulatory cascade that requires an unidentified chromosomally encoded component which directly activates transcription of the target genes. The study of global regulators of virulence genes has yielded significant information about the pathogenesis of many bacterial pathogens, and further characterization of the *per* regulon is likely to yield important insights into the pathogenesis of disease due to EPEC.

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