INFECTION AND IMMUNITY, Aug. 2003, p. 4516–4525 Vol. 71, No. 8
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.8.4516–4525.2003

Characterization of the Novel Factor Paa Involved in the Early Steps of the Adhesion Mechanism of Attaching and Effacing *Escherichia coli*

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Received 3 December 2002/Returned for modification 10 February 2003/Accepted 20 May 2003

Nonenterotoxigenic porcine *Escherichia coli* strains belonging to the serogroup O45 have been associated with postweaning diarrhea in swine and adhere to intestinal epithelial cells in a characteristic attaching and effacing (A/E) pattern. O45 porcine enteropathogenic *E. coli* (PEPEC) strain 86-1390 induces typical A/E lesions in a pig ileal explant model. Using Tn*phoA* transposon insertion mutagenesis on strain 86-1390, we found a mutant that did not induce A/E lesions. The insertion was identified in a gene designated *paa* (porcine A/E-associated gene). Sequence analysis of *paa* revealed an open reading frame of 753 bp encoding a 27.6-kDa protein which displayed 100, 51.8, and 49% homology with Paa of enterohemorrhagic *E. coli* O157:H7 strains (EDL933 and Sakai), PEB3 of *Campylobacter jejuni*, and AcfC of *Vibrio cholerae*, respectively. Chromosomal localization studies indicated that the region containing *paa* was inserted between the *yciD* and *yciE* genes at about 28.3 min of the *E. coli* K-12 chromosome. The presence of *paa* and *eae* sequences in the porcine O45 strains is highly correlated with the A/E phenotype. However, the observation that three *eae*-positive but *paa*-negative PEPEC O45 strains were A/E negative provides further evidence for the importance of the *paa* gene in the A/E activity of O45 strains. As well, the complementation of the *paa* mutant restored the A/E activity of the 86-1390 strain, showing the involvement of Paa in PEPEC pathogenicity. These observations suggest that Paa contributes to the early stages of A/E *E. coli* virulence.

Attaching and effacing (A/E) *Escherichia coli* (AEEC) induces distinctive histopathological lesions on the intestinal mucosa, known as the A/E lesions. These lesions are characteristic of enteric pathogens such as enteropathogenic *E. coli* (EPEC), responsible for severe childhood diarrhea in developing countries (14, 38), enterohemorrhagic *E. coli* (EHEC), causing hemorrhagic colitis and hemolytic-uremic syndrome, a diarrheagenic *E. coli* strain of rabbits (RDEC-1), strains of *Hafnia alvei* isolated from children with diarrhea, and *Citrobacter rodentium*, causing transmissible colonic hyperplasia in mice (4, 16, 53). A/E lesions have also been associated with diarrhea in different animal species such as rabbits, calves, dogs, cats, lambs, pigs, and tamarins (8, 9, 22, 32, 37, 55).

A/E lesions result from intimate bacterial adherence to the apical surfaces of enterocytes and activation of several chromosomal gene products that interact with components of the host cell, leading to host cell protein phosphorylation, effacement of target brush borders, and disruption of the underlying actin cytoskeleton (11, 38). The genes are clustered in a chromosomal pathogenicity island called the locus of enterocyte effacement (LEE). Its location and size vary in different strains. In EPEC strain E2348/69 and EHEC O157:H7 strains, the LEE is inserted in the *selC* locus at about 82 min on the *E. coli* K-12 chromosome, but its size varies from 35 kb for EPEC to 43 kb for EHEC. In strains of serotype O26:H-, the LEE is about 35 kb and is inserted in the *pheU* gene (12, 34, 46). One of the LEE genes (*eae*) encodes intimin, a 94-kDa outer membrane protein involved in intimate attachment to host cells (24). Another encodes a translocated intimin receptor called Tir, which interacts with intimin and allows the intimate attachment of the bacteria to the epithelial cells (27). Other genes encode the secreted proteins EspA, EspB, EspD, and EspF, which are responsible for signal transduction in epithelial cells (15, 23, 28, 29, 31, 33, 35, 50) and which are secreted through a type III secretion apparatus, which is also encoded in the LEE (33). The recently identified EspC enterotoxin, whose gene is located within a pathogenicity island at 60 min on the chromosome of *E. coli*, may also play a role as an accessory virulence factor in some EPEC strains (36).

A/E lesions in naturally occurring swine postweaning diarrhea cases are often associated with *E. coli* of the O45 serogroup (19, 21, 55). This pig AEEC, termed porcine EPEC (PEPEC), possesses all the genes in the LEE. The A/E activity of PEPEC O45 isolates is highly correlated with the presence of the LEE (21, 55, 56). Although there is some heterogeneity in PEPEC strains with respect to the LEE insertion, all of these strains possess a β-intimin subtype. In PEPEC strain 86-1390, sequences of the *eae*, *tir*, and *esp* regions are closely related to those of other AEEC strains, particularly of rabbit EPEC (REPEC) strains (3). The presence of the *eae* β variant gene in the porcine O45 strain 86-1390 (57) is associated with the ability of this strain to produce A/E lesions in experimentally

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inoculated newborn gnotobiotic piglets (55) and in an homologous in vitro model using newborn piglet ileal explants (56). We have created a bank of PEPEC strain 86-1390 Tn*phoA* mutants and screened for the loss of their capacity to induce the typical histopathological A/E lesions in pig intestinal ileal explants (2). One mutant, M155, did not induce A/E lesions, the Tn*phoA* insertion occurring in a gene that was called *paa*, for porcine A/E associated. The distribution of *paa* in PEPEC O45 strains revealed that its presence was associated with that of the *eae* gene and its A/E phenotype in vivo. On examination of enteric *E. coli* isolates from humans and various animal species, a strong correlation between the presence of *paa* and *eae* in EHEC O157:H7 and O26 isolates and dog, rabbit, and pig isolates, and a lesser correlation in human EPEC isolates, was found (2). The aim of this study was to characterize the *paa* gene and to study the contribution of Paa to the development of A/E lesions due to PEPEC in a pig ileal explant model.

MATERIALS AND METHODS

Bacterial strains and plasmids. The wild-type pathogenic *E. coli* strain 86-1390 (serogroup O45, tetracycline [Tc^r] and streptomycin [Sm^r] resistant) was isolated at the Faculté de Médecine Vétérinaire, Saint-Hyacinthe, Québec, Canada, from a 4-week-old pig with postweaning diarrhea. O45 strain 86-1390 induces typical A/E lesions both in vitro and in vivo and contains sequences homologous to the LEE (55, 56). A collection of 11 PEPEC strains was used for in vivo experiments. *E. coli* strain SM10λ*pir*(pRT733) was used to introduce Tn*phoA* into strain 86-1390 by conjugation (17). *E. coli* strain HB101 (*supE44 hsdS20* (r$^-$ m$^-$) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) (7) was used as host for recombinant plasmids in this study. The λZAPII^R system was used for construction of a genomic DNA library from strain 86-1390 (Stratagene, La Jolla, Calif.). The host strain *E. coli* XL1 Blue MRF′ {Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*[F′ *proAB lacI*^{q} ZΔM15] Tn*10*(Tet^r)} and *E. coli* strain SOLR {e14$^-$(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recB recJ umuC*::Tn*5*(Kan^r) *uvrC lac gyrA96 relA1* λ^R[F′ *proAB lacI*^{q} ZΔM15]Su$^-$ (nonsuppressing)}, as well as the helper phage M13 needed in the cloning procedure, were used according to the manufacturer's recommendations (Stratagene). E22 is an *eae* β-positive REPEC strain (40).

Tn*phoA* mutagenesis. Mutations were obtained from random insertion of the Tn*phoA* sequence into the chromosomal DNA of *E. coli* strain 86-1390 (Sm^r Tc^r). This was accomplished as described previously (17) by using the suicide vector pRT733, which carries the Tn*phoA* insertion and the kanamycin resistance (Km^r) gene in *E. coli* strain SM10λ*pir* (51). Exconjugants from the mating between *E. coli* strain SM10λ*pir*(pRT733) and *E. coli* strain 86-1390 were selected on Luria-Bertani (LB) agar (Difco Laboratories, Detroit, Mich.) containing kanamycin and streptomycin (40 μg ml^{-1}) and the alkaline phosphatase substrate XP (5-bromo-4-chloro-3-indolylphosphate) (Sigma Chemical Co., St. Louis, Mo.). Kanamycin- and streptomycin-resistant blue colonies resulting from the transposition of Tn*phoA* into the genome of the recipient strain *E. coli* 86-1390 were stored in glycerol at −70°C. Of the Km^r and Sm^r transposon insertions, 1% were found to produce blue colonies on agar in the presence of alkaline phosphatase substrate XP.

Cloning and DNA sequencing *phoA* fusion regions. Cloning the sequence flanking the 5′ end of *phoA* fusion regions was done with the kanamycin resistance gene as a selectable marker, and the appropriate DNA fragments were cloned into pBR322. One microgram of total DNA isolated from the mutant was digested by *Bam*HI and ligated with 0.1 μg of similarly digested pBR322. The ligation mixture was electroporated into the strain HB101 with a Gene Pulser according to the manufacturer's instructions (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada). Transformants carrying the desired recombinant plasmid were selected on LB agar plates supplemented with ampicillin (40 μg ml^{-1}) and kanamycin (40 μg ml^{-1}). To precisely identify the mutated gene, the double-stranded plasmid DNA at the junction of the site of Tn*phoA* insertion was sequenced with a T7 sequencing kit (Pharmacia LKB Biotechnology Inc., Baie d'Urfé, Québec, Canada) according to the manufacturer's instructions. An oligonucleotide (5′AATAATCGCCTGAGC3′) corresponding to nucleotides 72 to 86 of Tn*phoA*, synthesized on a Gene Assembler (Pharmacia LKB Biotechnology Inc.), was used as the primer. DNA sequence data were analyzed with the GeneWorks program (Intelligenetics, Inc., Mountain View, Calif.) and programs included in the GCG (Genetics Computer Group, Madison, Wis.) package (10). The deduced amino acid sequence was compared against the combined databases of the National Center for Biotechnology Information (Washington, D.C.) via the BLAST network service.

Cloning and sequencing *paa*. To clone the full length of the *paa* gene, corresponding to the gene of the mutant M155 with the Tn*phoA* insert, a genomic DNA library of PEPEC strain 86-1390 was constructed in the λZAPII^R vector. Chromosomal DNA was partially digested with *Eco*RI and pooled and fractionated through a 10 to 30% sucrose linear gradient. The desired fragments of 6 to 10 kb were isolated and ligated to dephosphorylated *Eco*RI-digested λZAPII^R vector arms and packaged with an in vitro packaging system (Stratagene). Bacteriophage particles were propagated in *E. coli* XL1 Blue and plated for plaque isolation. To screen the recombinant phages, a 350-bp PCR fragment derived from the 5′ end of the *paa* gene was generated by *paa*-specific primers M155-F1 (5′ATGAGGAAACATAATGGCAGG3′) and M155-R1 (5′TCTGGTCAGGT CGTCAATAC3′) annealed at nucleotides 91 to 110 and 428 to 447 of the *paa* gene, respectively. The 350-bp fragment was then radiolabeled with [α-^{32}P]CTP as a probe by using an oligonucleotide random priming labeling kit (Pharmacia LKB) according to the manufacturer's instructions. Positive plaques were selected and excised with the ExAssist helper phage (M13) and *E. coli* strain SOLR system according to the Stratagene λZAPII^R instruction manual. Plasmid DNA was isolated by alkaline lysis, and the insert was sequenced by an automated DNA sequencer (AFL DNA sequencer; Pharmacia LKB) using the *paa*-specific oligonucleotides synthesized on a Gene Assembler (Pharmacia LKB).

Transcomplementation of the M155 *paa*::Tn*phoA* mutant. The *paa* gene was amplified with its promoter regions from strain 86-1390 DNA with the PaaHO/F (5′GGATCCTTAAAGGGCAGG3′) and PaaHO/R (5′GGATCCGATGTCAA GTGC3′) primers and cloned into the pGEM-T vector. The *Bam*HI fragment was then inserted into the *Bam*HI-linearized pACYC184 plasmid, resulting in the pACYC184-PaaHO construct, containing the wild-type *paa* gene. This construct was used as a complementation plasmid for *paa* in the M155 Tn*phoA* mutant, leading to the M155c strain.

Quantification of A/E capacity of the mutants. The A/E capacities of the Tn*phoA* mutants generated in this study were examined by ileal explant culture as previously described (56). Briefly, overnight bacterial cultures were inoculated onto the villous surface of ileal explants from colostrum-deprived newborn piglets and incubated on a rocking platform at 37°C for 8 h in an atmosphere of 95% O_2 and 5% CO_2. RPMI 1640 culture medium (Gibco, BRL, Burlington, Ontario, Canada) was replaced with fresh medium at hourly intervals during the incubation to prevent acidic pH and overgrowth of bacteria. *E. coli* O45 strain 86-1390 and the porcine 862 strain, which does not possess the LEE, were used as positive and negative controls, respectively. Three or four ileal explants were used for each bacterial isolate, and the experiments were repeated three times. In some experiments, broth cultures were incubated at 37°C with an equal volume of lyophilized Paa antibody reconstituted with phosphate-buffered saline (PBS) for 30 min prior to the first explant inoculation. Tissues were processed for light microscopy examination as described previously (56); the intact villi with adherent bacteria were counted, and the number was expressed as a percentage of the total number of intact villi observed.

Southern analysis. The number of Tn*phoA* insertions was determined by Southern blot analysis as described previously (17). Briefly, total DNA was extracted from the strain by sodium dodecyl sulfate lysis, proteinase K treatment, phenol-chloroform extraction, and ethanol precipitation. Extracted DNA was digested with the restriction endonuclease *Sac*I or *Eco*RV, neither of which cuts within Tn*phoA*, under conditions described by the manufacturer (Pharmacia LKB). After separation by electrophoresis in a 0.7% agarose gel, DNA preparations were examined by Southern hybridization. An internal *Hin*dIII-*Bam*HI fragment of Tn*phoA* radiolabeled with [α-^{32}P]CTP by using an oligonucleotide random priming labeling kit (Pharmacia LKB) according to the manufacturer's instructions was used as a probe.

Chromosomal localization of the *paa* gene. First, the presence of *paa* between the *rem* and *rel* loci was investigated by PCR amplification. The remF (5′GAT GCCTGCCACATCAGAGG3′) and relR (5′CCTAAGCCAGTACGTGTGAC 3′) primers located at bp 2821 to 2840 and 3400 to 3420, respectively, were used to amplify a 580-bp fragment on the *E. coli* K-12 strain MG1655 chromosome. The PaaR primer (5′GCTACAAACCGATGAAGCGGC3′) was used in combination with remF to detect an insertion of the *paa* gene between the *rem* and *rel* loci, leading to a 605-bp amplicon. Second, the integrity of the *yciD-yciE* region was tested with the YciDF (5′AGTGGCGGCTTTGGCACTAA3′) and YciER (5′CGAATCTATGCTTGAATCCA3′) primers. They were used to amplify a 1,122-bp fragment on the MG1655 chromosome. The PCR mixture included 5 μl of template DNA, 5 μl of 2 mM deoxynucleoside triphosphate, 5 μl of 10× buffer (100 mM Tris-HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), 2.5 μl

of each primer pair (25 pmol), 1 U of *Taq* DNA polymerase (Pharmacia), and sterile distilled water to 50 μl. The following cycles were used: 1 cycle of 94°C for 2 min and 30 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 30 s, with a final extension of 72°C for 7 min. The PCR products were analyzed by agarose gel electrophoresis.

Nonpolar mutation in *paa*. A PCR fragment containing the gene and its promoter sequences was amplified with the PaaHO/F and PaaHO/R primers and cloned into the pGEM-T vector (Promega) according to the manufacturer's instructions. The construct was digested with *Kpn*I, and an *Hin*cII fragment from pSB315 containing a kanamycin resistance cassette was ligated in the *Kpn*I site, resulting in *paa*::*kan*. The construct was digested with *Bam*HI, and the *paa*::*kan* fragment was ligated to the pKNG101 suicide vector cut with the same enzyme. The resulting construct was transferred to strain S17 λ*pir*, from which it was mobilized into strain E22 by the membrane filter mating technique. Transconjugants were selected on M9 agar containing the appropriate antibiotic (kanamycin at 50 μg/ml). Selection for double-crossover allele replacement was obtained by *sacB* counterselection on LB agar plates without NaCl and containing 5% sucrose (25).

Pulsed-field gel electrophoresis. Strains 86-1390, M155, STJ348 (O157:H7), and E2348/69 (EPEC) were inoculated 1/100 in 20 ml of LB medium and incubated at 37°C overnight with agitation. Bacteria were washed two times in SE (75 mM NaCl, 25 mM EDTA; pH 7.5) by centrifugation and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA; pH 8). The optical density of the cells was adjusted to 1.5 to 1.8 at a wavelength of 600 nm. Low-melting-point agarose (Gibco, BRL) was prepared in distilled water to obtain a final concentration of 1.5%. Plugs were formed by mixing 500 μl of bacterial suspension with 500 μl of prewarmed (60°C) agarose. This mixture was then pipetted into plug molds (Bio-Rad Laboratories). After the plugs solidified, they were incubated at 50°C overnight in lysis buffer (1% [wt/vol] *N*-laurylsarcosine–0.5 M EDTA [pH 9.5] supplemented with 1 mg of proteinase K/ml). The lysis buffer was changed, and plugs were incubated for another 4 h. Plugs were washed three times for 1 h each time with 1 mM phenylmethylsulfonyl fluoride in 10 mM Tris-HCl, pH 8. Another set of three 30-min washes was done with 10 mM Tris-HCl, pH 8. The plugs were then preincubated for 30 min with 1 ml of the appropriate restriction enzyme buffer. The buffer was replaced by a fresh mixture containing 30 to 40 U of enzyme and incubated overnight at the appropriate temperature (37°C for *Xba*I and 50°C for *Sfi*I). The next day, 10 U of enzyme was added to the plugs for a 2- to 3-h incubation period. Electrophoresis of the samples was performed on the CHEF-DRII system by using a 1% pulsed-field grade agarose gel (Sigma) with 2 liters of modified 0.5× TBE running buffer (10× TBE is 89 mM Tris-borate plus 25 mM EDTA, pH 8.3). The running conditions were as follows: switch of 5 to 35 s, 6 V/cm, and a run time of 20 h. Finally, the gel was stained in 10 mg of ethidium bromide/ml in distilled water for 30 min. Digested genomic DNA separated in agarose gels was transferred to positively charged nylon membranes (Immobilon-Ny$^+$; Millipore Corporation, Bedford, Mass.) in accordance with the manufacturer's instructions and hybridized under stringent conditions as described by Sambrook et al. (45). The probe was labeled with biotin by PCR amplification using PaaF (5′GGATCCATGAGGAACATAA3′) and PaaR (5′CTCGAGAGTGCCTTTCCTGG3′).

Production of anti-Paa antibodies. The *paa* gene of strain 86-1390 was amplified by PCR using primer pairs PaaF and PaaR. The amplicon was inserted into the pQE-30 expression vector (Qiagen) by using appropriate cloning sites, and fusion was confirmed by sequencing. *E. coli* M15(pREP4) (Qiagen) was used as the host strain for the expression of recombinant His-Paa. The His-Paa was purified from a Ni-nitrilotriacetic acid affinity column (Qiagen). Laying hens 25 weeks of age were immunized intramuscularly with 500 μl of incomplete Freund's adjuvant and an equal volume of purified His-Paa, corresponding to 50 μg of protein. Paa-specific immunoglobulin Y (IgY) was then extracted from egg yolks by the method described by Akita and Nakai (1), with some modifications. Briefly, egg yolks were separated from the albumin. An equal volume of PBS was added to the egg yolks, and the mixture was then homogenized by Vortex agitation. An equal volume of chloroform was added, and a solid homogenate was then obtained by mixing. The preparation was centrifuged for 5 min at 14,000 × *g*, and the supernatant containing IgY was recovered, lyophilized, and conserved until use. The anti-Paa IgY titer was determined by enzyme-linked immunosorbent assay using microtiter plates (Immulon 2HB; Dynec) precoated with 100 ng of purified protein in carbonate buffer (pH 9.6) per well.

Electron microscopy. Electron microscopy and immunogold labeling were done as previously described (18) with modifications. Cultures of the wild-type strain 86-1390, complemented mutant strain M155c, and *paa*-negative control strain 862 were grown overnight at 37°C in tryptic soy broth (TSB) and washed three times in PBS. Bacterial pellets were obtained with an AirFuge air-driven ultracentrifuge (Beckman Instruments Inc., Palo Alto, Calif.) and were coated on 150-mesh Formvar-coated nickel grids (Marivac, St.-Laurent, Quebec, Canada). After a blocking step in 5% normal donkey serum, grids were labeled with appropriate dilutions of a chicken anti-Paa primary antibody, previously adsorbed against the *paa*-negative *E. coli* strain E2348/69, and a donkey anti-chicken IgY secondary antibody conjugated with 12-nm colloidal gold beads (Jackson ImmunoResearch Laboratories Inc.). Grids were negatively stained with 1% phosphotungstic acid at pH 6.0 and examined with a H-7100 transmission electron microscope at 75 kV (Hitachi High-Technologies, Rexdale, Ontario, Canada). The anti-Paa antibody adsorbed with the Paa-positive strain M155c was used as a negative control.

Ileal explant tissues were processed for transmission electron microscopy. Tissues were fixed for 2 h at room temperature in 2.5% glutaraldehyde and rinsed in 0.1 M cacodylate buffer at pH 7.3. Tissues were then postfixed in 2% osmium tetroxide, rinsed in water, dehydrated in a graded ethanol series, and finally embedded in Spurr resin (Marivac). Thin sections were mounted on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips 420 transmission electron microscope at 80 kV (Philips Electronics, Eindhover, The Netherlands).

Infection of gnotobiotic piglets. Eleven porcine O45 strains were tested for A/E activity in experimentally inoculated newborn gnotobiotic piglets as previously described (55). Briefly, aseptically collected piglets were inoculated intragastrically with 10 ml of an overnight culture of *E. coli* (approximately 10^9 CFU) and 10 ml of 0.1% peptone-water. They were examined several times daily for development of clinical signs, and necropsy was performed when diarrhea occurred or at 120 h postinoculation (p.i.) if clinically normal.

Statistical analysis. Results are presented as the means ± standard deviations of the means. A Kruskal-Wallis test was performed with commercially available software (SAS, version 8.1; SAS Institute, Cary, N.C.), and post hoc two-by-two comparisons were done to assess differences between the groups; a P value <0.0001 was taken to be significant.

Nucleotide sequence accession number. The complete nucleotide sequence of *paa* was lodged with GenBank under accession number U82533 (*paa* was previously named *anm*).

RESULTS

Identification of a transposon mutant deficient in A/E activity. After random insertion of Tn*phoA* into the genome of porcine O45 *E. coli* strain 86-1390, mutants containing translational fusions between bacterial genes for extracytoplasmic proteins and *phoA* were screened on LB agar supplemented with kanamycin and streptomycin and with substrate XP. A total of 180 Tn*phoA* mutants were then examined in a qualitative assay for adhesion to piglet ileal explants (56). Of these, 175 mutants were found to attach extensively to a similar extent as strain 86-1390, as observed by light microscopy (2) (Fig. 1A). In the five other mutants which attached less extensively to piglet ileal enterocytes, different insertion sites for Tn*phoA* were observed. Three insertions were in genes found in *E. coli* K-12 (one in *osmB*, two in *pstS*), one was in IS*100*, and one was in an as yet uncharacterized gene (the mutant with this gene was named M155). This gene was called *paa*. Hence, only M155 was retained for further characterization. The presence of a single copy of Tn*phoA* in the chromosomal DNA of the mutant M155 was demonstrated by Southern hybridization of genomic DNA digested by *Sac*I or *Eco*RV and probed with an internal *Bam*HI-*Hin*dIII fragment of Tn*phoA* (data not shown).

Effect of the Paa mutation and complementation on the adherence phenotype. The insertion of Tn*phoA* in the *paa* gene (M155 mutant) resulted in a significantly reduced number of ileal villi showing bacterial adherence to epithelial intestinal cells, compared to that observed for the wild-type 86-1390, in our explant culture model (Fig. 2A). As observed for negative-control strain 862 (Fig. 1B), there was a patchy, loose association of mutant M155 with the mucosal surfaces of a low

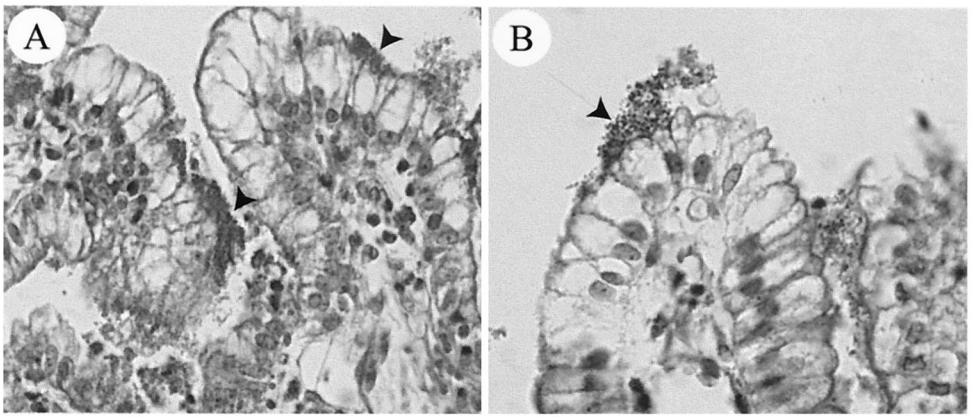

FIG. 1. Light microscopy micrographs of ileal explants inoculated with the wild-type O45 strain 86-1390 (A) or with the LEE-negative strain 862 (B). Strain 86-1390 showed a typical intimate-adherence pattern (arrowheads) with irregularity of the associated epithelial cells, whereas a loose association of bacteria with the intestinal mucosa of some villi with no obvious change in associated epithelial cells (arrow) was observed for negative-control strain 862. Magnification, ×400.

proportion of villi. Furthermore, the complementation of strain M155 with the pACYC184 plasmid carrying the *paa* gene and its promoter region (M155c strain) restored the adherence phenotype. On transmission electron microscopy, for the M155c and 86-1390 strains, bacteria demonstrated a tight attachment to epithelial cell surfaces, effacement of microvilli beneath the adherence site, and a dense region underneath the adherence site representing F-actin polymerization (Fig. 3A and 3B), whereas mutant M155 and control strain 862 showed no evidence of A/E lesion formation (Fig. 3C).

Sequence analysis of *paa* and associated loci. To further characterize the locus around the site of the Tn*phoA* insertion of mutant M155, a genomic DNA library of PEPEC strain 86-1390 was screened. On primary screening, it was found that several of the plaques hybridized to the 350-bp PCR probe fragment which corresponded to the sequence adjacent to the Tn*phoA* insertion of mutant M155 and which had been radiolabeled with [α-^{32}P]CTP. One of these, with an insert of 6 kbp and designated λZAPII^{R} AN1, was chosen for further study. Using primers obtained from the sequence adjacent to the Tn*phoA* insertion in M155, we determined the nucleotide sequence of the full length of the gene where *TnphoA* was inserted. It revealed an open reading frame of 753 bp. The region upstream of the first ATG was preceded by excellent matches to consensus sequences for −35 and −10 putative promoter regions and by a Shine-Dalgarno sequence (Fig. 4). Downstream of the TAG translational stop codon, a putative transcription terminator was evident (Fig. 4). The G+C content of *paa* was 44%, which is substantially lower than that of *E. coli* K-12 (50.8%) (6), suggesting that *paa* may have been acquired by 86-1390 through horizontal transfer. *paa* was predicted to encode a 251-amino-acid protein with an anticipated

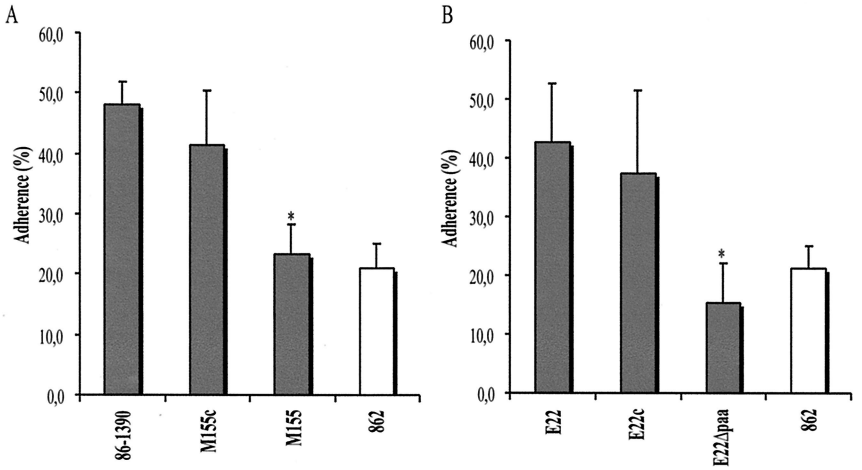

FIG. 2. Adherence of wild-type strains and their *paa* mutant strains. (A) *paa* mutant strain M155 (n = 18) showed a decreased number of intact ileal villi with bacterial adherence to epithelial cells, compared to wild-type PEPEC strain 86-1390 (n = 12) and to the complemented mutant strain M155c (n = 20). The porcine strain 862 (n = 15), which does not possess the LEE, was used as a negative control. (B) *paa* mutant strain E22Δpaa (n = 19) showed a decreased number of intact ileal villi compared to wild-type REPEC strain E22 (n = 19) and to the complemented mutant strain E22c (n = 10). Error bars, standard deviations of the means. Asterisk, statistically significant difference ($P < 0.0001$, when compared by Kruskal-Wallis test) from wild-type strains 86-1390 (A) and E22 (B).

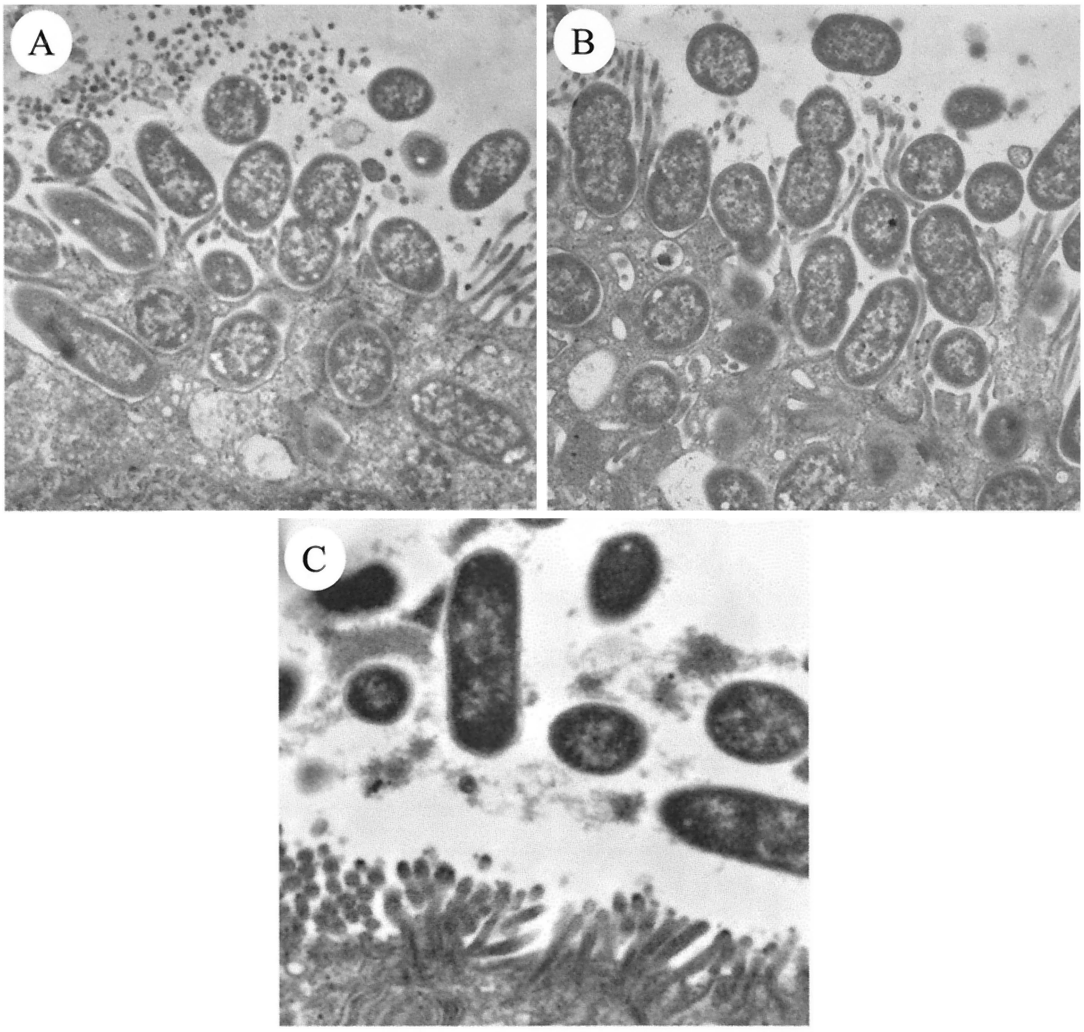

FIG. 3. Transmission electron micrographs of ileal explants inoculated with the wild-type O45 strain 86-1390 (A; magnification, ×20,664), the complemented mutant strain M155c (B; magnification, ×20,702), or Tn*phoA* mutant M155 (C; magnification, ×13,500). Typical A/E lesions were observed for both wild-type and complemented-mutant strains, whereas bacteria in the lumen without any direct contact with the epithelium were observed for the mutant M155.

molecular mass of 27.6 kDa (Fig. 4). The prediction of the Paa protein localization site with the Expasy software suggested that the Paa peptide may be cleaved after the first 18 residues (54) (Fig. 4). The hydrophobicity profile indicated the presence of a potential transmembrane region (amino acids 1 to 18) corresponding to a Sec-dependent signal sequence and hydrophilic segments in mature Paa. However, the Expasy program also predicted that the entire Paa protein could be unstable due to its N-terminal end. The Domain Architecture Retrieval Tool (DART) from the National Center for Biotechnology Information (NCBI) identified a sulfate-binding motif in the C-terminal half of the protein. The comparison with the SWISS-PROT database showed that the amino acid sequence deduced from the *paa* gene displayed an identity of 100% with those encoded by the *paa* genes of the O157:H7 EDL933 and Sakai strains, 51.8% with PEB3, a major antigen of *Campylobacter jejuni*, and 49% with AcfC, a *Vibrio cholerae* accessory colonization factor (Fig. 5). AcfC and PEB3 also contain the same sulfate-binding motif.

Localization of *paa* on the chromosome. The 3.5-kb region containing *paa* was also 100% identical to the same region in the O157:H7 EDL933 and Sakai strains. Upstream of *paa*, there was homology with the *prpH* gene, encoding a fimbrial protein precursor of Pap-related pilus H. Downstream of *paa* was a sequence displaying identity to the *rem* gene from *E. coli* K-12. This may indicate that *paa* has interrupted the *relB-rem* region. Indeed the amplification by PCR of *relB-rem* showed that this region is disrupted in many *paa*-positive strains (data not shown). On the other hand, amplification was successful when a set of *paa-rem* primers was used. Localization of *paa* in the genome of O157:H7 strains EDL933 and Sakai demonstrated that *paa* was inserted at 28.3 min within the *yciD-yciE* locus of the K-12 chromosome. PCR studies confirmed this result: 9 out of 14 (64%) EPEC strains isolated from pigs, 2 of

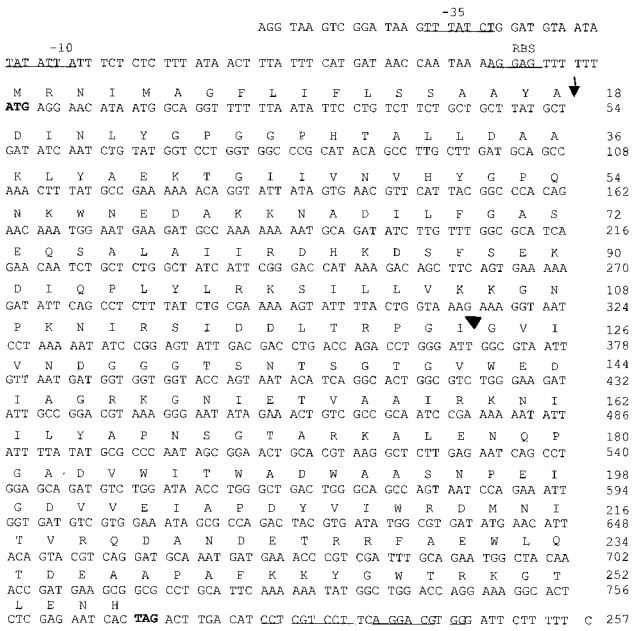

FIG. 4. The nucleotide sequence of *paa* and its flanking sequences and the deduced amino acid sequences. The putative −10 and −35 promoter sites, the ribosome binding site (RBS), and the putative transcription terminator are underlined. The translation initiation codon and the TAG translation termination codon are in boldface. Vertical arrow, potential peptide signal cleavage site; arrowhead, insertion site of Tn*phoA*.

7 (29%) REPEC strains, and 3 of 4 EHEC strains from humans (75%) were interrupted in the region between the *yciD* and *yciE* genes from *E. coli* K-12 (Table 1). Thus, in the O157:H7 strains EDL933 and Sakai, *paa* belongs to O island 57 and the Sp9 region, respectively, inserted within *yciD* and *yciE*. These islands contain incomplete lambda-like phage sequences (phage CP-933O for EDL933, phage Sp9 for Sakai).

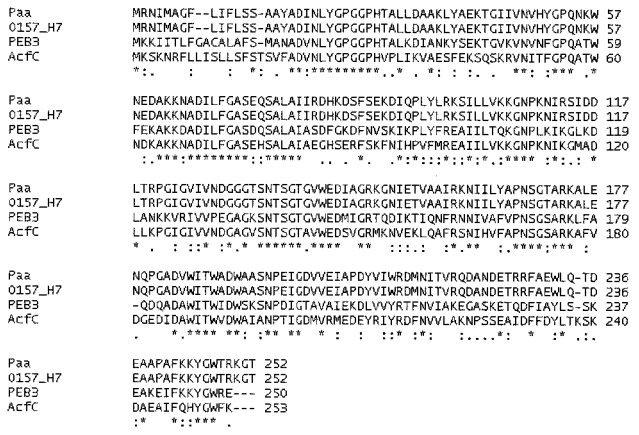

FIG. 5. Alignment of the deduced amino acid sequences of the Paa of the 86-1390 strain and the Paa proteins of the O157:H7 EDL933 and Sakai *E. coli* strains, the AcfC protein of *V. cholerae*, and the PEB3 protein of *C. jejuni*. ∗, identical or conserved residues in all sequences in the alignment; colons, conserved substitutions; periods, semiconserved substitutions. The amino acid sequence alignment was performed with the Clustal W program.

TABLE 1. Integrity of the *yciDE* region in *paa*-positive strains

Strain type	No. of strains[a]		
	Total	*yciDE* negative[b]	*yciDE* positive
EHEC	4	3	1
REPEC	7	2 (E22, RDEC-1)	5
PEPEC	14	9 (86–1390)	5
Total	25	14 (56%)	11 (44%)

[a] Strains considered positive amplified a 1,122-bp fragment, whereas no amplification was seen in the negative strains.

[b] Strains 86-1390 and E22 were used for testing the role of Paa; RDEC-1 is an REPEC strain.

In PEPEC strains, the *paa* region is also within *yciD-yciE*. Moreover, in these genomes the *relB-rem* region is disturbed and a copy of *rem* is found near the 3′ end of *paa*. Genome analysis of the two studied O157:H7 strains indicates that *paa* is in a region specific to these pathogenic strains which harbors the sequence of a lambda phage.

The chromosomes of different AEEC strains were digested with *Sfi*I or *Xba*I and examined by pulsed-field electrophoresis and Southern blotting. For both digests, a *paa* biotinylated probe hybridized with only one fragment of about 210 (*Xba*I) or 120 kbp (*Sfi*I) for strains 86-1390 and M155 and about 290 (*Xba*I) or 210 kbp (*Sfi*I) for the EHEC O157:H7 STJ348 strain, indicating that the chromosomal arrangement in the last strain was different from that in the other two. There was no hybridization for the *paa*-negative EPEC strain E2348/69. In *paa*-positive strains, *paa* was present in only one copy in the chromosome.

Creation of a *paa* mutant by allelic exchange and complementation. Creation of a *paa* mutant by allelic exchange on the chromosome of PEPEC strain 86-1390 was unsuccessful. However, a *paa* mutant (E22Δ*paa*) was obtained from strain E22, a *paa*-positive REPEC strain that induces A/E lesions in our porcine ex vivo model. Strain E22Δ*paa* showed a reduced-adherence phenotype (Fig. 2B) and was not able to induce A/E lesions in the ex vivo model. The complementation of E22Δ*paa* with the wild-type *paa* restored this phenotype (Fig. 2B).

Development of A/E lesions in vivo by *paa*-positive and *paa*-negative PEPEC strains. Most tested *eae*- and *paa*-positive porcine O45 isolates induced severe A/E lesions leading to diarrhea between 24 and 70 h after infection (Table 2). The severity and extent of the A/E lesions appeared to be related to the time of onset and severity of diarrhea in the inoculated piglets. On the other hand, *eae*-positive, *paa*-negative isolates induced less-severe or no A/E lesions and piglets developed no diarrhea or mild diarrhea after 83 h p.i.

Capacity of anti-Paa antibodies to block adhesion. Treatment with chicken egg yolk anti-Paa antibodies significantly reduced, by up to 53%, the proportion of intact villi showing intimate adherence, following inoculation of pig ileal explants with PEPEC strain 86-1390 ex vivo (Fig. 6), compared to treatment with egg yolk antibodies from hens immunized with a sonicated preparation from the Paa-negative host strain M15(pREP4).

The Paa protein is located at the bacterial surface. Immunogold labeling was performed in order to locate the Paa protein in the wild-type strain 86-1390 and the complemented mutant strain M155c labeled with the anti-Paa polyclonal an-

TABLE 2. Clinical and histopathological findings in piglets inoculated with porcine *E. coli* O45 isolates

Strain[e]	No. of pigs with diarrhea/no. inoculated	Onset of diarrhea (h)[b]	Extent of A/E lesions[d]	Presence of *paa* gene
81-4420	2/2	24	++++	+
86-1390	2/2	30	++++	+
91-19-172	2/2	35	++++	+
90-2061	2/2	39	+++	+
90-1513	2/2	41	+++	+
88-4299	1/1[a]	44	++	+
86-4733	2/2	70	+	+
83-2315	2/2	83	++	−
88-1861	1/1[a]	91	−	−
89-56-196	1/2	96	−	−
82-4378	0/2	NM[c]	+	−
81-1786	1/2	85	−	−

[a] One piglet died of causes unrelated to the infection within 20 h after birth.
[b] Mean time of onset of diarrhea p.i.
[c] NM, nonmeasurable data.
[d] ++++, extensive bacterial colonization and severe effacement of microvilli; +++, large areas of bacterial colonization and heavy effacement; ++, focal lesions; +, small scattered focal lesions; −, no lesions observed.
[e] Strains were *eae* and LEE positive, except for 81-1786, which was a control *eae*-negative strain.

tiserum. The Paa protein was uniformly distributed over the bacterial surface of the *trans*-complemented mutant strain M155c (Fig. 7A) and, to a lesser extent, on the bacterial surface of strain 86-1390 (data not shown). Moreover, the expression of the Paa protein was sevenfold higher in M155c than in the wild-type strain 86-1390 (data not shown). Low expression of Paa was confirmed by testing the PhoA activity of the fusion protein Paa-PhoA of the Tn*phoA* mutant M155 in similar conditions (data not shown). When strains were labeled with the Paa-adsorbed antibody preparation, only a few gold beads were present, mostly in the background, confirming the specificity of the labeled antibody (Fig. 7B).

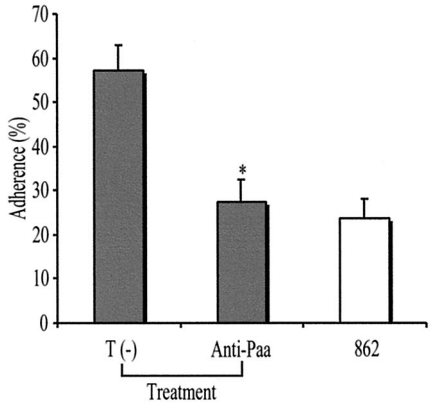

FIG. 6. Reduction of the percentage of intact villi showing intimate adherence when pig ileal explants are inoculated with PEPEC strain 86-1390 following treatment with anti-Paa antibodies, compared to percentages for explants inoculated with strain 86-1390 following treatment with antibodies from hens immunized with a sonicate preparation from host strain M15(pREP4) (T −). The porcine strain 862, which does not have the LEE, was used as a negative control. Asterisk, statistically significant difference ($P < 0.0001$, when compared by Kruskal-Wallis tests) from the T (−) treatment.

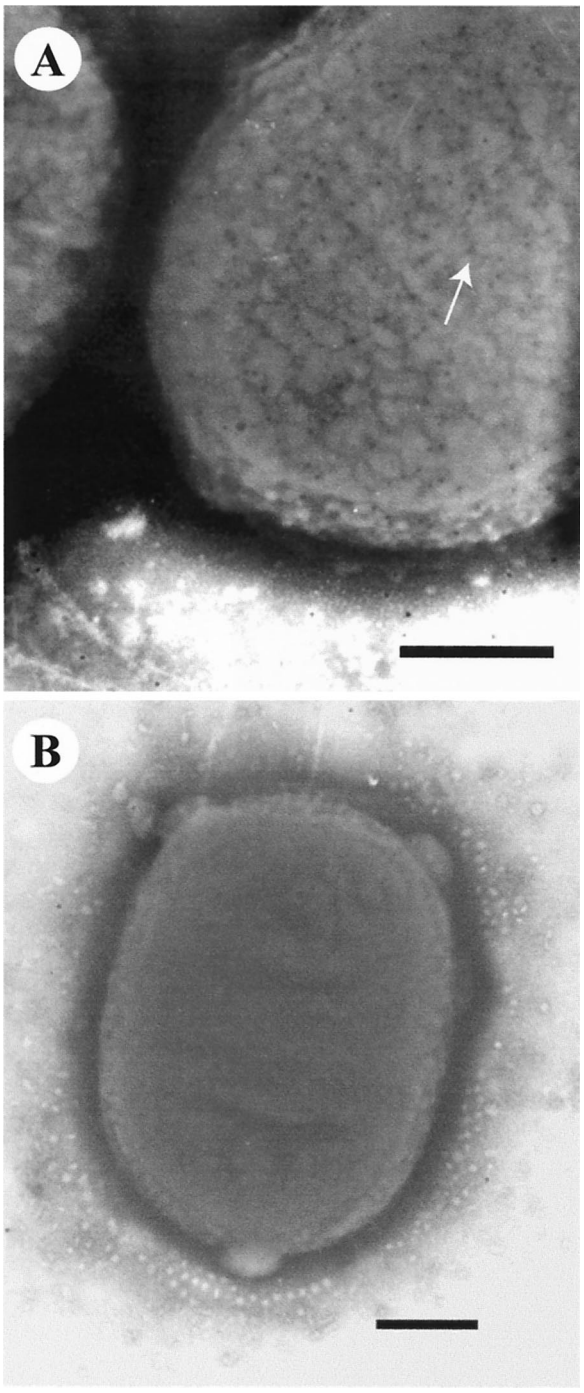

FIG. 7. Transmission electron micrographs showing the uniform distribution of immunogold-labeled Paa protein (arrow) over the bacterial surface of the complemented strain M155c (A) following overnight growth at 37°C in TSB. When anti-Paa serum was adsorbed against the Paa protein, only a few gold beads were observed for strain M155c, mostly in the background (B). Bars = 300 nm.

DISCUSSION

In this study, we have identified by transposon mutagenesis using a PEPEC strain a locus important for development of A/E lesions, which we named *paa*. The *paa*::Tn*phoA* mutant

was no longer able to adhere to microvilli of intestinal epithelial cells and to create A/E lesions. *paa* sequences are often present in A/E strains, especially O157:H7 strains. The predicted amino acid sequence of Paa is identical to those of the Paa proteins of O157:H7 strains EDL933 and Sakai (20, 41) and very similar to those of the PEB3 and AcfC proteins. PEB3 is a major surface antigen involved in the pathogenicity of *C. jejuni* and was shown to be very unstable at ambient temperature. This feature is also shared with Paa. The *acfC* gene is part of the toxin-coregulated pilus (*tcp*) *acf* gene cluster, which has previously been shown to be required for efficient intestinal colonization and biogenesis of the toxin-associated pilus of *V. cholerae* (42). Genomic analysis of numerous *V. cholerae* strains (O1, non-O1, and O139) revealed that only strains capable of causing epidemic Asiatic cholera possessed the TCP-accessory colonization factor colonization gene cluster (30). AcfC is possibly secreted by *V. cholerae* cells into the culture supernatant (13). It is not known if Paa plays a role similar to that of AcfC. Paa, in contrast to AcfC, was shown to be associated mostly with the bacterial pellet and was best expressed in LB broth at 30°C (data not shown). Furthermore, immunogold labeling indicated that the Paa protein is distributed on the bacterial surface in strains 86-1390 and M155c. Nevertheless, the *paa* gene encodes a protein involved in the mechanism of pathogenesis of infection due to strain 86-1390. This gene is absent in nonpathogenic *E. coli*, and the G+C content of *paa* (44%) differs from that of *E. coli* K-12 (50.8%).

The regions flanking *paa* in PEPEC 86-1390 were sequenced for a total of 3.5 kb. Upstream of *paa*, there is a truncated gene homologous to *prpH*, coding for a subunit of the H pilus, a member of the Pap family, and downstream of *paa* are two genes homologous to *gef* and *rem*. The *gef* gene encodes a putative toxic protein similar to the Hok/Gef family, and *rem* has no known function. The region containing *paa* in the 86-1390 strain is 100% identical to the region containing *paa* in the O157:H7 strains EDL933 and Sakai. In the Sakai strain, this region is enclosed in a region of 58.2 kb, specific to the pathogen, localized between *yciD* and *yciE* of *E. coli* K-12 MG1655. This 58.2-kb region contains a lambda prophage that harbors virulence-related genes encoding proteins such as Lom and TrcA homologues. Lom is a member of a family of outer membrane proteins associated with virulence in two enterobacterial species. Expressed in lysogens, this protein confers the ability to survive in macrophages (5). TrcA is reported to be a chaperone molecule in EPEC strains (52). The prophage contains insertions of insertion sequence elements and deletions and thus is presumably defective (39). In strain EDL933, the region containing *paa* is inserted in a larger region of 103.1 kb, also localized between *yciD* and *yciE*. We suppose that these flanking sequences are also found in the PEPEC 86-1390 strain. These data suggest that *paa* could be part of a new putative pathogenicity islet.

The distribution of *paa* in PEPEC O45 strains revealed that it was associated with the presence of *eae* and the A/E phenotype in vivo and in vitro (2). The correlation between the presence of *paa* and *eae* among the isolates from humans and animals suggests that *paa* may be more frequently required for the A/E activity of EHEC and dog isolates than for the A/E activity of rabbit, pig O45, and human EPEC isolates (2). The presence of the *paa* gene could reflect some differences in the mechanisms of A/E activity and/or the development of diarrhea for isolates from different animal species and categories such as EHEC and EPEC. The explant culture technique has proved to be an efficient way to study the A/E phenotype of PEPEC strains ex vivo (56). Moreover, use of ileal explants from the same animal species as those from which the isolates originated eliminates problems due to lack of species-specific recognition of receptors by bacterial adhesins. The observation that three *eae*-positive but *paa*-negative porcine O45 strains were A/E negative provides further evidence for the importance of the *paa* gene in the A/E activity of porcine O45 strains. These results are confirmed by demonstration in the ex vivo model of a clear decrease in the number of ileal villi showing bacterial intimate adherence for *paa* mutants compared to the numbers for wild-type PEPEC and REPEC strains. Complementation of the mutants with the *paa* gene restored adherence capacity to a level similar as that for the wild-type strain (Fig. 1), confirming the importance of *paa* in PEPEC O45 strain 86-1390 and REPEC strain E22. However, we observed that the growth rate of strain M155 complemented with the high-copy-number plasmid pCRII carrying the *paa* gene and its promoter sequence was lower than that of M155 complemented with *paa* carried by the low-copy-number plasmid pACYC184. This suggests that overexpression of Paa may be toxic in the wild-type *E. coli* strain. In experimental infection of newborn piglets, the *paa*-negative isogenic strain was less adherent than the wild-type strain in the ileum but as adherent as the wild-type strain in the cecum and colon (data not shown). This reflects the results obtained with the ileal ex vivo model, in which the *paa*-negative strain is less adherent than the wild type. This also could indicate that *paa* has a more important role in early colonization of the ileum. Moreover, the localization of the Paa protein at the bacterial surface and the ability of Paa-specific antibodies to reduce the adherence level of the PEPEC strain 86-1390 clearly demonstrate the involvement of the Paa protein in A/E lesion formation, possibly in the initial-adherence process. These results also indicate that the Paa protein could be a potential candidate for a vaccine, together with Eae and Tir.

Interestingly, Paa contains a sulfate-binding domain; such motifs are also associated with microbial adherence. For instance, numerous pathogens such as *Neisseria gonorrhoeae*, *Helicobacter pylori*, and *Pseudomonas aeruginosa* bind to the host cell surface via heparan sulfate (HS). Gram-positive bacteria, viruses, and parasites also bind HS on host cells (43, 44). Furthermore, infection studies of gnotobiotic piglets also suggested that the Paa protein is involved in the first step of PEPEC pathogenicity, particularly in initial bacterial adherence, since *paa*-defective strains showed a reduced adherence and infected piglets had no, or delayed-onset, diarrhea. All these data indicate that Paa contributes to the intimate-adherence phenotype and might be a new adhesin. Its receptor could be HS as with other pathogens. Paa may have a role similar to that of other adherence-conferring molecules of *E. coli* such as Efa1, Iha, ToxB, and Afa. Efa1 influences colonization of the bovine intestine by Shiga toxin-producing *E. coli* (47), while Iha facilitates the adherence of *E. coli* O157:H7 to epithelial cells (48). ToxB is important for full expression of adherence by affecting the production and secretion of some virulence factors required for the development of A/E lesions with

O157:H7 strains (49), and it was suggested that EPEC Afa functions as an initial adhesin (26). The more precise role of *paa*, which is associated not only with AEEC but also with some pig enterotoxigenic *E. coli* strains (2), is under investigation.

ACKNOWLEDGMENTS

This work was supported in part by the Fonds pour la Formation des Chercheurs et l'Aide à la Recherche du Québec, grant 0214, by the Natural Sciences Engineering Research Council of Canada, grant 215841-98, and by EU-Community Quality of Life QLK2 2000-0060.

We thank Bernadette Foiry for her technical assistance, Guy Beauchamp for statistical analysis, Diane Montpetit and Robert Alain for electron microscopy, Hojabr Dezfulian for pulsed-field gel electrophoresis, and John Leong for critical reading of the manuscript.

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Editor: B. B. Finlay