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

Isabelle Batisson, Marie-Pierre Guimond, Francis Girard, Hongyan An, Chengru Zhu, Eric Oswald, John M. Fairbrother, Mario Jacques, and Josée Harel

Laboratoire de Biologie des Protistes, UMR 6023, Université Blaise Pascal, Aubière, and Unité INRA-ENVT de Microbiologie Moléculaire, École Vétérinaire de Toulouse, 31076 Toulouse Cedex, France; Groupe de Recherche sur les Maladies Infectieuses du Porc, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec J2S 7C6, Canada; Advantage International USA, Inc., Westport, Connecticut 06880; and Center for Vaccine Development, Division of Geographic Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201

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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 TnphoA 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 (33), and AcfC of *Campylobacter jejuni*, 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:H1-, 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...
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 TphoA4 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 TphoA4 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]’ and streptomycin [Sm]’ 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 SM10 pir (pRT733) was used to introduce TphoA into strain 86-1390 by conjugation (17). E. coli strain HB101 (supE44 hsdS20 (r− m−) recA1 ara-14 proA2 lacY1 galK2 rpsL20 xyl) (7) was used as host for recombinant plasmids in this study. The xZAPII’ system was used for construction of a genomic DNA library from strain 86-1390 (Strategene, La Jolla, Calif.). The host strain E. coli XL1 Blue MRF’ (Δ[mecA]83 Δ(mcrB-hudSMR-nrr)175 endA1 supE44 thi-1 recA43 galK1 relA1 lacI2 proAB (Kmr) ΔmcrCB hsdsMR K-12 [Φ80d-] Tn10 [Tc]) and E. coli strain SQR (c14’ [mcrC] Δ(mcrB-hudSMR-nrr)171 sbcC recB recF umuC::Tn5[Kan’]) lacI2 galK1 relA1 k11 proAB lacI2 ΔM15SU1 [nonsuppressing]), as well as the helper phage M13 needed in the cloning procedure, were used according to the manufacturer’s recommendations (Strategene). E22 is an eur β-positive REPEC strain (40).

**TphoA4 mutagenesis.** Mutations were obtained from random insertion of the TphoA4 sequence into the chromosomal DNA of E. coli strain 86-1390 (Sm’ Tc’). This was accomplished as described previously (17) by using the suicide vector pRT733, which carries the TphoA4 insertion and the kanamycin resistance (Kmr) gene in E. coli strain SM10pir (51). Enconjugants from the mating between E. coli strain SM10pir (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) and the porcine 862 strain, which does not possess the LEE, were used as 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 hophylated Paa antibody reconstituted with phosphate-buffered saline (PBS) for 30 min prior to the first explant inoculation. Tissues were processed for light microscopy and immunohistochemical 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 TphoA4 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 endonucleases ScaI and EcoRV, neither of which cuts within TphoA4, under conditions described by the manufacturer (Pharmacia LKB). After separation by electrophoresis in a 0.7% agarose gel, DNA fragments were transferred to a nylon membrane and hybridized to a radioactive probe. The deduced amino acid sequence was compared against the combined data-bases 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 TphoA4 insert, a genomic DNA library of PEPEC strain 86-1390 was constructed in the xZAPII’ vector. Chromosomal DNA was partially digested with EcoRI 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 EcoRI-digested xZAPII’ vector and packaged in vitro with packaging system (Strategene). Helper phage 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’’ATGGAGGATAACATAGGAGC’’) and M155-R1 (‘‘5’’TCTGTGCAAGGCGTCAATAG’’) annealed at nucleotides 111 to 142 and 425 to 447 of the paa gene, respectively. The 350-bp fragment was then radiolabeled with [α-32P]dCTP 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 Strategene xZAPII’ 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).

**Transposition of the M155 paa: TphoA4 mutant.** The paa gene was amplified with its promoter regions from strain 86-1390 DNA with the PaaHO/F (‘‘5’’GATCCCTTAAAGGGCGAGG’’) and PaaHR (‘‘5’’GATCCTCGATGCTCAATAG’’) primers and cloned into the pGEM-T vector, resulting in the pGEM-T’ plasmid. The BamHI fragment was then inserted into the BamHI-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 TphoA4 mutant, leading to the M155 strain.

**Quantification of A/E capacity of the mutants.** The A/E capacities of the TphoA4 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 colostomy-deprived newborn piglets and incubated on a rocking platform at 37°C for 8 h in an atmosphere of 95% O2 and 5% CO2. 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 hophylated Paa antibody reconstituted with phosphate-buffered saline (PBS) for 30 min prior to the first explant inoculation. Tissues were processed for light microscopy and immunohistochemical 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.

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**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 GCCGCGCAACATAGGAG’’) and relR (‘‘5’’CTTAAAGCGCACTGTCGTA 3’) primers located at bp 2821 to 2840 and 3400 to 3420, respectively, were used to amplify a 590-bp fragment on the E. coli K-12 strain MG1655 chromosome. The PaaR primer (‘‘5’’GCTACAAAAAGCATGGAAGCG’’) was used in combination with a probe using an oligonucleotide random priming labeling kit (Pharmacia LKB) according to the manufacturer’s instructions was used as a probe.
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 KpnI and a HinflII fragment from pSB315 containing a kanamycin resistance cassette was ligated in the KpnI site, resulting in pPaaHO. The construct was digested with BamHI and the puc19 fragment was ligated to the PKNG101 suicide vector cut with the same enzyme. The resulting construct was transferred to strain S17Δpir, 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 twice 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) with the optical density adjusted at 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 mL of 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 XbaI and 50°C for SfiI). 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-DR II system by using a 1% pulsed-field grade agarose gel (Sigma) with 2 liters of modified 0.5 TBE running buffer (0.5× TBE is 89 mM Tris- HCl, 89 mM boric acid, and 2 mM EDTA) at 14°C and a field strength of 68 V/cm. The running conditions were as follows: for PaaHO, a 2- to 3-h incubation period at 14°C, after which the gel was scanned and photographed.

RESULTS

Identification of a transposon mutant deficient in A/E activity. After random insertion of TphoA 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 TphoA 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 TphoA were observed. Three insertions were in genes found in E. coli K-12 (one in osmB, two in ptsS), one was in IS100, 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 TphoA in the chromosomal DNA of the mutant M155 was demonstrated by Southern hybridization of genomic DNA digested by SacI or EcoRV and probed with an internal BamHI-HindIII fragment of TphoA (data not shown).

Effect of the Paa mutation and complementation on the adherence phenotype. The insertion of TphoA 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
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 TnphoA insertion of mutant M155 and which had been radio-labeled with [$\alpha$-$^32$P]CTP. One of these, with an insert of 6 kbp and designated λZAPIIR AN1, was chosen for further study. Using primers obtained from the sequence adjacent to the TnphoA 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 $\alpha$-35 and $\alpha$-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. 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.](http://iai.asm.org/)

![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).](http://iai.asm.org/)
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. 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 TnphoA 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.
and the Sp9 region, respectively, inserted within yciD of E. coli O157:H7 strains EDL933 and Sakai, in the yciD-yciE region. These islands contain incomplete lambda-like phage sequences (phage CP-933O for EDL933, phage Sp9 for Sakai).

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 SfiI or XhoI and examined by pulsed-field electrophoresis and Southern blotting. For both digests, a probe hybridized with only one fragment of about 210 kbp (SfiI) or 290 kbp (XbaI) for strains 86-1390 and M155 and about 210 kbp (SfiI) or 290 kbp (XbaI) 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.

The Paa protein is located at the bacterial surface. 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.

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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 (E22Δpaa) was obtained from strain E22, a paa-positive PEPEC strain that induces A/E lesions in our porcine ex vivo model. Strain E22Δpaa showed a reduced-adherence phenotype (Fig. 2B) and was not able to induce A/E lesions in the ex vivo model. The complementation of E22Δpaa 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 anti-
serum. 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 TnphoA 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).

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of pigs with diarrhea/no. inoculated</th>
<th>Onset of diarrhea (h)</th>
<th>Extent of A/E lesions</th>
<th>Presence of paa gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>81-4420</td>
<td>2/2</td>
<td>24</td>
<td>++++</td>
<td>+</td>
</tr>
<tr>
<td>86-1390</td>
<td>2/2</td>
<td>20</td>
<td>++++</td>
<td>+</td>
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<td>88-4299</td>
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<td>+</td>
</tr>
<tr>
<td>86-4733</td>
<td>2/2</td>
<td>70</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>83-2315</td>
<td>2/2</td>
<td>83</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>88-1861</td>
<td>1/4†</td>
<td>91</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>89-56-196</td>
<td>1/2</td>
<td>96</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>82-4378</td>
<td>0/2</td>
<td>NM†</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>81-1786</td>
<td>1/2</td>
<td>85</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

† One piglet died of causes unrelated to the infection within 20 h after birth.

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::PhoA mutant

**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::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 aecC gene is part of the toxin-coregulated pilus (tcp) aef 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 pCRRI 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**

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