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Pet, an Autotransporter Enterotoxin from Enteroaggregative *Escherichia coli*

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Enteroaggregative *Escherichia coli* (EAEC) is an emerging cause of diarrheal illness. Clinical data suggest that diarrhea caused by EAEC is predominantly secretory in nature, but the responsible enterotoxin has not been described. Work from our laboratories has implicated a ca. 108-kDa protein as a heat-labile enterotoxin and cytotoxin, as evidenced by rises in short-circuit current and falls in tissue resistance in rat jejunal tissue mounted in an Ussing chamber. Here we report the genetic cloning, sequencing, and characterization of this high-molecular-weight heat-labile toxin. The toxin (designated the plasmid-encoded toxin [Pet]) is encoded on the 65-MDa adherence-related plasmid of EAEC strain 042. Nucleotide sequence analysis suggests that the toxin is a member of the autotransporter class of proteins, characterized by the presence of a conserved C-terminal domain which forms a β-barrel pore in the bacterial outer membrane and through which the mature protein is transported. The Pet toxin is highly homologous to the EspP protease of enterohemorrhagic *E. coli* and to EspC of enteropathogenic *E. coli*, an as yet cryptic protein. In addition to its potential role in EAEC infection, Pet represents the first enterotoxin within the autotransporter class of secreted proteins. We hypothesize that other closely related members of this class may also produce enterotoxic effects.

Enteroaggregative *Escherichia coli* (EAEC) is an emerging cause of pediatric diarrhea and has been associated with persistent enteric symptoms (8, 9, 14, 16, 29, 39, 44, 63). The pathogenesis of EAEC diarrhea is not completely defined; however, two prominent histopathologic features have been described: (i) formation of a thick mucus gel on the intestinal mucosa (60) and (ii) mucosal damage, apparently via the elaboration of mucosa-damaging toxin(s) (22, 28, 61). Clinical observations, including EAEC outbreaks (14, 30, 55), studies of endemic EAEC diarrhea, and adult volunteer studies, suggest that EAEC diarrhea is predominantly secretory in nature. Patient stools have been noted to contain mucus and often blood but generally not polymorphonuclear cells (16, 40). Such observations have led investigators to search for an EAEC enterotoxin(s).

Candidate EAEC enterotoxins have been reported. Savarino et al. (51, 52) described a heat-stable enterotoxin (EAST1), which is related to enterotoxigenic *E. coli* ST; EAST1 is present in ca. 40% of EAEC strains and is also found in strains of other diarrheagenic categories and in nonpathogenic *E. coli* (53). The role of EAST1 in diarrhea is questionable given the lack of diarrhea in volunteers challenged with EAST1-producing EAEC strains that colonized the intestine at high levels (40). Baldwin et al. (4) described a 120-kDa heat-labile EAEC protein which elicited rises in intracellular calcium in HEp-2 cells. No in vivo effect of this protein has been shown.

We have observed two severe outbreaks of EAEC diarrhea in Mexican hospitals (22) and have found that infants who died in these outbreaks manifested necrotic lesions of the ileal mucosa. We have also found that supernatants from the outbreak strains express two high-molecular-mass proteins (predicted molecular masses of 108 and 116 kDa) which, when injected into rat ileal loops, induce fluid accumulation and cytotoxic effects on the mucosa (22). These proteins were the predominant species in the supernatants of the outbreak strain and were recognized by the sera of the infected patients. It has been shown recently that the 108-kDa protein elicits rises in short-circuit current (Isc) in rat mucosal Ussing chambers (42), an effect which is accompanied by a fall in tissue resistance and damage to the tissue when examined under light microscopy (43).

In this work, we report the molecular cloning and nucleotide sequence analysis of the 108-kDa EAEC enterotoxin derived from a proven pathogenic strain. The toxin gene is located on the 65-MDa EAEC virulence plasmid (the AA plasmid) and is clustered within a locus of putative virulence-associated genes. The toxin is a member of a family of autotransporter proteins which feature serine protease motifs and are related to the immunoglobulin A proteases of *Neisseria* and *Haemophilus* species (33). Several proteins in this class have been described recently; however, this is the first instance of an autotransporter protein with enterotoxic activity and represents what may be a critical virulence factor of EAEC.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. Strain 042 was isolated from a child with diarrhea in the course of an epidemiological study in Lima, Peru, in 1983; this strain has been shown to cause diarrhea in adult volunteers in Baltimore (40). Strain 049766 was implicated in an outbreak of EAEC infection in Mexico City; JM221 was obtained from J. Mathewson. *E. coli* HB101 and DH5α were used as recipient strains for genetic manipulations. Strains were passed routinely on Luria-Bertani broth (L broth) or agar with the following antibiotics where appropriate: ampicillin (100 mg/ml), kanamycin (50 mg/ml), streptomycin (100 mg/ml), tetracycline (15 mg/ml), and chloramphenicol (20 mg/ml). All strains were stored at −70°C in Trypticase soy broth with 15% glycerol.

Molecular cloning and nucleotide sequence analysis. All genetic manipulations were performed by standard methods (2). Plasmid DNA was extracted by

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TABLE 1. *E. coli* strains and plasmids used in this work

Strain or plasmid	Description[a]	Reference
Strains		
042	Wild-type EAEC strain from Peru	40
049766	Wild-type EAEC strain from outbreak in Mexico	22
HB101	K-12–B hybrid	10
DH5α	K-12 strain	2
UT5600	*proC leu6 trpE38 entA403 tsx* Δ*ompP* Δ*ompT*	34
KS474	F^- Δ*lacX74 galE galK thi rpsL* (*strA*) Δ*phoA* (*Pvu*II) *degP41* (Δ*Pst*I-*Kan*^{r})	58
JCB517	*dsbA*::Kan-1	5
Plasmid		
pSPORT1	High-copy-number cloning vector (Ap)	18
pBluescriptII SK	High-copy-number cloning vector (Ap)	1
pJRD215	Low-copy-number cloning vector (Ap)	19
pRK415	Low-copy-number cloning vector (Tc)	35
pJPN201	13-kb *Mlu*I fragment from pAA2 cloned into pJRD215 (Ap)	This work
pJPN204	11-kb *Pst*I fragment from pAA2 cloned into pRK415 (Tc)	This work
pJPN205	2.8-kb *Mlu*I-*Pst*I fragment from pAA2 cloned into pSPORT1	This work
pJPN208	4.0-kb *Mlu*I-*Kpn*I fragment from pJPN201 cloned into pSPORT1; expresses both Pet and EAST1 proteins (Ap)	This work
pCEFN1	3.9-kb PCR-derived fragment expressing Pet protein cloned into pSPORT1 (Ap)	This work

[a] Abbreviations: Ap, ampicillin resistance; Tc, tetracycline resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance.

using a Plasmid Midi kit (Qiagen Inc., Chatsworth, Calif.). Purification of DNA fragments and extraction from agarose gel slices were performed with Geneclean (Bio 101, La Jolla, Calif.). Plasmid DNA was introduced into *E. coli* HB101 by transformation of competent cells (Gibco/BRL, Gaithersburg, Md.) according to the method of Hanahan (26). Colony blot hybridization was performed by standard methods (2), using as a probe the insert from clone pJPN205 (Fig. 1).

The minimal clone of *pet* was constructed by PCR using oligonucleotide primers with the following sequences: 5′-ATGGATCCGGAAGACGGTTGTT GCGC-3′ (upstream) and 5′-GGGGTACCGGTTAGCTCTGAATTAAG-3′ (downstream).

DNA sequence determination was performed on an Applied Biosystems model 373A automated sequencer via dye terminator cycle sequencing with *Taq* polymerase (Perkin-Elmer Corp., Norwalk, Conn.) according to manufacturer's instructions; sequencing was performed in the Biopolymer Laboratory, Department of Microbiology and Immunology, University of Maryland School of Medicine. Nucleotide sequence was analyzed with GENEPRO sequence analysis software (version 5.00; Riverside Scientific, Bainbridge Island, Wash.) and the Wisconsin GCG (Genetics Computer Group) sequence analysis package available through the Center of Marine Biotechnology, University of Maryland. The predicted amino acid sequence of each open reading frame (ORF) was compared with sequences of proteins listed in EMBL/GenBank by using the GCG TFASTA program and the BLAST algorithm (National Center for Biotechnology Information). Secondary-structure predictions were performed by Jähnig (32) or Emini et al. (21) algorithms, which are available in the HUSAR program package of the Deutsches Krebsforschungszentrum (Heidelberg, Germany).

Cosmid library construction. Plasmid DNA was purified from strain 042 and digested partially with the restriction endonuclease *Sau*3a. The resulting fragments (15 to 30 kb in size) were ligated into the *Bam*HI site of the cosmid vector pCVD301, and the ligation mix was packaged into phage by using the Gigapack packaging extract (Stratagene, Inc.). Recombinant phage were transfected into *E. coli* HB101. The library comprising 768 clones was maintained at −70°C in L broth containing 15% glycerol.

Protein methods. Late-logarithmic-phase nutrient broth culture supernatant of strain 042 was subjected to 60 and 75% ammonium sulfate fractionation for 18 h at 4°C. Precipitates collected by centrifugation were dissolved and dialyzed in 0.07 M sodium phosphate buffer, pH 8.2. This suspension was treated with a 3.5 M solution of potassium phosphate, pH 6.8; the precipitate obtained was fractionated by chromatography in DEAE-cellulose and Sephadex columns (LKB Biotechnology, Uppsala, Sweden) and concentrated 10-fold by ultrafiltration through a Diaflo YM100 membrane (Amicon, Lexington, Mass.). The protein separated in polyvinylidene fluoride by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (36) was transferred to Immobilon membranes (Millipore, Bedford, Mass.) prior to amino-terminal sequencing. Amino-terminal sequencing was performed by automated Edman degradation at the Protein and Nucleic Acid Facility, Stanford University, Palo Alto, Calif.

Outer membrane preparations were performed by concentrating overnight cultures of HB101(pCEFN1) and solubilizing the membranes in Triton X-100 as previously described (12). Cytoplasmic and periplasmic fractions were prepared as previously described (12). Preparations were separated by SDS-PAGE and were visualized by Coomassie blue staining.

Immunologic methods. The purified Pet protein was injected subcutaneously in complete Freund's adjuvant to New Zealand White rabbits weighing between 2.5 and 3.0 kg. Rabbits received subcutaneous boosters of 200 mg of total protein on days 0, 15, and 20. Rabbits were exsanguinated on day 25, and the serum collected was stored at −20°C until use.

Western immunoblots of the Pet protein were performed with the purified Pet protein (see above). Samples containing 100 mg of total protein were separated by SDS-PAGE, and the protein bands obtained were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, N.H.) as described by Towbin et al. (59). The membranes were incubated overnight with rabbit antisera (anti-Pet) diluted 1:500. The Pet protein was visualized with goat anti-rabbit antibodies conjugated with alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

Ussing chamber experiments. Six pieces of rat jejunum removed from adult male Sprague-Dawley rats under sodium pentobarbital anesthesia were placed in ice-cold Ringer's solution for mammals and gassed with an O_2-CO_2 (95%:5%) mixture. The excised segments were cut open along the mesenteric border, washed with cold Ringer's solution, and mounted between the circular openings of six Ussing hemichambers. Each hemichamber was filled with 10 ml of gassed Ringer's solution and kept at 37°C under constant O_2-CO_2 bubbling (41). Chambers were equilibrated for 30 min before experiments were initiated. After addition of the test sample, transepithelial electrical potential difference (PD) was measured at 10-min intervals under current-clamped conditions. Tissue conductance was determined at an applied current of 100 μA, and Isc was calculated by using Ohm's law (25).

Samples used in Ussing chamber experiments consisted of 100 ml of L-broth cultures grown overnight at 37°C without shaking. After centrifugation at 12,000 × *g* for 10 min, supernatants were concentrated and size fractionated (>50 kDa) by passage through Biomax-50 Ultrafree filters (Millipore). Samples were adjusted to a concentration of 25 μg of protein/ml, and 100 μl of each sample was added to the mucosal hemichamber of rat jejunum preparations.

Nucleotide sequence accession number. The sequence of the *pet* gene has been submitted to GenBank under accession no. AF056581.

RESULTS

Cloning and sequencing of the 108-kDa toxin gene. We had previously raised polyclonal antiserum to the 108-kDa EAEC protein derived from strain 049766 (implicated in an EAEC outbreak in Mexico). We used this antiserum to localize the toxin gene in strain 042. Use of the anti-108-kDa protein antiserum in Western immunoblotting of concentrated culture supernatants from *E. coli* HB101 containing the 65-MDa plasmid pAA2 (from strain 042) revealed the presence of the 108-kDa protein, whereas culture supernatants from HB101 lacking pAA2 were negative for the toxin. To clone the plasmid-encoded toxin gene, a cosmid library of plasmid pAA2 was constructed in vector pCVD301. A portion of the cosmid bank was subjected to restriction analysis in order to identify a small subset of clones which were representative of the entire parent plasmid. This series of experiments resulted in the selection of 11 overlapping cosmid clones which encompassed the large majority of the plasmid. Subsequent Western immunoblot analysis of this cosmid subset revealed that two of the 11 cosmid clones expressed high-molecular-weight bands that reacted with anti-108-kDa protein antiserum (not shown). Restriction mapping of these two cosmid clones demonstrated an overlapping region of approximately 20 kb.

The two toxin-encoding cosmids shared a common 13-kb

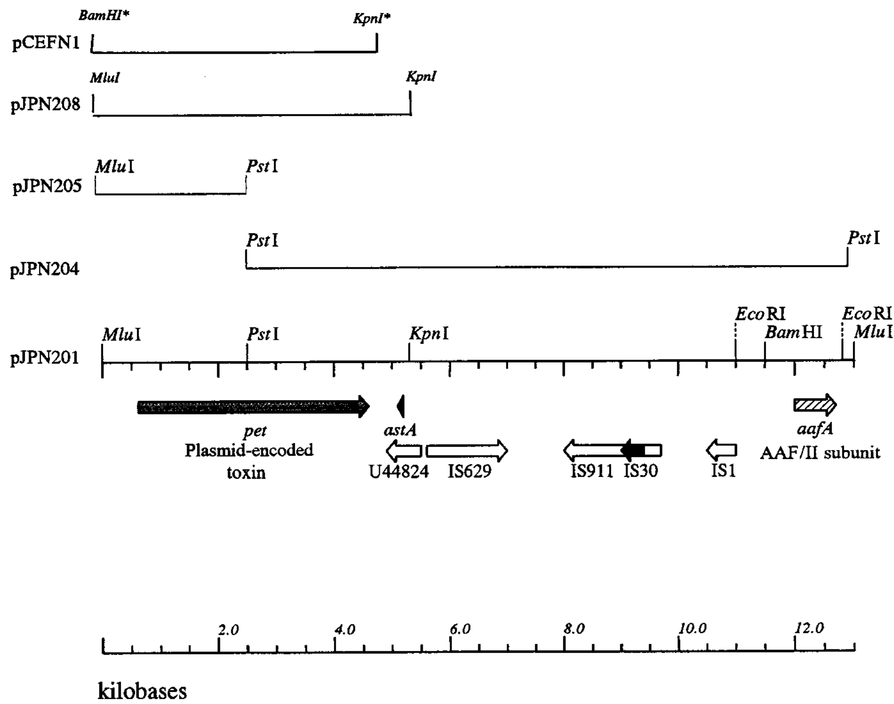

FIG. 1. Map of the cloned insert of pJPN201. The fimbrial subunit of the AAF/II antigen is encoded on the far right end of the fragment (17). At the left end of the insert is the *pet* gene, followed in opposite orientation by the *astA* gene embedded within an IS-like element and then by four other IS-homologous sequences (see text). Subclones used for sequencing and expression of *pet* are indicated. Restriction sites introduced by PCR are indicated by asterisks.

*Mlu*I fragment. A series of subclones was constructed from this region (Table 1 and Fig. 1). Western immunoblot analysis suggested that pJPN201, carrying the full 13-kb *Mlu*I fragment, expressed the 108-kDa protein, whereas the nested clone pJPN204 did not.

Sequence analysis of the toxin gene. Single-stranded nucleotide sequencing was performed on the insert of pJPN201. Due to the absence of the 108-kDa protein in supernatants of HB101(pJPN204), we expected to find an ORF near the left terminus of the cloned insert. Indeed, analysis of the nucleotide sequence of pJPN201 revealed a large ORF (3,885 bp in length) starting 617 bp from the *Mlu*I site at the left end of the pJPN201 insert (Fig. 1). The G+C content of this ORF was 43.6%, significantly lower than average for the *E. coli* genome. Sequence analysis of the cloned insert did not reveal any other ORFs which could potentially encode a protein greater than 80 kDa in size. In recognition of the fact that the large ORF apparently encodes the high-molecular-weight toxin described by Eslava et al. (22) and by Navarro-Garcia et al. (43), this gene has been designated *pet* (EAEC plasmid-encoded toxin). Figure 1 illustrates the map of pJPN201 including the position of *pet* and the subclones that were used for sequencing and phenotypic analysis.

We identified a potential *pet* promoter which had a −10 region (TTTAAT) and a −35 region (GTAACA) positioned 48 and 70 bp, respectively, upstream from the ATG start codon. A possible *rho*-independent stem-loop transcriptional termination signal was also identified 6 bp downstream of the TGA termination codon of the *pet* gene. The presence of the promoter is consistent with the ability of clone HB101 (pJPN201) to express the Pet product.

Downstream from the *pet* gene are five insertion sequence (IS)-homologous ORFs (Fig. 1). Immediately downstream from *pet* is a potential ORF of 581 bp (in the antisense direction), the predicted product of which exhibits 49% identity with a transposase of *Burkholderia cepacia* (accession no. U44828). Within this ORF, in the same orientation as the transposase and 647 bp downstream of the 3′ end of the *pet* gene, lies a gene homologous to the *astA* gene, which encodes the 38-amino-acid EAST1 heat-stable enterotoxin (51). Interestingly, the *astA* gene of strain 042 is 100% identical at the amino acid level with the predicted sequence of the *astA* gene from enterotoxigenic *E. coli* strain H10407 (64; accession no. S81691); this EAST1 differs in only one residue from the EAST1 protein of EAEC strain 17-2. Immediately upstream of the *B. cepacia* IS-like element, and in the opposite orientation, is a sequence of 1,310 bp which is 97% identical to an IS*629* element of *Shigella sonnei* (37). IS*629* is 95% identical to the IS*1203* element found recently in pathogenic *E. coli* O111:H$^-$ (45). Further downstream from *pet* lies an element identical to *Shigella dysenteriae* IS*911* (47), the sequence of which is interrupted by a complete IS*30* element (13). Upstream of this element lies a complete IS*1* element (50).

To facilitate further analyses, a minimal clone of the *pet* gene (pCEFN1) was constructed by PCR and cloned into pSPORT1 (Fig. 1). The insert was flanked by the native *Mlu*I site (upstream) and an engineered *Kpn*I site (downstream) and spanned from 610 bp upstream of *pet* to 50 bp downstream of the termination codon. This fragment included the predicted *pet* promoter but not the *astA* gene. Of note, all known promoters of pSPORT1 are aligned in opposite orientation to the cloned *pet* gene in pCEFN1.

Assuming that the first ATG codon of the ORF corresponds to the translational start codon, the *pet* gene would encode a 1,295-aminio-acid protein with a predicted molecular mass of 140.0 kDa and a calculated isoelectric point of 6.71. Comparison of the deduced amino acid sequence with those listed in GenBank databases revealed 58% overall identity (83% simi-

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                                                                     *
Pet  MNKIYSIKYS AATGGLIAVS ELAKKVICK- TNRKISAALL SLAVISYTNI IY--AANMDI 57
EspP MNKIYSLKYS HITGGLIAVS ELSGRVSSRA TGKKKHKRIL ALCFLGLLQS SYSFASQMDI
EspC MNKIYALKYC HATGGLIAVS ELASRVMKKA AR--GSLLAL FNLSLYGAFL SASQAAQLNI
SepA MNKIYYLKYC HITKSLIAVS ELARRVTCKS HRRLSRRVIL TSVAALSLSS AWPALSATVS

Pet  SKAWARDYLD LAQNKGVFQP GSTHVKIKLK DGTDF-SFPA LPVPDFSSAT ANGAATSIGG 116
EspP SNFYIRDYMD FAQNKGIFQA GATNIEIVKK DGSTL-KLPE VPFPDFSPVA NKGSTTSIGG
EspC DNVWARDYLD LAQNKGVFKA GATNVSIQLK NGQTF-NFPN VPIPDFSPAS NKGATTSIGG
SepA AEIPYQIFRD FAENKGQFTP GTTNISIYDK QGNLVGKLDK APMADFSSAT ITTGSLPPGD

Pet  AYAVTVAHNA KNKSSANYQT ----YGSTQY TQINRMTTGN -DFSIQRLNK YVVETRGAD- 170
EspP AYSITATHNT KNHHSVATQN ----WGNSTY KQTDWNTSHP -DFAVSRLDK FVVETRGAT-
EspC AYSVTATHNG TTHHAISTQN ----WGQSSY KYIDRMTNGD FAVT--RLDK FVVETTGVK-
SepA HTLYSPQYVV TAKHVSGSDT MSFGYAKNTY TAVGTNNNSG LDIKTRRLSK LVTEVAPAEV

Pet  TSFNYNENNQ NIIDRYGVDV GNGKKEIIGF RVGSGNTTFS GIKTSQTYQA DLLSASLFHI 230
EspP EGADISLSKQ QALERYGVNY -KGEKKLIAF RAGSGVVSVK KNGRITPFNE VSYKPEMLNG
EspC NSVDFSLNSH DALERYGVEI -NGEKKIIGF RVGAGTTYTA QNGNTYSTGQ VYNPL--LLS
SepA SDIGAVSGAY QAGGRFTEFY -RLGGGMQYV KDKNGNRTQV YTNGGFLVGG TVSALNSYNN

Pet  TNLRANTVGG NKVEYEND-S YFTNLTTNGD SGSGVYVFDN KEDKWVLLGT THGIIGNGKT 289
EspP SFVHIDDWSG WLILTNNQFD EFNNIASQGD SGSALFVYDN QKKKWVVAGT VWGIYNYANG
EspC ASMFQLNWDN KRPYNNT--T PFYNETTGGD SGSGFYLYDN VKKEWVMLGT LFGIASSGAD
SepA GQMITAQTGD IFLIPPN--G PLANYLNMGD SGSPLFAYDS LQKKWVLIGV LSSGTNYGNN

Pet  QKTYVTPFDS KTTNELKQLF IQNVNIDNNT A-TIGGGKIT IGNTTQDIEK NKNNQNKDLV 348
EspP KNH--AAYSK WNQTTIDNL- KNKYSYNVDM S-GAQVATIE NGKLTGTGSD TTDIKNKDLI
EspC VGSILNQYDE NTVNGLKNKF TQ-------- --KVQLNNNT MSLNSDSFTL AGNNTAVAKN
SepA WVVTTQDFLG QQPQNDFDKT IAYTSGEGVL QWKYDAANGT GTLTQGNTTW GYAWKERKLI

Pet  FSGGGKISLK ENLDLGYGGF IFDENKKYTV SAEGN----- NNVTFKGAGI DIGKGSTVDW 403
EspP FTGGGDILLK SSFDNGAGGL VFNDKKTYRV NGD------- -DFTFKGAGV DTRNGSTVEW
EspC NNNYKDLSFS GGGSINFDND VNIGSGGLIF DAGHHYTVTG NNKTFKGAGL DIGDNTTVDW
SepA LNAGKNLLFT GNNGEVVLQN SVNQGAGYLQ FAGDYRVSAL NGQTWMGGGI ITDKGTHVLW

Pet  NIKYASNDAL HKIGEGSLNV IQA--QNTNL KTGNGTVILG AQKTFNNIYV A-GGPGTVQL 460
EspP NIRYDNKDNL HKIGDGTLDV RKT--QNTNL KTGEGLVILG AEKTFNNIYI T-SGDGTVRL
EspC NVKGVVGDNL HKIGAGTLNV NVS--QGNNL KTGDGLVVLN SANAFDNIYM A-SGHGVVKI
SepA QVNGVAGDNL HKTGEGTLTV NGTGVNAGGL KVGDGTVILN QQADRDGKVQ AFSSVGIASG

Pet  NAENALGEGD Y---AGIFFT ENGGKLDLNG HNQTFKKIAA TDSGTTITNS NTTKESVLSV 517
EspP NAENALSGGE Y---NGIFFA KNGGTLDLNG YNQSFNKIAA TDSGAVITNT ST-KKSILSL
EspC NHSAALNQNN DYR--GIFFT ENGGTLDLNG YDQSFNKIAA TDIGALITNS AV-QKAVLSV
SepA RPTVVLSDSQ QVNPDNISWG YRGGRLELNG NNLTFTRLQA ADYGAIITNN SE-KKSTVTL

Pet  NNQNNYIYHG NV-------- DGNVRLEHHL DTKQDNARLI LDGD------ ---------- 553
EspP NNTADYIYHG NI-------- NGNLDVLQHH ETKKENRRLI LDGG------ ----------
EspC NNQSHYMYHG SVS------- -GNTEINHQF DTQKNNSRLI LDG------- ----------
SepA DLQTLKASDI NVPVNTVSIF GGRGAPGDLY YDSSTKQYFI LKASSYSPFF SDLNNSSVWQ
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FIG. 2. Alignment of the predicted Pet protein with its closest homologs, EspP (accession no. X97542), EspC (U69128), and SepA (Z48219). Shaded residues represent identity. Coordinates are shown for Pet only. The asterisk at residue 53 indicates the first amino acid of the mature Pet protein. The arrowhead at residue 1018 indicates the position of cleavage of the β domain. The serine protease motif is in boldface.

larity) with the recently described EspP protein of enterohemorrhagic *E. coli* (11) (Fig. 2). In addition, *pet* displayed 55% identity (70% similarity) and 44% identity (60% similarity) with the *espC* gene product of enteropathogenic *E. coli* and with SepA, the major secreted protein of *Shigella flexneri*, respectively. Significant homology was also seen with other members of the so-called autotransporter family of bacterial virulence factors. Notably, the homologies displayed are not uniformly distributed over the sequences; the N-terminal passenger domain (encoding the mature protein) of *pet* displays 49, 45, and 31% identity to the EspP, EspC, and SepA passenger domains, respectively, whereas the C-terminal β domains (the C-terminal β barrel) exhibited 90, 80, and 78% identity, respectively.

Several features of the autotransporter family were evident within the predicted *pet* gene product. First, analysis of the predicted Pet amino acid sequence revealed the presence of a putative Walker A box (62) nucleotide binding motif (G^{281}IIGNGK) which has been described for a number of other members of this class, though a function for these motifs has not yet been shown (11, 48, 54, 56). Second, a serine protease motif (GDSGSP) has been found in several of the closest homologs of Pet. Although this site has been shown to act in proteolysis in *Haemophilus* and *Neisseria* autotransporters, (3, 27), a function has not been determined for this motif in the autotransporters of members of the family *Enterobacteriaceae*. At the corresponding site in Pet, the sequence was determined to be G^{256}DSGSGV. Computer-aided analysis of the deduced amino acid sequence of Pet indicated that the protein possessed the characteristics of a signal sequence (31), with positively charged amino acids followed by a hydrophobic region and a signal peptidase cleavage site at residue 52. The length of this signal sequence would be unusually long for *E. coli* but similar to those predicted for other autotransporters. To confirm the site of cleavage of the mature protein, Pet was isolated from culture supernatants of *E. coli* 049766 and the

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Pet   -IQA-NSISI KNAPLVMQGH ATDHAIFRT- --TKTN---- NCPEFLCGVD WVTRIKNAEN  604
EspP  -VDTTNDISL RNTQLSMQGH ATEHAIYRD- --GAFSCSLP APMRFLCGSD YVAGMQNTEA
EspC  NVDITNDINI KNSQLHHAGT CLHLMLFLER VGVT-----C MLPGVICEKD YVSGIQQQEN
SepA  NVGKDHNKAI DTVKQQKIEA SSQPYMYHGQ LNGNMDVNIP QLSGKDVLAL DGSVNLPEGS

Pet   SVNQKNKTTY KSNNQVSDLS QPDWETRKFR FDNLNIEDSS LSIARNADVE GNIQAKNSVI  664
EspP  DAVKQNGNAY KTNNAVSDLS QPDWETGTFR FGTLHLENSD FSVGRNANVI GDIQASKSNI
EspC  SANKNNNTDY KTNNQVSSFE QPDWENRLFK FKTLNLINSD FIVGRNAIVV GDISANNSTL
SepA  ITKKSGTLIF QGHPVIHAGT TTSSSQSDWE TRQFTLEKLK LDAATFHLSR NGKMQGDINA

Pet   NIGDKT--AY IDLYSGKNIT GAGFTFRQDI KSGDSI--GE SKFTGGIMAT D---------  711
EspP  TIGDTT--AY IDLHAGKNIT GDGFGFRQNI VRGNSQ--GE TLFTGGITAE D---------
EspC  SLSGKDTKVH IDMYDGKNIT GDGFGFRQDI KDGVSVSPES SSYFGNVTLN NHSLLDIGNK
SepA  TNGSTVILGS SRVFTDRSDG TGNAVSSVEG SATATTVGDQ SDYSGNVTLE NKSSLQIMER

Pet   --------GS ISIGDKAIVT LNTVSSLDRT ALTIHKGANV TASSSLFTTS --NIKSGGDL  761
EspP  --------ST IVIKDKAKAL FSNYVYLLNT KATIENGADV TTQSGMFSTS --DISISGNL
EspC  FTGGIEAYDS SVSVTSQNAV FDRVGSFVNS SLTLEKGAKL TAQGGIFSTG AVDVKENASL
SepA  FTGGIEAYDS TVSVTSQNAV FDRVGSFVNS SLTLGKGAKL TAQSGIFSTG AVDVKENASL

Pet   TLTGATESTG EITPSMFYAC RRYELTEDGA NFTAKNQASV TGDIKSEKAA KLSFGSADKD  821
EspP  SMTGNPDKDN KFEPSIYLND ASYLLTDDSA RLVAKNKASV VGDIHSTKSA SIMFGHDESD
EspC  ILTGTPSAQK QEYYSPVIST TEGINLGDKA SLSVKNMGYL SSDIHAGTTA ATI---NLGD
SepA  TLTGMPSAQK QGYYSPVIST TEGINLEDNA SFSVKNMGYL SSDIHAGTTA ATI---NLGD

Pet   NSATR---YS QFALAMLDGF DTSYQGSIKA AQSSLAMNNA LWKVTGNSEL KKLNSTGSMV  878
EspP  LSQLSDRTSK GLALGLLGGF DVSYRGSVNA PSASATMNNT WWQLTGDSAL KTLKSTNSMV
EspC  GDAE---TDS PLFSSLIRGY NAVLSGNITG EQSTVNMNNA LWYSDGNSTI GTLKSTGGRV
SepA  SDADAGKTDS PLFSSLMKGY NAVLRGSITG AQSTVNMINA LWYSDGKSEA GALKAKGSRI

Pet   LFNGG--KNI FNTLTVDELT TSNSAFVMRT NTQQADQLIV KNKLEGANNL LLVDFIEKKG  936
EspP  YFTDSANNKK FHTLTVDELA TSNSAYAMRT NLSESDKLEV KKHLSGENNI LLVDFLQKPT
EspC  ELGGG---KD FATLRVKELN ANNATFLMHT NTSQADQLNV TNKLLGSNNT VLVDFLNKPA
SepA  ELGDG---KH FATLQVKELS ADNTTFLMHT NNSRADQLNV TDKLSGSNNS VLVDFLNKPA

Pet   NDKNGLNIDL VKAPENTSKD VFKTETQTIG FSDVTPEIKQ QEKDGKSVWT LTGYKTVANA  996
EspP  PEKQ-LNIEL VSAPKDTNEN VFKASKQTIG FSDVTPVITT RETDDKITWS LTGYNTVANK
EspC  SE---MNVTL ITAPKGSDEK TFTAGTQQIG FSNVTPVIST EKTDDATKWM LTGYQTVSDA
SepA  SE---MSVTL ITAPKGSDEK TFTAGTQQIG FSNVTPVIST EKTDDATKWV LTGYQTTADA
                        ▼
Pet   DAAKKATSLM SGGYKAFLAE VNNLNKRMGD LRDINGEAGA WARIMSGTGS AGGGFSDNYT  1056
EspP  EATRNAAALF SVDYKAFLNE VNNLNKRMGD LRDINGEAGA WARIMSGTGS ASGGFSDNYT
EspC  GASKTATDFM ASGYKSFLTE VNNLNKRMGD LRDTQGDAGV WARIMNGTGS ADGGYSDNYT
SepA  GASKAAKDFM ASGYKSFLTE VNNLNKRMGD LRDTQGDAGV WARIMNGTGS ADGDYSDNYT

Pet   HVQVGADNKH ELDGLDLFTG VTMTYTDSHA GSDAFSGETK SVGAGLYASA MFESGAYIDL  1116
EspP  HVQVGVDKKH ELDGLDLFTG FTVTHTDSSA SADVFSGKTK SVGAGLYASA MFDSGAYIDL
EspC  HVQIGADRKH ELDGVDLFTG ALLTYTDSNA SSHAFSGKTK SVGGGLYASA LFDSGAYFDL
SepA  HVQIGVDRKH ELDGVDLFTG ALLTYTDSNA SSHAFSGKNK SVGGGLYASA LFNSGAYFDL

Pet   IGKYVHHDNE YTATFAGLGT RDYSSHSWYA GAEVGYRYHV TDSAWIEPQA ELVYGAVSGK  1176
EspP  IGKYVHHDNE YTATFAGLGT RDYSTHSWYA GAEAGYRYHV TEDAWIEPQA ELVYGSVSGK
EspC  IGKYLHHDNQ YTASFASLGT KDYSSHSWYA GAEVGYRYHL SEESWVEPQM ELVYGSVSGK
SepA  IGKYLHHDNQ HTANFASLGT KDYSSHSWYA GAEVGYRYHL TKESWVEPQI ELVYGSVSGK

Pet   QFSWKDQGMN LTMKDKDFNP LIGRTGVDVG KSFSGKDWKV TARAGLGYQF DLFANGETVL  1236
EspP  QFAWKDQGMH LSMKDKDYNP LIGRTGVDVG KSFSGKDWKV TARAGLGYQF DLLANGETVL
EspC  SFIWEDRGMA LSMKDKDYNP LIGRTGVDVG RTFSGDDWKI TARAGLGYQF DLLANGETVL
SepA  AFSWEARGMA LSMKDKDYNP LIGRTGVDVG RAFSGDDWKI TARAGLGYQF DLLANGETVL

Pet   RDASGEKRIK GEKDGRMLMN VGLNAEIRDN VRFGLEFEKS AFGKYNVDNA INANFRYSF   1295
EspP  RDASGEKRIK GEKDSRMLM- VGLNAEIRDN VRFGLEFEKS AFGKYNVDNA VNANFRYSF
EspC  RDASGEKRFE GEKDSRMLMN VGMNAEIKDN MRFGLELEKS AFGKYNVDNA INANFRYSF
SepA  QDASGEKRFE GEKDSRMLMT VGMNAEIKDN MRLGLELEKS AFGKYNVDNA INANFRYVF
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FIG. 2—*Continued.*

N-terminal amino acid sequence was determined. The derived amino acid sequence (ANMDISKAWARD......) indeed localized the site of cleavage between residues A^{52} and A^{53}.

Processing and export of Pet. Since the ca. 108-kDa protein present in culture supernatant fluids is smaller than predicted from the N-terminal processed ORF (1,243 amino acids; molecular mass of 134.4 kDa), the Pet protein apparently undergoes a posttranslational processing step, namely, cleavage of the passenger domain from the β domain. Members of the autotransporter family of proteins are exported through the outer membrane of the bacterium via the presence of a characteristic C-terminal amphipathic region (β domain) comprising an even number of antiparallel β sheets; this region of the protein forms a β-barrel structure in the outer membrane

through which the passenger domain of the protein passes. The high homology between the β domains of Pet and the EspP, EspC, and SepA proteins suggests that the β domain of Pet functions as an outer membrane translocator and that cleavage of the passenger domain occurs during this step. Based on the sequence homology with other autotransporters the cleavage site was predicted to be between N^{1018} and N^{1019}.

To localize accurately the site of C-terminal processing, the outer membrane β domain was visualized by SDS-PAGE analysis of envelopes from HB101(pCEFN1) extracted with Triton X-100 and compared with similar extracts of HB101 (pSPORT1). These analyses revealed the presence of a 30-kDa species in the fractions obtained from HB101(pCEFN1) that was absent from the similar fractions of the control strain (Fig. 3a). As expected, the N-terminal amino acid sequence of this protein (NLNKRMGDLR...) placed the site of cleavage between N^{1018} and N^{1019}. Cleavage at this point, and at the site of cleavage of the signal sequence (A^{52}-A^{53}), would result in a secreted Pet product of 104.2 kDa, a mass which agrees well with the mass of 108 kDa predicted for Pet on SDS-PAGE analysis.

Structural predictions of the β domain of Pet, from the N^{1019} cleavage site to the terminal phenylalanine residue, were performed by using the algorithm $\{Hb(i) = [h(i \pm 4) + h(i \pm 2) + h(i)]/5\}$ described by Jähnig (32). According to these predictions, the β domain of Pet consists of at least 14 membrane-spanning amphipathic β strands interrupted by large external loops and generally short periplasmic loops, spanning amino acid positions 1032 to 1295 of the Pet precursor. These results were confirmed by calculating the regions of high surface probability as described by Emini et al. (21), using the GCG program from the Wisconsin sequence analysis package, since such regions are always located between the β strands. An alpha helix was not predicted upstream of the amphipathic strands.

To test the hypothesis that the β domain is involved in translocation of the Pet passenger domain to the external milieu, the deletion mutant pJPN205 (truncated at residue 770) was analyzed for expression of the mature Pet protein. By Western immunoblotting, HB101(pJPN205) supernatants did not reveal a protein consistent with this truncated passenger domain (Fig. 3b, lane F), although an appropriate-size protein species was detected in the bacterial periplasm (Fig. 3b, lane B) and, to a much lesser extent, in the cytoplasm (Fig. 3b, lane D). These data confirm that the C-terminal β domain is required for Pet translocation to the external milieu.

The possible role of several endogenous membrane-associated enzymes in the processing and export of Pet was investigated. The pCEFN1 clone was transformed into *E. coli* UT5600 (*ompP ompT*), KS474 (*degP*), and JCB517 (*dsbA*). The resulting constructions were screened for processing of Pet by SDS-PAGE analysis of concentrated culture supernatants. Each strain yielded a 104-kDa species, suggesting that normal processing of the Pet precursor occurs in the absence of the DegP, OmpP, and OmpT proteases and in the absence of the DsbA isomerase.

Phenotypic analysis of the Pet protein. We have shown that concentrated supernatant from strain 049766 produces an increase in jejunal PD and Isc (43). The >50-kDa fraction of supernatants from HB101(pJPN201) also induced rises in jejunal PD and Isc which were not induced by concentrated supernatants from HB101(pJRD215) or HB101(pJPN204). Supernatants derived from the minimal clone of *pet* were also found to induce rises in Isc (Fig. 4), which were significantly higher than those induced by the cloning vector, suggesting that Pet is the enterotoxic moiety. Rises in Isc and PD induced by the *pet* clone were similar in timing and degree to those induced by the parent strain 042.

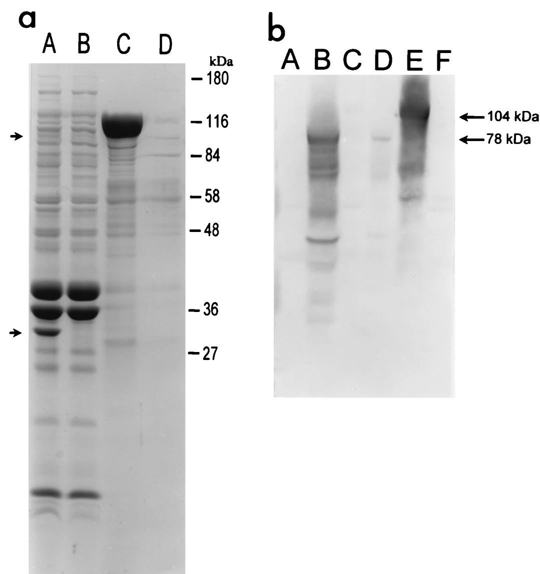

FIG. 3. (a) SDS-PAGE analysis of clone pCEFN1, encoding the complete *pet* gene. Lanes: A, Triton X-100-insoluble fraction of HB101(pCEFN1); B, Triton X-100-insoluble fraction of HB101 harboring the cloning vector pSPORT1; C, supernatant of HB101(pCEFN1); D, supernatant of HB101(pSPORT1). The arrow at 104 kDa represents the Pet passenger domain (the mature form of the protein); the arrow at ca. 30 kDa represents the β domain inserted into the bacterial outer membrane. (b) Western immunoblot of bacterial supernatants reacted with anti-Pet antiserum. Lanes: A, periplasmic fraction of HB101 (pCEFN1); B, periplasmic fraction of HB101(pJPN205) harboring the C-terminal deletion mutant of the *pet* gene; C, cytoplasmic fraction of HB101(pCEFN1); D, cytoplasmic fraction of HB101(pJPN205); E, supernatant of HB101(pCEFN1); F, supernatant of HB101(pJPN205). Arrows denote expected sizes of mature Pet and the expected truncated species produced by pJPN205. Smaller species reacting with antibodies in lanes B and E most likely represent breakdown products of the mature toxin.

The ability of the Pet protein to act as a protease was tested by separating concentrated supernatants through casein or gelatin zymogram gels (NOVEX). Strains 042, HB101(pCEFN1), and HB101(pJPN201) all yielded zones of clearing at 108 kDa, whereas HB101 and HB101(pSPORT) supernatants did not reveal proteolytic activity at this molecular mass (data not shown).

Regulation of Pet expression. The EspP protein has been shown to be regulated by temperature (11). To obtain information on the regulation of *pet* expression, we analyzed the expression of Pet from EAEC strain 042 grown at different temperatures (20, 37, and 42°C). The bacteria were grown to an optical density at 600 nm of 1.0, and concentrated culture supernatants were analyzed by SDS-PAGE for the presence of Pet. The mature Pet protein was observed in similar amounts from supernatants of 042 grown at all three temperatures (not shown), suggesting that the *pet* gene is not strictly temperature regulated.

Prevalence of the pet gene among EAEC. To determine the prevalence of the *pet* gene among clinical isolates, colony blot hybridization studies were performed with a restriction fragment internal to the *pet* gene, corresponding to the region encoding residues 62 to 781 of the Pet protein. Against a collection of EAEC strains from various epidemiological studies around the world, 5 of 34 strains (15%) yielded a positive hybridization signal with the probe (Table 2).

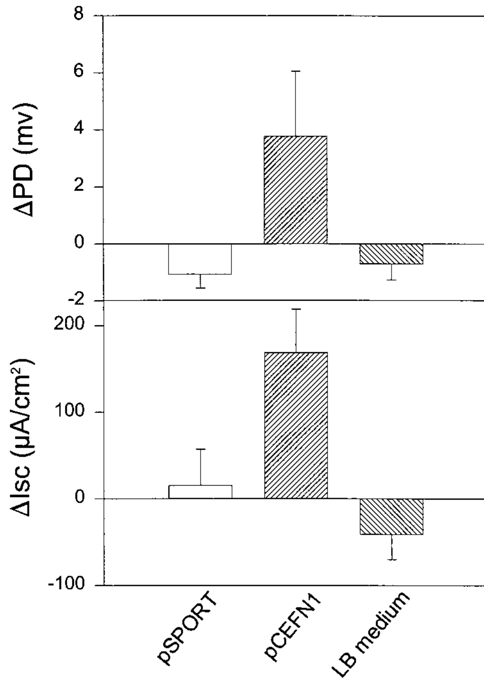

FIG. 4. Enterotoxic activity of the Pet protein derived from pCEFN1. Supernatants from overnight cultures were size fractionated (>50 kDa), and 5 μg of protein was added per Ussing chamber into which was mounted full-thickness rat jejunal tissue. The supernatants of HB101(pCEFN1) and HB101(pSPORT1) are illustrated in lanes C and D, respectively of Fig. 3a. Data points represent the means of at least three experiments; error bars represent standard errors of the means. The insert of pCEFN1 generates significant rises in PD and Isc compared with negative controls ($P < 0.05$ by Student's t test).

DISCUSSION

The pathogenesis of EAEC diarrhea is poorly understood. Clinical descriptions suggest that EAEC diarrhea is secretory in nature and therefore perhaps due to the presence of an as yet unidentified enterotoxin. The low-molecular-weight putative enterotoxin EAST1 has been found in some EAEC strains, but its role in diarrhea has yet to be proven. Eslava et al. (22) have described a ca. 108-kDa EAEC protein that is able to elicit fluid accumulation and mucosal destruction in rat ileal loops. Navarro-Garcia et al. (43) suggested that this protein elicits rises in Isc and decreases of electrical resistance in rat jejunal tissue mounted in an Ussing chamber, accompanied by damage to the epithelial cells (43). Using molecular methods, we have characterized this high-molecular-weight protein and have demonstrated that it is a plasmid-encoded autotransporter enterotoxin of EAEC.

Our analysis of the gene encoding the Pet enterotoxin shows homology with members of the autotransporter family of bacterial proteins. This family comprises a rapidly growing number of virulence determinants of gram-negative bacteria (33). The class takes its name from its so-called type IV secretion mechanism (24), in which an N-terminal amino acid leader sequence directs secretion through the general secretory pathway into the bacterial cytoplasm; once in the periplasm, a C-terminal amphipathic region forms a β barrel in the outer membrane, allowing the processed N-terminal protein to pass through into the extracellular milieu. In some cases, the protein remains anchored in the outer membrane (7), whereas in other cases, the protein is released into the supernatant. Secondary structure analyses of the predicted Pet product by the method of Jähnig (32) suggested the presence of 14 amphipathic β strands, each strand consisting of 10 to 14 amino acids. An even number of antiparallel transmembrane segments would place the first and last strands in opposite orientation and would allow closing of the β barrel, a feature observed for the trimeric porins such as OmpF (15). Thus, our analyses suggest that the β domain forms a pore through which the passenger domain is translocated to the surface, as is typical of the type IV mechanism.

The release of the Pet passenger domain apparently occurred by proteolytic cleavage between residues N^{1018} and N^{1019}. In the case of the immunoglobulin A1 proteases of *Neisseria gonorrhoeae*, this processing step is a result of autoproteolysis involving the serine protease site of the molecule (46). The presence in Pet of a putative serine protease active site suggests that a similar step could also occur in the case of Pet. To characterize further the processing step involved in the maturation of Pet, export of the passenger domain was investigated in *E. coli* strains lacking either the periplasmic protease DegP, the OmpT and OmpP proteases of the outer membrane, or DsbA, the disulfide bond isomerase. The results indicated that formation of the passenger and β domains was independent of these four enzymes and implied that either another unidentified protease is involved or autoprocessing may occur.

A number of autotransporter proteins from *Enterobacteriaceae* have been reported recently. Among these are EspP from enterohemorrhagic *E. coli* (11), EspC from enteropathogenic *E. coli* (57), She (49), and SepA (6) from *S. flexneri* and Tsh (48). Each of these proteins is >100 kDa, is processed and exported by the type IV mechanism, and features a serine protease active site motif. The precise roles of these proteins have not been determined, however; only Pet has been tested rigorously for enterotoxic activity.

Analysis of nucleotide sequence data identified a number of IS-like elements flanking the genes encoding members of the autotransporters of *Enterobacteriaceae*. Of note is the presence of an IS*629* (IS*1203*) element downstream of the *pet* gene. Other workers have shown that IS*629* elements are linked to the presence of putative virulence loci, but of specific interest is the association of this IS-like element with the *espP*, *sepA*, and *she* genes. Furthermore, *espP* of *E. coli* O157:H7 is flanked by both an IS*629* element and an IS*1* element (although in the opposite orientation to the *pet* gene), while the identical gene from O26:H$^-$ is flanked by IS*629* and remnants of an IS*911* element (20). These data coupled to the fact that *pet* has a G+C content significantly lower than the average for *E. coli* (51%) suggests that the gene may have been acquired by strain 042 via horizontal transfer. Certainly, the association of most

TABLE 2. Prevalence of the *pet* gene among EAEC strains

Site	*pet* probe result	Strain(s)
Chile	Negative	17-2
Mexico	Negative	60A, 93A, JM221
India	Negative	34b
Peru	Negative	H46-2, H92-1, H32-1, H223-1, H11-1, H232-1, H145-1, H191-1, H38-1, Peru-10
	Positive	Peru-49
Philippines	Negative	DS61R2, DS65R3, DS67R2
	Positive	DS244R3
Thailand	Negative	44-1-1, 6-1-1, 103-1-1, 144-1-1, 501-1-1, 309-1-1, 253-1-1, 239-1-1, 278-1-1, 495-1-1, 232-1-1
	Positive	216-1-1, 199-1-4, 435-1-1

autotransporters in *E. coli* and *Shigella* with the IS*629*-like elements suggests a role for this element in the evolution and spread of these homologs among the *Enterobacteriaceae*.

The enterotoxic activity induced by Pet is consistent with the secretory diarrhea seen in most patients with EAEC enteritis. However, the rises in Isc induced by the Pet protein are accompanied by a fall in tissue resistance, and light microscopic examination of the tissue after exposure to the toxin in an Ussing chamber reveals damage to the tissue (43). In light of observations suggesting that EAEC causes cytotoxic effects in in vitro intestinal culture and T84 cells, it is tempting to speculate that the Pet toxin may have cytopathic effects as well (28, 38). This possibility is currently under investigation. As well, we have observed that while only a small fraction of EAEC strains express the *pet* gene, it is quite possible that only these strains are in fact diarrheagenic. Indeed, human volunteer studies suggest that strain 042 induced diarrhea in healthy adults whereas three other strains did not induce enteric symptoms (40). Our DNA probe analysis for the *pet* gene suggests that of the four strains fed, only strain 042 expresses Pet. Moreover, Pet was initially isolated from EAEC strain 049766, which was implicated in a highly virulent outbreak of diarrhea in Mexico. The hypothesis that only Pet-producing EAEC strains are capable of inducing diarrhea is currently being tested in epidemiological studies.

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