Identification of a Novel Fimbrial Gene Cluster Related to Long Polar Fimbriae in Locus of Enterocyte Effacement-Negative Strains of Enterohemorrhagic *Escherichia coli*

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Enterohemorrhagic *Escherichia coli* (EHEC) is a food-borne cause of bloody diarrhea and the hemolytic-uremic syndrome (HUS) in humans. Most strains of EHEC belong to a group of bacterial pathogens that cause distinctive lesions on the host intestine termed attaching-and-effacing (A/E) lesions. A/E strains of EHEC, including the predominant serotype, O157:H7, are responsible for the majority of HUS outbreaks worldwide. However, several serotypes of EHEC are not A/E pathogens because they lack the locus of enterocyte effacement (LEE) pathogenicity island. Nevertheless, such strains have been associated with sporadic cases and small outbreaks of hemorrhagic colitis and HUS. Of these LEE-negative organisms, O113:H21 is one of the most commonly isolated EHEC serotypes in many regions. Clinical isolates of LEE-negative EHEC typically express Shiga toxin 2 and carry an ~90-kb plasmid that encodes EHEC hemolysin, but in the absence of LEE, little is known about the way in which these pathogens colonize the host intestine. In this study we describe the identification of a novel fimbrial gene cluster related to long polar fimbriae in EHEC O113:H21. This chromosomal region comprises four open reading frames, *lpfA* to *lfpD*, and has the same location in the EHEC O113:H21 genome as O island 154 in the prototype EHEC O157:H7 strain, EDL933. In a survey of EHEC of other serotypes, homologues of *lpfA*$_{O113}$ were found in 26 of 28 LEE-negative and 8 of 11 non-O157:H7 LEE-positive EHEC strains. Deletion of the putative major fimbrial subunit gene, *lpfA*, from EHEC O113:H21 resulted in decreased adherence of this strain to epithelial cells, suggesting that *lpf*$_{O113}$ may function as an adhesin in LEE-negative isolates of EHEC.

Shiga toxin-producing strains of enterohemorrhagic *Escherichia coli* (EHEC) are a prominent cause of acute gastroenteritis, hemorrhagic colitis, and the hemolytic-uremic syndrome (HUS) in humans (17, 23). HUS is characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, and those most at risk of developing renal disease are children under 10 years and the elderly. In approximately 3 to 5% of affected children, HUS is fatal, while 12 to 30% suffer serious impairment of renal function or neurologic damage (17, 23).

Although the carriage of bacteriophage-encoded Shiga toxin genes (*stx*) is a defining feature of virulence, strains of EHEC also harbor a large ~90-kb plasmid, pEHEC, and/or a large chromosomal pathogenicity island, termed the locus for enterocyte effacement (LEE) (6, 14). Strains of EHEC carrying LEE belong to a group of bacterial pathogens that cause distinctive lesions on the host intestine termed attaching-effacing (A/E) lesions. This group of organisms also includes the human pathogen enteropathogenic *E. coli* (EPEC) and the animal pathogens rabbit-specific enteropathogenic *E. coli*

(REPEC) and *Citrobacter rodentium*. A/E lesions are characterized by localized destruction (effacement) of intestinal brush border microvilli, intimate attachment of the bacteria to the host cell membrane, and the formation of underlying actin-rich, pedestal-like structures in the host cell (11, 17). The formation of A/E lesions is essential for bacterial colonization of the intestinal mucosa by LEE-positive pathogens. Knockout mutations in *eae*, *espB*, *tir*, and *espA* abolish the ability of A/E pathogens to cause A/E lesions and seriously impair the capacity of these organisms to colonize the host and cause disease (1, 8, 15, 16, 28, 31). Nevertheless, some serotypes of EHEC do not carry LEE and are not A/E pathogens (9, 22). These strains have regularly been associated with sporadic cases and small outbreaks of hemorrhagic colitis and HUS, but little is known about the way in which these LEE-negative EHEC adhere to and colonize the human intestine. Clinical isolates of LEE-negative EHEC typically express Shiga toxin type 2 and also harbor an ~90-kb plasmid which encodes EHEC hemolysin (22). However, restriction fragment length polymorphism analysis of the *ehxA* gene has shown that the large plasmids from LEE-negative EHEC comprise an evolutionarily distinct group compared with the similarly sized plasmids of LEE-positive EHEC (5). EHEC O113:H21 is one of the most commonly isolated LEE-negative EHEC serotypes worldwide, but until recently no virulence-associated genes apart from *stx* and *ehx* in these bacteria had been documented. Recently, however, Paton et al. identified a novel plasmid-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
E. coli strains		
ORN103	*thr-1 leu-6 thi-1* Δ(*argF-lac*) *U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 fhuA2 minA monB recA13* Δ(*fimABCDEFGH*)	33
XL-1 Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac* F′ [*proAB*+ *lacI*q *lacZ* ΔM15 Tn*10*] (Tet^r)	Stratagene
EH41	Wild-type EHEC O113:H21 HUS isolate (Sm^r)	This study
EH41*lpfA*−	*lpfA* deletion mutant of EH41 (Sm^r Km^r)	This study
15–26	EH41 *pstS*::Tn*phoA* (Sm^r Km^r)	This study
83/39	Wild-type REPEC O15:H−	2
Plasmids		
PCR-Script	High-copy-number cloning vector (Amp^r)	Stratagene
pWSK29	Low-copy-number cloning vector (Amp^r)	35
pWSK:*lpf*	pWSK29 carrying *lpfABCD*$_{O113}$	This study
pRT733	Suicide plasmid carrying Tn*phoA* (Amp^r)	32
pKD46	Low-copy-number thermosensitive plasmid encoding red recombinase	7
pKD4	Template for PCR amplification of kanamycin cassette for red recombinase-mediated recombination	7

encoded autoagglutinating adhesin, termed Saa, in LEE-negative EHEC O113:H21 (21). Saa is a 516-amino-acid protein with a low degree of similarity to the adhesin YadA of *Yersinia enterocolitica*. Mutation of the *saa* gene or curing of the pO113 plasmid resulted in reduced adhesion to epithelial cells (21). Dytoc et al. have demonstrated the pili on the surfaces of EHEC O113:H21 cells, but the genes coding for pili in these strains or any LEE-negative EHEC have not been described (9).

The most common serotype of EHEC associated with outbreaks and sporadic disease worldwide is O157:H7 (14). Recently, the genome sequences of two EHEC O157:H7 outbreak strains were completed. Analysis of these sequences showed that EHEC O157:H7 has 1.34 Mb of DNA that is not present in nonpathogenic *E. coli* K-12 (12, 24). The additional regions of DNA (termed O islands) are inserted into a common "backbone" shared by *E. coli* K-12 and EHEC O157:H7 and vary in size from a few hundred base pairs up to 88 kb. About 33% of the 177 O islands contain only genes of unknown function. Many genes in the other O islands have been classified according to their homology with known virulence factors, although their role in disease is not proven (24). Several O islands contain putative fimbrial biosynthesis operons, including two regions predicted to encode long polar fimbriae (LPF). LPF are related to type I fimbriae in genetic organization and were first identified in *Salmonella enterica* serovar Typhimurium (3). They form surface structures 7 to 8 nm in diameter and 2 to 10 μm in length extending from the poles of the bacterial cell. In *S. enterica* serovar Typhimurium, LPF have been shown to facilitate attachment of the bacteria to murine Peyer's patches. Synthesis of LPF is controlled by a phase-variable on/off switch that regulates expression of LPF in different tissues and organs (4). Phase variation of LPF is also a mechanism by which *S. enterica* serovar Typhimurium evades cross immunity among different *Salmonella* serotypes (19). In EHEC O157:H7, two putative *lpf* operons are present as O islands 141 and 154; however, the expression and production of LPF in EHEC O157:H7 has not yet been demonstrated, and their contribution to virulence is unknown (24). In this study, we report the identification of a novel fimbrial gene cluster related to LPF in LEE-negative EHEC serotype O113:

H21. We also describe the contribution of these genes to the adherence of EHEC O113:H21 to epithelial cells and the relationship of *lpf*$_{O113}$ to *lpf* gene clusters in other bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains (except those used for hybridization studies) and plasmids used in this study are listed in Table 1. EHEC O113:H21 (EH41) was isolated from a child with HUS in New South Wales, Australia, and expresses Stx-2 and EHEC hemolysin. Bacteria were grown in Luria-Bertani (LB) broth at 37°C with shaking or on LB agar at 37°C. Where required, antibiotics were added to media at the following concentrations: kanamycin, 100 μg/ml; streptomycin, 50 μg/ml; ampicillin, 100 μg/ml. For the selection of Tn*phoA* mutants, XP (5-bromo-4-chloro-3-indolylphosphate) was added to media at 100 μg/ml. For the induction of lambda red recombinase from plasmid pKD46, 100 mM arabinose was added to LB broth and competent cells were prepared by growth at 30°C.

Bacterial adhesion to epithelial cells. Qualitative assessment of bacterial adherence to tissue culture cells was performed as described by Vial et al. (34). Washed semiconfluent monolayers of CHO-K1 cells were infected with bacterial strains grown overnight at 37°C in LB broth supplemented with 0.5% glucose. Approximately 10^5 CHO-K1 cells on glass coverslips were incubated with around 10^6 bacteria for 4 h in the presence of 0.5% mannose. Following the incubation period, cells were washed five times with phosphate-buffered saline to remove nonadherent bacteria, fixed in methanol, and stained with Giemsa. Mounted coverslips were viewed by bright-field microscopy using a Leica DMLB compound microscope, and digital images were captured with a Leica DC300 digital camera.

Quantitative assessment of bacterial association with CHO-K1 cells was determined as described previously (26). Briefly, washed semiconfluent monolayers of CHO-K1 cells were infected in the presence of 0.5% mannose with around 10^6 CFU of different bacterial strains for 4 h. Cell monolayers were washed five times with phosphate-buffered saline and lysed in 0.1% digitonin. Following lysis, bacteria were resuspended in LB broth and quantified by serial dilution. Assays were carried out in duplicate, and the results from at least three experiments were expressed as a percentage of the original inoculum (mean ± standard deviation). Differences in adherence were assessed for significance by using Student's two-tailed *t* test.

DNA manipulations and DNA sequencing. Routine DNA manipulations (restriction digestion, ligation, agarose gel electrophoresis, transformation, labeling, and Southern and dot blot hybridizations) were performed according to standard techniques described by Sambrook et al. (27). Genomic DNA was prepared by using a DNeasy extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Marker rescue of a 7.1-kb *Bam*HI fragment containing the Tn*phoA* insertion site into PCR-Script cut with *Bam*HI was performed as described previously (32). Plasmid DNA for sequencing was purified using a QIAprep spin miniprep kit (Qiagen). Both strands of the rescued fragment were sequenced with custom-made oligonucleotide primers using a PRISM ready-reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems).

The DNA sequence was determined by automated DNA sequencing using an Applied Biosystems model 373A DNA sequencing system. DNA sequences were assembled by using Sequencher 3.1.1 (GeneCodes Corp., Ann Arbor, Mich.). BLAST programs were used to determine DNA homology and amino acid similarities and identities in pairwise comparisons of DNA sequences. The neural network promoter prediction (NNPP) program for prokaryotes was used to predict promoter sequences upstream of *lpfA* (25). The CLUSTAL W program was used to generate multiple amino acid alignments (13).

Identification and cloning of the *lpf*$_{O113}$ gene cluster. The transposon Tn*phoA* was introduced into EHEC O113:H21 (EH41) on the suicide plasmid pRT733 by conjugation as described previously (32). Blue (in-frame) Tn*phoA* mutants derived from a streptomycin-resistant derivative of EH41 were selected from LB agar containing streptomycin, kanamycin, and XP and tested for their ability to adhere to a semiconfluent monolayer of CHO-K1 cells. Of the 412 Tn*phoA* mutants screened, only one mutant was identified that showed an apparent reduction in adherence to CHO-K1 cells. The transposon insertion site of the mutant, 15-26, was cloned by marker rescue of a *Bam*HI fragment into PCR-Script. The entire *lpf* gene cluster from EHEC O113:H21 was amplified from genomic DNA with the Expand long-template PCR system (Roche Diagnostics, Mannheim, Germany) and the oligonucleotide primers O-154F (5′-CTGGCAA AATCGGTAACGGT-3′) and O-154R (5′-CCACCGGAAGAACCGAT-3′). PCR conditions were as follows: 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, and 68°C for 6 min and then an extension period of 7 min at 68°C. The final 5.5-kb product was purified with the Concert nucleic acid purification system (Gibco BRL) and cloned into the T-tailed pDK101 for DNA sequencing. The complete DNA sequence was determined as described above. For adherence studies, the *lpf* locus was cloned into pWSK29 digested with *Eco*RV. O-island amplifications on other bacterial strains were performed with O-154F and O-154R or the primers O-141F (5′-AAAAGTGTGGGGAAAGA GTG-3′) and O-141R (5′-AGCAGAAAGTATTGCGTGAG-3′). PCR conditions were identical except that an annealing temperature of 45°C was used for O-141F and O141-R.

Dot blot hybridizations and generation of an *lpfA*$_{O113}$ probe. Hybridization studies were performed with strains from our culture collection as reported previously (18). These strains were isolated from clinical specimens and from animal, food, and environmental sources in a variety of geographic locations. Genomic DNA extracted from these strains was spotted onto Hybond-N membranes (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and hybridized under stringent conditions with a digoxigenin (DIG)-labeled *lpfA*$_{O113}$ probe. Oligonucleotide primers used to generate the *lpfA*$_{O113}$ probe were lpfA-F (5′-ATGAAGCGTAATATTATAG-3′) and lpfA-R (5′-TTATTTCTTATATTC GAC-3′), with genomic DNA from EHEC O113:H21 (EH41) as the template. Primer sequences were chosen on the basis that they did not match any DNA sequences in GenBank, and specificity was confirmed by successful amplification of *lpfA*$_{O113}$ from EH41 and no amplification from EHEC O157:H7 (EDL933), which is known not to carry *lpf*$_{O113}$. By using these primers, DIG-labeled nucleotides (Roche) were incorporated by PCR using the following conditions: 94°C for 2 min followed by 30 cycles of 94°C for 1 min, 52°C for 50 s, and 72°C for 1 min, with an extension period of 5 min at 72°C. Strains that tested positive by hybridization for *lpfA*$_{O113}$ were confirmed by PCR analysis using the above-described primers and conditions.

Construction of an *lpfA* deletion mutant of EHEC O113:H21 (EH41). To generate an *lpfA* deletion mutant of strain EH41, the *lpfA* gene was replaced by a gene encoding kanamycin resistance by using the lambda red recombinase system (7). Long oligonucleotide primers designed for introducing the mutation were lpfAKOF (5′ATAAGTCGATGATTCATGGTAAAGGATATA TTATATCAATGTGTAGGCTGGAGCTGCTTC-3′) and lpfAKOR (5′-CT GGCCAAAGCCAACTGAATAAAAAGGCCCTTGATAAATTCATATGA ATATCCTCCTTAG-3′). Each primer included 20 bp of sequence homologous to the template plasmid, pKD4, and 40 bp of sequence homologous to regions flanking the *lpfA* gene. The kanamycin resistance gene was amplified from pKD4 by PCR. The product was treated with *Dpn*I to remove the pKD4 template and purified by using the Concert nucleic acid purification system. The purified PCR product was electroporated into EH41 which had previously been transformed with the lambda red recombinase expression vector, pKD46. Electrocompetent EH41 cells were prepared by culturing the bacteria at 30°C in 100 mM arabinose to induce expression of lambda red recombinase. Following electroporation, transformants of EH41 were recovered at 30°C for 2 h in LB broth supplemented with 100 mM arabinose and plated onto LB agar containing kanamycin for overnight growth at 37°C to induce the loss of pKD46. Kanamycin-resistant colonies were then tested by PCR for replacement of *lpfA* by the kanamycin resistance marker. The *lpfA*

deletion mutant of EH41 was complemented in *trans* by introduction of the entire *lpf*$_{O113}$ gene cluster on pWSK:*lpf*.

Nucleotide sequence accession number. The DNA sequence determined in this study has been assigned GenBank accession no. AY057066.

RESULTS

Identification and cloning of a novel fimbrial gene cluster. To identify novel factors required for the adherence of LEE-negative EHEC to epithelial cells, we performed Tn*phoA* mutagenesis on a streptomycin-resistant derivative of EHEC O113:H21 (EH41). EH41 was originally isolated from a child with HUS in southeastern Australia. We identified one blue Tn*phoA* mutant (designated 15-26) that showed reduced adherence to CHO-K1 cells compared to the parent strain. We subsequently cloned the Tn*phoA* insertion site from this mutant into PCR-Script by marker rescue of a *Bam*HI fragment. DNA sequencing of the 7.1-kb rescued fragment revealed that 15-26 contained an in-frame insertion of Tn*phoA* into *pstS*, an *E. coli* gene predicted to encode a periplasmic phosphate-binding protein. Translated BLAST analysis of the other end of the rescued fragment revealed an open reading frame (ORF) with predicted amino acid similarity to LpfD from *S. enterica* serovar Typhimurium. In *S. enterica* serovar Typhimurium, *lpfD* is part of an operon involved in the expression of LPF and is believed to encode a minor structural component of the fimbriae (3). The genome of EHEC O157:H7 also contains two novel, putative *lpf* operons. These are present as O islands 141 and 154, where O island 154 is positioned next to *pstS* and O island 141 (as well as the *lpf* operon from *S. enterica* serovar Typhimurium) is located next to *yhjW* (Fig. 1) (24). The presence of *lpfD* in the chromosome of EH41 adjacent to *pstS* suggested that we had identified an LPF operon in EH41. To clone the putative EHEC O113:H21 O-island insertion containing LPF, we designed primers to flanking regions of the *E. coli* chromosome in the genes *pstS* and *glmS* (encoding L-glutamine:D-fructose-6-phosphate aminotransferase) and amplified the O-island insertion by PCR. The 5.5-kb product was cloned into PCR-Script, and DNA sequencing revealed four complete ORFs, *lpfA*, *lpfB*, *lpfC*, and *lpfD* (Fig. 1A). The overall G+C content was 43.7% (compared with 50.8% for the *E. coli* K-12 genome), suggesting that the *lpf* gene cluster (*lpf*$_{O113}$) in the chromosome of EHEC O113:H21 (EH41) was acquired by lateral gene transfer. However, we could find no evidence of insertion elements or integrases or evidence of other mobile genetic elements adjacent to the *lpf*$_{O113}$ locus.

Using the NNPP promoter search program for prokaryotes, we identified a putative ribosome binding site and potential −10 and −35 promoter sequences in the nucleotide region upstream from the first ORF, *lpfA*$_{O113}$ (Fig. 1B). Potential ribosome binding sites were also identified between coding regions for *lpfB*$_{O113}$, *lpfC*$_{O113}$, and *lpfD*$_{O113}$ (data not shown). The distances of 10 to 50 nucleotides between the four ORFs suggested that these fimbrial biosynthesis genes were part of an operon.

Homology of LpfABCD$_{O113}$ to other fimbrial proteins. The *lpf* operons of *S. enterica* serovar Typhimurium, EHEC O157:H7, and EHEC O113:H21 carry genes predicted to encode chaperone-usher fimbrial biosynthesis systems and resemble members of the type I pilus family in their organization. By

A
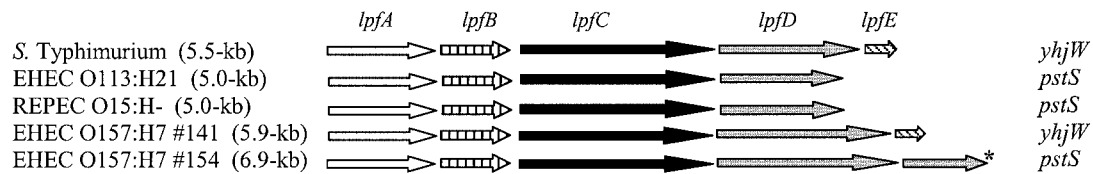
* O-island 154 contains two copies of *lpfD*

B

```
atctttctatataaatatattctgaattatcattaatgtctgataacaaacttgatt

ttttactgtttaaataatatttaaacaataaatttatattttattttttgcgtaatta

atattatttttagataaagctgtctctttggtgtactattcctt Tgaatcaaaaata
        -35                          -10
cacttattaatataagtgatgattcatggtaaaggatatattatatcaatggagttt

  M   K   R   N   I   I
ttatgaagcgtaatattata
  lpfA
```

FIG. 1. (A) Genetic organization of the *lpf* gene clusters from strains of *E. coli* and *S. enterica* serovar Typhimurium showing the approximate sizes and number of ORFs of the gene clusters and the *E. coli* K-12 gene adjacent to the chromosomal insertion site. (B) Analysis of the region upstream from the lpf_{O113} gene cluster. Putative −10 and −35 promoter sites are underlined, and a putative ribosome binding site is shaded. The amino acid translation is shown above the nucleotide sequence. A potential transcription start point is in bold type and uppercase, and the coding region of *lpfA* is in bold type. The promoter region was analyzed with the NNPP program for prokaryotes.

homology to other fimbrial proteins, $LpfA_{O113}$ is predicted to be a major fimbrial subunit protein, $LpfB_{O113}$ is predicted to be a putative chaperone, $LpfC_{O113}$ is predicted to be an outer membrane usher, and $LpfD_{O113}$ is predicted to be a putative minor fimbrial component. In addition to *lpfABCD*, the *lpf* operons from *S. enterica* serovar Typhimurium and O island 141 from EHEC O157:H7 contain *lpfE*, which is predicted to encode a minor tip adhesin (Fig. 1A). The properties of the four putative lpf_{O113} gene products and their amino acid identities and similarities with other related fimbrial proteins are given in Table 2. The putative fimbrial components $LpfA_{O113}$

and $LpfD_{O113}$ from EHEC O113:H21 (EH41) exhibited the highest amino acid identity to homologues encoded by O island 154 from EHEC O157:H7, where the genetic loci have the same location in the bacterial chromosome. Across the entire lpf_{O113} O island, however, no DNA homology to any sequences in GenBank was detected. An amino acid alignment between $LpfA_{O113}$ and homologues from EHEC O157:H7 and *S. enterica* serovar Typhimurium is shown in Fig. 2. Like other major fimbrial subunits of the type I family, the putative $LpfA_{O113}$ product has conserved cysteine residues at positions 19 and 59 of the mature pilin which form a disulfide bridge in

TABLE 2. Predicted molecular masses and pI of the putative lpf_{O113} gene products and amino acid identities (and similarities) with related LPF proteins from EHEC O157:H7 EDL933 and *S. enterica* serovar Typhimurium LT-2

Gene	Predicted characteristic of product			% Amino acid identity (similarity) of lpf_{O113} with:		
	No. of amino acids	Mass (kDa)	pI	EDL933 O island 154 (AE005604[a])	EDL933 O island 141 (AE005581[a])	LT-2 (AE008868[a])
lpfA	190	19.7	4.9	54 (68)	30 (44)	28 (42)
lpfB	243	27.2	9.6	30 (38)	32 (53)	34 (50)
lpC	840	92.8	4.8	64 (81)	23 (35)	39 (61)
lpfD	265	29.1	6.2	34 (49)[Z5220], 30 (44)[Z5221][b]	24 (37)	28 (39)

[a] Accession number.
[b] O island 154 contains two copies of LpfD.

LpfA

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S. Typhimurium      MEFLMKKVVFALSALAVVSTSAFAAESGDGTIKFTGEIVDAPCVVSTDSQ  50
EHEC O157:H7 #141   MEFFMKKVVFALTALALTSGTVFAAESGDGTVKFTGEIVDSPCVLSVDSQ  50
EHEC O157:H7 #154   MKPNMIVGALALTSVFMAG----HLQAADGTVHFRGEIIDSTCEVTPETK  46
EHEC O113:H21       MKRNIIGGAFTLASLMLAG----HALAEDGVVHFVGEIVDTTCEVTSDTA  46
                                                              *

S. Typhimurium      NQEVVLGQVKKNIFKAIGDKSSSKPFQIKLEDCDILSNTKVNVSFNCVGD 100
EHEC O157:H7 #141   NQEVVLGQVQKSVFAAVGDKSPAKPFEIKLEDCDTTTMKKANVSFSCVGD 100
EHEC O157:H7 #154   DQVVDLGKVNRTAFSGVDDVAAPTAFSIDLTQCPET-FKSAAIRFDCNED  95
EHEC O113:H21       DQIVPLGKVSKNAFSGVGSLASPQKFSIKLENCPAT-YTQAAVRFDCTEA  95
                                                      *

S. Typhimurium      TDDATLVSVNTEAG-----------------------AATGVGIGIYD 125
EHEC O157:H7 #141   ABKSDLISVSTEAG-----------------------AAKGVGIGIYD 125
EHEC O157:H7 #154   AHGNGNLAIGTPLDNSNDAAAGISPSDNSGDYTGAGAVSAAKGVAIRLYN 145
EHEC O113:H21       PGGDGDLKVGTPLT-----------AGNPGDFTGTGQAIAATGVGIRIFN 134

S. Typhimurium      NA-NKLVEMNTGKSTTTLA-AGQTVLYYTANYVATKDTVTTGYGNAEVDF 173
EHEC O157:H7 #141   NS-NTLVALNGGKASVDLS-KGQTVLYFTANYVSTLATVTTGYGNAQVDF 173
EHEC O157:H7 #154   RADNTQVKLYENSASTPIS-NGNASMKFMARYIATETTIDPCTANADSQF 194
EHEC O113:H21       QSDNSQVKLYNDSAYTAIDAEGKAEMKFIARYVATNATVTAGTANADSQF 184
                                                              #

S. Typhimurium      NLSYE- 178
EHEC O157:H7 #141   NLSYE- 178
EHEC O157:H7 #154   TVEYIK 200
EHEC O113:H21       TVEYKK 190
                       #
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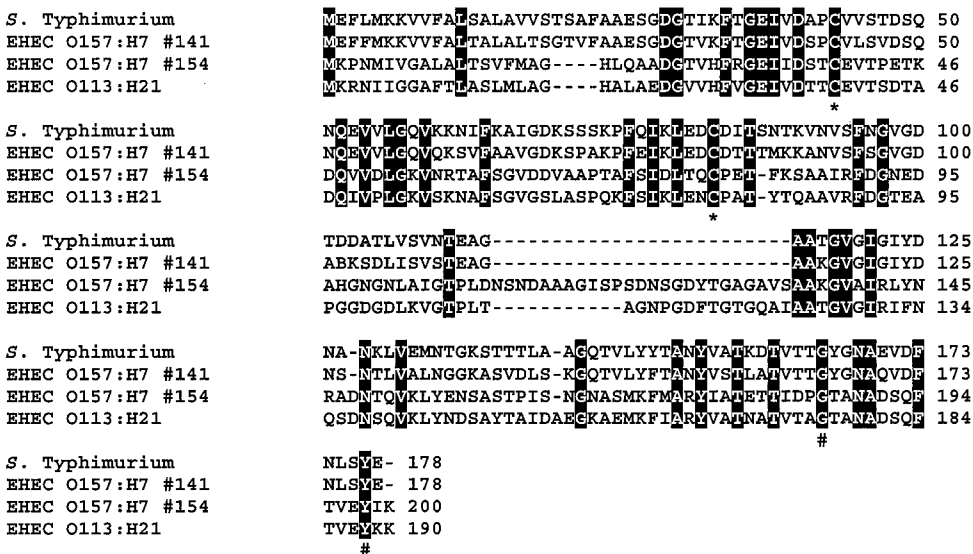
FIG. 2. Amino acid alignment of the predicted $lpfA_{O113}$ product and related fimbrial proteins. The alignment was performed with CLUSTAL software, and amino acid numbers are on the right. Gaps are indicated by a dashed line, and conserved amino acids are shaded in black. Cysteine residues (Cys39 and Cys79) predicted to form a disulfide bridge are indicated by an asterisk. The invariant glycine (Gly176) and aromatic (Tyr188) residues predicted to comprise a chaperone-binding domain are also indicated (#).

other pilins (Cys39 and Cys79) (Fig. 2). $LpfA_{O113}$ also carries a predicted chaperone-binding domain characterized by an invariant glycine residue (Gly176) and aromatic residue (Tyr188) separated by a series of alternating hydrophobic residues (Fig. 2).

Distribution of $lpfA_{O113}$ among other pathogenic *E. coli* and enteric pathogens. The identification of a novel fimbrial gene cluster in an EHEC strain prompted us to examine the distribution of $lpfA_{O113}$ among other strains of EHEC, EPEC, and REPEC and other pathogenic *E. coli* strains and enteric pathogens. $lpfA_{O113}$-specific primers were used to amplify $lpfA_{O113}$ from genomic preparations of DNA derived from test strains or used to generate a DIG-labeled $lpfA_{O113}$ probe by PCR from EH41 which was then used for dot blot hybridization. Overall, $lpfA_{O113}$ was detected by PCR and dot blot hybridization in 26 of 28 LEE-negative EHEC strains tested, including all strains of O113:H21 (Table 3). Of the EHEC O113:H21 strains included in this study, seven strains were clinical isolates from independent cases of HUS, thrombotic thrombocytopenic purpura, or hemorrhagic colitis, three were isolated from food sources, and one was of bovine origin. $lpfA_{O113}$ was also detected in 8 of 11 non-O157:H7 LEE-positive EHEC strains. In contrast, only 1 of 11 EPEC strains contained $lpfA_{O113}$. Interestingly, the $lpfA_{O113}$-positive EPEC strain was O26:H−, which is a serotype common to the EHEC pathotype. $lpfA_{O113}$ was also detected in four of nine REPEC strains tested (Table 3). In further testing by dot blot hybridization, we found $lpfA_{O113}$ in one of five strains of enterotoxigenic *E. coli* but could not detect $lpfA_{O113}$ in any strains of enteroaggregative *E. coli* (zero of five), enteroinvasive *E. coli* (zero of five), *S. enterica* serovar Typhimurium (zero of five), *Y. enterocolitica* (zero of three), *Pseudomonas aeruginosa* (zero of two), *Shigella* spp. (zero of four), or *Klebsiella* spp. (zero of three).

Contribution of LPF_{O113} to adherence of EHEC O113:H21 (EH41) to CHO-K1 epithelial cells. To examine the capacity of LPF_{O113} to mediate adherence to epithelial cells, the lpf_{O113} gene cluster was introduced into a nonpiliated *fim* deletion mutant of *E. coli* K-12, strain ORN103, on the low-copy-number vector pWSK29. The resulting strain, *E. coli* ORN103(pWSK:*lpf*), and a control strain, *E. coli* ORN103(pWSK29), were used to infect CHO-K1 cell monolayers for 4 h. In a qualitative assay designed to reveal patterns of bacterial adherence, *E. coli* ORN103(pWSK:*lpf*) adhered to CHO-K1 cells in clusters and the bacteria aggregated on the cell surface (Fig. 3A). Although the adherence pattern of *E. coli* ORN103(pWSK:*lpf*) was not uniform across the microscopic field, this type of adherence was not seen at all for *E. coli* ORN103(pWSK29) (Fig. 3B). These observations implied that the presence of LPF_{O113} could alter the surface properties of *E. coli* ORN103 and promote adherence to epithelial cells and interbacterial interactions. The suggested increase in adherence was confirmed in quantitative adherence assays, where *E. coli* ORN103(pWSK:*lpf*) was twice as adherent to CHO-K1 cells as *E. coli* ORN103(pWSK29) (Table 4).

To investigate the putative contribution of LPF_{O113} to the adherence of EHEC O113:H21 (EH41) to CHO-K1 cells, we introduced an $lpfA_{O113}$ deletion using the recently described lambda red recombinase system. Successful replacement of $lpfA_{O113}$ with a gene encoding kanamycin resistance was confirmed by PCR analysis (data not shown). We could find no clear difference in the adherence patterns of wild-type EHEC O113:H21 (EH41) and EH41*lpfA*− (Fig. 3C and D), although fewer bacteria per cell were observed for EH41*lpfA*. Quantitative adherence assays confirmed that EHEC O113:H21 (EH41) was significantly more adherent to CHO-K1 cells than the *lpfA* mutant (Table 4). *trans* complementation of

TABLE 3. Prevalence of $lpfA_{O113}$ among serogroups of EHEC, EPEC, and REPEC

Pathotype	Serogroup	Presence of eae^a	No. of strains positive for $lpfA_{O113}$/no. tested
EHEC	O157:H7	+	0/2
	O111:H−	+	3/4
	O15:H−	+	1/1
	O26:H11	+	1/2
	O147:H−	+	1/1
	O145:H25	+	1/1
	O5:H−	+	1/1
	O113:H21	−	11/11
	O116:H21	−	1/1
	O130:H11	−	1/1
	O5:H−	−	2/2
	O1:H7	−	0/1
	NT:H7	−	1/1
	NT:H−	−	1/1
	O48:H21	−	1/1
	O76:H7	−	0/1
	O128:H2	−	3/3
	O91:H−	−	2/2
	O87:H16	−	1/1
	OR:H−	−	1/1
	O123:H−	−	1/1
EPEC	O127:H6	+	0/1
	O111:H2	+	0/2
	O114:H2	+	0/1
	O125:H2	+	0/1
	O128:H2	+	0/1
	O142:H6	+	0/2
	O55:H7	+	0/1
	O26:H−	+	1/1
	O86:H−	+	0/1
REPEC	O15:H−	+	2/2
	O103:H2	+	0/3
	O153:H7	+	1/1
	O20:H7	+	1/1
	O8	+	0/1
	O109:H2	+	0/1

^{a} Marker for LEE pathogenicity island.

EH41*lpfA*− with the lpf_{O113} gene cluster carried on pWSK:*lpf* restored adherence of the mutant to CHO-K1 cells to a level comparable to that of wild-type EH41 (Table 4).

Analysis of the genome insertion site of lpf_{O113} and the presence of O-island insertions near *pstS* and *yhjW* in other *E. coli* pathogens. DNA sequence comparison between the chromosomal positions of the *lpf* loci from EHEC O113:H21 and EHEC O157:H7 revealed that the positions of lpf_{O113} and O island 154 in the *E. coli* backbone were virtually identical, with only a 2-bp difference at the *pstS* end of the O island (Fig. 4). In view of this coincidence, we looked for the presence of an O-island insertion in the chromosome of EHEC O113:H21 next to *yhjW* which corresponds to O-island position 141 in EHEC O157:H7. Primers were designed based on flanking regions of the *E. coli* chromosome in the genes *yhjW* and *yhjX*. A 200-bp product corresponding to the *E. coli* K-12 sequence was amplified, indicating that this site in EHEC O113:H21 does not contain an O island. We then tested a number of other serotypes of LEE-negative and LEE-positive EHEC strains and the related pathogens EPEC and REPEC for O-island insertions next to *pstS* and *yhjW* and were able to amplify

O islands from one or both of these regions in 10 of 11 strains (Table 5). Cloning and sequencing of the O island next to *pstS* from REPEC 83/39 revealed that this region in the chromosome also contained an *lpf* gene cluster identical to lpf_{O113}, which we have designated lpf_{R154}. Nucleotide sequencing of the remaining O islands is currently in progress.

DISCUSSION

Although a wide variety of Shiga toxin-producing strains of *E. coli* are associated with disease in humans, the predominant causative agent of hemorrhagic colitis and HUS worldwide is EHEC O157:H7 (14, 23). As a result, much of the research effort into EHEC has focused on the characterization of virulence determinants in this pathogen, in particular the role of the O157:H7 LEE pathogenicity island in colonization and disease. Nevertheless, a significant proportion of EHEC strains isolated from patients with hemorrhagic colitis and HUS do not carry the LEE pathogenicity island yet are capable of causing severe, even life-threatening disease (9, 10, 22). Aside from the production of EHEC hemolysin and Stx-2, little is known about the virulence determinants of these pathogens. In particular, in the absence of LEE, the mechanisms by which these strains adhere to the host mucosal epithelium are unknown. Recently, Srimanote et al. described a pO113-encoded type IV pilus that promotes plasmid conjugation but does not appear to contribute to bacterial adherence (30). In contrast, mutation of the *saa* gene encoded by pO113 or curing of the large pO113 plasmid from wild-type EHEC O113:H21 resulted in a reduction in bacterial adherence to HEp-2 cells. Adherence was not completely abolished, however, indicating that pO113-independent factors also contribute to the adherence phenotype in vitro (21).

In another recent study of LEE-negative Shiga toxin-producing *E. coli* (STEC), Schmidt et al. uncovered a novel genomic island in STEC O91:H− (29). This region is located next to the *selC* locus and encodes a serine protease autotransporter of *Enterobacteriaceae* protein EspI, and a homologue of the putative adhesin, Iha. However, the contribution of these genomic factors to bacterial adherence was not examined, and despite having tested a large number of LEE-negative STEC strains, the authors did not report the presence of this putative pathogenicity island in EHEC O113:H21 (29).

In this study, we describe the identification of a novel chromosomal fimbrial gene cluster in LEE-negative EHEC O113:H21 that is related to LPF. Nucleotide sequencing of this novel region, lpf_{O113}, revealed four putative ORFs predicted to encode homologues of long polar fimbrial proteins LpfA, LpfB, LpfC, and LpfD. In *S. enterica* serovar Typhimurium, LPF evidently contribute to the tropism of the bacteria for Peyer's patches of mice (4). The contribution of the homologues of these genes to the virulence of EHEC O157:H7 and O113:H21, however, is not known. Like other *lpf* gene clusters, lpf_{O113} exhibited similarity to the type I chaperone-usher family of fimbriae but lacked genes encoding regulatory elements (3). A comparison of the genetic organization among different *lpf* gene clusters showed that those located next to *yhjW* carry an additional gene, *lpfE*, encoding a putative fimbrial adhesin, which is not present in the fimbrial gene clusters adjacent to *pstS* (24).
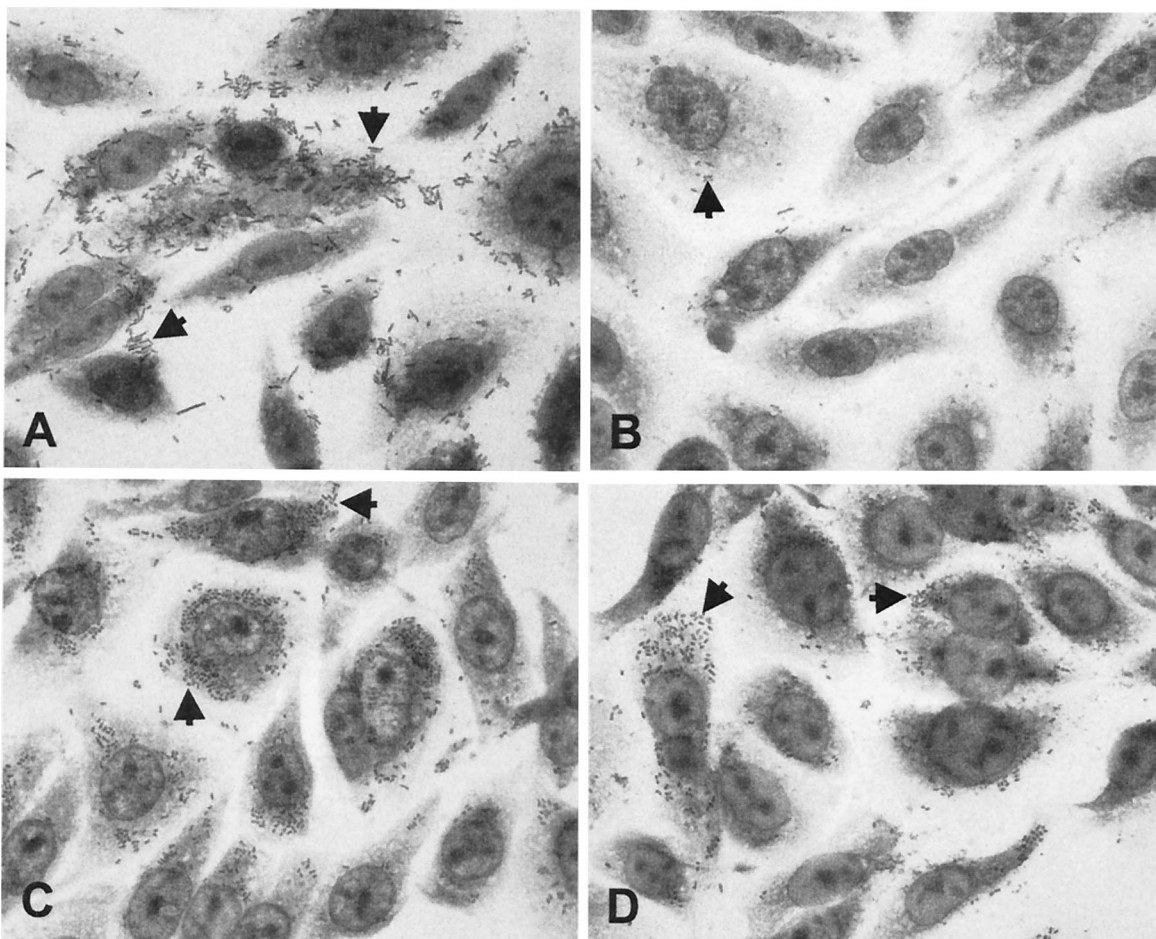
FIG. 3. Adherence of derivatives of *E. coli* ORN103 and EHEC O113:H21 (EH41) to Chinese hamster ovary cells (CHO-K1). (A) *E. coli* ORN103(pWSK:*lpf*); (B) *E. coli* ORN103(pWSK29); (C) parent strain EHEC O113:H21 (EH41); (D) EH41*lpf*−. Arrows indicate adherent bacteria.

Evidence that the lpf_{O113} genes are functional was obtained by performing site-specific deletion of the putative major fimbrial subunit gene, $lpfA_{O113}$, which resulted in significantly reduced adherence of EHEC O113:H21 (EH41) to CHO-K1 cells. Complementation of the EH41 *lpfA* deletion mutant with lpf_{O113} in *trans* restored adherence to wild-type levels. We attempted to visualize LPF_{O113} on the surface of *E. coli* ORN103(pWSK:*lpf*) by using negative staining and electron microscopy, but without success. This may be explained by poor expression of LPF_{O113} by ORN103, as evidenced by the

TABLE 4. Adherence of derivatives of *E. coli* strains EH41 (parent) and ORN103 (nonfimbriated) to CHO-K1 cells

Strain	% Adherence to CHO-K1 cells[a]
ORN103(pWSK29)	1.1 ± 0.54
ORN103(pWSK:*lpf*)	2.2 ± 1.3[b]
EH41 (wild type)	49.2 ± 34.2
EH41*lpfA*−	10.6 ± 6.7[c]
EH41*lpfA*−(pWSK:*lpf*)	47.1 ± 22.6

[a] Percentage of original inoculum; mean ± standard deviation of at least three experiments in duplicate wells.
[b] Significantly more than ORN103(pWSK29) ($P = 0.01$).
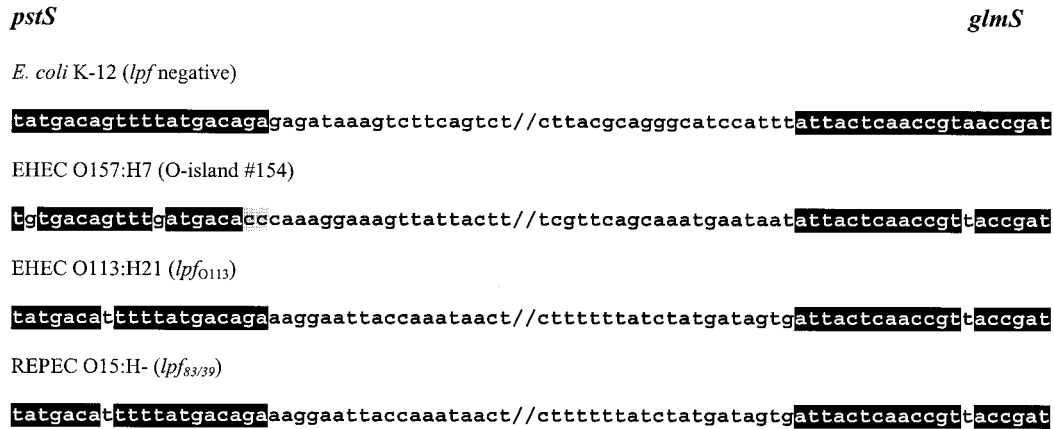[c] Significantly less than EH41 (wild type) ($P = 0.005$).

relatively weak adherence of *E. coli* ORN103(pWSK:*lpf*) to CHO-K1 cells, despite being significantly more adherent than *E. coli* ORN103(pWSK29). In addition, *E. coli* ORN103 may not be capable of supporting the correct assembly of LPF_{O113} on its surface, because genes other than the four ORFs described here are required for the full expression and normal assembly of the fimbriae. This is in contrast to the *lpf* operon from *S. enterica* serovar Typhimurium, which, when introduced into a nonfimbriated strain of *E. coli*, ORN172, encoded structures 7 to 8 nm in width and 2 to 10 μm in length in approximately one-third of the bacterial population (3). However, these structures were not confirmed as LPF by immunogold electron microscopy and may represent the activation of a cryptic fimbrial pathway in *E. coli* ORN172. The production of specific antibodies recognizing native $LpfA_{O113}$ may aid the visualization of LPF_{O113} in EHEC O113:H21 as well as in *E. coli* ORN103(pWSK:*lpf*).

The *lpf* gene clusters from EHEC, REPEC, and *S. enterica* serovar Typhimurium are present in one or two locations in the *E. coli* chromosome, namely, next to *yhjW* (*S. enterica* serovar Typhimurium and EHEC O157:H7 O island 141) or abutting *pstS* (EHEC O113:H21, REPEC O15:H−, EHEC O157:H7 O island 154, and *S. enterica* serovar Typhi). The presence in

pstS   *glmS*

E. coli K-12 (*lpf* negative)

```
tatgacagttttatgacagagagataaagtcttcagtct//cttacgcagggcatccatttattactcaaccgtaaccgat
```

EHEC O157:H7 (O-island #154)

```
tgtgacagtttgatgacacccaaaggaaagttattactt//tcgttcagcaaatgaataatattactcaaccgttaccgat
```

EHEC O113:H21 (*lpf*$_{O113}$)

```
tatgacattttatgacagaaaggaattaccaaataact//ctttttttatctatgatagtgattactcaaccgttaccgat
```

REPEC O15:H- (*lpf*$_{83/39}$)

```
tatgacattttatgacagaaaggaattaccaaataact//ctttttttatctatgatagtgattactcaaccgttaccgat
```

FIG. 4. Nucleotide sequence of the regions flanking different *lpf* sites adjacent to *pstS*. Conserved nucleotides are shaded in black, and two nucleotides unique to EHEC O157: H7 (EDL933) are shaded in grey.

several pathogens of additional DNA at these two positions suggested that they might be common sites for chromosomal insertions. Indeed, PCR amplification of these regions in several EHEC, EPEC, and REPEC isolates revealed insertions in one or other of these positions in 10 of 11 strains tested. While several of the insertions next to *pstS* are likely to be identical to *lpf*$_{O113}$, insertions adjacent to *yhjW* were also present in several EHEC and REPEC strains, but the DNA sequence of these regions has yet to be determined. Sequence analysis of the *lpf* operons positioned next to *pstS* in three different pathogens revealed that the sites of insertion in the *E. coli* chromosome were identical. This suggests that the putative lateral acquisition of *lpf* in different bacterial chromosomes was precise and specific for this site, or that *lpf* was an integral part of ancestral *E. coli* strains but was subsequently lost from some of its progeny.

In phylogenetic analyses, the predicted LpfA$_{O113}$ subunit clusters with a putative fimbrial subunit from *S. enterica* serovar Typhi, StgA, and the putative major fimbrial subunit from

EHEC O157:H7 O island 154 (data not shown) (20, 24). The three fimbrial gene clusters are all located between *pstS* and *glmS* in the chromosomal backbone and thus are related in amino acid sequence and genome position. However, the putative usher gene, *stgC*, is a pseudogene, suggesting that this fimbrial operon is not functional in *S. enterica* serovar Typhi (20). Likewise, LpfA from *S. enterica* serovar Typhimurium is less related to LpfA$_{O113}$ than it is to the putative major fimbrial subunit from EHEC O157:H7 O island 141, where these two gene clusters have the same position in the chromosome.

An examination of the distribution of *lpf*$_{O113}$ among other pathogenic *E. coli* strains and enteric pathogens revealed that *lpf*$_{O113}$ is closely associated with EHEC strains and in particular with different serogroups of LEE-negative EHEC. This is in contrast to *saa*, which was associated only with LEE-negative strains of EHEC (21). *lpf*$_{O113}$ was also found in four strains of REPEC. The presence of a *lpf* gene cluster identical to *lpf*$_{O113}$ in REPEC O15:H- (83/39) provides us with an opportunity to test the contribution of these fimbriae to virulence. The construction of a REPEC (83/39) *lpfA* mutant is currently in progress for testing in a rabbit model of infection (2). We propose that the putative LPF of EHEC may facilitate colonization of humans and/or the persistence of *E. coli* pathogens in animal reservoirs of infection. Further studies are required to elucidate fully the role of *lpf* gene clusters in the pathogenesis of *E. coli*-mediated diarrheal disease.

TABLE 5. Presence of insertions in the chromosome of different strains of *E. coli*

Strain	Presence of *eae*	Product size (kb)[a]	
		O island 141 position (*yhjW*)	O island 154 position (*pstS*)
E. coli K-12 (HB101)	−	—[b]	—
EHEC O113:H21 (EH41)	−	—	5.5
EHEC O113:H21 (EH53)	−	—	5.5
EHEC O113:H21 (EH71)	−	—	5.5
EHEC O116:H21 (EH42)	−	—	5.5
EHEC O130:H11 (EH43)	−	6.0	5.5
EHEC O157:H7 (EDL933)	+	6.0	6.9
EHEC O111:H− (EH39)	+	6.0	5.5
EPEC O55:H7	+	6.0	6.9
EPEC O127:H6 (E2348/69)	+	—	—
REPEC O103:H2 (84/110)	+	6.0	—
REPEC O15:H− (83/39)	+	6.0	5.5

[a] Approximate sizes from amplification of the regions corresponding to O islands 141 and 154 in the genome of EHEC O157:H7. In both PCRs, a 200-bp product is generated from *E. coli* K-12.
[b] —, no O island was detected.

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