Characterization of the second long polar (LP) fimbriae of
Escherichia coli O157:H7 and distribution of LP fimbriae in
other pathogenic E. coli strains

Alfredo G. Torres a,b,*, Kristen J. Kanack c, Christopher B. Tutt a,
Vsevolod Popov b, James B. Kaper c,d

a Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555, USA
b Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555, USA
c Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD 21201, USA
d Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, MD 21201, USA

Received 20 April 2004; received in revised form 7 July 2004; accepted 23 July 2004
First published online 4 August 2004

Abstract

A second region containing five genes homologous to the long polar fimbrial operon of Salmonella enterica serovar Typhimurium is located in the chromosome of enterohemorrhagic Escherichia coli (EHEC) O157:H7. A non-fimbriated E. coli K-12 strain carrying the cloned EHEC lpf (lpf2) genes expressed thin fibrillae-like structures on its surface and displayed reduced adherence to tissue culture cells. Neither mutation in the lpfA2 gene in either the parent or lpfA1 mutant strains showed an effect in adherence or in the formation of A/E lesions on HeLa cells. lpfA2 isogenic mutant strains adhere to Caco-2 cells almost as well as the wild-type at 5 h, but they were deficient in adherence at early time points. A collection of diarrheagenic E. coli strains were investigated for the presence of lpfA1 and lpfA2 and results showed that these genes are present in specific serogroups which are phylogenetically related. Our results suggest that LP fimbriae 2 may contribute to the early stages of EHEC adhesion and that genes encoding the major LP fimbrial subunits are present in a small group of EHEC and EPEC serotypes.

Keywords: Enterohemorrhagic Escherichia coli; Long polar fimbriae; FAS assay

1. Introduction

The first step of enterohemorrhagic Escherichia coli (EHEC) pathogenesis involves the initial adherence of the bacterium to the intestinal epithelium and the subsequent formation of intimin-mediated attaching and effacing (A/E) lesions on intestinal epithelial cells (reviewed in [1]). The recent completion and analysis of the genomic sequence from two prototype EHEC strains of serotype O157:H7 indicated that at least 24 regions encode putative adhesins: 10 loci corresponded to potential fimbrial adhesin gene clusters, 13 regions encode putative non-fimbrial adhesins and one corresponds to the Locus of Enterocyte Effacement (LEE)-encoded intimin [2,3]. At least 13 of the 24 regions are conserved in E. coli K-12, and two other regions contain ORFs that are truncated relative to K-12. The remaining ORFs encode EHEC-specific genes not found in E. coli K-12. Five of these regions...
encode putative fimbrial operons. The presence of so many different and, in the vast majority, uncharacterized ORFs in the chromosome of EHEC, raised the possibility that, in addition to intimin, EHEC O157:H7 encodes novel factors that mediate binding and colonization of the intestinal mucosa and these factors might also be present in other Shiga Toxin-Producing E. coli (STEC) strains. STEC strains associated with human infection are generally referred to as EHEC, and serotype O157:H7 is considered to be the definitive EHEC strain due to its common association with morbidity and the presence of the LEE pathogenicity island. Therefore, there is a possibility that the putative EHEC O157:H7 colonization factors are also present in other STEC strains.

Some of these regions that potentially encode adhesins have been characterized but their roles in adhesion are uncertain. For example, one of the regions contains genes closely related to the long polar fimbriae (lpf) operon of Salmonella enterica serovar Typhimurium [4]. The introduction of the EHEC lpf operon into a non-fimbriated E. coli K-12 strain resulted in expression of long polar (LP) fimbriae and increased adherence to tissue culture cells. However, only a slight reduction in adherence was observed when lpf was mutagenized in EHEC O157:H7 [4].

A second fimbrial operon with homology to LP fimbriae of S. typhimurium exists in the chromosome of EHEC O157:H7 and a homologous region has recently been characterized in EHEC O113:H21 [5]. These fimbrial operons are found in the same chromosome location in both EHEC strains and mutation of this locus in O113:H21 LP fimbriae resulted in decreased adherence to epithelial cells, suggesting that LP fimbriae in this particular isolate function as adhesive factors [5]. To further characterize the second EHEC O157:H7 LP fimbriae, the lpf2 region was cloned, EHEC lpfA2 mutants were created, and their adherence properties were tested in tissue culture assays. Furthermore, the distribution of the putative major fimbrial subunit genes (lpfA1 and lpfA2) were assayed in a collection of diarrheagenic E. coli (DEC) strains.

2. Materials and methods

2.1. Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. Strains were routinely grown in Luria–Bertani (LB) broth or on L agar at 37 °C [6]. When indicated, the bacterial strains were grown in Dulbecco’s minimal Eagle medium (D-MEM) (Gibco/Invitrogen Cat. No. 11885–084) at 37 °C. Additional media used to grow bacteria were: CFA agar [7] and MacConkey agar (Difco). Antibiotics were added to media at the following concentrations: kanamycin (Km) 50 μg/ml, ampicillin (Ap) 100 μg/ml, chloramphenicol (Cm) 30 μg/ml, streptomycin (Sm) 100 μg/ml, tetracycline (Tc) 12.5 μg/ml, and nalidixic acid (Nal) 20 μg/ml.

2.2. Recombinant DNA techniques

Plasmid DNA was isolated by the method of Kado and Liu [8]. Alternatively, QIAGEN QIAprep™ plasmid preparation kit was used to isolate plasmid DNA from 3 ml of overnight bacterial culture. Restriction endonuclease analyses, ligation and transformation of plasmid DNA, and isolation of chromosomal DNA from bacteria were performed following standard methods [6,9].

2.3. Cloning of the lpf2 chromosomal region

To clone the EHEC O157:H7 lpf2 region, the total genomic DNA from strains EDL933 and 86–24 was isolated and used as template for PCR amplification with platinum Pfx DNA polymerase (Gibco BRL, Life Technologies) with the primer pair 5LPF2 (5'-GCAAGCTTGTGTATACCCGTAAGTAT-3') and 3LPF2 (5'-ATGGATCCAAAGGAAAGTTATTTCTATTG-3'). The PCR product was purified and the 6806 bp amplicon, containing the EHEC lpfABCDD2 region, was cloned in the plasmid pGEM®-T Easy (Promega), following the manufacturer’s instructions, to create plasmids pLPF201 (lpfABCDD2 from strain EDL933) and pLPF202

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Serotype, genotype or relevant information</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>86–24</td>
<td>EHEC O157:H7 strain 86–24, Sm' Na'</td>
<td>[27]</td>
</tr>
<tr>
<td>EDL933</td>
<td>Prototype EHEC O157:H7</td>
<td>[28]</td>
</tr>
<tr>
<td>SM10 (P Pir)</td>
<td><em>thi</em> <em>thr</em> <em>leuB</em> <em>tonA</em> <em>lacY</em> <em>supE</em> <em>recA</em>:RP4-2-Tc::Mu-Km, Km'</td>
<td>[11]</td>
</tr>
<tr>
<td>CVD451</td>
<td>86–24, <em>excN</em>:aphA3, Km'</td>
<td>[29]</td>
</tr>
<tr>
<td>AGT201</td>
<td>86–24, <em>lpfA2</em>:tet, Sm' Te'</td>
<td>This study</td>
</tr>
<tr>
<td>AGT210</td>
<td>86–24, <em>lpfA1</em>:<em>cat</em> <em>lpfA2</em>:tet, Sm' Te' Cm'</td>
<td>This study</td>
</tr>
</tbody>
</table>
(lpfABCDD’2 from strain 86–24). The ligated products were transformed into E. coli K-12 strain ORN172 and recombinant clones containing the lpf2 region were selected by PCR and plasmid screening and confirmed by restriction enzyme profiling. The lpfA2 gene in plasmid pLPF202 was mutated by amplifying the tet gene from plasmid pACYC184 and inserting it into the unique NsiI site found in the coding sequence of lpfA2, to create plasmid pLPF202::tet.

2.4. Construction of lpfA2 isogenic mutants

EHEC strains defective in LP fimbriae 2 expression were constructed in the chromosome of strains 86–24 and CVD468 (Table 1) by allelic marker exchange as follows. The lpfA2::tet gene from pLPF202::tet was amplified by PCR with Taq polymerase (Invitrogen) using the primer pair 5′LPFA2 (5′-GGTCGTTTTTGCCTTAACCGC-3′) and 3LPFA2 (5′-ATCC-CGGGCAAAGCAGATATG-3′) and cloned into the suicide vector pCVD442 [10]. The pCVD442/lpfA2::tet was introduced into EHEC strains 86–24 and CVD468 by conjugation using the donor strain SM10 (λ pir) [11]. Colonies resistant to tetracycline and sucrose, or tetracycline, chloramphenicol and sucrose, were tested for ampicillin sensitivity. The mutant strains AGT201 (86–24 lpfA2::tet) and AGT210 (CVD468 lpfA2::tet) were selected as described before [4]. The presence of the tet cassette within the chromosomal lpfA2 gene of AGT201 and AGT210 was confirmed by PCR with the primers listed above.

2.5. Bacterial adhesion to epithelial cells

HeLa cells were seeded to semi-confluence at 37 °C and 5% CO2 in 24-well plates (Corning) and the adhesion assay was performed as described before [4]. Briefly, the strains were grown in LB broth overnight at 37 °C and the monolayers were infected with approximately 1 × 10⁷ bacteria per well (bacterial concentration was estimated by OD at 600 nm and plating serial dilutions on LB agar plates) for 3 h (recombinant strains) or 5 h (wild-type and isogenic mutants) and adherence was evaluated qualitatively by Giemsa staining and quantitatively by plating adherent bacteria on 3% agar plates with the proper antibiotic. For the FAS assay, HeLa cells were grown to 75–90% confluence on cover slips in 6-well tissue culture plates and 30 μl of overnight bacterial cultures was added per well and the infection was extended for 6 h. For adhesion studies with Caco-2 cells, monolayers were seeded with 1 × 10⁵ cells/well and incubated for 48 h and after the cell monolayers were washed twice with phosphate-buffered saline (PBS, pH 7.4), the infection was carried out with the wild-type and isogenic mutants for 3, 4 and 5 h and quantification was performed as described above.

2.6. Florescent actin staining assay

HeLa cells infected with different bacterial cultures and incubated for a total of 6 h were washed, permeabilized with 0.1% Triton X-100/PBS and actin was stained with AlexaFluor® 488-phalloidin (Molecular Probes, Eugene, Oregon) and both eukaryotic and prokaryotic DNA were stained with DAPI – (4′,6-diamidino-2-phenylindole, dihydrochloride) (Molecular Probes, Eugene, Oregon). The preparations were analyzed by confocal microscopy using a Zeiss LSM 510 Meta, Laser Scanning Microscope.

2.7. Electron microscopy

Strains were grown overnight at 37 °C in Dulbecco’s minimal Eagle medium (DMEM, Gibco/Invitrogen) under static conditions, suspended in phosphate-buffered saline (PBS, pH 7.4) and allowed to adhere to Formvar-carbon-coated copper grids (200 mesh, Electron Microscopy Sciences). The fimbriae were visualized by negative staining with 2% potassium–phosphotungstic acid, pH 6.8 and the grids analyzed in a Phillips 201 electron microscope.

2.8. Detection of genes encoding LP fimbriae major subunits

The phylogenetically well-characterized diarrheagenic E. coli (DEC) strain collection [12] was screened by PCR for the presence of the genes encoding the putative major fimbrial subunits of LP fimbriae 1 (lpfA1) and 2 (lpfA2). The lpfA genes were amplified using Taq polymerase and primer pairs 5LPFA and 3LPFA for lpfA1 gene [4] and 5LPFA2 and 3LPFA2 for lpfA2 gene. The amplification was performed during 30 cycles at 94 °C for 45 s, 45 °C for 1 min and 72 °C for 2 min.

2.9. Growth curves

Strains ORN172 (pGEM-T Easy), ORN172 (pLPF201), ORN172 (pLPF202), 86–24, CVD468, AGT201, and AGT210 were grown for 18 h in LB at 37 °C, diluted 1:500 in either fresh LB or DMEM, and grown at 37 °C with shaking at 250 rpm. OD₆₀₀ measurements were taken every h, and 100 μl of the cultures was diluted and plated on LB agar plates to obtain CFU counts.

2.10. DNA hybridization

Fragment probes generated by PCR using the primer pairs for lpfA1 (5′-GTTCGTTTTTGCCTTAACCGC-3′ and 5′-AGTTGAAATCGACCTGCGC-3′) and for lpfA2 (5′-TTATGCGACCAGTCACCTAGGC-3′ and 5′-TCAACTGAAACTGCGAGTCGG-3′) were used to screen colony blots of all diarrheagenic E. coli (DEC) isolates, prepared by using 82 mm nylon
membranes (Roche Applied Science, Indianapolis, IN). Membranes were denatured in 0.5 M NaOH–1.5 M NaCl, neutralized in 1.5 M NaCl–0.5 M Tris–HCl–1 mM EDTA, dried, and fixed by UV exposure. All probes were purified by gel extraction and DIG labeled by using a commercially available DIG DNA Labeling and Detection Kit (Roche Applied Science), following manufacturer’s instructions. Following 2 h of prehybridization at 42 °C, the membranes were hybridized with a denatured probe at 42 °C, with continuous, gentle agitation in a hybridization solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5% blocking reagent, 0.1% N-lauryl sarcosine, and 0.02% sodium dodecyl sulfate (SDS). Membranes were washed three times in 2× SSC–0.1% SDS and then three times in 0.1× SSC–0.1% SDS at 65 °C (high stringency) or 55 °C (low stringency). Signals were detected by using the DIG nucleic acid detection kit (Roche) in accordance with the manufacturer’s instructions.

2.11. Autoaggregation assay

Overnight cultures of the recombinant strains were adjusted to approximately the same OD600 as described by Hasman et al. [13], and 25 ml of each culture was placed in sterile 50 ml conical tubes. The cultures were vortexed for 10 s and incubated statically at room temperature. Samples of 100 µl were taken from the top of the tube at different time points, and the OD600 was determined.

3. Results

3.1. Features of the nucleotide sequence of lpf2 region

The second region in the chromosome of the EHEC O157:H7 strain EDL933 (GenBank Accession No. AE005604) with homology to lpf genes is located in the O-island #154(OI#154), a region of the EDL933 chromosome not homologous to E. coli K-12 MG1655 [3]. We have designated this region as lpf2 to distinguish it from the EHEC EDL933 lpf operon located in the OI#141 that we have previously characterized [4]. The lpf2 region contains five open reading frames (ORFs) predicted to encode fimbrial proteins that share significant homology to proteins encoded by the lpf operon in EHEC O113:H21 and to a lesser extent to the LP fimbriae of S. typhimurium and LP fimbriae 1 of EHEC O157:H7 (Fig. 1). Based on their homologies and their location within the region, we designated the five ORFs as lpfA2, lpfB2, lpfC2, lpfD2 and lpfD’. lpfA2 encodes the putative major fimbrial subunit; lpfB2, a chaperone; lpfC2, a chaperone; lpfD2, a chaperone; and lpfD’, a chaperone.

![Fig. 1. Comparison of the genetic organization and the deduced amino acid sequences of the lpf regions from EHEC O157:H7, O113:H21, S. enterica serovar Typhimurium, REPEC O15:H’, EPEC O127:H6, and S. typhi. Genes are shown as arrows with patterns representing their putative function: solid black (major fimbrial subunit), vertical stripes (chaperone), white (outer membrane usher chaperone), gray (minor fimbrial subunit) and hatched (fimbrial subunit). Genes disrupted are indicated with two crossed lines. The proteins encoded by the EHEC O157:H7 lpf2 region and some of their features are listed in the table.](https://academic.oup.com/femsle/article-abstract/238/2/333/490775)
protein; lpfC2, the outer membrane usher chaperone; and lpfD2 and lpfD’2, the minor fimbrial subunits. A unique feature of the lpf2 region when compared with the other lpf operons, is that this region encodes two copies of the lpfD gene and it lacks a gene with homology to lpfE, which is found in S. typhimurium lpf and EHEC lpf1 operons. Another feature of the EHEC lpf2 region is that it is found in a similar chromosomal location as the EHEC O113:H21 lpf operon (next to the pstS gene) [5] and in a different location than the EHEC lpf1 and S. typhimurium lpf operons (next to the yhjW gene). The percentage of homology, similar gene arrangement and same chromosomal location shared by the EHEC O113:H21 lpf and EHEC O157:H7 lpf2 operons suggest that these genes belong to a family of fimbrial operons which also display some homology to, but are different from a second group of lpf operons located adjacent to the yhjW gene.

In BLAST analysis, the predicted EHEC Lpf2 proteins clustered with putative proteins encoded in the chromosome of S. enterica serovar Typhi (GenBank Accession No. NC_004631). The S. typhi lpf genes are located in what appears to be an operon and these ORFs share homology with the lpfA2, lpfB2 and lpfD2 genes (Fig. 1). In the case of the ORF homologous to lpfC2, a premature stop codon disrupts the expected ORF after codon 170 and this gene is therefore considered a pseudogene. Although the S. typhi LP fimbriae is probably not functional due to the inactivation of the putative outer membrane usher protein, it belongs to the family of LP fimbriae located next to the pstS gene, which also includes the lpf operon from the rabbit enteropathogenic E. coli (EPEC) strain O15:H− [5].

Additional comparison of the EHEC lpf2 DNA sequence with the E. coli genome sequencing projects at the Sanger Institute (http://www.sanger.ac.uk/Projects/Escherichia_Shigella/), indicated that the predicted EHEC lpf2 genes also clustered with a previously unidentified chromosomal region of the prototype EPEC O127:H6 strain E2348/69. The EPEC O127:H6 ORFs encoding the putative LP fimbriae, are located adjacent to the yhjW gene, which suggest that the E2348/69 lpf genes could be more closely associated in their characteristics and properties to the proteins encoded by the S. typhimurium and EHEC (lpf1) LP fimbriae. Interestingly, the region located adjacent to the pstS in EPEC O127:H6 is not occupied by ORFs encoding putative fimbriae, instead this region is occupied by genes encoding proteins associated with an ATPase system (data not shown).

3.2. Cloning and adherence properties of the LP fimbriae 2

The EHEC O157:H7 lpf2 region from strains EDL933 and 86–24 was cloned in the plasmid pGEM®-T Easy and the resulting plasmids, pLPF201 (EDL933 lpfABCD’D’2) and pLPF202 (86–24 lpfABCD’D’2), were transformed into the E. coli K-12 strain ORN172 (E. coli Δtim, Km+) [14], which has been previously used to study expression of the EHEC lpf1 [4]. We subcloned the lpf2 region from EHEC 86–24, the strain used in subsequent adhesion assays (see below), to compare it with the lpf2 region of the prototype EHEC strain EDL933. To determine whether the lpf2 region encodes fimbrial structures, the ORN172 strains carrying plasmids pGEM®-T Easy (control), pLPF201 (lpfABCD’D’2 from EHEC strain EDL933) or pLPF202 (lpfABCD’D’2 from EHEC strain 86–24), were grown in D-MEM media (37 °C and static conditions) and analyzed by transmission electron microscopy after negative staining (Fig. 2, panels a–d). We observed on the surface of ORN172 (pLPF201) and ORN172 (pLPF202), fibril-like structures that were absent in strain ORN172 (pGEM®-T Easy). The morphology of the recombinant EHEC LP fimbriae 2 are structurally different from those fimbriae observed when the EHEC O157:H7 LP fimbriae 1, S. typhimurium LP fimbriae or REPEC O15:H− LP fimbriae were expressed in a non-fimbriated E. coli strain [4,15,16]. These organelles surrounding the bacteria were very difficult to visualize by negative staining. We were unable to detect any fimbriae-like organelles when bacteria were grown on CFA, LB or MacConkey agar plates. Under all the conditions tested, we did not detect any fimbriae produced by strain ORN172 either carrying pGEM®-T Easy or pLPF202::tet (see below), even after we examined multiple fields under the EM (data not shown). The purification and visualization of these types of fimbriae has become a difficult task [5] and our attempts to purify the major LP fimbrial subunit have been unsuccessful.

We then examined the capacity of the LP fimbriae 2 to mediate adherence to tissue culture cells. E. coli strain ORN172 containing the lpf2 operon cloned either from strain EDL933 or 86–24, displayed a decrease in adherence to HeLa cells of 89.06% (P < 0.05) and 65.7% (P < 0.05), respectively, when compared to ORN172 (pGEM®-T Easy) (Fig. 2, panel e). This surprising result is in contrast to the increase in adherence observed with the recombinant lpf1 operon [4]. To show that the decrease in adherence was directly associated with the LP fimbriae 2, the plasmid pLPF202::tet, containing a disrupted lpfA2::tet gene was created. ORN172 (pLPF202::tet) adhere as well as ORN172 (pGEM®-T Easy), suggesting that the protein products encoded by this region are associated with the phenotype observed on the epithelial cells. To confirm that the decrease in adherence was not associated with a defect in growth, the samples were diluted in either LB or DMEM and their growth rate monitored for 8 h by optical density at 600 nm (see Section 2). The bacterial strains grew at the same rate regardless of the plasmid (containing the recombinant lpf operons or empty vector) or the media
Fig. 2. (a–d) Fimbriae expression was visualized by transmission electron microscopy (Bars, 0.2 μm) and (e) adherence of the recombinant *E. coli* strains quantified in HeLa cell assays. (a) ORN172 (pGEM-T Easy) (*E. coli* Δ*fim* strain); (b) ORN172 (pLPF201) (*lpf*ABCDD'2 from EHEC strain EDL933) (c and d) ORN172 (pLPF202) (*lpf*ABCDD'2 from EHEC strain 86-24); (e) Percentages of ORN172 (pGEM-T Easy), ORN172 (pLPF201), ORN172 (pLPF202) and ORN172 (pLPF202::tet) (*lpf*A::tet from pLPF202) adherent to cultured HeLa cells after 3 h of incubation. The error bars indicate standard deviations. Asterisk indicates $P < 0.05$. (f) Autoaggregation quantification was performed from static LB or DMEM liquid cultures of strains ORN172 (pGEM-T Easy), ORN172 (pLPF201), and ORN172 (pLPF202).
tested (data not shown). Furthermore, to determine whether the decrease in adherence was not associated with an autoaggregation phenotype, the strain ORN172 containing plasmids pGEM-C210-T Easy, pLPF201, or pLPF202 were grown in either LB or DMEM and its rate of aggregation was determined by spectrophotometry. The autoaggregation phenotype was not affected regardless of the plasmid or the media used (Fig. 2, panel f). Although the LP fimbriae 2 must be further characterized, the initial data suggest that the lpf2 region is displaying a different adherence phenotype in vitro than the lpf1 operon, at least when cloned in an E. coli K-12 strain ORN172 background.

3.3. Role of EHEC lpfA2 and lpfA1 lpfA2 mutants in adherence and A/E lesion formation

Our initial experiments suggested a role for LP fimbriae 1 in adherence of EHEC O157:H7 [4]. We therefore created an EHEC 86–24 lpfA2 single and lpfA1 lpfA2 double mutant (AGT201 and AGT210, respectively), and assayed their role in adherence on HeLa cells. The use of the lpfA2 isogenic mutants of EHEC, however, demonstrated that there appeared to be no role for LP fimbriae 2 in adherence to HeLa cells. No differences in the number of bacteria recovered after infection were observed between the EHEC strain 86–24 (1.9 × 10^8 CFU ± 2.1) and the isogenic mutants AGT201 (2.0 × 10^8 CFU ± 2.1) and AGT210 (1.8 × 10^8 CFU ± 2.6). We previously showed that CVD468, the isogenic EHEC lpf1 mutant strain, showed a slight reduction in adherence to tissue culture cells and formed fewer microcolonies when compared with the wild-type parent strain [4], suggesting a role for Lpf1 in adherence. HeLa cells have been used to determine that EHEC strains adhere in a characteristic localized adherence pattern and whether they develop the intimin-mediated A/E lesion [1]. A/E lesion formation has been shown to be required for pathogenicity in all animal models tested [17–19] and although it is known that the genes encoding the A/E phenotype map to the LEE pathogenicity island, it is highly likely that other EHEC...
colonization factors distinct from the LEE also participate directly or indirectly in the formation of the A/E lesion [20]. Therefore, we determine whether the LP fimbriae 1 or 2 affect the formation of A/E lesions on tissue culture cells. When compared with the EHEC wild type strain 86–24, the isogenic strains CVD468, AGT201 and AGT210 were able to accumulate actin underneath the site of bacterial attachment, which is characteristic of the A/E lesions (Fig. 3, panels a–l). The strain CVD451, unable to secrete type III effector proteins was used as a negative control for the formation of A/E lesions (Fig. 3, panels m–o). Although LP fimbriae are expressed during the course of infection in vitro from cloned \( lpf2 \) genes, a clear role in adherence is yet to be demonstrated because the presence or absence of LP fimbriae 2 in the isogenic strains had no apparent effect in adhesiveness or A/E lesion formation on HeLa cells.

We therefore assayed the role of Lpf2 fimbriae in adhesion to a more relevant cell line. Monolayers of Caco-2 cells (human colonic adenocarcinoma cells) were seeded and infected with the wild-type or the isogenic mutant strains and the number of adherent bacteria was quantified at different time points after infection (Table 2). Initially, we determine the number of adherent bacteria at 5h post infection, which we have used as a standard time point to observe microcolonies and A/E lesion formation on the surface of the infected cells [4]. When compared with the wild-type strain, the \( lpfA2 \) single and double mutants adhere almost as well as the wild type strain, although they showed a difference which is statistically significant. The single \( lpfA1 \) mutant also showed a slight reduction in adherence when compared with the wild type strain. We then compare the wild type and the \( lpf \) mutant strains at early time points of adhesion to Caco-2 cells. Our results indicate that the strain
AGT601 (lpfA2⁻) was clearly less adherent than the wild type strain at 3 and 4 h (Table 2, trail 1), which suggest that LP fimbriae 2 may be required for initial adherence. On the other hand, strain CVD468 (lpfA1/C0) did not show any defect in adherence at early time points, indicating that LP fimbriae 1 may not be required for initial adherence. To our surprise, strain AGT602 (lpfA1/C0 lpfA2/C0) displayed an intermediate phenotype. While the strain showed a defect in adherence when compared with the wild type and CVD468 strains, it also adhered better than the lpfA2 single mutant strain AGT601 (Table 2, trail 1). To confirm that the phenotype observed was associated to the lack of LP fimbriae expression instead of an artifact caused by the mutagenesis protocol, strains AGT601 and AGT602 were complemented with plasmid pLPF202. When the strains were tested in the adhesion assay, the complemented strains adhere as well as the wild type strain and no differences in adhesion at 3h of infection were observed as in the case of AGT601 or AGT602 (Table 2, trail 2). To confirm that the decrease in adherence observed with the EHEC strains AGT201 and AGT210, was not associated with a defect in their growth, we grew the strains in LB or DMEM and monitored their growth for 8 h by spectrophotometry (see Section 2). The EHEC wild type and its isogenic mutants grew at the same rate regardless of the media tested (Fig. 3, panel p).

3.4. Distribution of LP fimbrial genes in other STEC strains

We wished to establish whether LP fimbriae genes are present in other pathotypes, particularly EHEC and STEC strains isolated worldwide from humans and cattle. The presence of the genes encoding the putative major fimbrial subunits of LP fimbriae 1 (lpfA1) and 2 (lpfA2) was investigated by PCR in representatives of the phylogenetically well-characterized DEC strain collection [12]. The DEC strains were selected because this collection includes strains representing common clones of EPEC and EHEC where the evolution of virulence and pathogenic mechanisms has been previously established [21]. Our results indicate that while the lpfA2 gene is found in strains of DECs 4 (EHEC O157:H7) and 5 (EPEC O55:H7), the lpfA1 gene is present in two different branches of the dendogram, including strains from DECs 3 (EHEC O157:H7), 4 and 5, and DECs 1 (EPEC O55:H6) and 2 (EPEC O55:H6) (Fig. 4). In the case of lpfA2, sequences homologous to this gene were almost present in all O157:H7 strains tested (see Fig. 4). lpfA2 was found apparently restricted to O157:H7 (DECs 3 and 4) and O55:H7 (DEC 5) strains. To confirm the PCR results, we performed colony blot hybridization using probes to the lpfA1 and lpfA2 genes. The hybridization data confirmed our previous PCR results. Interestingly, using low stringency, we detected positive clones of strains belonging to DECs 13, 14 and 15 for the lpfA1 gene and DECs 6, 7 and 8 when the lpfA2 probe was used (Fig. 4).

4. Discussion

In this paper we performed the initial characterization of a chromosomal region of EHEC O157:H7 containing genes encoding fimbriae which share homology to the long polar fimbriae found in S. typhimurium, EHEC O113:H21 and EHEC O157:H7. Although the gene order and the percentages of homology and similarity indicate that these fimbriae are members of the LP fimbrial family, it is worth noting, however that LP fimbriae 2 did not display the same morphology as other LP fimbriae when expressed as a recombinant clone. Furthermore, E. coli K-12 expressing LP fimbriae 2 showed a reduction in adherence to tissue culture cells, suggesting that these fimbriae may perform a different

| Table 2 | Adherence of EHEC strain 86–24 and its isogenic mutants to Caco-2 cells |
|-------------------------------|-----------------|-----------------|-----------------|
| EHEC strains                  | CFU (x10⁸) ± SD (P)* recovered after Caco-2 infection for: | |
|                               | 3 h             | 4 h             | 5 h             |
| Trial 1                       |                 |                 |                 |
| 86–24                         | 1.52 ± 0.06     | 1.68 ± 0.05     | 1.93 ± 0.04     |
| CVD468                        | 1.41 ± 0.05 (<0.05) | 1.55 ± 0.03 (>0.05) | 1.79 ± 0.06 (>0.05) |
| AGT201                        | 0.56 ± 0.08 (<0.01) | 1.07 ± 0.09 (<0.05) | 1.61 ± 0.07 (<0.01) |
| AGT210                        | 1.13 ± 0.05 (<0.05) | 1.41 ± 0.04 (<0.05) | 1.74 ± 0.06 (<0.05) |
| Trial 2                       |                 |                 |                 |
| 86–24                         | 1.51 ± 0.05     | 1.66 ± 0.05     | 1.81 ± 0.05     |
| AGT201                        | 0.65 ± 0.08 (<0.01) | 1.46 ± 0.07 (>0.05) | 1.61 ± 0.07 (0.05) |
| AGT201 (pLPF202)              | 1.26 ± 0.16 (>0.05) | 1.41 ± 0.16 (>0.05) | 1.56 ± 0.16 (>0.05) |
| AGT210                        | 1.00 ± 0.04 (<0.01) | 1.41 ± 0.04 (>0.05) | 1.56 ± 0.04 (>0.05) |
| AGT210 (pLPF202)              | 1.45 ± 0.05 (>0.05) | 1.60 ± 0.04 (>0.05) | 1.75 (>0.05)     |

The P value (t test) was obtained by comparing the wild-type with the isogenic mutant at the same time point.
role in adhesion in vitro when compare with other LP fimbriae. The possibility also exist that expression of the LP fimbriae 2 down regulated the expression of endogenous E. coli K-12 adhesins, leading to the phenotype that we observed.

When compared with other lpf operons, the lpf2 region encodes an additional ORF, that we renamed lpfD2, encoding for a second putative minor fimbrial subunit. The role of this additional ORF in O157:H7 LP fimbriae 2 biogenesis is unknown but its presence is unique as compared to other lpf operons previously described [4,15,16]. In EHEC O113:H21 lpf and in the cryptic operon in S. typhi, this ORF is absent, while in EHEC O157:H7 and S. typhimurium, the lpfD2 gene is replaced by lpfE. Whether the Lpf2-structure or -adhesive properties are influenced by the presence of this additional ORF or the expression of LP fimbriae 2 affects the expression of other fimbriae, are topics of future investigation.

The role of chromosomally-encoded LP fimbriae 2 in the biology of EHEC O157:H7 strains needs further investigation. The function of LP fimbriae in in vitro infections of tissue culture cells has been investigated in this work. Previous reports had showed that LP fimbriae have a role in adherence to different cell lines [5]. Unfortunately, the cell lines used in these studies (HeLa and CHO-K1 cells) were not obtained from intestinal cells and therefore, the adherence phenotype observed in these assays may not be relevant to the actual interaction between EHEC and the intestinal epithelial cells. We now showed that LP fimbriae is required for adherence to Caco-2 cells and proposed that LP fimbriae 2 may have a role in adherence during early stages of infection.

Previous studies have shown that fimbriae expression can have a profound effect in the expression of other adhesins. For example, type 1 fimbriae expression per se constitutes a signal transduction mechanism that affects a number of unrelated genes, including Antigen 43 (Ag43), a self-recognizing surface-displayed protein that confers autoaggregation of bacteria by an intercellular handshake mechanism [22]. Changes in fim expression correlated directly with alterations in colony morphology and deletion of the entire fim gene cluster resulted in the converse expression of Ag43. Furthermore, it has been shown that deletion of the type 1 fimH gene does not affect expression of other fim genes or Ag43, but cause a dramatic reduction in the number of fimbriae expressed on the cell surface [23]. These observations had led to propose that fimbriae expression within a bacterial population is the result of a sequential or co-ordinate control at the level of the individual bacterium. This coordinated expression or cross-talk between fimbriae loci can lead to a programmed set of events within a bacterium that is of benefit during infection [24]. In the case of our study and regardless of the

<table>
<thead>
<tr>
<th>DEC Predominant serotype</th>
<th>lpfA1</th>
<th>lpfA2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 EPEC O55:H6</td>
<td>+ (5/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>2 EPEC O55:H6</td>
<td>+ (5/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>3 EHEC O157:H7</td>
<td>+ (5/5)</td>
<td>+ (5/5)</td>
</tr>
<tr>
<td>4 EHEC O157:H7</td>
<td>+ (5/5)</td>
<td>+ (5/5)</td>
</tr>
<tr>
<td>5 EPEC O55:H7</td>
<td>+ (5/5)</td>
<td>+ (5/5)</td>
</tr>
<tr>
<td>6 EPEC O111:H12</td>
<td>- (0/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>7 ETEC O128:H11</td>
<td>- (0/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>8 EHEC O26:H11</td>
<td>- (0/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>9 EHEC O26:H11</td>
<td>- (0/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>10 EHEC O111:H8</td>
<td>- (0/5)</td>
<td>+ (3/5)</td>
</tr>
<tr>
<td>11 EPEC O128:H21</td>
<td>+ (3/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>12 EPEC O111:H21</td>
<td>+ (2/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>13 ETEC O128:H7</td>
<td>+ (4/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>14 EPEC O111:H2</td>
<td>- (0/5)</td>
<td>- (0/5)</td>
</tr>
<tr>
<td>15 EPEC O111:H21</td>
<td>- (0/5)</td>
<td>- (0/5)</td>
</tr>
</tbody>
</table>

Fig. 4. Dendogram showing the relationship of diarrheagenic E. coli (DEC) strains and the distribution of the lpfA1 and lpfA2 genes. This phylogenetic tree is adapted from [12] and [30]. The presence (+) or absence (−) of sequences homologous to the lpfA1 genes is indicated. * These DEC strains hybridized with the lpfA1 and lpfA2 probes at low stringency. The number of samples tested of each DEC (in parentheses) is also included. EHEC, enterohemorrhagic E. coli; EPEC, enteropathogenic E. coli; ETEC, enterotoxigenic E. coli.
proposed hypothesis, the final adherence and formation of intimin-mediated A/E lesions was not affected by the deletion of LP fimbriae, suggesting that the overall EHEC adhesion is mediated by multiple adhesins (e.g., LP fimbriae 1 and 2, intimin or other EHEC adhesins not characterized, yet) which may be expressed and/or regulated in an independent manner.

Although the studies presented in this manuscript were performed in an in vitro system, they are useful and could provide important data to interpret the results in a more complex scenario. For example, the role played by LP fimbriae 1 and 2 in follicle-associated epithelium (FAE) adhesion in human intestine using in vitro organ culture (IVOC) has been recently investigated. The results indicated that rather than reducing FAE adhesion, the intestinal regional range of the EHEC O157:H7 expanded to include both FAE and the small intestine when lpfA1, lpfA2 or both genes were mutated (Fitzhenry, R. et al. manuscript in preparation). This finding suggests that the EHEC LP fimbriae influences human intestinal tissue tropism and interaction with putative receptors found in the FAE and that the altered distribution could have an impact on the virulence of the organism, possibly resulting from expression of other fimbriae that might mediate adhesion to other intestinal regions.

Furthermore, the role in vivo of the EHEC O157:H7 LP fimbriae 1 and 2 have also been investigated in the pig and sheep models (Jordan, D.M. et al. manuscript in preparation). No difference was observed between the wild type and lpfA1 single mutant with respect to fecal shedding and the formation of A/E lesions. In contrast, the lpfA1 lpfA2 double mutant was shed in significantly lower number than the parent strain, and tissues from infected gnotobiotic pigs had a significant reduction in A/E lesions. These results argue in favor of LP fimbriae 2 as contributors to the colonization and persistence of EHEC O157:H7 in swine and sheep. Furthermore, Newton et al. [16] recently investigated whether the LP fimbriae contribute to the virulence of a rabbit-specific EPEC (REPEC) O15:H/C0 strain. They observed that disruption of the lpf loci resulted in significant attenuation of the REPEC strain for rabbits with respect to early stages of colonization and severity of diarrhea and they conclude that LP fimbriae is important for the development of diarrhea in susceptible animals.

In this manuscript, we have also established that LP fimbriae genes are present in related DEC strains. It has been established that these strains had co-evolved from a parental strain by acquiring different virulence factors and therefore, making them members of different categories of pathogenic E. coli strains. Our data support previous findings indicating that strains belonging to the O157:H7 serotype or closely related strains (particularly O55:H7), may have acquired putative virulence factors as mobile elements via horizontal transfer and recombination. Selective pressure favoring the maintenance of specific combinations of those factors would presumably enhance the virulence of such organisms [12,21]. However, we could not find evidence of insertion elements, integrases, or any other evidence of other mobile genetic elements adjacent to the lpf1 or lpf2 regions that would suggest a mechanism for such a transfer. Our results also suggest that these fimbrial genes appear to be restricted to the O55 and O157 serogroups. A separate study examining the distribution of lpfA1 genes in a collection of human and bovine EHEC and EPEC strains belonging to different serogroups supports this observation. The investigators found that all bovine and human EHEC strains belonging to the O145 and O157 serogroups possess a sequence homologous to the lpfA1 gene [25]. Their study did not include strains of the O55 serogroup and we did not have any representative of the O145 serogroup. Therefore, our studies complement each other and since O157:H7, O55:H7 and O145 strains seem to belong to the same LEE evolutionary group pathotype (with LEE inserted at the selC locus and containing the intimin type γ) [21,25], we are proposing that the lpf1 operon is marking a subset of strains associated with a particular phylogenetic lineage. This hypothesis is currently under investigation.

A recent study by Osek et al. [26], indicated that lpfA from serotype O113:H21, which shows the highest homology to EHEC lpfA2, is widely distributed among LEE (eae)-negative STEC strains isolated from humans and animals, and they suggest that this putative fimbrial adhesin is contributing to the in vitro adherence of these strains. We could not demonstrate a clear difference in adherence of the EHEC O157:H7 lpfA2 single and double mutants at later time points of infection but we showed that LP fimbriae 2 are required for the initial stages of adhesion. Based on the above findings, we suggest that the LP fimbriae 2 may play a role in adhesion at early time points where intimin is not required to be expressed for the initial adherence or for the formation of the A/E lesion. Nevertheless, further studies are needed to elucidate the role of the lpf2 operon and its products in the pathogenesis of EHEC O157:H7.

Acknowledgement

We thank Susana Oaxaca-Torres and Jessenya Martinez for technical assistance and Iruka N. Okeke for critical reading of the manuscript. This work was supported by institutional funds from the UTMB John Sealy Memorial Endowment Fund for Biomedical Research and the Gastro Intestinal Research Interdisciplinary Program to A.G.T., and Grants AI41325 and...
DK58957 to J.B.K. K.J.K. was supported by NIH institutional training Grant 2T32AI007540-06.

References


Downloaded from https://academic.oup.com/femsle/article-abstract/238/2/333/490775 by guest on 25 January 2019