The long polar fimbriae (*lpf*) operon and its flanking regions in bovine *Escherichia coli* O157:H43 and STEC O136:H12 strains

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ABSTRACT

Long polar fimbriae (Lpf) are intestinal adhesins and important virulence factors of pathogenic *Escherichia coli* strains. We cloned and sequenced the *lpf2-1* operon (*lpf2ABCD)* and its flanking regions of an intimin- and Shiga toxin-negative *E. coli* O157:H43 strain from bovine origin, and also sequenced the *lpf2-1* operon of 6 additional atypical O157 bovine *Escherichia coli* strains of various serotypes Nucleotide sequence comparison of these *lpf* operons showed sequence conservation as they contain only four polymorphic nucleotide positions. Investigation of these O157 strains as well as 13 *Escherichia coli* Reference Collection (ECOR) strains carrying the *lpf2-1* allele revealed high degree of sequence conservation in the *lpf2* flanking regions. The *lpf2-1* allele is also present in a bovine Shiga toxin-producing *E. coli* STEC O136:H12 strain and *in vitro* adherence assays revealed that the absence of *lpf2-1* in this strain did not affect its host cell-binding properties. Our data indicate that *lpf2* loci is highly conserved in *E. coli* isolates, but its role in adherence might be masked by other uncharacterized adhesins.

INTRODUCTION

Escherichia coli is an important member of the commensal intestinal microflora in mammals, but there are a large number of isolates, which have acquired a variety of virulence factors and are capable of causing serious diseases in humans and animals, including those classified as enterohemorrhagic *E. coli* (EHEC; Kaper et al., 2004). The frequent emergence of new isolates with new combinations of virulence genes is exemplified by the appearance of the strain responsible for the recent outbreak of hemolytic uremic syndrome (HUS) in Germany (Mellmann et al., 2011), and underlines the importance of the various possible lateral gene transfer mechanisms in the spread of virulence genes. Typical EHEC O157:H7/NM strains carry *stx* genes encoding Shiga-toxin and also harbour a pathogenicity island (PAI) known as the locus of enterocyte effacement (LEE), encoding the intimin adhesin, among other virulence factors (Kaper et al., 2004).

In addition to several extensively studied virulence factors carried by pathogenic *E. coli*, there are several additional factors such as the long polar fimbriae (Lpf), which represent a relatively recently described adhesin and virulence determinant in EHEC (Doughty et al., 2002). The exact mechanism by which Lpf contributes to the virulence of each pathogenic *E. coli* strain is currently under investigation, but there is well-documented evidence that Lpf promote adhesion of EHEC strains to the intestinal epithelium (Jordan et al., 2004; Fitzhenry et al., 2006; Torres et al., 2008) as well as showing that Lpf interacts with extracellular matrix proteins (Farfan et al., 2011).

Initially, two genetic variants of Lpf (Lpf1 and Lpf2) were identified in *E. coli*, and with the availability of additional sequence data, more variants have been discovered, some of which show a degree of association with certain serotypes and/or pathogroups (Torres et al., 2009). All known *lpf1* and *lpf2* operons are encoded on genomic islands, termed O islands,

integrated in specific chromosomal locations (Fitzhenry et al., 2006; Mellmann et al., 2011). The Lpf variant encoded by the operon first named *lpfA*$_{O113}$ (Ideses et al., 2005) and later termed as allele 1 of *lpf2* (*lpf2-1*) (Torres et al., 2009) is the most prevalent genetic variant of Lpf according to our present knowledge, as it has been detected in several strains from various serotypes (Toma C, 2004; Toma et al., 2006; Torres et al., 2009; Galli et al., 2010; Monaghan et al., 2011). In comparison, the integration site of the *lpf2* operon is found between the genes coding for the L-glutamine:D-fructose-6-phosphate aminotransferase (*glmS)* and that of phosphate-binding periplasmic protein (*pstS)*. Recently, seven *E. coli* strains of the O157 serogroup (including strain T22), isolated from healthy cattle and without key EHEC virulence factors were found to harbour the Lpf2 variant (Sváb & Tóth, 2012). Three isolates were from the serotype O157:H43 (Tóth et al., 2009), and some of these members have been the focus of a recent study dealing with the evolution of the O157 serogroup (Iguchi et al., 2011).

In the current study, we report the sequence of the *lpf2* operon and its flanking regions in strain T22, a non-sorbitol-fermenting (NSF) O157:H43 with an atypical pathotype (*stx-, eae),* monitor the presence of the operon and its flanking regions in a collection of *E. coli* strains of various serotypes carrying the *lpf2-1* allele, and investigate the possible function of Lpf2 in adherence *in vitro* of a STEC O136:H12, that does not possess intimin or other known adhesins found in bovine STEC strains.

MATERIAL AND METHODS

Bacterial strains. *E. coli* strains used in this study are listed in Table 1. The ECOR strains were provided by Mónika Kerényi (Department of Medical Microbiology and Immunology, Medical School, University of Pécs, Hungary). Strains were grown in lysogeny broth (LB), as well as on LB and bromothymol-blue agar plates. For isolation of genomic and cosmid DNA, strains were grown in tryptic soy broth (TSB).

Cosmid clone library construction. Genomic DNA was isolated from strain T22 with the phenol-chlorophorm method (Sambrook et al., 1982) after growing overnight in TSB. The preparation of the cosmid clone library was performed with pWEB-TNC Cosmid Cloning Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions, with the modification that instead of mechanical shearing, genomic DNA was subjected to a partial digestion with restriction endonuclease *Mbo*I (Fermentas, Vilnius, Lithuania). Altogether, 1000 transformant colonies were kept as cosmid library.

PCR screening for the presence of *lpf2* flanking regions. The cosmid library was screened by PCR for the presence of *lpf2*. The primers and annealing temperatures used in the reactions are listed in Table 2. The strains listed in Table 1 were screened for the presence of flanking regions.

Reverse Transcriptase PCR for *lpfA* of *E. coli* T22. RNA was isolated from cells of a 48 h culture of *E. coli* strain T22 with RNEasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with the modification that cells were collected with

centrifugation at 13,000 xg for 1 minute. After discarding the supernatant, the bacterial pellet was processed according to the manufacturers' instructions. RNA samples were treated with Sigma DNase I Amplification Grade (Sigma-Aldrich, St. Louis, MO, USA). The DNAse treated samples were used as template for reverse transcription with Fermentas Maxima Reverse Transcriptase (Fermentas, Vilnius, Lithuania) according to the manufacturer's protocol. The product of this reaction was used as template in a regular PCR with the primers defined in Table 2.

Sequencing. A cosmid carrying the whole *lpf* operon was identified. DNA was isolated with the Sigma GenElute BAC DNA kit (Sigma-Aldrich, St. Louis, MO, USA), and was sequenced at Baygen Institute (Szeged, Hungary) using the combination of Life Tech's SOLiD 4, IonTorrent sequencing and the dideoxynucleotide methods. The products of the RT-PCR were also sequenced with the dideoxynucleotide method. Nucleotide sequence analysis and searches for open reading frames (ORFs) and homologous DNA sequences in the EMBL and GenBank database libraries were performed with the tools available from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), with Vector NTI and the CLC Bio DNA Workbench.

Construction of an *lpfA2-1* deletion mutant of STEC O136:H12. To generate an *lpfA2-1* deletion mutant of strain 187/06 (22), the *lpfA2-1* gene was replaced by a gene encoding kanamycin resistance using the lambda red recombinase system (Datsenko & Wanner, 2000). The long oligonucleotide primers used for introducing the mutation were those described by (Doughty et al., 2002). Each primer included 20 bp of sequence homologous to the kanamycin resistance gene, and 40 bp of sequence homologous to regions flanking the *lpfA2-1* gene. The kanamycin resistance gene was amplified from pKD4 by PCR. The purified PCR

136 product (1 µg DNA) was electroporated into 187/06 (22) strain which had previously been
137 transformed with the lambda red recombinase expression vector, pKM201. Following
138 electroporation, transformants of 187/06 (22) were recovered at 30ºC for 2 h in LB broth and
139 plated onto LB agar with kanamycin for overnight growth at 37ºC to induce the loss of
140 pKM201. Kanamycin-resistant colonies were then confirmed by PCR for replacement of
141 *lpfA2-1*. The *lpfA2-1* deletion mutant of 187/06 (22) was complemented in trans by
142 introduction of the entire *lpf2* operon on pWSK:lpf (kindly provided by Dr. E. Hartland).
143
144 **Bacterial adhesion.** The potential adherence capacity of strain T22 was investigated on
145 primary bovine kidney and testis cells, which were kindly provided by Emília Szállás
146 (Veterinary Diagnostic Directorate, National Food Chain Safety Office, Budapest, Hungary).
147 The cells were grown to semi-confluency at 37ºC in 5% CO_2 in 24 well plates in Roswell
148 Park Memorial Institute 1640 (RPMI 1640) medium without supplements. Prior to use, cells
149 were washed once with PBS. Strain T22 was grown for 48 hours with shaking at 200 rpm,
150 and the cell monolayers were incubated for 5 hours with ca. 10^{10} bacteria per well. The
151 infected monolayers were washed two times with PBS, fixed with methanol and stained with
152 Giemsa reagent.
153 The ability of *E. coli* O136:H12 *lpfA2-1*$^+$ strain and its deletion mutant to adhere to
154 Hep-2, Caco-2 and T84 cell lines was assessed as previously described (Doughty et al., 2002),
155 with minor modifications. The cells were grown to semi-confluency at 37ºC in 5% CO_2 in 24
156 well plates (Falcon^{TM} BD) in Dulbecco´s minimal essential medium (DMEM), DMEM/F12
157 (Gibco, Carlsbad, CA, USA) or MEM, depending of the cell line, with 10% or 20% (vol/vol)
158 heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 1% (vol/vol) of a mixture of
159 antibiotics/antimycotics (Gibco). Before use, the cells were washed twice with phosphate-
160 buffered saline (PBS, Gibco) and replenished with the corresponding medium with no

supplements, as it was observed previously that mannose could inhibit Lpf mediated adherence (Farfan et al., 2011). The strains were grown static in LB broth overnight at 37ºC, tissue culture cells were incubated with ca. 10^7 bacteria per well for 3 h at 37ºC and 5% CO_2. To quantify adherence, the infected monolayers were washed two times with PBS, and the adherent bacteria were recovered with 200 µl of 0.1% Triton X-100 in PBS and plated on LB agar plates containing the proper antibiotic. Data were expressed as the percentage of the bacterial inoculum recovered from triplicate wells and are the mean of at least two separate experiments. Statistical difference was expressed as the *P* value determined by a *t* test analysis. The *in vitro* competition assays were performed as described above except that cells were inoculated with 5 x 10^6 cells each of mutant and wild type bacteria (total number of bacteria/well 10^7 cells) and competition index (CI) was calculated.

Nucleotide sequence accession number. The sequence of the *E. coli* T22 *lpf2* operon and its neighbouring regions has been deposited in the GenBank database under accession number AHZD01000104. The sequences of the *lpf2* operons of *E. coli* O157 strains B47, B54, T16, T34, T49 and T50 (see Table 1) were deposited under accession numbers KC207119, KC207120, KC207121, KC207122, KC207123 and KC207124, respectively.

RESULTS AND DISCUSSION

Sequence characteristics of the *lpf2* operon in the atypical bovine O157 strains. We cloned and sequenced a 15.3 kb region from the genome of *E. coli* O157:H43 strain T22, including the *lpf2* operon. The sequence is deposited in GenBank under the accession number AHZD01000104. The schematic representation of the sequenced region is shown in Figure 1. According to our knowledge, this is the first time that the allelic variant *lpf2-1* operon and its flanking regions were sequenced in a non-sorbitol fermenting (NSF) strain of the serotype O157:H43. The *lpf2-1* operon itself has been detected earlier by PCR in other *E. coli* strains (Torres et al., 2009; Farfan et al., 2011).

In the *lpf2-1* operons of T22 and six other atypical O157 strains, there are only 4 positions that show polymorphism. One of them is a synonymous point mutation in the gene *lpfC* of strain T49, the others produce amino acid switches in the respective genes. The *lpf2A* gene is uniformly conserved in the investigated strains, and this is also true for the majority of the strains with whole genomes available in GenBank, an exception is the *lpf2A* gene of strain 55989 (Table 3.), which contains an isoleucine instead of a leucine in position 116. The *lpf2B* has an alanine instead of serine in position 99 in all the atypical O157 strains as opposed to the strains from GenBank listed in Table 3.

The *lpf2C* gene proved to be uniform in all the sequenced strains with the exception of T49, which has a cysteine in position 809 instead of tryptophane – the second polymorphism within the *lpf* operons of the atypical O157 strains. The only strain with an amino acid switch in *lpf2C* available in GenBank is E24377A, which has a leucine instead of proline in position 122. In the case of *lpf2D* gene, strain T22 has serine instead of alanine in position 341, this switch is shared with strains SE11, 55989 and 11368, and is the third polymorphism within the sequenced atypical strains. The fourth polymorphism can be observed in strains T49 and

T50, which encode a leucine instead of a methionine in position 313. The nucleotide sequence comparison of the *lpf* genes of the atypical O157 bovine strains is shown in Figure S1 (Supporting Material).

The existence of these polymorphisms indicate a similar level of variation in the otherwise conserved *lpf2* sequences. However, currently there is no data available on the potential or actual effect of these differences on the expression and/or function of Lpf2. The GC content of the sequenced *lpf* operons was 44%, while that of the flanking regions in T22 was 52%, close to the average GC content in the *E. coli* genome (McLean et al., 1998). This fact, together with the generally conserved sequence of *lpf2* in strains of various sero- and pathotypes (Table 3) suggests that these *lpf* variants might be located in genomic islands.

Dissemination and characteristics of the flanking regions of *lpf2*. The results of the PCR scanning of previously characterized strains carrying allele *lpf2-1* are listed in Table 1. The fact that the *lpf2-1* operon is flanked by the same set of genes in the majority of the strains sequenced so far (Table 3) is further demonstrated by the results of our PCR scanning. This uniformity indicates that the site between the *pstS* and *glmS* genes served as an integration hotspot at some point during the evolution of these strains. The genetic analysis of this region was performed in an earlier study, in which the authors designed primers specific for the flanking regions, and investigated whether the site between *pstS* and *glmS* is intact or interrupted by the *lpf2* operon (Toma et al., 2006). It must be noted however, that in the case of prototypic enteroaggregative strain *E. coli* (EAEC) strain 042 (O44:H18), a Tn*21* transposon sequence is inserted between *lpfA* and *glmS* genes. This transposon element contains among other features transposases and genes encoding antibiotic resistance (Chaudhuri et al., 2010). Interestingly, four out of five strains which have the closest homologues to the *lpf2-1* operon of strain T22, are commensal isolates (Table 3).

228

229 **Expression of Lpf2.** The RT-PCR specific for the *lpfA* genes from T22 yielded positive
230 results, confirming transcription of Lpf2 in 48-hour cultures. In an earlier study with EHEC
231 strain EDL933, one of the authors of the current manuscript found that the H-NS protein has a
232 silencer role, while the regulatory protein Ler acts as an anti-silencer during the expression of
233 Lpf1 (Torres et al., 2008). Our findings, as well as the lack of LEE (which includes the *ler*
234 gene) in strain T22 suggests a different regulatory mechanism controlling Lpf2 expression
235 relative to Lpf1.

236

237 **Contribution of LpfA2-1 to adherence of STEC O136:H12 *in vitro*.** The wide distribution
238 of this particular allele of Lpf in pathogenic *E. coli* strains, especially in LEE-negative strains
239 (Doughty et al., 2002) underlines its potential role as an important adhesin. There is both *in*
240 *vitro* (Doughty et al., 2002; Newton et al., 2004; Torres et al., 2008; Farfan et al., 2011) and *in*
241 *vivo* (Jordan et al., 2004) experimental evidence that Lpf enhances the adherence of intimin-
242 positive and negative strains. Construction of an *lpf2* mutant in strain T22 resulted more
243 challenging than expected; therefore, we created an *lpf2-1* mutant in STEC O136:H12 strain
244 187/06 (22), a bovine isolate that possesses the same *lpf2-1* allele as strain T22 (data not
245 shown) and flanked with the same genes (Table 3). The role of Lpf2 in adherence of strain
246 187/06 (22) was evaluated using different tissue cultured cells lines; however, no clear
247 differences could be observed in the quantitative adherence assays between O136:H12 and its
248 corresponding *lpfA2-1* mutant. The strain 187/06 (22) did not show a significant reduction in
249 the adherence neither to Hep-2 (P=0.10), nor to Caco-2 (P=0.42) cell lines when was
250 compared to its deletion mutant, but exhibited a significant reduction in adherence to T84 cell
251 line (P<0.0002) (Figure 2). However, when competition adhesion assays were performed
252 using the wild type and its corresponding *lpfA2-1* deletion mutant, the mean CI (3.27 ± 1.35)

253 was significantly greater than 1 (P=0.043). This finding, where the mutant strain is adhering
254 more than the wild type suggested that the strain lacking Lpf might produce additional
255 adhesion factors that provided a subtle advantage in the *in vitro* adherence assay.
256 In the case of strain T22, no specific adhesion could be observed in bovine testis and
257 kidney cell cultures (data not shown), which is in harmony with the finding that strains
258 possesing Lpf2 are not defective in adhesion but further analysis is needed to define the role
259 of this fimbriae or other adhesion factors in these subset of strains.
260 In summary, we cloned and sequenced for the first time the long polar fimbriae-
261 encoding operon and its flanking regions in an atypical, NSF O157:H43 *E. coli* strain. The
262 Lpf operon itself is nearly identical to those of several other pathogenic and non-pathogenic
263 strains from various serotypes and pathogroups, and represents the genetic variant termed
264 allele 1 of *lpf2*. The integration site of the operon also shows high similarity to that on the
265 aforementioned strains. Characteristic flanking regions were also found in other O157 non H7
266 strains carrying the same operon. Given the experimental evidence for the role of Lpf in
267 adherence of *E. coli* O157:H7 and other pathogenic *E. coli* strains, it is plausible to propose
268 that the Lpf2-1 fimbriae are important factors mediating adherence of our bovine *E. coli*
269 strains. However, we did not observe any difference in the adherence profile of STEC 187/06
270 (22) or STEC O136:H12 strains. Based on recent published report, we speculate that in
271 absence of an adhesin such as Lpf, the strains synthesized alternate surface structure as a
272 compensatory mechanism for colonization (Lloyd et al., 2012). Further, the complete
273 regulatory mechanisms of *lpf2* still need to be fully elucidated. Finally, the highly conserved
274 sequence and integration site of the *lpf2* operon suggests that *lpf2* loci belongs to a conserved
275 genomic island, and an interesting future task will be to elucidate the mechanism of genetic
276 acquisition of this operon in pathogenic and commensal *E. coli* strains.
277

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285 **REFERENCES**

286 Appleyard RK (1954) Segregation of New Lysogenic Types during Growth of a Doubly
287 Lysogenic Strain Derived from *Escherichia coli* K12. *Genetics* **39:**440-452.

288

289 Archer CT, Kim JF, Jeong H, Park JH, Vickers CE, Lee SY, Nielsen LK (2011) The genome
290 sequence of *E. coli* W (ATCC 9637): comparative genome analysis and an improved genome-
291 scale reconstruction of *E. coli*. *BMC Genomics* **12:**9.

292

293 Chaudhuri RR, Sebaihia M, Hobman JL, Webber MA, Leyton DL, Goldberg MD,
294 Cunningham AF, Scott-Tucker A, Ferguson PR, Thomas CM, Frankel G, Tang CM, Dudley
295 EG, Roberts IS, Rasko DA, Pallen MJ, Parkhill J, Nataro JP, Thomson NR, Henderson IR
296 (2010) Complete genome sequence and comparative metabolic profiling of the prototypical
297 enteroaggregative *Escherichia coli* strain 042. *PLoS One* **5:**e8801.

298

299 Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia*
300 *coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97:**6640-6645.

301

302 Doughty S, Sloan J, Bennett-Wood V, Robertson M, Robins-Browne RM, Hartland EL
303 (2002) Identification of a novel fimbrial gene cluster related to long polar fimbriae in locus of
304 enterocyte effacement-negative strains of enterohemorrhagic *Escherichia coli*. *Infect Immun*
305 **70:**6761-6769.

306

307 Farfan MJ, Cantero L, Vidal R, Botkin DJ, Torres AG (2011) Long Polar Fimbriae of
308 Enterohemorrhagic *Escherichia coli* O157:H7 Bind to Extracellular Matrix Proteins. *Infect*
309 *Immun* **79:**3744-3750.
310
311 Fitzhenry R, Dahan S, Torres AG, Chong Y, Heuschkel R, Murch SH, Thomson M, Kaper
312 JB, Frankel G, Phillips AD (2006) Long polar fimbriae and tissue tropism in *Escherichia coli*
313 O157:H7. *Microbes Infect* **8:**1741-1749.
314
315 Galli L, Torres AG, Rivas M (2010) Identification of the long polar fimbriae gene variants in
316 the locus of enterocyte effacement-negative Shiga toxin-producing *Escherichia coli* strains
317 isolated from humans and cattle in Argentina. *FEMS Microbiol Lett* **308:**123-129.
318
319 Hayashi T, Makino K, Ohnishi M, Kurokawa K, Ishii K, Yokoyama K, Han CG, Ohtsubo E,
320 Nakayama K, Murata T, Tanaka M, Tobe T, Iida T, Takami H, Honda T, Sasakawa C,
321 Ogasawara N, Yasunaga T, Kuhara S, Shiba T, Hattori M, Shinagawa H (2001) Complete
322 genome sequence of enterohemorrhagic *Escherichia coli* O157:H7 and genomic comparison
323 with a laboratory strain K-12. *DNA Res* **8:**11-22.
324
325 Ideses D, Biran D, Gophna U, Levy-Nissenbaum O, Ron EZ (2005) The *lpf* operon of
326 invasive *Escherichia coli*. *Int J Med Microbiol* **295:**227-236.
327
328 Iguchi A, Shirai H, Seto K, Ooka T, Ogura Y, Hayashi T, Osawa K, Osawa R (2011) Wide
329 Distribution of O157-Antigen Biosynthesis Gene Clusters in *Escherichia coli*. *PLoS One*
330 **6:**e23250.

Jordan DM, Cornick N, Torres AG, Dean-Nystrom EA, Kaper JB, Moon HW (2004) Long polar fimbriae contribute to colonization by *Escherichia coli* O157:H7 *in vivo*. *Infect Immun* **72:**6168-6171.

Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic *Escherichia coli*. *Nat Rev Microbiol* **2:**123-140.

Lloyd S, Ritchie J, Torres AG (2012) Fimbriation and curliation in *Escherichia coli* O157:H7: A paradigm of intestinal and environmental colonization. *Gut Microbes* **3:** 272-6.

McLean MJ, Wolfe KH, Devine KM (1998) Base composition skews, replication orientation, and gene orientation in 12 prokaryote genomes. *J Mol Evol* **47:**691-696.

Mellmann A, Harmsen D, Cummings CA, Zentz EB, Leopold SR, Rico A, Prior K, Szczepanowski R, Ji Y, Zhang W, McLaughlin SF, Henkhaus JK, Leopold B, Bielaszewska M, Prager R, Brzoska PM, Moore RL, Guenther S, Rothberg JM, Karch H (2011) Prospective genomic characterization of the German enterohemorrhagic *Escherichia coli* O104:H4 outbreak by rapid next generation sequencing technology. *PLoS One* **6:**e22751.

Monaghan A, Byrne B, Fanning S, Sweeney T, McDowell D, Bolton DJ (2011) Serotypes and Virulence Profiles of non-O157 Shiga-Toxin Producing *Escherichia coli* (STEC) from Bovine farms. *Appl Environ Microbiol* **77:**8662-8668.

354 Newton HJ, Sloan J, Bennett-Wood V, Adams LM, Robins-Browne RM, Hartland EL (2004)
355 Contribution of long polar fimbriae to the virulence of rabbit-specific enteropathogenic
356 *Escherichia coli*. *Infect Immun* **72:**1230-1239.
357
358 Ochman H, Selander RK (1984) Standard reference strains of *Escherichia coli* from natural
359 populations. *J Bacteriol* **157:**690-693.
360
361 Ogura Y, Ooka T, Iguchi A, Toh H, Asadulghani M, Oshima K, Kodama T, Abe H,
362 Nakayama K, Kurokawa K, Tobe T, Hattori M, Hayashi T (2009) Comparative genomics
363 reveal the mechanism of the parallel evolution of O157 and non-O157 enterohemorrhagic
364 *Escherichia coli*. *Proc Natl Acad Sci U S A* **106:**17939-17944.
365
366 Oshima K, Toh H, Ogura Y, Sasamoto H, Morita H, Park S, Ooka T, Iyoda S, Taylor TD,
367 Hayashi T, Itoh K, Hattori M (2008) Complete genome sequence and comparative analysis of
368 the wild-type commensal *Escherichia coli* strain SE11 isolated from a healthy adult. *DNA Res*
369 **15:**375-386.
370
371 Rasko DA, Rosovitz MJ, Myers GSA, Mongodin EF, Fricke WF, Gajer P, Crabtree J,
372 Sebaihia M, Thomson NR, Chaudhuri R, Henderson IR, Sperandio V, Ravel J (2008) The
373 pangenome structure of *Escherichia coli*: comparative genomic analysis of *E. coli* commensal
374 and pathogenic isolates. *J Bacteriol* **190:**6881-6893.
375
376 Sambrook J, Fritsch EF, Maniatis T (1982) *Molecular Cloning: A Laboratory Manual*. Cold
377 Spring Harbor Laboratory Press, New York.
378

379 Sváb D, Tóth I (2012) Allelic types of long polar fimbriae in human and bovine *Escherichia*
380 *coli* O157 strains. *Acta Vet. Hung.* **60:**1-15.
381
382 Toma C, Higa N, Iyoda S, Rivas M, Iwanaga M (2006) The long polar fimbriae genes
383 identified in Shiga toxin-producing *Escherichia coli* are present in other diarrheagenic *E. coli*
384 and in the standard *E. coli* collection of reference (ECOR) strains. *Res Microbiol* **157:**153-
385 161.
386
387 Toma C, Martínez Espinosa E, Song T, Miliwebsky E, Chinen I, Iyoda S, Iwanaga M and
388 Rivas M (2004) Distribution of Putative Adhesins in Different Seropathotypes of Shiga
389 Toxin-Producing *Escherichia coli*. *J Clin Microb* **42:**4937-4946.
390
391 Torres AG, Blanco M, Valenzuela P, Slater TM, Patel SD, Dahbi G, López C, Barriga XF,
392 Blanco JE, Gomes TAT, Vidal R, Blanco J (2009) Genes related to long polar fimbriae of
393 pathogenic *Escherichia coli* strains as reliable markers to identify virulent isolates. *J Clin*
394 *Microbiol* **47:**2442-2451.
395
396 Torres AG, Slater TM, Patel SD, Popov VL, Arenas-Hernández MMP (2008) Contribution of
397 the Ler- and H-NS-regulated long polar fimbriae of *Escherichia coli* O157:H7 during binding
398 to tissue-cultured cells. *Infect Immun* **76:**5062-5071.
399
400 Touchon M, Hoede C, Tenaillon O, Barbe V, Baeriswyl S, Bidet P, Bingen E, Bonacorsi S,
401 Bouchier C, Bouvet O, Calteau A, Chiapello H, Clermont O, Cruveiller S, Danchin A, Diard
402 M, Dossat C, Karoui ME, Frapy E, Garry L, Ghigo JM, Gilles AM, Johnson J, Le Bouguénec
403 C, Lescat M, Mangenot S, Martinez-Jéhanne V, Matic I, Nassif X, Oztas S, Petit MA, Pichon

C, Rouy Z, Ruf CS, Schneider D, Tourret J, Vacherie B, Vallenet D, Médigue C, Rocha EPC, Denamur E (2009) Organised genome dynamics in the *Escherichia coli* species results in highly diverse adaptive paths. *PLoS Genet* **5:**e1000344.

Tóth I, Schmidt H, Kardos G, Lancz Z, Creuzburg K, Damjanova I, Pászti J, Beutin L, Nagy B (2009) Virulence genes and molecular typing of different groups of *Escherichia coli* O157 strains in cattle. *Appl Environ Microbiol* **75:**6282-6291.

412 **Table 1. List of strains used in this study and results of the PCR scanning of the flanking regions of the *lpf2* operon.**

Strain	Serotype	Phylogenetic group	*bgIG-phoU*	*phoU-pstB*	*pstB-pstA*	*pstA-pstC*	*pstC-pstS*	*pstS-lpfD*	*lpfA-glmS*	*glmS-glmU*	*glmU-atpC*	Reference
T16	O157:H43	B1[c]	+	+	+	+	+	+	+	+	+	(Tóth et al., 2009)
T22	O157:H43	B1[c]	+	+	+	+	+	+	+	+	+	(Tóth et al., 2009)
T34	O157:H43	B1[c]	+	+	+	+	+	+	+	+	+	(Tóth et al., 2009)
T49	O157(rough):H9	B1[c]	+	+	+	+	+	+	+	+	+	(Tóth et al., 2009)
T50	O157(rough):H37r	B1[c]	+	+	+	+	+	+	+	+	+	(Tóth et al., 2009)
B47	O157:NM	B1[c]	+	+	+	+	+	+	+	+	+	(Tóth et al., 2009)
B54	O157(rough):H12	A[c]	-[a]	+	-[a]	+	+	+	+	+	+	(Tóth et al., 2009)
ECOR7	O85:HN	A	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR23	O86:H43	A	+	+	-[a]	+	-	+	+	+	+	(Ochman & Selander, 1984)
ECOR26	O104:H21	B1	+	+	+	+	+	+	-	+	+	(Ochman & Selander, 1984)
ECOR30	O113:H21	B1	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR32	O7:H21	B1	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR33	O7:H21	B1	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR34	O88:NM	B1	+	+	-	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR36	O79:H25	D	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR57	ON:NM	B2	-[b]	-[b]	+	+	-[b]	-[b]	-[b]	-[b]	-[b]	(Ochman & Selander, 1984)
ECOR58	O112:H8	B1	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR67	O4:H43	B1	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR69	ON:NM	B1	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
ECOR72	O144:H8	B1	+	+	+	+	+	+	+	+	+	(Ochman & Selander, 1984)
187/06 (22)	O136:H12	B2	+	+	-[a]	+	+	+	+	+	+	this study
C600	K12	A	-	+	+	+	-	+	+	+	+	(Appleyard, 1954)

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414 [a] These strains yielded consistently longer product with the given primer pair.

415 [b] Strain ECOR57 yielded unspecific or weak products in all these reactions.

[c] The phylogenetic group of these strains was determined in Sváb & Tóth, 2012.

Abbreviations:

HN: H antigen non-typeable

NM: non-motile

ON: O antigen non-typeable

422 **Table 2. Primers used for the amplification of regions flanking the *lpf2* operon.**

Primer	Sequence (5'->3')	Genes amplified	Genbank number	Position amplified	Annealing temperature (°C)	Reference
bgIGfw	CCCAAGCGCTCCTGCGCTAAA	*bgIG-phoU*	CU928160.2	3977599-3978492	60	this study
phoUrev	TCTGGAGTCGCTGGGCCGTC					this study
phoUfw	CGATCACGCGCTTCGCCAGA	*phoU-pstB*	CU928160.2	3978707-3979162	59	this study
pstBrev	GGTATCGCCATTCGCCCGGA					this study
pstBfw	GGACGGCCCGATAAACGCCG	*pstB-pstA*	CU928160.2	3979526-3979912	60	this study
pstArev	CAGCCGATCGCCAACCTGCC					this study
pstAfw	AGCCAGAACAGGCCGAAGGC	*pstA-pstC*	CU928160.2	3980508-3980919	59	this study
pstCrev	ATCGGCGGCATCATGCTGGG					this study
pstCfw	ACCGTAGATCGGCACCAGCG	*pstC-pstS*	CU928160.2	3981370-3981924	58	this study
pstSrev	CCAGAAAGGCGAAGATGCATGGC					this study
pstSfw	CAGACAGCGGCGCGTCAGAG	*pstS-lpfD*	CU928160.2	3982472-3983233	58	this study
lpfDrev	TGCTACCGAACCCAATACGGACAA					this study
lpfAfw	TGTCGACAATTTCACCGACGAAGTG	*lpfA-glmS*	CU928160.2	3987849-3988769	58	this study
glmSrev	GCTGCCGAGCCGTATTGAGCA					this study
glmSfw	CGTGTGTCGCCCAGCGAGTA	*glmS-glmU*	CU928160.2	3989854-3990519	58	this study
glmUrev	GGCGATGCGGAAATTGGCGA					this study
glmUfw	AGCAGATCGCCGCCGTGAC	*glmU-atpC*	CU928160.2	3991436-3992119	59	this study
atpCrev	ACGAAGCGCGAGCCATGGAA					this study
lpfA F	ACCGCTATCGATGCTGAAGG	*lpfB-lpfA*	AY057066	678-1349	63	(Ideses et al., 2005)

lpfB R	GCGCAACATCTTCGGGAATA					
lpfC F2	CGCCGGGTTAGAAATAGATA	*lpfD-lpfC*	AY057066	3658-4421	63	(Ideses et al., 2005)
lpfD R2	TGCCTGGTTTATTTTTGACGTA					
lpfA_inside_fw[a]	TCGACAGTAAATTGTGAATC	Part of *lpfA*	AY057066	233-790	50	this study
lpfA_inside_rev[a]	GAAGCGTAATATTATAGGCG					

[a] Primers used in RT-PCR for the detection of *lpfA* expression.

Table 3. ***Escherichia coli*** **strains with whole genomes in GenBank containing continuous** ***lpf2*** **operons with high homology to the** ***lpf2-1*** **of strain T22.**

Strain	Serotype	Pathotype	SNPs in the Lpf operon	Genbank number	Reference
SE11		commensal	6	AP009240.1	(Oshima et al., 2008)
11128	O111:NM	EHEC	7	AP010960.1	(Ogura et al., 2009)
55989		EAEC	8	CU928145.2	(Touchon et al., 2009)
KO11		commensal	9	CP002516.1	unpublished, JGI Project ID: 4085738
W		commensal	9	CP002185.1	(Archer et al., 2011)
11368	O26:H11	EHEC	9	AP010953.1	(Ogura et al., 2009)
IAI1		commensal	10	CU928160.2	(Touchon et al., 2009)
E24377A		ETEC	11	CP000800.1	(Rasko et al., 2008)

FIGURE LEGENDS

Figure 1. Schematic representation of the genetic region from *E. coli* strain T22 containing *lpf2*. The relative length of the arrows is proportional to the relative length of the genes. Arrows representing genes from the same functional cluster are filled with the same pattern. The *pst* cluster encodes a phosphate ABC transporter, *phoU* encodes a phosphate transport regulator, and the product of *bgIG* is a putative transcriptional regulator. The *glm* cluster encodes an N-acetyl glucosamine-1-phosphate uridyltransferase, and *atpC* encodes the epsilon subunit of ATP synthase.

Figure 2. Comparative chart of *in vitro* bacterial adhesion assays to Caco-2, Hep-2 and T84 cell lines. The results are expressed as the percentage of cell-associated bacteria from the original inoculum [(final CFU/ml / initial CFU/ml) x 100] and are the means ± the standard error of at least two independent experiments in triplicate wells.

Figure S1. Sequence comparison of the *lpf2* operons of atypical bovine *Escherichia coli* O157 strains. The yellow arrows indicate the ORFs of the individual *lpf* genes. Point mutations are marked in red. The sequences of the *lpf2* operons were deposited under the following accession numbers: *E. coli* B47 (O157:NM) **KC207119**, *E. coli* B54 (O157:H12) **KC207120**, *E. coli* T16 (O157:H43) **KC207121**, *E. coli* T34 (O157:H9) **KC207122**, *E. coli* T49 (O157:H37) **KC207123**, *E. coli* T50 (O157:H43) **KC207124**

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448 **FIGURES**

449

450 **Figure 1. Schematic representation of the genetic region from *E. coli* strain T22 containing *lpf2*.**

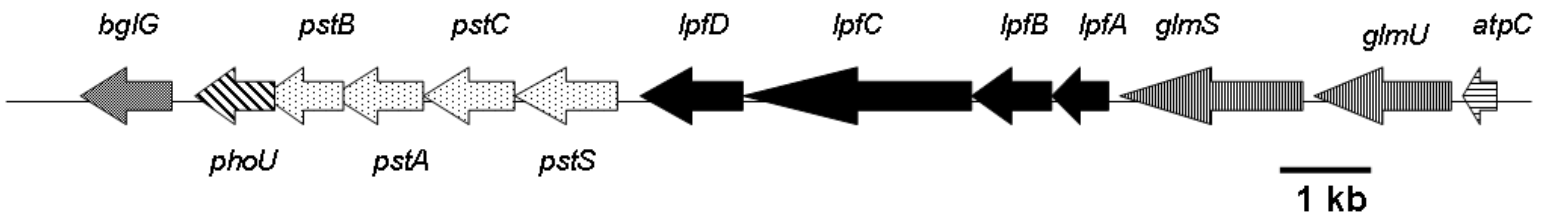

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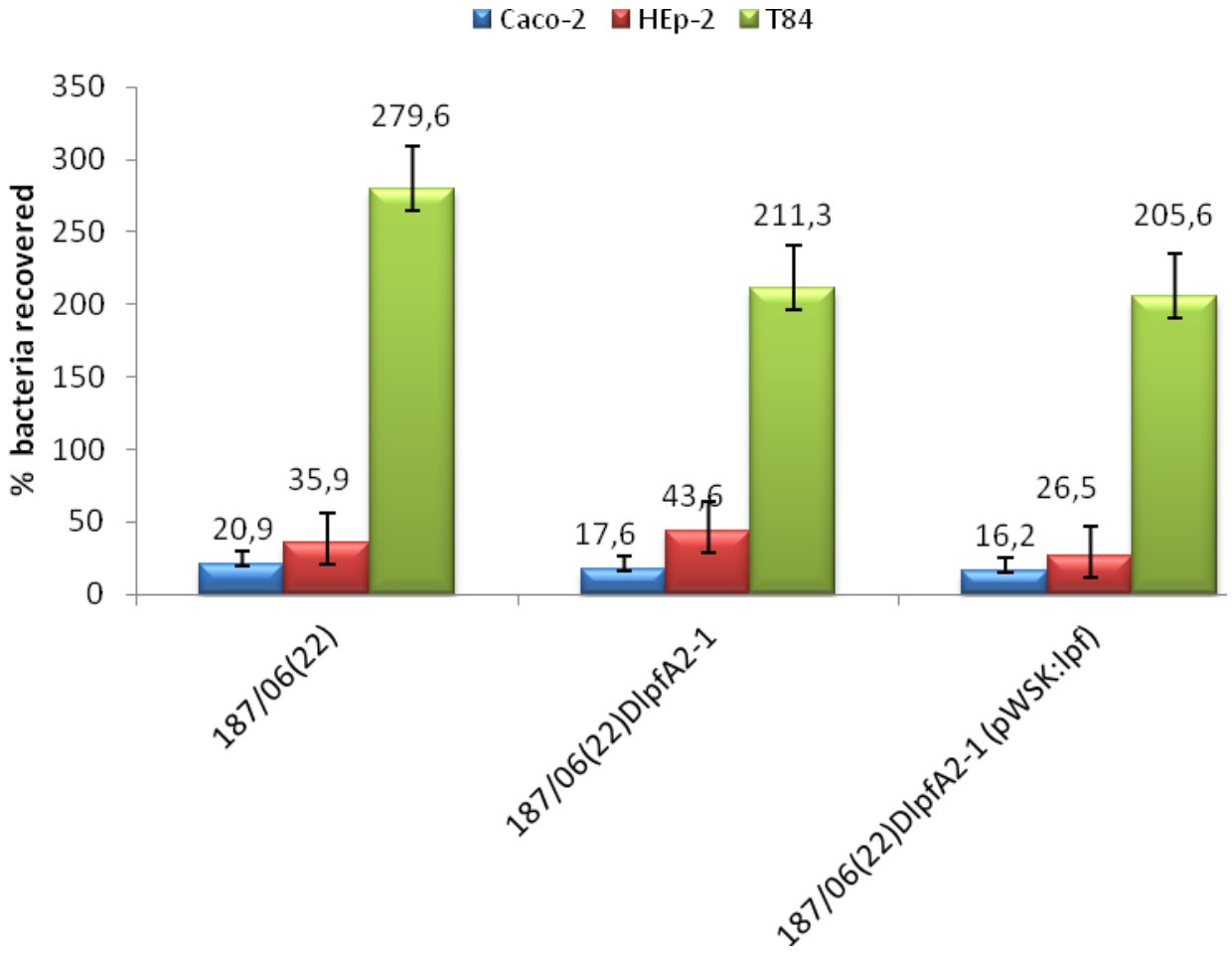
Figure 2. Comparative chart of *in vitro* bacterial adhesion assays to Caco-2, Hep-2 and T84 cell lines.



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