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Genes Related to Long Polar Fimbriae of Pathogenic *Escherichia coli* Strains as Reliable Markers To Identify Virulent Isolates▽†

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Lpf (stands for long polar fimbriae) is one of the few adhesive factors of enterohemorrhagic *Escherichia coli* O157:H7 associated with colonization of the intestine. *E. coli* O157:H7 strains possess two *lpf* loci encoding highly regulated fimbrial structures. Database analysis of the genes encoding the major fimbrial subunits demonstrated that they are present in commensal as well as pathogenic (both intestinal and extraintestinal) *E. coli* strains and in *Salmonella* strains and that the *lpfA1* and *lpfA2* genes are highly prevalent among LEE (locus of enterocyte effacement)-positive *E. coli* strains associated with severe and/or epidemic disease. Further DNA sequence analysis of the *lpfA1* and *lpfA2* genes from different attaching-and-effacing *E. coli* strains has led us to the identification of several polymorphisms and the classification of the major fimbrial subunits into distinct variants. Using collections of pathogenic *E. coli* isolates from Europe and Latin America, we demonstrated that the different *lpfA* types are associated with the presence of specific intimin (*eae*) adhesin variants and, most importantly, that they are found in specific *E. coli* pathotypes. Our results showed that the use of these fimbrial genes as markers, in combination with the different intimin types, resulted in a specific test for the identification of *E. coli* O157:H7, distinguishing it from other pathogenic *E. coli* strains.

During the infection process, enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 adheres to the intestinal epithelium, where it produces Shiga toxins responsible for the hemorrhagic symptoms associated with bloody diarrhea or during development of hemolytic-uremic syndrome (HUS). The adhesion of *E. coli* O157:H7 to enterocytes induces the formation of the attaching and effacing (A/E) lesion (reviewed in references 14 and 39). The A/E phenotype is conferred mainly by the locus of enterocyte effacement (LEE), a pathogenicity island containing genes encoding structural components of a type III secretion apparatus, translocator and secreted effector proteins, an adhesin (intimin), and the intimin receptor, Tir (reviewed in reference 35). The association of intimin with Tir triggers a host cell response leading to pedestal formation, and although this phenotype is best characterized in vitro, its expression correlates with the ability of the A/E organisms to colonize the intestine and cause disease in human and other animal hosts (reviewed in reference 20). Interestingly, it has been postulated that different intimin types (differences in the amino acid sequence of the intimin proteins) influence the pattern of colonization and tissue tropism in the host (10, 24). Therefore, initial experimental approaches provided evidence for the existence of at least four distinct types, known as intimin α, β, γ, and δ (1, 2). Subsequent studies have proposed that additional intimin types exist, and based on differences at the nucleotide level, they have been classified as intimins ζ, η, θ, ι, and κ, etc. (3, 4, 13, 18, 29, 41).

While the correlation between the expression of some of the intimin types and the tissue tropism of different *E. coli* strains has been demonstrated experimentally using in vitro human intestinal organ cultures (6, 10, 11, 25), very little is known about other *E. coli* O157:H7 colonization factors, including those controlling the expression of fimbriae. EHEC O157:H7 contains two nonidentical *lpf* loci homologous to the long polar fimbriae (LPF) of *Salmonella enterica* serovar Typhimurium (33, 34). Expression of *E. coli* O157:H7 *lpf* operon 1 (*lpf1*) in *E. coli* K-12 has been associated with increased adherence to tissue-cultured cells and with the appearance of long fimbriae (33, 38). The *lpf2* operon has also been linked to adherence to epithelial cells (34), and its expression in other pathogenic *E. coli* strains is believed to be important for the development of severe diarrhea (8, 23). *E. coli* O157:H7 strains harboring mutations in one or both of the *lpf* loci have diminished colonization abilities in animal models (swine and sheep) (12) and also display an altered human intestinal tissue tropism (9). Furthermore, the role of LPF as a colonization factor associated with persistence in the intestine was elucidated using a lamb model of infection (37). Recently, we established the connection between regulatory proteins and expression of the *lpf1* loci in response to environmental cues, and we found that these fimbriae are regulated by H-NS, a protein that binds to

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the regulatory sequence of *lpfA1* and "silences" transcription, while the LEE-encoded Ler regulator binds to the regulatory sequence and inhibits the action of H-NS (36). Further, we found that deregulation of the *lpf1* operon produced constitutive expression of the fimbriae, a phenotype associated with adherence and hemagglutination phenotypes in *E. coli* O157:H7 (38).

Because our data indicated that LPF constitute an important colonization factor of EHEC O157:H7 strains and because cumulative evidence indicates that homologues to the *lpf* genes are found in other pathogenic *E. coli* and *Salmonella* strains (7, 8, 28, 31, 34), in the current study, we identified several polymorphisms within the *lpfA* genes, which were used to classify the major fimbrial subunit genes into distinct variants. Further, we showed that *lpf* genes, in combination with the different intimin types, are reliable markers for the differentiation of *E. coli* O157:H7 and other pathogenic *E. coli* strains.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. Diarrheagenic and extraintestinal pathogenic *E. coli* (ExPEC) strains from reference laboratories in Spain, Chile, and Brazil were employed in this study (4, 18, 19, 22, 40). The Spanish collection comprised 100 strains including 18 Shiga toxin-producing *E. coli* (STEC), 30 enteropathogenic *E. coli* (EPEC), and 52 atypical EPEC (aEPEC) strains. The Chilean collection comprised 125 strains, including 64 STEC, 39 EPEC, and 22 aEPEC strains. Finally, the collection from Brazil comprised 4 EPEC and 33 aEPEC strains. For the PCR tests, EHEC strain EDL933 and *E. coli* K-12 MG1655 were used as positive and negative controls, respectively. Strains were maintained at −80°C, and when needed, they were grown in Luria-Bertani broth (17) at 37°C.

Recombinant DNA techniques. Standard methods were used to perform genomic DNA isolation, PCR, and gel electrophoresis (27). Recombinant *Taq* polymerase enzyme (1 U) was used in combination with 2 mM $MgCl_2$ and 1 μM oligonucleotide primer in each reaction. All amplifications began with a 5-min hot start at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing for 30 s in a range of 52°C to 72°C (depending on the *lpfA* variant amplified), and extension at 72°C for 30 s. In some cases, PCRs were performed with boiled bacterial colonies. On the basis of multiple sequence alignments, the polymorphic regions in the *lpfA* genes were chosen (see below), and PCR primers were derived from those regions with the help of OLIGO primer analysis software. All oligonucleotide primers are listed in Table 1.

Phylogenetic analysis and gene accession numbers. The *E. coli* and *Salmonella* *lpfA* gene sequences available from public databases were analyzed using the Discovery Studio gene program (version 1.5; Accelrys). Multiple sequence alignments were performed using ClustalW with open and extended gap penalties of 10.0 and 5.0, respectively. Bootstrap subsets (1,000 sets) and phylogenetic trees were generated with the neighbor-joining algorithm, and the distance model used was the Kimura two-parameter model (15).

LpfA1 protein NCBI GenBank accession numbers for *E. coli* serotype O157:H7 strains are as follows: for EDL933, AAG58695; for EC4115, ACI36002; for Sakai, BAB37854. For other *E. coli* serotypes (with strains given in parentheses), LpfA1 protein accession numbers are as follows: for O55:H7 (DEC5A), BAE48422; for ONT:H26 (ECOR42), BAE48423; for O119:NM (O119-53), BAE48424; for O127:H6 (E2348/69), CAS11346; for O8 (IAI1), CAR00508; for O26:H11, BAD69589; for O81 (ED1a), CAR10220; for O4:H43 (ECOR67), BAE48419; for O111:H21 (DEC15A), BAE48418; for O111:H8 (DEC8B), BAE48417; for O104:NM (ECOR28), BAE48416; for O86:H43 (ECOR23), BAE48415; for O128:H2 (DEC11A), BAE48420; for ONT:H10 (ECOR65), BAE48421; for rabbit EPEC (REPEC) O15:H− (83/39), AAO22843; for enteroaggregative *E. coli* (EAEC) (55989), CAV00478. For *Salmonella enterica* strains, LpfA1 protein accession numbers are as follows: for *Salmonella enterica* serovar Enteritidis P125109, CAR35040; for *Salmonella enterica* serovar Dublin CT02021853, ACH74212; for *Salmonella enterica* serovar Newport SL254, ACF63868; for *Salmonella enterica* serovar Heidelberg SL476, ACF70317; for *Salmonella* serovar Typhimurium LT2, AAL22500.

LpfA2 protein accession numbers for *E. coli* serotype O157:H7 strains are as follows: for EDL933, AAG58930; for EC4115, ACI39341; for Sakai, BAB38093. For other *E. coli* serotypes (with strains given in parentheses), LpfA2 protein accession numbers are as follows: for O55:H7 (DEC5A), BAE48400; for O119:NM (O119-53), BAE48402; for ONT:H26 (ECOR42), BAE48401; for O113:H21 (EH41), AAL18161; for O152:H28 (SE11), BAG79542; for O78 (789), AAY18076; for O78:H9 (chi7122), AAS99229; for O13:H21 (ECOR30), BAE48408; for O7:H21 (ECOR33), BAE48407; for O26:H11 (DEC10A), BAE48410; for O111:H8 (DEC8B), BAE48409; for O86:H43 (ECOR23), BAE48406; for O157:H43 (DEC7A), BAE48405; for O104::NM (ECOR28), BAE48404; for O85:HNT (ECOR7), BAE48403; for ONT:HNT (ECOR48), BAE48413; for O1:H6 (ECOR46), BAE48412; for O7:NM (ECOR40), BAE48411; for O79:H25 (ECOR36), BAE48414; for O8 (IAI1), CAR00706; for enterotoxigenic *E. coli* (ETEC) O139:H28 (E24377A), ABV19201; for verotoxigenic *E. coli* O15, AAT76975; for EAEC (55989), CAV00812; for O44 (O44-20), BAE48399.

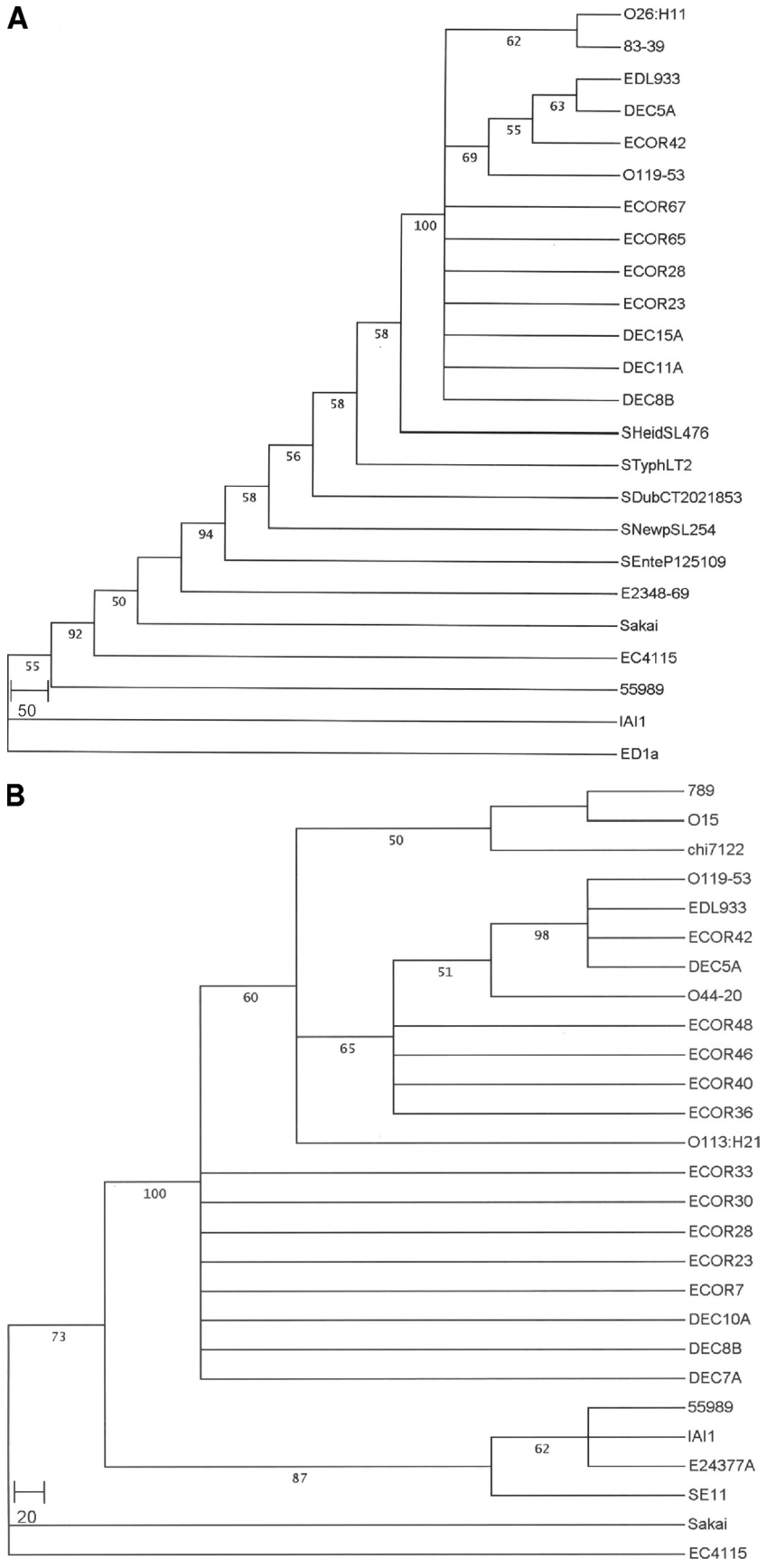
Statistical analysis. Analysis of variance and Pearson's chi-square test were used to test associations between the clinical courses of *E. coli* O157:H7 infections (acute diarrhea, bloody diarrhea, or HUS) and the presence of the *lpfA* genes.

TABLE 1. PCR primer pairs for the amplification of the different *lpfA* types

Gene type (predominant serotype) and primer	Sequence (5′–3′)	Position	Amplicon length (bp)
lpfA1			
1 (O127:H6)			
LPFA1-AF	AGTTGGTGATAAATCACCAT	186–205	222
LPFA1-AR	GTGCTGGATTCACCACTATTC ATCG	383–407	
2 (O26:H11)			
LPFA1-B1F	AAGTCTGTATTTACTGCTATG	169–189	273
LPFA1-B1R	GAAATACAGAACGGTCTGA	423–441	
3 (O157:H7)			
LPFA1-CF	GGTTGGTGACAAATCCCCG	186–204	244
LPFA1-CR1	CGTCTGGCCTTTACTCAGA	411–429	
4 (ONT:H10)			
LPFA1-B2F	AAGTCTGTGTTTACCACTACT	64–84	273
LPFA1-B2R	AAAATACAGAACAGTCTGG	318–336	
5 (ONT:H26)			
LPFA1-CF	GGTTGGTGACAAATCCCCG	81–99	250
LPFA1-CR1	GAGAACCGTCTGGCCTGTTT	311–330	
lpfA2			
1 (O113:H21)			
LPFA2-B1F	GGTAGTCTGGCGTCGCCACAGA	130–151	207
LPFA2-B1R	AATACGAATACCAACGCCG	318–336	
2 (O157:H7)			
LPFA2-CF	CTACAGGCGGCTGATGGAACA	61–81	297
LPFA2-CR	GCTAATACCAGCGGCAGCATCGT	335–357	
3 (O44)			
LPFA2-B2F	GGTAGTCTGGCGTCACCACAGC	190–211	207
LPFA2-B2R	AATACGAATACCGACACCC	378–396	

RESULTS AND DISCUSSION

Phylogenetic trees based on *lpfA1* and *lpfA2* gene sequences of *E. coli* and *Salmonella* strains. Previously, it was demonstrated that the *lpfA1* and *lpfA2* genes are highly prevalent among LEE-positive *E. coli* strains, including EHEC O157:H7 strains associated with severe and/or epidemic disease (28, 31, 34). Further, homologues of *lpf* genes have also been detected in non-O157:H7 LEE-positive *E. coli* strains, LEE-negative pathogenic *E. coli* strains, and REPEC strains (8, 21, 23, 32). Therefore, we performed BLAST analysis to identify the DNA sequences currently available in the database that display homology to the *lpfA1* and *lpfA2* genes of EHEC O157:H7 strain EDL933. Using phylogenetic analysis of DNA sequences, distinct clades could be distinguished corresponding to the diversity of *lpfA1* and *lpfA2* genes (Fig. 1). In the *lpfA1* tree analysis, we found that genes with 69 to 99% identity to the EDL933 *lpfA1* gene were present in a range of *E. coli* strains, including


A
O26:H11
83-39
EDL933
DEC5A
ECOR42
O119-53
ECOR67
ECOR65
ECOR28
ECOR23
DEC15A
DEC11A
DEC8B
SHeidSL476
STyphLT2
SDubCT2021853
SNewpSL254
SEnteP125109
E2348-69
Sakai
EC4115
55989
IAI1
ED1a
62
63
55
69
100
58
58
56
58
94
50
92
55
50
B
789
O15
chi7122
O119-53
EDL933
ECOR42
DEC5A
O44-20
ECOR48
ECOR46
ECOR40
ECOR36
O113:H21
ECOR33
ECOR30
ECOR28
ECOR23
ECOR7
DEC10A
DEC8B
DEC7A
55989
IAI1
E24377A
SE11
Sakai
EC4115
50
98
51
60
65
100
73
62
87
20

EPEC strains DEC11A, DEC5A, O119-53, and E2348/69, STEC strain DEC8B, EAEC strains DEC15A and 55989, EHEC strains Sakai, EC4115, and O26:H11, REPEC strain 83-39, *E. coli* Reference Collection (ECOR) strains ECOR67, ECOR65, ECOR28, ECOR23, and ECOR42, ExPEC strain IAI1, and commensal *E. coli* strain ED1a, as well as in *Salmonella enterica* serovars Dublin (CT02021853), Newport (SL254), Enteritidis (P125109), Heidelberg (SL476), and Typhimurium (LT2). As previously described, we now confirmed that the EDL933 *lpfA1* gene is phylogenetically related to the *lpfA* genes found in EPEC DEC5A, O119-53, and ECOR42 (96 to 99% identity) and is less related (69% identity) to the *lpfA1* genes found in the different serovars of *Salmonella* (Fig. 1A). To our surprise, the *lpfA1* genes from O157:H7 strains Sakai and EC4115 were more phylogenetically related at the nucleotide level to EPEC O127:H6 strain E2348/69 than to O157:H7 strain EDL933. Our tree also showed that the *lpfA1* gene is also present in several ECOR strains and that these genes shared close phylogenetic relationships to the genes found in the other ECOR strains and strains DEC15A, DEC11A, and DEC8B. Three new genome sequences recently included in the database indicated that the *lpfA1* gene is also present in EAEC strain 55989, ExPEC strain IAI1, and commensal *E. coli* strain ED1a (Fig. 1A). Finally, the two strains of *E. coli* that carried the more distantly related (72 to 73% identity) *lpfA1* genes are REPEC strain 83-39 and EHEC strain O26:H11.

The tree analysis of the *lpfA2* genes revealed a totally distinct distribution of the genes and indicated that the EDL933 *lpfA2* gene is also closely related at the nucleotide level (98 to 99% identity) to the genes found in EPEC strains DEC5A and O119-53 and strain ECOR42 (Fig. 1B). Genes with homology to EDL933 *lpfA2* are also found in STEC strains O15, DEC10A, and DEC8B; EHEC strains Sakai, EC4115, and O113:H21; and other members of the ECOR reference collection (ECOR48, ECOR46, ECOR40, ECOR36, ECOR33, ECOR30, ECOR28, ECOR23, and ECOR7). As in the *lpfA1* tree analysis, addition of new genome sequences to the database demonstrated that genes with homology to *lpfA2* are also present in other categories of pathogenic *E. coli*, such as EAEC strains O44-20 and 55989, ETEC strains E24377A and DEC7A, avian-pathogenic *E. coli* strain chi7122, ExPEC strains IAI1 and 789, and commensal *E. coli* strains ED1a and SE11 (Fig. 1B). Our database search and analysis also revealed that genes with homology to *lpfA2* were also present in *Shigella sonnei*, *Shigella flexneri*, and *Shigella boydii* (data not shown).

We selected the *lpfA* genes for our analysis for several reasons. (i) The LPF are a novel determinant of EHEC O157:H7 tropism for the human intestinal tract (9). The expression of the LPF in LEE-negative strains of EHEC is believed to be important for the development of severe diarrhea and hence is potentially clinically relevant (8, 23). (ii) A large number of intestinal pathogenic *E. coli* strains associated with severe and/or epidemic disease possess the *lpf* genes, and it is postulated that they express the LPF (28, 31, 34). A study analyzing *lpf* genes in a collection of pathogenic *E. coli* strains of different categories isolated from the intestine found that the *lpf* genes are not specific to EHEC O157:H7; they are present in other diarrheagenic *E. coli* (DEC) strains and in the standard collection of ECOR strains (28, 31, 34). These findings suggest that there is a relationship between the *lpfA* gene variant and the phylogenetic group. (iii) Our current data confirmed that *lpfA* genes are present in intestinal pathogens, such as *E. coli*, *Salmonella*, and *Shigella* spp.; however, elucidation of genome sequences recently deposited in GenBank has now demonstrated that *lpfA1* and *lpfA2* homologues are also present in the genomes of ExPEC strains, in *E. coli* strains of other pathotypes causing infection in animals, and, unexpectedly, in some isolates from healthy humans that are considered to be commensal *E. coli* strains. The presence of these genes seems to be widespread in pathogenic *E. coli* strains of different origins, which justified their study as putative markers to identify outbreak strains of specific pathotypes that occur in specific locations around the world.

Prevalence of *lpf1* and *lpf2* genes in reference collections of pathogenic *E. coli* strains. Because a large portion of *lpfA* DNA sequences available in the database belong to pathogenic *E. coli* strains producing A/E lesions (A/E *E. coli* [AEEC]), we hypothesized that the *lpfA* genes might contain conserved regions useful for classifying these genes into different types (variants) and that these variants might be present in specific virulent serotypes. We aligned all the available DNA sequences and found several conserved regions (see Fig. S1 in the supplemental material), allowing us to group the *lpfA1* genes into at least five different types (we named them alleles 1, 2, 3, 4, and 5) and the *lpfA2* genes into three distinct types (alleles 1, 2, and 3). Using these conserved regions, we designed pairs of oligonucleotides (Table 1) that specifically amplified these segments in the different *lpfA* types, and then we determined by PCR analysis whether these *lpfA* variants were present in all strains or only in specific subsets of AEEC strains as well as in *E. coli* strains in reference collections. As indicated in Table 2, by using the DEC reference collection (strains were kindly provided by the late Thomas Whittam [Michigan State

FIG. 1. Trees based on sequence data from the *lpfA1* (A) and *lpfA2* (B) genes. Shown are the phylogenetic positions of the 525-bp and 603-bp *E. coli* O157:H7 *lpfA1* and *lpA2* genes from strain EDL933, respectively, and the corresponding *lpfA1* and *lpfA2* DNA sequences from *E. coli* and *Salmonella* strains currently available in GenBank (for accession numbers, see Materials and Methods). The occurrence (percentage) of the branching order in 1,000 bootstrapped trees is given at each branch. *E. coli* strains (with serotypes given in parentheses) listed include EDL933, EC4115, and Sakai (O157:H7), DEC5A (O55:H7), DEC7A (O157:H43), DEC8B (O111:H8), DEC10A (O26:H11), DEC11A (O128:H2), DEC15A (O111:H21), ECOR7 (O85:HNT), ECOR23 (O86:H43), ECOR28 (O104:NM), ECOR30 (O13:H21), ECOR33 (O7:H21), ECOR36 (O79:H25), ECOR40 (O7:NM), ECOR42 (ONT:H26), ECOR46 (O1:H6), ECOR48 (ONT:HNT), ECOR65 (ONT:H10), ECOR67 (O4:H43), O119-53 (O119:NM), E2348/69 (O127:H6), EH41 (O113:H21), O44-20 (O44), IAI1 (O8), 83/39 (O15:H−), O26:H11 (O26:H11), ED1a (O81), 789 (O78), chi7122 (O78:H9), SE11 (O152:H28), O15 (verocytotoxigenic *E. coli* O15), E24377A (ETEC O139:H28), and 55989 (EAEC). *Salmonella enterica* strains listed (with serovars given in parentheses) include P125109 (*S*. Enteritidis), CT02021853 (*S*. Dublin), SL254 (*S*. Newport), SL476 (*S*. Heidelberg), and LT2 (*S*. Typhimurium).

TABLE 2. Different *lpfA* types and their associations with intimin types in reference collections of virulent *E. coli* strains

lpfA type	Predominant serotype(s) (strain[s]) possessing the *lpfA* type	Specific intimin (*eae*) type(s) associated
lpfA1-1	EPEC O127:H6 (E2348/69)	α1 (alpha), δ (delta), κ (kappa), η1 (eta), η2, λ (lambda), μ (mu), π (pi)
lpfA1-2	REPEC O15:H− (83/39), EPEC2 O128:H2 (DEC11a), O86:H43 (ECOR23), EPEC O128:H21 (DEC15A), O4:H43 (ECOR67), EHEC2 O111:H8 (DEC8B), O104:NM (ECOR28), O26:H11 (AB161111)	β1 (beta), θ1 (theta), ε1 (epsilon), ε2
lpfA1-3	EHEC1 O157:H7 (EDL933), EPEC O55:H7 (DEC5A)	γ1 (gamma)
lpfA1-4	ONT:H10 (ECOR65)	NA[a]
lpfA1-5	ONT:H26 (ECOR42), O119:NM (O119-53)	NA
lpfA2-1	EHEC O113:H21, O15 (O15), O78:K80:H9 (chi7122), O78 (789), O85:H$^-$ (ECOR07), O104:NM (ECOR28), ETEC O157:H43 (DEC7A), O86:H43 (ECOR23), O7:H21 (ECOR33), EHEC2 O111:H8 (DEC8B), EHEC2 O26:H11 (DEC10A)	β1, θ1, ε2, ζ1 (zeta), ι1B (iota), ι1C
lpfA2-2	EHEC1 O157:H7 (EDL933), EPEC O55:H7 (DEC5A), ONT:H26 (ECOR42), O119:NM (O119-53)	γ1
lpfA2-3	O44 (O44-20), O1:H6 (ECOR46), ONT:HNT (ECOR48), O7:NM (ECOR40), O79:H25 (ECOR36)	NA

[a] NA, no association; these types of *lpfA1* or *lpfA2* genes were not found in any of the intimin-positive strains from the *E. coli* Reference Laboratory (Lugo, Spain) analyzed.

University]) and ECOR, as well as other prototypic AEEC strains, we determined that the different *lpfA* types are present in a wide variety of serotypes, and we observed no apparent correlation between the type of *lpfA1* and/or *lpfA2* gene and the bacterial pathotype. Such observations have been reported previously by C. Toma and colleagues (31); however, their study also suggested the existence of a relationship between the *lpfA* type and the bacterial phylogenetic group. Because it has been determined previously that AEEC strains possess distinct variants of intimin and that some of the genes encoding these proteins are associated with specific pathotypes (26), we investigated whether there was an association between intimin (*eae*), the *lpfA* types, and the different pathotypes. As shown in Table 2, an interesting correlation emerged from this association; e.g., we found that the *lpfA1-1* variant was present only in those *E. coli* strains carrying the intimin gene types α1, δ/κ, η1, η2, λ, μ, and π and that EPEC O127:H6 was the predominant serotype representative of that group. The *lpfA1-2* gene is associated with *E. coli* strains carrying intimin types β1, γ2/θ1, ε1, and ε2. To our surprise, the *lpfA1-3* gene was found only in AEEC strains belonging to serotype O157:H7 and in O55:H7 strains (both of these serotypes possess γ1 intimin, a type of intimin found only in EHEC O157 strains and in some of the phylogenetically related serotype O55:H7 and O145 strains). In contrast, no association with any intimin type was found for strains carrying the *lpfA1-4* and *lpfA1-5* gene types.

For the *lpfA2* genes, the associations were not as defined as those observed for the *lpfA1* genes. We found that the *lpfA2-1* gene was associated with *E. coli* strains carrying intimin types β1, γ2/θ, ε2, ζ, and ι1 and that the *lpfA2-2* gene was associated with *E. coli* strains carrying intimin type γ1 (EPEC O55:H7 and EHEC O157:H7) (Table 2). Interestingly, a combination of the *lpfA1-3* and *lpfA2-2* types was observed only for serotypes O55:H7 and O157:H7. In contrast, no association with intimin types was found for strains carrying the *lpfA2-3* gene variant. Our data strongly suggest that a correlation exists between the intimin and *lpfA* gene variants carried by different pathogenic *E. coli* strains. In the case of EHEC O157:H7, because the O55:H7 clinical isolates are rarely found, the use of *lpfA* genes as probes in combination with the use of the intimin types could result in a specific test for the O157:H7 strains and for other pathogenic *E. coli* strains.

The idea of distinguishing pathogenic *E. coli* strains belonging to different pathotypes through sequence-based comparison of their virulence-associated genes has been demonstrated previously (30). In that study, 12 putative virulence genes from ExPEC strains were evaluated based on single-nucleotide polymorphisms. The investigators found that only polymorphisms in the *fimH* gene (which encodes a minor component of the type 1 fimbriae) were able to distinguish uropathogenic *E. coli* strains from other ExPEC organisms. With those concepts in mind, we performed a comprehensive analysis of a large collection of EPEC and STEC strains for the presence of the different intimin and *lpfA* gene variants.

Specific combinations of *lpfA* and intimin gene types are present in STEC and EPEC strains. To determine whether the *lpfA* gene types could be used as a simple, inexpensive screening test for epidemiological studies of pathogenic *E. coli* strains, we analyzed collections of EPEC and STEC strains located in our reference laboratory in Spain, mainly representing isolates from Europe and Brazil (Table 3). The identification of the different intimin and *lpfA* gene variants in these strains produced the following results. The *lpfA1-3* and *lpfA2-2* alleles were present only in strains carrying the intimin γ1 gene (STEC O157:H7, EPEC O55:H7, and two rare aEPEC isolates of serotypes O33:H7 and O163:H7). These combinations of alleles are not present in other STEC strains and can be used as a discriminatory tool because, while other STEC strains from serotypes O26:H11, O111:H−, and O111:H8 possessed the *lpfA1* and *lpfA2* genes, they carried the *lpfA1-2* and *lpfA2-1* alleles in combination with intimin gene types β1 and θ1 (Table 3). Among the EPEC strains (this pathotype represents typical EPEC strains that possess the EPEC adherence factor [EAF] virulence plasmid and carry the *bfp* fimbrial genes), the majority possess only one of the two *lpfA* genes. The majority of the EPEC strains analyzed possess the *lpfA1-1* allele in combination with *eae* type α1 (O55:H6, O127:H6), δ (O86:H34), κ (O86:H34), η1 (O125:H−), η2 (ONT:H45), or μ

TABLE 3. Correlation of *lpfA* types with STEC and EPEC serotypes in a collection of strains from the LREC[a]

LREC no.	Pathotype[b]	Serotype[c]	Country of origin	Intimin (*eae*) type	*lpfA1* type	*lpfA2* type
IH30873A-03	aEPEC	O51:H41	Spain	α1	1	
IH52368A/03	aEPEC	O51:H49	Spain	α1	1	
FV10087	EPEC	O55:H6	United Kingdom	α1	1	
FV10088-2348III	EPEC	O127:H6	Unknown	α1	1	
EPEC-21	EPEC	O127:H−	Unknown	α1	1	
EPEC-23	EPEC	O142:H6	Unknown	α1	1	
IH1658-A	EPEC	O157:H45	Spain	α1	1	
FV10089	EPEC	O157:H45	Switzerland	α1	1	
IH26845A-05	aEPEC	O5:H6	Spain	α2		
IH9661A-04	aEPEC	O20:H6	Spain	α2		
FV10090	aEPEC	O125:H6	Spain	α2		
FV10091	aEPEC	O63:H33	Spain	α2		
FV10092	aEPEC	O132:H34	Spain	α2		
FV10094-IH27256-03-A	STEC	O26:H11	Spain	β1	2	1
O26-7	STEC	O26:H11	Spain	β1	2	1
O26-22	STEC	O26:H11	Spain	β1	2	1
VTH-62	STEC	O118:H16	Spain	β1	2	1
FV10095	EPEC	O111:H2	Uruguay	β1		
EPEC-11	EPEC	O111:H−	Unknown	β1		
IH22561A-04	EPEC	O111:H−	Spain	β1		
FV10096	aEPEC	O177:H11	Spain	β1	2	1
VTB-266	STEC	O177:H11	Spain	β1	2	1
VTB-163	STEC	O177:H11	Spain	β1	2	1
FV12055-IH40495-06-A	aEPEC	O103:H−	Spain	β1	2	1
FV12056-IH46769-07-A	aEPEC	O103:H−	Spain	β1	2	1
FV11582-IH11922A07	aEPEC	O104:H2	Spain	β1	2	
FV5751	aEPEC	O104:H2	Brazil	β1	2	
FV11697-T2932-2	aEPEC	ONT:H7	Brazil	β1	2	1
IH51463A-04	EPEC	O88:H6	Spain	β2		
FV10097	EPEC	O119:H6	Uruguay	β2		
FV10098	aEPEC	O113:H6	Spain	β2		
FV4575	aEPEC	O139:H14	Brazil	β2		
IH2056A-03	EPEC	O167:H6	Spain	β2		
FV10099	EPEC	O167:H6	Brazil	β2		
FV10101-IH34136-03-C	aEPEC	O128:H7	Spain	β3		1
FV10102-IH42584-03-A	aEPEC	O128:H−	Spain	β3		
FV5667	aEPEC	O33:H7	Brazil	γ1	3	2
FV10105-IH28143-03-A	aEPEC	O55:H7	Spain	γ1	3	2
IH44336A-04	aEPEC	O55:H7	Spain	γ1	3	2
IH5027/06A	aEPEC	O55:H7	Spain	γ1	3	2
FV5701	aEPEC	O55:H7	Brazil	γ1	3	2
FV5665	aEPEC	O55:H7	Brazil	γ1	3	2
O157-847	STEC	O157:H7/SF−	Spain	γ1	3	2
O157-881	STEC	O157:H7/SF−	Spain	γ1	3	2
FV10103	STEC	O157:H7/SF−	Spain	γ1	3	2
FV10104-EDL933	STEC	O157:H7	Canada	γ1	3	2
FV10108-FV5570	STEC	O157:H−/SF+	Germany	γ1	3	2
FV10107-FV5569	STEC	O157:H−/SF+	Germany	γ1	3	2
FV5668	aEPEC	O163:H7	Brazil	γ1	3	2
IH7548-05A	aEPEC	ONT:H−	Spain	γ1	5	
IH15752-07A	aEPEC	O2	Spain	γ1	5	
FV10106	aEPEC	O145:H28	Spain	γ1	5	
IH11218-04A	aEPEC	O145:H28	Spain	γ1		
IH38354A/05	STEC	O145:H−	Spain	γ1	5	
IH34365-05A	aEPEC	O145:H−	Spain	γ1		3
IH10248A-05	aEPEC	O2:H40	Spain	θ1		
FV11585-IH8663A06	aEPEC	O2:H49	Spain	θ1		
FV10109	STEC	O111:H−	Spain	θ1	2	1
FV10110	STEC	O111:H8	Germany	θ1	2	1
IH45218A-06	aEPEC	O111:H25	Spain	θ1		
FV10111	EPEC	O127:H40	Uruguay	θ1		
IH5098A-07	EPEC	O131:H46	Spain	θ1		1
FV11695-T1871-1	aEPEC	O34:H−	Brazil	θ2	2	1
IH25556A-03	EPEC	O49:H10	Spain	κ		
FV10114	EPEC	O86:H34	Brazil	δ	1	
FV10112	EPEC	O86:H34	Unknown	κ	1	
IH48480A-06	EPEC	O88:H−	Spain	κ		
FV10113	EPEC	O118:H5	Germany	κ		
FV5713	aEPEC	O1:H45	Brazil	ε1		
FV5718	aEPEC	O21:H38	Brazil	ε1		1
FV10116	aEPEC	O26:H−	Brazil	ε1		
FV5715	aEPEC	O80:H26	Brazil	ε1		
FV10115	STEC	O103:H2	Spain	ε1	2	
FV5676	EPEC	O111:H40	Brazil	ε1		
FV10117	aEPEC	O157:H16	Spain	ε1		
FV10118	aEPEC	O6:H19	Spain	ε2	2	1
FV10119	aEPEC	O103:H19	Brazil	ε2		
FV10120	aEPEC	O123:H19	Spain	ε2	2	1
FV12050-IH9456-07-A	EPEC	O88:H25	Spain	ε2	2	1
FV12051-IH37508-06-A	EPEC	O88:H25	Spain	ε2	2	1

Continued on following page

TABLE 3—*Continued*

LREC no.	Pathotype[b]	Serotype[c]	Country of origin	Intimin (*eae*) type	*lpfA1* type	*lpfA2* type
FV11698–73382-9	aEPEC	O109:H9	Brazil	ε2		1
FV11680-T2332-7	aEPEC	O123:H−	Brazil	ε2		1
FV11681-T1482-11	aEPEC	O123:H−	Brazil	ε2		1
FV11678-T0811-4	aEPEC	ONT:H−	Brazil	ε2		1
FV10121-IH31923-03-A	aEPEC	O181:H−	Spain	ε3		
FV10123-IH37159-03-A	EPEC	O109:H−	Spain	ε4		
FV10142	STEC	O80:H−	Spain	ε5/ξ		
FV10143	aEPEC	O80:H2	Slovakia	ε5/ξ		
FV10144	aEPEC	O157:H−	Spain	ε5/ξ		
FV10124	STEC	O156:H−	Spain	ζ1		1
FV10125	aEPEC	O156:H−	Spain	ζ1		1
FV11212-E110019	aEPEC	O111:H9	Finland	ζ2		1
FV11584-IH46488A07	EPEC	O111:H−	Spain	ζ2		1
FV11677-T2381-8	aEPEC	O154:H9	Brazil	ζ2		1
FV11682-T2232-6	aEPEC	O49:H−	Brazil	ζ2		1
FV10126	aEPEC	O85:H31	Spain	ζ3		
FV10127	EPEC	O125:H−	Burundi	η1	1	
FV10128-IH53199-03-A	aEPEC	ONT:H45	Spain	η2		
FV10129	EPEC	ONT:H45	Switzerland	η2	1	
FV10130	aEPEC	O145:H4	Germany	ι1A		
FV5750	aEPEC	O145:H2	Brazil	ι1A		-
FV11583-IH47502A07	aEPEC	O2:H49	Spain	ι1A		
FV10131	EPEC	O153:H8	Spain	ι1B		1
FV11679-T3281-6	aEPEC	O85:H−	Brazil	ι1B/C		1
FV11868-IH40760A/06	EPEC	O55:H8	Spain	ι1C		1
FV10132	EPEC	O119:H8	Switzerland	ι1C		1
FV10133	aEPEC	ONT:H45	Spain	ι2		
FV11873-IH39717A/03	aEPEC	O101:H−	Spain	ι2		
FV11874-LORENY217.2	aEPEC	O101:H−	Brazil	ι2		
FV10134	aEPEC	O34:H−	Brazil	λ		
FV10135	aEPEC	O33:HNT	Brazil	λ	1	
FV10136	aEPEC	O101:H33	Uruguay	λ		
FV5737	aEPEC	O101:H33	Brazil	λ		
FV5752	aEPEC	O124:H11	Brazil	λ	1	
FV10137	EPEC	O55:H−	Uruguay	μ	1	
FV10138	EPEC	O55:H51	Uruguay	μ	1	
LR-88-1	EPEC	O55:H51	Brazil	μ	1	
FV10139	EPEC	O55:H−	Uruguay	μ	1	
FV10140	aEPEC	O10:H−	Spain	ν		
FV10141	aEPEC	ONT:H−	Spain	ν		
FV10145	aEPEC	O129:H−	Spain	ο		
FV10146	aEPEC	O84:H−	Spain	ο		
FV12052-IH28343-03-A	aEPEC	O84:H−	Spain	ο		
FV12053-IH36299-04-A	aEPEC	O105:H4	Spain	ο	2	1
IH21822A	aEPEC	ONT:H−	Spain	ο		
FV10430-T1551-2	aEPEC	ONT:H−	Brazil	ο		
FV10147	aEPEC	O14:H5	Brazil	π	1	
FV10148	aEPEC	ONT	Brazil	π	1	
FV5724	aEPEC	ONT:H5	Brazil	π	1	
FV10149	aEPEC	O149:H−	Spain	ρ		
FV10150	aEPEC	O149:H−	Spain	ρ		
FV10151	aEPEC	O180:H−	Spain	ρ		
FV10152	aEPEC	O86:H−	United Kingdom	σ		
FV10153	aEPEC	O86:H−	United Kingdom	σ		
FV10425-T0621-6	aEPEC	ONT:H−	Brazil	σ		
FV11772A-T4281-7	aEPEC	O104:H−	Brazil	τ		
FV11696-T1632-7	aEPEC	O26:H−	Brazil	υ		

[a] LREC, *E. coli* Reference Laboratory, Lugo, Spain.
[b] Pathotypes were established based on the presence of virulence factors: EPEC strains were positive for the *bfpA* gene and the presence of the EAF plasmid; aEPEC strains were negative for *bfpA* and EAF; and STEC strains carried the *stx* gene.
[c] SF− and SF+, negative and positive for sorbitol fermentation, respectively.

(O55:H− and O55:H51). In contrast, the majority of the aEPEC strains (which lack the EAF virulence plasmid and the *bfp* genes) possess the *lpfA1-2* and *lpfA2-1* genes in combination with *eae* types β1 and ε2 (Table 3). Overall, these results indicated that a strong correlation exists between the intimin types and the *lpfA* gene variants, and they also suggested that the presence of both *lpfA1* and *lpfA2* alleles is associated with pathogenic *E. coli* strains, particularly with those belonging to the STEC pathotype.

We then independently analyzed a collection of strains isolated predominantly in Chile (collection located at the Center for Vaccine Development, Santiago, Chile), with no relationship to our other reference strains. Sixty-two STEC O157:H7 strains were evaluated for the presence of the *lpfA* and intimin genes. As shown in Table 4, we found a perfect correlation (100%) between the EHEC serotype O157:H7 strains and the intimin (*eae*) types and *lpfA* gene variants. All 62 strains possessed the intimin γ1 gene, and they all carried the *lpfA1-3* and *lpfA2-2* gene combination, strongly supporting the idea that these three genes are reliable markers for the identification of this highly virulent serotype. Then we determined whether a correlation exists between the presence of these genes and the pathotypes in a collection of EPEC strains. As shown in Table 4, different types of *lpfA1* and *lpfA2* genes were found in com-

TABLE 4. Correlation of *lpfA* types with EHEC and EPEC serotypes in a collection of strains from the Center for Vaccine Development, Santiago, Chile

Strain reference no.	Pathotype	Serotype[a]	Intimin (*eae*) type[b]	*lpfA1* type	*lpfA2* type
O157:H7 isolates (n = 62)	STEC	O157:H7	γ1	3	2
Di-304	aEPEC	O63:H6	α2		
PH-78	aEPEC	O125	α2		
Di-12	aEPEC	O15:H25	β1		1
Di-126	aEPEC	O20:H7	β1	2	1
Di-140	aEPEC	O20:H7	β1	2	1
Di-91	aEPEC	O26:H−	β1	2	1
O26-187	STEC	O26:H11	β1	2	1
O26-215	STEC	O26:H11	β1	2	1
J-80	aEPEC	O119:H−	β1	2	1
FV10037	aEPEC	O153:H7	β1	2	1
Di-358	aEPEC	O137:H6	β1		1
Di-178	EPEC	ND	β1		
Di-210	EPEC	O98:HNT	β1	2	1
Di-282	EPEC	O158	β1		1
Di-305	EPEC	O119	β1	2	
Di-333	EPEC	ND	β1	2	
J-11	EPEC	O126	β1	2	1
J-145	aEPEC	ND	β1	2	
J-215	EPEC	ND	β1		
J-275	EPEC	ND	β1		
J-294	EPEC	ND	β1	5	
FV9988	EPEC	O145:H8	γ1	5	
Di-141	aEPEC	O145:H8	γ1	5	1
J-104	EPEC	ND	γ1	2	1
FV9965	EPEC	O23:H8	θ1	2	1
FV9986	aEPEC	O23:H8	θ1	2	1
Di-4	aEPEC	O23:H8	θ1	2	1
J-9	EPEC	O23:H8	θ1	2	1
FV9967	aEPEC	O55:H40	θ1		
FV10039	EPEC	O111:H25	θ1		
J-97	aEPEC	ONT:H40	θ1		
J-98	aEPEC	O55	θ1		
J-151	EPEC	ND	θ1		
Di-174	EPEC	ONT:H19	ε	2	1
Di-219	EPEC	ND	ε	2	1
J-168	EPEC	ND	ε		
PH-11	EPEC	ND	ε		
FV9968	EPEC	O109:H−	ε4		
Di-45	EPEC	ONT:H1,H12	ζ1		
Di-224	EPEC	ND	ζ1		
PH-92	EPEC	O1:H1,H12	ζ1		3
FV9970	EPEC	O1:H1,H12	ζ3		
FV9991	EPEC	O1:H1,H12	ζ3		3
Di-101	EPEC	O86:H8	ι1		1
Di-307	aEPEC	O145:H34	ι1	2	
FV9969	aEPEC	ONT:H4	ο	2	1
J-222	aEPEC	ONT:H4	ο	2	1
FV9984	aEPEC	O76:H2	ρ		
Di-124	aEPEC	O76:H2	ρ		
Di-192	aEPEC	O145:H34	NT		
J-70	EPEC	ND	NT		
J-206	EPEC	ND	NT	1	
J-280	EPEC	ND	NT	5	
J-303	EPEC	ND	NT		
J-349	EPEC	ND	NT		
J-351	EPEC	ND	NT		
J-94	EPEC	ND	NT		3
J-281	EPEC	ND	NT		
PH-32	EPEC	ND	NT		3
PH-74	EPEC	ND	NT	2	3
PH-112	EPEC	ND	NT		
PH-120	EPEC	ND	NT	2	1
S-28	EPEC	ND	NT		

[a] ND, not determined.
[b] NT, not typeable.

bination with intimin gene variants. However, some trends were evident. (i) The majority of *lpfA* genes belong to the *lpfA1-2* and/or the *lpfA2-1* variant. (ii) The strains possessing *lpfA1-2* and *lpfA2-1* type genes also carried intimin gene types β1, θ1, ε, ο, and γ1. (iii) The majority of the Chilean EPEC strains, whether they belonged to the typical or the atypical EPEC pathotype, either possessed both *lpfA1* and *lpfA2* genes or lacked both of these genes. Interestingly, novel trends also emerged from our analysis; for example, we identified strains of serotype O145:H8 carrying a unique combination of *lpfA* genes (*lpfA1-5* with *lpfA2-1*).

Finally, we determined what the probability of association was between the presence of the *lpfA* gene and the type of disease produced by the different pathotypes isolated in Chile (acute diarrhea, bloody diarrhea, or HUS). Our statistical analysis indicated that a strong association existed between the *lpfA* gene and *E. coli* strains carrying the *stx* genes (STEC strains). Further, we found that the *lpfA* (P = 0.0058) and *stx2* (P = 0.0014) genes were significantly associated with HUS. Neither *lpfA* nor *stx* was significantly associated with acute diarrhea or bloody diarrhea. Therefore, we can conclude that *lpfA* and *stx2* are STEC virulence factors showing strong associations with the HUS pathology that can result from a STEC infection; however, additional research is needed to confirm this association with a larger collection of pathogenic *E. coli* strains.

Our own initial studies, combined with observations by other groups, suggested that the *lpf* genes are associated with particular serotypes and/or specific genotypes (16, 28, 32–34). However, further studies indicated that the EHEC O157:H7 *lpfA* genes are widely distributed among DEC strains (31). Our current study confirmed and expanded these observations, because we now demonstrated that certain variants of the *lpfA1* and *lpfA2* genes are restricted to strains carrying intimin type γ (mainly EHEC O157:H7 and EPEC O55:H7). The study by Toma and colleagues tried to understand the relationship between *lpfA* variants and phylogenetic groups (31); unfortunately, at the time of that study, the number of variants available in the database was limited, and the phylogenetic trees obtained were incongruent with the strain phylogeny. Although it is evident that the *lpf* gene clusters are widely distributed in different *E. coli* lineages, and the *lpfA* genes seem not to be specific to EHEC strains, the availability of additional sequences in the database and the incorporation of the different intimin types into our analysis led us to identify specific combinations of genes present only in AEEC strains that are associated with severe and/or epidemic disease. Overall, our results indicate that the combination of these three gene markers (*eae*, *lpfA1*, and *lpfA2*) could be sufficient for performing a quick identification of AEEC isolates, specifically for the quick identification of the highly virulent serotype O157:H7.

One additional task that we are currently undertaking is the complete elucidation of the evolutionary history of the *lpf* gene clusters in DEC strains, as well as in ExPEC, commensal *E. coli*, *Shigella*, and *Salmonella* strains. This analysis includes mapping of the chromosomal location of the *lpf* gene clusters (in EHEC O157:H7, *lpfA1* is linked to O-island 141 [OI-141], while *lpfA2* is located in OI-154) and characterization of the other open reading frames within the *lpf* operons, because studies with other fimbrial gene clusters have suggested that different regions within an operon have diverse evolutionary histories (5). But it is now evident that the acquisition of different *lpf* gene clusters in specific lineages of *E. coli* might be contributing to the emergence of highly virulent strains derived

from commensal organisms, which also possess unique *lpf* variants.

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