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Type 1 Fimbriation and Its Phase Switching in Diarrheagenic *Escherichia coli* Strains

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Type 1 fimbriae can be expressed by most *Escherichia coli* strains and mediate mannose-sensitive (MS) adherence to mammalian epithelial cells. However, the role of type 1 fimbriae in enteric pathogenesis has been unclear. Expression of type 1 fimbriae in *E. coli* is phase variable and is associated with the inversion of a short DNA element (*fim* switch). Forty-six strains of diarrheagenic *E. coli* were examined for the expression of type 1 fimbriae. Only four of these strains were originally type 1 fimbriated. Seventeen strains, originally nonfimbriated, expressed type 1 fimbriae in association with off-to-on inversion of the *fim* switch, after serial passages in static culture. The switching frequencies of these strains, from fimbriate to nonfimbriate, were greater than that of the laboratory strain *E. coli* K-12. None of the 16 strains of serovar O157:H7 or O157:H$^-$ expressed type 1 fimbriae after serial passages in static culture. The nucleotide sequence analysis of the *fim* switch region revealed that all of the O157:H7 and O157:H$^-$ strains had a 16-bp deletion in the invertible element, and the *fim* switch was locked in the "off" orientation. The results suggest that expression of type 1 fimbriae may be regulated differently in different *E. coli* pathogens causing enteric infections.

Adherence is generally an initial, prerequisite step for some strains of *Escherichia coli* in successful colonization of a specific host mucosal tissue (10, 19, 25, 39, 44).

Type 1 fimbriae, which are found on the majority of clinical isolates of *E. coli* (5), bind to mannose-containing receptors on epithelial cells (33) and on leukocytes (2). Type 1 fimbriae may be important in the pathogenesis of urinary tract infections (24, 41) and play an important role in enterobacterial communicability (3). However, the role of type 1 fimbriae in enteric infection remains unclear. A series of investigations using streptomycin-treated mice colonized with a human fecal *E. coli* isolate demonstrated that type 1 fimbriae are expressed in the intestinal tract, in vivo, and may be involved in the colonization of the intestinal tract by *E. coli* (22, 23). It has been shown that type 1 fimbriae are excellent immunogens (13, 37). Thus, the capacity to rapidly switch their expression from "on" to "off" would be advantageous to the organism and consequently important in pathogenesis.

Type 1 fimbriae are encoded by a *fim* gene cluster, including at least nine genes required for its biosynthesis (20, 35), and are composed primarily of the structural subunit, FimA (18). The mannose-sensitive adhesive function is provided by a small amount of the adhesin subunit, FimH, located at the tip of the fimbrial shaft (15). The expression of these fimbriae is phase variable, depending on the orientation of the 314-bp invertible element located between two 9-bp inverted repeats (1). This element contains a promoter which drives the transcription of the *fim* subunit genes in one orientation (on) but not the other (off). This inversion is catalyzed by two site-specific recombinases, FimB and FimE, encoded upstream of this invertible element. FimB can catalyze inversion in both directions (on to off, off to on), but FimE can catalyze inversion in only one direction (on to off) (12, 29).

In this study, we examined the expression and switching frequencies of type 1 fimbriae of diarrheagenic *E. coli* strains. We also showed that all of the O157:H7 and O157:H$^-$ strains tested in this work abolish expression of type 1 fimbriae due to a 16-bp deletion in the invertible element and the locking of the *fim* switch in the "off" orientation.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* strains used in this study are described in Table 1. The strains were collected from Japan (Fukuoka, Kumamoto, Osaka, and Tokyo), Thailand, the United States, Canada, Great Britain, and Denmark. Bacteria were grown in Luria-Bertani (LB) broth (40) containing 5 g of sodium chloride (Wako, Osaka, Japan), 5 g of yeast extract (Difco, Detroit, Mich.), and 10 g of tryptone (Difco) per liter and in LB agar, which was LB broth containing 1.5% agar (Wako). To promote expression of type 1 fimbriae, organisms were serially passaged in a static, nonaerated broth culture of brain heart infusion (BHI) broth (Eiken Chemical, Tokyo, Japan) at 37°C for 10 days in small test tubes as described previously (34). Expression of type 1 fimbriae by bacteria was monitored by the mannose-sensitive hemagglutination (MSHA) test.

Hemagglutination assay. MSHA was determined in phosphate-buffered saline (PBS) with a 2% (wt/vol) suspension of guinea pig erythrocytes, with or without 1% (wt/vol) D-mannose (Sigma, St. Louis, Mo.). Twenty microliters of the erythrocyte suspension with or without D-mannose was placed on a glass slide, and an equal volume of a bacterial suspension (10^8 CFU) was added. The slide was gently rotated for 2 min while monitoring for visible HA was conducted.

PCR amplification of the invertible element and its flanking regions. Chromosome DNA was isolated by using a GenomicPrep Cells and Tissue DNA Isolation Kit according to the manufacturer's instructions (Amersham Pharmacia Biotech, Piscataway, N.J.). PCR amplifications, for the total volume of 100 μl, contained 1× standard PCR buffer (Promega, Madison, Wis.), 2.5 mM of $MgCl_2$, 200 μM each of the four deoxyribonucleotides, 0.25 μM each primer, 2.5 μg of chromosome DNA, and 2.5 U of *Taq* DNA polymerase (Promega). PCR was carried out for a total of 30 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, with a final extension of 5 min at 72°C, using a PC-700 thermal cycler (ASTEC, Fukuoka, Japan). Oligonucleotides used in PCR amplification were as follows. P-3 (5′GT GCATCGAAATATTCGCCATACT-3′) (5′-biotin labeled) and P-2 (5′ACGTC

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CCTGAACCTGGGTAGGTTA-3′) were used for sequencing analysis, and nonbiotin-labeled P-3 and P-4 (5′-AGTCGTTCTGTACACTTTGTTTTG-3′) were used for the orientation analysis of the invertible element. All synthetic oligonucleotides were purchased from Nippon Flour Mills (Tokyo, Japan).

Determination of invertible element orientation. The orientation in the chromosome of the invertible DNA element containing the *fimA* promoter was determined using a PCR-based assay. This method exploited a restriction fragment length dimorphism, which arises out of the orientation-dependent location of a unique *Sna*BI restriction site within the amplified DNA (12) (see Fig. 1A).

Bacteria (10^8 CFU) were harvested from a cell suspension before and after being serially passaged 10 times. This suspension was boiled for 10 min to release genomic DNA. An upstream sequence of the *fimA* gene, including the *fim* switch region, was amplified with oligonucleotides P-3 and P-4 to generate a 554-bp DNA product for the K-12 strain JE2571 (18). Amplified products (2 μl; approximately 0.1 μg) were digested with 5 U of *Sna*BI (TAKARA, Shiga, Japan). Digested PCR products were resolved on 2% agarose gels. Phase-oh populations of bacteria yielded two DNA fragments of 442 and 112 bp, whereas phase-off populations yielded two fragments of 200 and 354 bp (Fig. 1). Mixed populations of both phase "on" and phase "off" contained a mixture of all four fragments.

DNA sequencing analysis. After PCR amplifications, 40 μl of Dynabeads streptavidin (Dynal, Oslo, Norway) was added to an equal volume of PCR products, and amplified DNA was collected in a magnet stand (Dynal). After separation of DNA strands by addition of 40 μl of 0.1 N NaOH, sense strands were collected in a magnet stand. DNA sequencing analysis was performed with a Sequencing PRO Kit (Toyobo, Tokyo, Japan) using single-stranded DNA, primer P-4, and the radioactive deoxyribonucleotide [α-^{33}P]dCTP (Amersham Pharmacia Biotech). Sequencing reactions were carried out according to the manufacturer's instructions.

Measurement of frequency of production of MSHA$^-$ colonies by the MSHA$^+$ colony on plate culture. After promotion of type 1 fimbriation by 10 passages in a static culture, the cell suspension was serially diluted in PBS and spread on an LB plate. After overnight growth, three individual blocks of agar, each bearing a single MSHA$^+$ colony, were cut out, transferred to 5 ml of PBS in separate test tubes, and agitated vigorously. Bacterial-cell suspensions were serially diluted in PBS and spread on the LB plates. The MSHA statuses of 50 randomly selected colonies were determined after overnight growth. The switching frequencies were determined as the means of frequencies from the three separate cultures, as described previously (11).

Phylogenetic characterization. Phylogenetic analysis was conducted with the nucleotide sequence of the 314-bp invertible element. A phylogenetic tree based on DNA sequences of this element was constructed by using the CLUSTAL program (version 1.5) (45).

RESULTS

Expression of type 1 fimbriae and orientation of the *fim* switch. Expression of type 1 fimbriae was monitored by MSHA. At the start of this study, only four strains (96-39, 1646-3, 85/91-5, and CH28991) exhibited the MSHA-positive phenotype. After 2 to 8 consecutive passages in static broth, 17 strains expressed type 1 fimbriae. None of the O157:H7 or O157:H$^-$ strains produced type 1 fimbriae; neither did F76193 (O55:H6), TEC1101, TEC1117, and E98445 (O55:H10), BK005 and O29 (enterotoxigenic *E. coli* [ETEC]), or TL100, E2, and 452-1-1 (enteroaggregative *E. coli* [EAggEC]) (Table 1).

The expression of type 1 fimbriae is phase variable and depends on the orientation of an invertible 314-bp DNA switch (1). In *E. coli* strain K-12, an asymmetric *Sna*BI restriction site within the 314-bp invertible element, upstream of the *fimA* gene, allows for the determination of the orientation of the switch responsible for phase variation (12). When DNAs were amplified by PCR with one set of primer sequences upstream of the *E. coli* K-12 *fimA* gene including an invertible sequence and then digested with *Sna*BI, DNA from MSHA$^-$ strains showed fragments of 354 and 200 bp, representing the "off" orientation, while MSHA$^+$ strains showed fragments of 442 and 112 bp, representing the "on" orientation (Fig. 1A and Fig. 1B, lanes 15 and 16). DNA from MSHA$^-$ strains (before and after passages) amplified with the same primers and digested with *Sna*BI indicated that *fim* switches were in the "off" orientation (Fig. 1B, lanes 1, 2, 3, 4, 5, 6, 7, 9, 11, and 13). Restriction analysis of PCR products from the strains which became MSHA$^+$ after serial passages showed the presence of all four *Sna*BI bands, indicating that MSHA$^+$ strains were composed of a mixture of MSHA$^+$ and MSHA$^-$ cells. (Fig. 1B, lanes 8, 10, 12, and 14).

These results confirm that the expression of type 1 fimbriae and the orientation of the *fim* switch are related as described previously (1).

DNA sequencing analysis. PCR products flanking the switch region in the phase-off orientation were directly sequenced. Sequences are shown in Fig. 2. The PCR products of eight strains (TEC1101, TEC1117, E98445, BK005, O29, TL100, 17-2, and 452-1-1) were not amplified, and DNA sequences of these strains could not be determined.

All 16 strains of serovar O157:H7 or O157:H$^-$ had perfectly identical sequences and had a unique 16-bp deletion (bp 66 to 81) in the switch region (Fig. 2). This deletion included 2 bp (bp 80 to 81) of a putative binding site of integration host factor (IHF), which is required for inversion of the *fim* switch (4). This deletion was not observed in the other strains tested in this study.

DNA sequence comparison of invertible elements of *E. coli* K-12 and clinical isolates used in this study revealed that nucleotide changes (A to C at bp 110; C to A at bp 218, and T to G at bp 278) and insertions (A at bp 259; A at bp 283) were observed in most of the pathogenic strains (Fig. 2).

Switching frequency of MSHA$^+$ to MSHA$^-$ on plate culture. The frequencies of MSHA$^+$-to-MSHA$^-$ switching were measured for strains expressing the MSHA$^+$ phenotype. Data are shown in Table 2. The pathogenic strains expressing the MSHA$^+$ phenotype showed MSHA$^+$-to-MSHA$^-$ changes on plate culture; however, the K-12 strain DH5α did not produce any MSHA$^-$ colonies.

The frequency of MSHA$^+$-to-MSHA$^-$ phenotype switching was high (>0.1 per cell per generation) in non-O157 Shiga-like toxin-producing *E. coli* (STEC) strains. Enteropathogenic *E. coli* (EPEC) strains showed both higher and lower frequencies than STEC strains. The switching frequencies for ETEC and EAggEC strains were lower than those for other pathogenic *E. coli* strains.

Phylogenetic analysis based on sequences of the switching element. A phylogenetic tree based on the DNA sequences of the *fim* switch region was constructed (Fig. 3). Strains of the same serotypes were clustered into closely related or identical branches.

This phylogenetic tree indicates that the O157:H7, O157:H$^-$, and O55:H7 strains were clustered into a single branch, while O111:H$^-$ strains formed another distinct branch. The O26:H11 strains and strains CH5667 and O22 (ETEC) formed a third branch. The other strains represented a fourth branch. The O157:H45 and O55:H6 strains were clustered together and were related to the O111:H12 and EAggEC strains, but they were quite divergent from the O157:H7 and O55:H7 strains.

TABLE 1. Bacterial strains used in this study

Strain	Serotype	Description	MSHA[a]	16-bp deletion in invertible element[b]	Source or reference[c]
STEC strains					
96-7	O157:H7	ST1, ST2	−	+	FIHES
96-24	O157:H7	ST1, ST2	−	+	FIHES
98E1	O157:H7	ST1, ST2	−	+	FIHES
98E2	O157:H7	ST2	−	+	FIHES
98E3	O157:H7	ST1, ST2	−	+	FIHES
98E5	O157:H7	ST1, ST2	−	+	FIHES
CL-49	O157:H7	ST2	−	+	6
86-24	O157:H7	ST2	−	+	14
467	O157:H7	ST2	−	+	FCIHE
468	O157:H7	ST2	−	+	FCIHE
Sakai	O157:H7	ST1, ST2	−	+	31
E333	O157:H$^-$	ST2	−	+	FCIHE
98E6	O157:H$^-$	ST2	−	+	FIHES
E32511	O157:H$^-$	ST2	−	+	28
97E12	O26:H11	ST1	+	−	FIHES
97E16	O26:H11	ST1	+	−	FIHES
97E2	O111:H$^-$	ST1	+	−	FIHES
97E22	O111:H$^-$	ST1	+	−	FIHES
Non-ST-producing O157 strains					
FSE5	O157:H7		−	+	FIHES
FSE13	O157:H7		−	+	FIHES
96-39	O157:H45		+*	−	FIHES
96-11	O157:H45		+	−	FIHES
EPEC strains					
1646-3	O55:H$^-$		+*	−	IMSUT
2	O55:H6		+	−	SSI
F76193	O55:H6		−	−	SSI
85/91-5	O55:H7		+*	−	SSI
70	O55:H7		+	−	SSI
F57076	O55:H7		+	−	SSI
PE163	O55:H7		+	−	OPIPH
PE182	O55:H7		+	−	OPIPH
807-257	O55:H7		+	−	OPIPH
F43341	O111:H12		+	−	SSI
118	O111:H12		+	−	SSI
TEC1101	O55:H10		−	ND	TMRLPH
TEC1117	O55:H10		−	ND	TMRLPH
E98445	O55:H10		−	ND	TMRLPH
ETEC strains					
BK005			−	ND	RIMDOU
CH5667			+	−	CSTRI
CH28991			+*	−	CSTRI
O22			+	−	This laboratory
O29			−	ND	This laboratory
EAggEC strains					
TL100			−	ND	51
17-2			+	ND	46
E2			−	−	47
253-1-1			+	−	RIMCJ
452-1-1			−	ND	RIMCJ
Laboratory strain (DH5α)			+	−	40

[a] Determined by hemagglutination of guinea pig erythrocytes with or without 2% D-mannose. +, hemagglutinating strain after serial passages; +*, originally hemagglutinating strain (at start of this study).

[b] ND, not determined; no amplification in PCR with primers used in this study.

[c] FIHES, Fukuoka Institute of Health and Environmental Sciences; FCIHE, Fukuoka City Institute for Hygiene and Environment; IMSUT, The Institute of Medical Science, The University of Tokyo; SSI, The State Serum Institute, Copenhagen, Denmark; OPIPH, The Osaka Prefectural Institute of Public Health; TMRLPH, The Tokyo Metropolitan Research Laboratory of Public Health; RIMDOU, The Research Institute for Microbial Diseases, Osaka University; CSTRI, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan; RIMCJ, The Research Institute, International Medical Center of Japan.

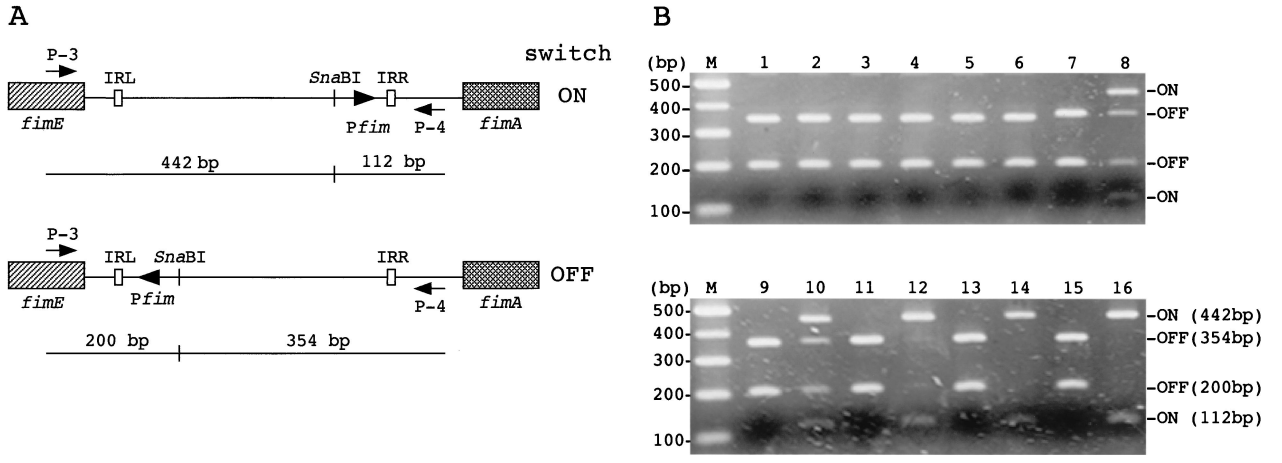

FIG. 1. Orientation of the switch-regulating phase variation in *E. coli*. (A) Schematic representation of the *fim* switch showing the positions of primers and restriction sites used in this study. The orientation of the *fim* switch can be determined by combined PCR and restriction analysis, since *Sna*BI cuts asymmetrically. IRL and IRR, left and right inverted repeats, respectively. The *fimA* promoter (P*fim*) is indicated. (B) Agarose gel (2%) electrophoresis of *Sna*BI digests of PCR products. Lanes M, molecular weight markers; lanes 1 and 2, 96-7 (O157:H7); lanes 3 and 4, CL-49 (O157:H7); lanes 5 and 6, E333 (O157:H$^-$); lanes 7 and 8, 96-11 (O157:H45); lanes 9 and 10, 97E12 (O26:H11); lanes 11 and 12, 97E2 (O111:H$^-$); lanes 13 and 14, 70 (O55:H7); lanes 15 and 16, DH5α. Lanes 1, 3, 5, 7, 9, 11, 13, and 15 are from strains amplified at the start of this study (MSHA$^-$); lanes 2, 4, and 6 (MSHA$^-$) and lanes 8, 10, 12, 14, and 16 (MSHA$^+$) are from strains amplified after 10 static serial passages.

DISCUSSION

We investigated the expression of type 1 fimbriae in *E. coli* isolates from gastrointestinal tract infections. Of the 46 strains used in this study, only 4 strains were originally type 1 fimbriated. It has been shown that certain growth conditions favor the isolation of fimbriate bacteria (growth in static broth, anaerobic growth), whereas other conditions favor afimbriate bacteria (exponential growth in well-aerated broth, growth on agar) (34). After serial passages in static culture, 17 strains expressed type 1 fimbriae in association with *fim* switch off-to-on inversion (Table 1 and Fig. 1). None of the 16 strains belonging to serotype O157:H7 or O157:H$^-$ expressed type 1 fimbriae after more than 20 passages in static culture (Table 1). Frequencies of switching from an MSHA$^+$ to an MSHA$^-$ phenotype were greater in EPEC and STEC strains than in the K-12 strain (Table 2).

Oral challenge of humans with EPEC strain E2348/69 resulted in an immune response to EPEC type 1 fimbriae (16). Type 1 fimbriae might be expressed during the course of EPEC infections. The type 1 fimbriated strain was more susceptible to phagocytosis than the nonfimbriated strain (32), and type 1 fimbriae were found to be a good immunogen (21, 37). There is no consensus, on the role of type 1 fimbriae, either in susceptibility to phagocytosis or in pathogenicity. Type 1 fimbriae may be disadvantageous for bacteria in the presence of phagocytes, but other reports indicate that they could be advantageous for bacteria which are entering the circulatory system or are located within phagocytes (17, 27).

Type 1 fimbriae might be disadvantageous for *E. coli* strains which colonize the mucosa by an attaching and effacing (A/E) lesion, because they induce an immune response (16). In the present study, STEC strains other than O157:H7 and O157:H$^-$ serotypes produced type 1 fimbriae after serial passages in static culture. Serogroup O157:H7 strains are most prevalent among STEC strains. The type 1 afimbriated situation might be advantageous for O157:H7 strains.

Sherman et al. (42) and Durno et al. (6) reported that the *E. coli* O157:H7 strain CL-49 expressed type 1 fimbriae after serial passages in static culture and could adhere to human and rabbit epithelial cells, while binding could not be demonstrated for type 1 afimbriated O157:H7 strains. However, we could not isolate any type 1 fimbriated O157:H7 or O157:H$^-$ strains, after serial passages of 16 O157:H7 and O157:H$^-$ strains, including strain CL-49, in static culture. DNA sequence analysis revealed that all 16 strains had a 16-bp deletion in the invertible element and that the *fim* switch was locked in the "off" orientation. It would be of interest to elucidate the mechanisms of the type 1 fimbrial expression of CL-49 in the studies cited above.

Li et al. (26) demonstrated that a 16-bp sequence 5′ to *fimA* was absent in O157 strains and suggested that a PCR assay using the primer flanking the 16-bp deletion offers a simple, rapid, and reliable means to detect *E. coli* strains of the O157:H7 serotype. However, they did not investigate if fimbrial expression was affected by this 16-bp deletion.

Enami et al. (9) reported that expression of type 1 fimbriae was not observed in verotoxin-producing *E. coli* O157, though the genetic mechanism for the lack of expression of type 1 fimbriae was not studied.

We demonstrated that the 16-bp deletion in the *fim* switching region is a possible molecular mechanism responsible for the lack of type 1 fimbrial expression of serovars O157:H7 and O157:H$^-$. Pathogenic strains have obtained virulence genes such as *stx* or *eae* in the case of STEC. Such bacteria may abandon or destroy some gene systems while acquiring others. Type 1 fimbrial expression might be subject to such alterations in the genetic determinant in O157:H7 strains.

A phylogenetic tree was constructed based on the DNA

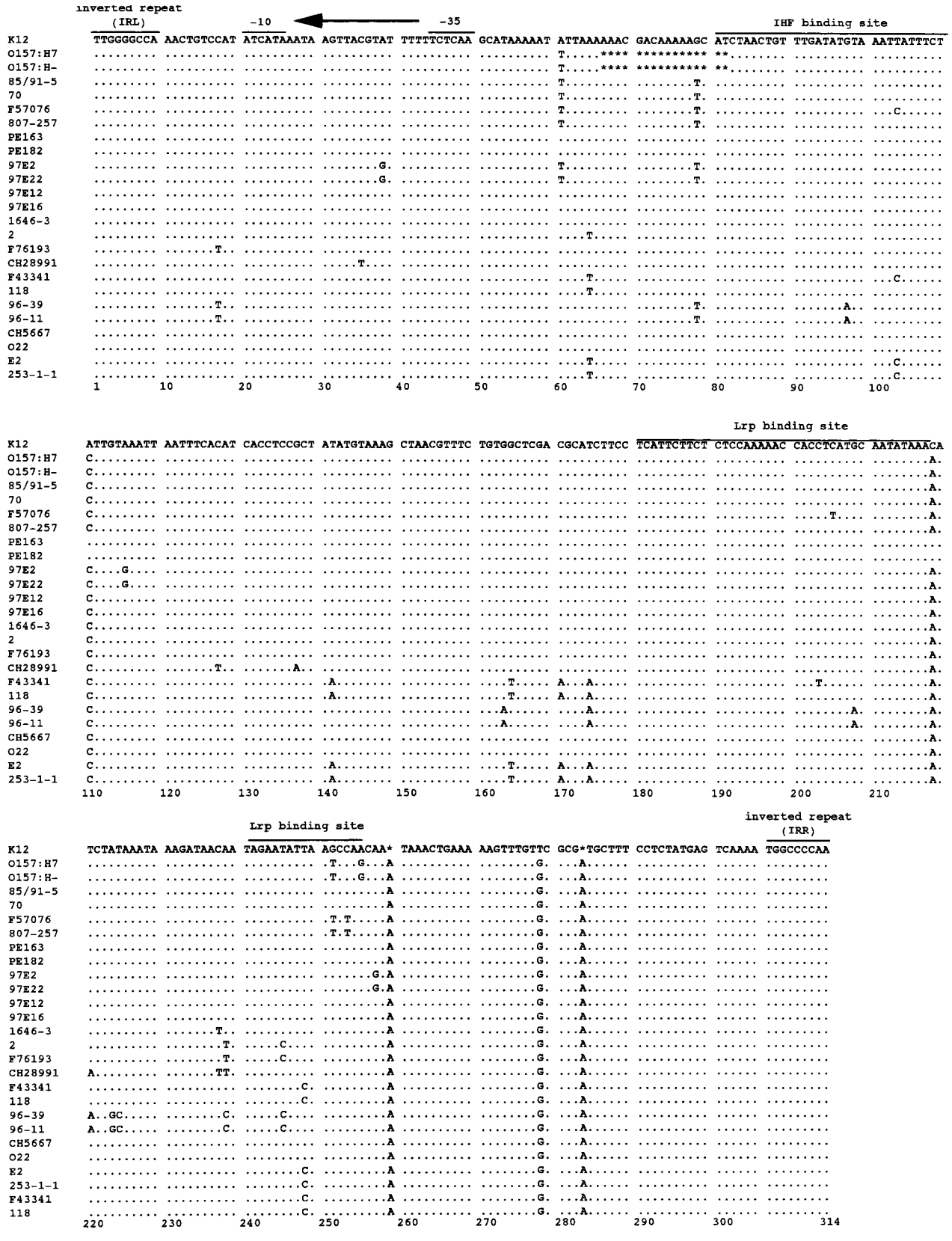

FIG. 2. DNA sequence comparison of the invertible element of *E. coli* K-12 (GenBank accession number X00981) and strains used in this study. O157:H7 and O157:H$^-$ strains have completely identical sequences. Dots indicate identity; stars indicate gaps. These sequences are shown in phase-off orientation. The inverted repeats that border the *fim* switch and the *fimA* promoter (1) are overlined and allow for the indication of the direction of transcription. Binding sites for IHF (4) and Lrp (38) are also shown.

TABLE 2. On-to-off switching in strains used in this study

Strain	Serotype	Switching frequency[a]
STEC strains		
97E12	O26:H11	0.101
97E16	O26:H11	0.147
97E2	O111:H$^-$	0.168
97E22	O111:H$^-$	0.154
Non-ST-producing O157 strains		
96-39	O157:H45	0.110
96-11	O157:H45	0.101
EPEC strains		
1646-3	O55:H$^-$	0.186
2	O55:H6	0.035
85/91-5	O55:H7	0.134
70	O55:H7	0.021
F57076	O55:H7	0.101
PE163	O55:H7	0.001
PE182	O55:H7	0.029
807-257	O55:H7	0.077
F43341	O111:H12	0.077
118	O111:H12	0.134
ETEC strains		
CH5667		0.027
CH28991		0.001
O22		0.004
EAggEC strains		
17-2		0.022
253-1-1		0.026
K-12 strain (DH5α)		0[b]

[a] Switching frequency per cell per generation. Rates were determined as the means of the frequencies calculated from three separate colonies based on a method described previously (11).

[b] The transition rates from fimbriate to nonfimbriate of the K-12 strain CSH50 were reported as 0.001 per cell per generation (7).

sequence of the *fim* switching region (Fig. 3). Strikingly, the DNA sequence used as a basis for construction of the phylogenetic tree with the strains used in this study almost perfectly reflected the clonal relationships among *E. coli* strains associated with enteric disease, as defined by Whittam et al. (49), who compared the mutilocus enzyme profiles of *E. coli* strains. We are convinced that the ubiquity of type 1 fimbriae and the moderate evolutionary divergence of their DNA sequences make them very useful molecular chronometers for phylogenetic analyses.

It has not been known whether *E. coli* type 1 fimbriae play a direct role in enteric pathogenesis (8, 22, 23, 30). Type 1 fimbrial adhesin specifically binds mannose, which is ubiquitous in mammalian cell membranes. Thus, these structures have the potential to attach to a wide variety of host cells. It is possible that the environment within the host induces a transient expression of fimbrial structures. It has also been reported that type 1 fimbriae or MSHA fimbriae are required for biofilm formation in *E. coli* (36) and *Vibrio cholerae* (48). Type 1 fimbriae may promote survival in aquatic environments, such as wells and ponds, by allowing fimbriated cells to form biofilms at the water-air interface, thereby contributing to bacterial survival and the outbreak of disease.

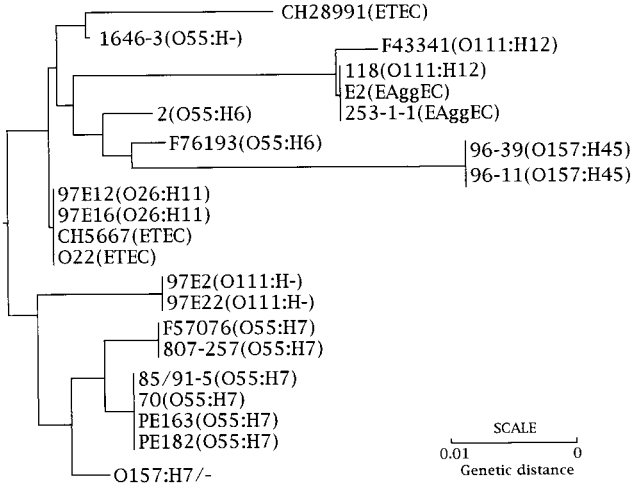

FIG. 3. Phylogenetic tree showing the genetic relationship among the strains used in this study on the basis of DNA sequences of invertible elements. The phylogenetic tree was constructed with CLUSTAL, version 1.5 (45). The program initially calculates pairwise similarity scores based on the method of Wilbur and Lipman (50). Based on these scores, the program constructs a phylogenetic tree using the average linkage cluster analysis method (43).

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