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Detection and Sequences of the Enteroaggregative *Escherichia coli* Heat-Stable Enterotoxin 1 Gene in Enterotoxigenic *E. coli* Strains Isolated from Piglets and Calves with Diarrhea

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Enterotoxigenic *Escherichia coli* (ETEC) strains isolated from piglets and calves with diarrhea were tested for the presence of the enteroaggregative *E. coli* enterotoxin 1 (EAST1) gene sequences by PCR and colony hybridization. The EAST1 gene was found in most porcine ETEC strains with adherence factor K88, especially in those elaborating heat-labile enterotoxin. One porcine ETEC strain with adherence factor K99 was also positive for the EAST1 gene. In contrast, 987P-positive (987P$^+$) ETEC strains from piglets, K99$^+$ ETEC strains from calves, and K99$^+$ F41$^+$ or F41$^+$ ETEC strains from piglets and calves were negative for the EAST1 gene. The K88ab$^+$ or K88ac$^+$ ETEC strains tested possessed the EAST1 gene on a plasmid that was distinct from a K88-encoding plasmid. The EAST1 gene sequences of the K88$^+$ ETEC strains were identical to each other and 99.1 and 98.3% homologous to the previously reported sequences of ETEC strains colonizing humans and enteroaggregative *E. coli* strains, respectively. The data indicate that the EAST1 gene is distributed among porcine ETEC strains in association with the adherence factor type.

Escherichia coli is a normal inhabitant of the intestinal tracts of animals. However, particular *E. coli* strains such as enterotoxigenic *E. coli* (ETEC) (10, 18, 32) and Vero cytotoxin-producing *E. coli* (30) are associated with diarrhea; ETEC has been proved to be a dominant bacterial agent of diarrheal diseases in piglets and calves (1, 14).

Adherence to mucosa is the initial, essential step in the development of pathogenicity for diarrhea-associated *E. coli* (16). Most ETEC strains from piglets with diarrhea possess the pilus adherence factor K88 (including K88ab, K88ac, and K88ad), 987P, K99, or F41 (10, 32); of those, K88 is the predominant adherence factor (27, 34, 36). In contrast, ETEC strains from calves with diarrhea, in most cases, possess K99 alone or a combination of K99 and F41 (10, 32).

ETEC strains elaborate heat-labile enterotoxin (LT) that is similar to cholera toxin and/or heat-stable enterotoxin I (STI) or II (STII) (3, 11). K88-positive (K88$^+$) ETEC strains are usually LT$^+$, LT$^+$, STI$^+$, or LT$^+$ STII$^+$, and K99$^+$ or 987P$^+$ ETEC strains are STI$^+$ in most cases (12, 22, 34).

Some strains of enteroaggregative *E. coli* (EAggEC), the most recently recognized category of human diarrheagenic *E. coli*, elaborate an enterotoxin of 38 amino acids named EAggEC heat-stable enterotoxin 1 (EAST1) (28, 29). EAST1 of EAggEC and STI of ETEC are genetically and immunologically distinct enterotoxins (29). EAST1 is detected in the rabbit intestinal model (28), and STI is detected in suckling mice (4). EAST1 activates guanylate cyclase, as does STI (28). The EAST1 gene was also found in ETEC strains pathogenic for humans (37) or in a subgroup of diffusely adhering *E. coli* (DAEC) strains isolated from patients with diarrhea (39).

In this study, we investigated the presence of the EAST1 gene in ETEC strains isolated from piglets and calves with diarrhea and determined the EAST1 gene sequences distributed among those ETEC strains.

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MATERIALS AND METHODS

Bacterial strains. The ETEC strains examined in this study were all isolated from piglets or calves with diarrhea. Seventeen K88$^+$ strains were used. One K88ab$^+$ strain (E68, serotype O141:H4 [25]) was isolated in England and was provided by I. Ørskov (International Escherichia Centre, World Health Organization, Copenhagen, Denmark). Of 12 K88ac$^+$ strains, strains Abbotstown (serotype O149:H10 [26]) from Ireland, A2 (serotype O157:H19 [9]) from Ireland, and G1253 (serotype O147:H19 [25]) from England were provided by I. Ørskov. The remaining nine K88ac$^+$ strains were isolates from the National Institute of Animal Health, Tsukuba, Japan. Four K88$^+$ strains (isolates from the National Institute of Animal Health, Japan) were not tested for their K88 subtypes (ab, ac, or ad). Of eight 987P$^+$ strains used, strain 987 (an isolate from the United States [serotype O9:HNM [13]) was provided by I. Ørskov. The remaining seven 987P$^+$ strains were isolates from the National Institute of Animal Health, Japan. K99$^+$ strains (11 strains) included strain B117 from England (serotype O8:HNM [24]), which was provided by I. Ørskov, and strains H1914 (serotype O101:HNM), H1915 (serotype O8:H9), H1918 (serotype O141:HNM), H1763 (serotype O9:HNM), and H1764 (serotype O9:HNM) from The Netherlands, which were provided by P. A. M. Guinée (National Institute of Public Health, Bilthoven, The Netherlands); they were all isolates from bovines (5). The remaining five K99$^+$ strains were isolates from bovines or piglets from the National Institute of Animal Health, Japan. Three K99$^+$ F41$^+$ strains included bovine strain B41 (serotype O101:HNM [21]) from England, which was provided by P. A. M. Guinée, porcine strain 431 (serotype O101:HNM [21]) from the United States, and one bovine strain from the National Institute of Animal Health, Japan. F41$^+$ strains (two strains) were porcine strain 1706 (serotype O101:HNM [2]) from the United States, which was provided by H. W. Moon (National Animal Disease Center, Ames, Iowa) and one bovine isolate from the National Institute of Animal Health, Japan. Bovine F17$^+$ strain 25KHO9 (serotype O101:HNM [19]) from Belgium was provided by P. Pohl (National Institute of Veterinary Research, Brussels, Belgium). The F165$^+$ nontoxigenic, porcine strain 4787 (serotype O115:H51 [8]) from Canada was provided by J. M. Fairbrother (University of Montreal, Montreal, Quebec, Canada).

EAggEC strain 17-2 (29) and diffusely adhering *E. coli* strain 73-1 (39) were used as reference strains in the PCR assay; they were isolated from patients with diarrhea and were provided by J. B. Kaper (University of Maryland School of Medicine, Baltimore). *E. coli* HB101 is a hybrid of *E. coli* K-12 and *E. coli* B and lacks restriction ability.

PCR assay. For the detection of the EAST1 gene sequences by PCR, *E. coli* strains grown on nutrient agar (Eiken Chemical Co., Tokyo, Japan) overnight at 37°C were suspended in sterile water at a concentration of ca. 5×10^7 cells per ml and were boiled for 5 min. A 2.5-μl portion of the DNA solution was subjected to PCR. *E. coli* DNA for PCR was also prepared essentially as de-

scribed previously (20). Bacterial cells grown overnight at 37°C in L broth (17) (1.5 ml each) were lysed by the addition of sodium dodecyl sulfate (0.5%) and proteinase K (100 μg/ml). After treatment with cetyltrimethylammonium bromide (in the presence of 0.7 M NaCl) and subsequently with chloroform-isoamyl alcohol (24:1) and phenol-chloroform-isoamyl alcohol (25:24:1), DNA in the aqueous solution was precipitated with 0.6 volume of isopropanol. DNA was then rinsed with 70% ethanol and was redissolved in 100 μl of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The DNA solution was diluted 10-fold with sterile water and a 1- to 2.5-μl portion of the diluted DNA solution was used for PCR.

PCR primers were designed on the basis of the reported sequence of EAggEC strain 17-2 (29): EAST11a, 5′-CCATCAACACAGTATATCCGA (corresponding to the 2nd to 8th codons), and EAST11b, 5′-GGTCGCGAGTGACGGCT TTGT (corresponding to the 38th [C terminus] to 32nd codons). This primer set generated a 111-bp product. PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM $MgCl_2$) and DNA polymerase (*Thermus aquaticus* DNA polymerase; Takara Shuzo, Kyoto, Japan) were used according to the manufacturer's instructions; the error rate of the DNA polymerase was 10^{-5}/nucleotide. The same DNA polymerase was used for all tests in this study. Cycling conditions were denaturation for 30 s at 95°C, annealing for 120 s at 55°C, and polymerization for 120 s at 72°C (30 cycles). The amplified products were analyzed by electrophoresis in 2% agarose gel or a 5% polyacrylamide gel. ϕX174 replicative-form, *Hae*III-digested DNA fragments (Life Technologies, Gaithersburg, Md.) were used as molecular size standards.

Two additional primer sets were also used: EAST12a (5′-ACGATATCCTC ATCGCCTGTG) and EAST12b (5′-CTGCTGGCCTGCCTCTTCCGT), generating a 203-bp product, and EAST13a (5′-AGAACTGCTGGGTATGTGG CT) and EAST13b (5′-GTTGGATAAGCGAAGAACGTG), generating a 393-bp product. The primer sequences EAST12a and EAST13a are located 24 and 110 nucleotides, respectively, upstream from the initiation ATG sequence of the EAST1 gene, and the primer sequences EAST12b and EAST13b are located 20 and 124 nucleotides, respectively, downstream from the stop TGA sequence of the EAST1 gene; these primers were constructed on the basis of the determined nucleotide sequence of the EAST1 gene cloned from DAEC strain 73-1 (39).

Plasmid analysis. Plasmids in *E. coli* were analyzed essentially by a previously described method (15), with the following modifications (38). Bacterial cells grown in L broth (5 ml) were suspended in 100 μl of 40 mM Tris-acetate (pH 7.9) containing 2 mM EDTA (pH 7.9) in a 1.5-ml microcentrifuge tube. This was followed by the addition of 200 μl of lysis solution (3% sodium dodecyl sulfate, 50 mM Tris, 0.128 N NaOH) at room temperature. After being mixed by brief agitation, the solution was heated to 55°C for 20 min. The solution was then mixed with 600 μl of phenol-chloroform (1:1; vol/vol) by shaking briefly. It was then centrifuged at room temperature, and the resultant upper aqueous phase (~160 μl) was retained. Plasmid DNA thus prepared (10 μl) was electrophoresed in 0.3 or 0.7% agarose with reference plasmid DNAs of known molecular size (including the NR1 plasmid of 94.5 kbp [35]).

Raffinose-positive, K88$^+$ plasmid analysis. The plasmid DNA (100 μl), obtained as described above from K88$^+$ strains, was precipitated with ethanol and suspended in 50 μl of 10 mM Tris-HCl (pH 7.5). This was mixed with 200 μl of 50 mM $CaCl_2$-treated competent cells of *E. coli* HB101 (raffinose-negative), and raffinose-positive transformants (red colonies on a colorless cell lawn of HB101 cells) were selected on raffinose (1%)-containing MacConkey agar (Eiken Chemical Co.). The raffinose-positive colonies were then tested for K88 antigens by using antiserum to K88. Raffinose-positive, K88$^+$ plasmid DNA (7, 31) was analyzed as described above.

DNA hybridization. The PCR product generated from strain 17-2 with the primer set EAST11a and EAST11b was purified, labeled with digoxigenin by using a DNA labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and used as a probe. Southern hybridization was done as described previously (6) by using a nylon membrane (Life Technologies). DNA hybrids on the membrane were treated with alkaline phosphatase-conjugated antidigoxigenin antibody and then with 3-(2′-spirodamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane (substrate) by using the DIG luminescent detection kit (Boehringer Mannheim Biochemicals). The resultant chemiluminescence was detected by exposing the membrane to X-ray film. Colony hybridization (6) was also done as described above.

DNA sequencing. For sequencing of the EAST1 gene, the PCR products were cloned into a cloning vector (pT7Blue; Novagen Inc., Madison, Wis.) in accordance to the manufacturer's instruction, and the nucleotide sequence was determined by using the *Taq* dye deoxy terminator cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and the 373A DNA sequencer (Applied Biosystems) according to the manufacturer's instructions.

RESULTS

PCR assay of K88$^+$ strains. K88ab$^+$ strain E68 and K88ac$^+$ strain Abbotstown were tested for the EAST1 gene by PCR. In both strains, primer sets EAST11a-EAST11b and EAST12a-EAST12b gave PCR products of the expected sizes (Fig. 1, lanes 1 and 2, respectively). Although primer set EAST13a-EAST13b produced negative results, primer set EAST13a-EAST12b produced positive results (Fig. 1, lane 3).

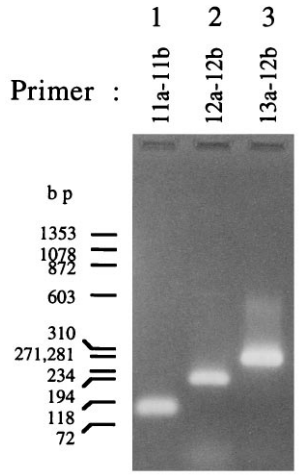

FIG. 1. Analysis of K88$^+$ enterotoxigenic *E. coli* strains for the EAST1 gene by PCR. PCR was conducted with the primer sets EAST11a-EAST11b, EAST12a-EAST12b, and EAST13a-EAST12b (see text); the expected PCR products were 111, 203, and 289 bp, respectively. The strain used for this figure was K88ab$^+$ strain E68. PCR products were analyzed by electrophoresis in a 2.0% agarose gel and were stained with ethidium bromide. The ϕX174 replicative-form, *Hae*III-digested DNA fragment sizes are given on the left. The same results shown here were also obtained with K88ac$^+$ strain Abbotstown.

Location of the EAST1 gene. Plasmids of strains E68 and Abbotstown were examined for the EAST1 gene by hybridization. For this, the PCR product of EAggEC strain 17-2 obtained with primer set EAST11a-EAST11b was labeled with digoxigenin and was used as a probe. Although strains E68 and Abbotstown possessed multiple plasmid species of different molecular sizes (Fig. 2A), only one plasmid species was positive for the EAST1 gene in each strain (Fig. 2B): they were the 58-kbp plasmid for strain E68 and the 85-kbp plasmid for strain Abbotstown (indicated by the arrows in Fig. 2A).

When *E. coli* HB101 was transformed with plasmid DNAs from strains E68 and Abbotstown, the raffinose-positive, K88$^+$ plasmids from the two strains were found to be 75 and 77 kbp in molecular size, respectively (indicated by arrowheads in Fig. 2A).

Nucleotide sequence of the EAST1 gene. For sequencing of the EAST1 gene, the PCR products were generated from strains E68 and Abbotstown with primer set EAST13a-EAST12b and were then cloned into a cloning vector. Sequence analysis of the PCR products was done by using four clones of the recombinant plasmids, and determination of the sequences of both DNA strands was done for each clone. The EAST1 gene sequences of strains E68 and Abbotstown were identical to each other. They differed from the EAST1 gene sequences of ETEC or DAEC strains isolated from patients with diarrhea by one base at the eighth codon position (CG<u>A</u> →CG<u>G</u>) (Fig. 3). They also differed from the EAST1 gene sequence of EAggEC strain 17-2 by two bases at the 8th codon position (CG<u>A</u>→CG<u>G</u>) and 21st codon position (<u>A</u>CA→<u>G</u>C A), resulting in a deduced amino acid change at the 21st position (Thr→Ala) (Fig. 3).

To confirm the 21st codon sequence (GCA), PCR products generated from strains E68 and Abbotstown with the primer set EAST12a-EAST12b were purified and digested with re-

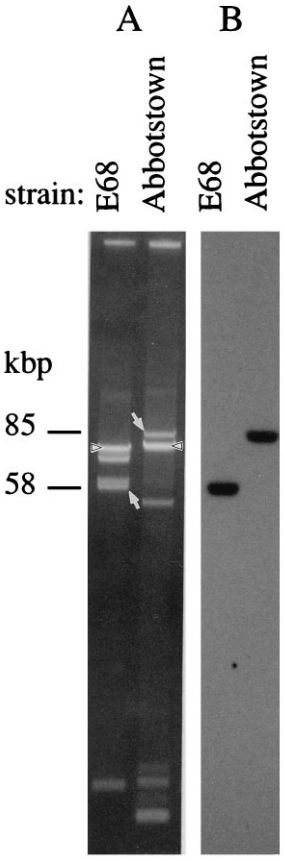

FIG. 2. Southern hybridization analysis of plasmids of K88ab$^+$ and K88ac$^+$ ETEC strains E68 and Abbotstown. (A) Agarose gel (0.3%) after electrophoresis and ethidium bromide staining; (B) results of Southern hybridization with 111-bp PCR products generated with primer set EAST11a-EAST11b and labeled with digoxigenin as a probe. The arrows in panel A indicate the EAST1 plasmids (58 kbp for strain E68 and 85 kbp for strain Abbotstown), and the arrowheads indicate the K88-encoding plasmid (75 kbp for strain E68 and 77 kbp for strain Abbotstown).

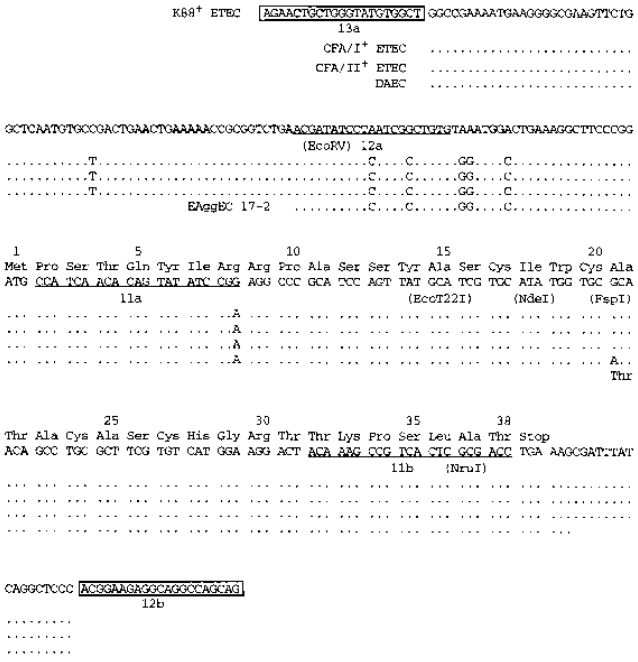

FIG. 3. Nucleotide sequences of the PCR products generated from K88ab$^+$ and K88ac$^+$ ETEC strains E68 and Abbotstown with primer set EAST13a-EAST12b. The nucleotide sequence determined for strains E68 and Abbotstown is shown in the first (top) line of the nucleotide sequences; the deduced amino acid sequence of EAST1 is shown above the nucleotide sequence (the sequences of the two strains were identical to each other). The EAST1 nucleotide sequences of a human-colonizing CFA/I$^+$ strain (H10407), a CFA/II$^+$ strain (H10176), a DAEC strain (73-1), and EAggEC strain 17-2 are from references 37, 37, 39, and 29, respectively, and are shown on the second, third, fourth, and fifth lines of the nucleotide sequences, respectively; nucleotides that are identical to those for K88$^+$ enterotoxigenic *E. coli* are indicated as dots. In the case of strain 17-2, only amino acids of EAST1 that differ from those for the K88$^+$ strains are shown below the nucleotide sequence. The sequences and positions of primers EAST13a and EAST12b are marked with boxes. Underlines indicate the positions of primers EAST11a, EAST11b, and EAST12a.

striction enzyme *Fsp*I. The two digestion experiments showed a single *Fsp*I site on each PCR product (data not shown).

The nucleotide sequences upstream from the EAST1 gene of strains E68 and Abbotstown were also identical to each other, but somewhat divergent from those of ETEC, DAEC, and EAggEC (17-2) strains isolated from patients with diarrhea (Fig. 3).

Distribution of the EAST1 gene among porcine and bovine ETEC strains. The distribution of the EAST1 gene among ETEC strains with various adherence factors was examined by PCR with the primer set EAST11a-EAST11b (Table 1). The EAST1 gene was strongly associated with K88$^+$ strains (88.2% positive; $n = 17$). Twelve K88ac$^+$ strains tested were all EAST1 gene positive, irrespective of the toxin type. The two EAST1 gene-negative strains were LT$^-$ and STI$^+$, although one K88ac$^+$ LT$^-$ STI$^+$ strain was EAST1 gene positive.

In contrast, most K99$^+$ strains were negative for the EAST1 gene (9.1% positive; $n = 11$). One EAST1$^+$ K99$^+$ strain found was a porcine isolate.

The K99$^+$ F41$^+$ strains, F41$^+$ strains, 987P$^+$ strains, and one F17$^+$ strain tested were all negative in the PCR assay. One F165$^+$ nontoxigenic strain was also negative. Lack of the EAST1 gene sequence in the PCR-negative strains was also confirmed by colony hybridization (Table 1).

DISCUSSION

In this study, we clearly demonstrated a strong association of the EAST1 gene with porcine-colonizing ETEC strains that possessed K88 adherence factors. One porcine-colonizing ETEC strain that possessed K99 was also found to be positive for the EAST1 gene.

K88 is a major adhesin, in many cases, of ETEC strains isolated from piglets with diarrhea. For instance, Wray et al. (36) have shown that the proportions of K88$^+$, K99$^+$, 987P$^+$, and F41$^+$ ETEC strains were, 21.3, 1.5, 1.3, and 0.2%, respectively. The high incidence of K88$^+$ ETEC was also reported by Osek and Svennerholm (27) (incidence, 56.3%) and by Wilson and Francis (34) (incidence, 48%). The association of the EAST1 gene with ETEC strains possessing a major adherence factor suggests that the EAST1 gene is an important virulence constituent of ETEC strains pathogenic for piglets.

The EAST1 gene was also found in ETEC strains isolated from patients with diarrhea and was again found to be distributed among strains with major adherence factors such as colonization factor antigen (CFA)/II (37). Thus, it is concluded that the EAST1 gene is distributed among ETEC strains isolated from both humans and domestic animals and that the distribution is associated with the adherence factor type (although human strains carried the EAST1 and CFA genes on the same plasmid, in contrast to porcine strains, which carried the EAST1 and K88 genes on separate plasmids).

TABLE 1. Distribution of EAST1 gene among ETEC strains possessing various adhesins, toxin genes, and O:H serotypes

Adhesin type	Isolation source (host)	Toxin gene type	O:H serotype (no. of strains)[a]	No. EAST1 positive/total no. by:	
				PCR[b]	Colony hybridization[c]
K88					
K88ab	Piglets	LT, VT	O141:H4 (1)	1/1	1/1
K88ac	Piglets	LT, STI	O149:HNM (3), O157 (1)	4/4	4/4
	Piglets	LT	O147:HNM (1), O149:H10 (1), O149:H19 (3), O149:HNM (1), O157:H19 (1)	7/7	7/7
	Piglets	STI	O149:HNM (1)	1/1	1/1
K88[d]	Piglets	LT	O8:HNM (2)	2/2	2/2
	Piglets	STI	O9:H16 (1), O9:H19 (1)	0[e]/2	0[e]/2
987P	Piglets	STI	O9:HNM (3), O20:HNM (2), O141:HNM (3)	0/8	0/8
K99	Calves, piglets	STI	O8:H9 (1), O8:HNM (2), O9:HNM (2), O9:H9 (1), O101:HNM (2), O141:HNM (2), O141:H2 (1)	1[f]/11	1[f]/11
K99, F41	Calves, piglets	STI	O101:HNM (3)	0/3	0/3
F41	Calves, piglets	STI	O101:HNM (2)	0/2	0/2
F17	Calves	STI1	O101:HNM (1)	0/1	0/1

[a] NM, nonmotile.
[b] Presence of the EAST1 gene was tested by PCR with primer set EAST11a-EAST11b (see text).
[c] Presence of the EAST1 gene was tested by colony hybridization by using digoxigenin-labeled 111-bp PCR products (generated with primer set EAST11a-EAST11b) as a probe (see text).
[d] K88 subtypes (ab, ac, and ad) were not tested.
[e] The EAST1-negative strains are isolates from Japan.
[f] The EAST1-positive strain, AD-5 (a porcine isolate from Japan), had a serotype of O8:HNM.

The EAST1 gene sequence of $K88^+$ ETEC strains determined in this study was different from that of human-colonizing ETEC strains; the 8th codon (from the initiation codon) is CGG for $K88^+$ ETEC and CGA for human-colonizing ETEC (37). The deduced EAST1 amino acid sequences of $K88^+$ ETEC (this study), human-colonizing ETEC (37), and DAEC (39) were identical to each other and differed from that of EAggEC strain 17-2 (29). Nataro et al. (23) reported the heterogeneity of the virulence of EAggEC demonstrated in volunteers, in that strain O42 was more virulent than some other strains (including strain 17-2). We have determined the EAST1 gene sequence of this strain, strain O42, that was pathogenic for volunteers; the gene sequence (and thus the deduced amino acid sequence of EAST1) was identical to those of human-colonizing (CFA/I^+ or CFA/II^+) ETEC (37) and DAEC (39) strains and thus differed from that of EAggEC strain 17-2 (unpublished data). We therefore speculate that the EAST1 gene of $K88^+$ ETEC, which is identical to that of strain O42 pathogenic for volunteers, could play a role in pathogenicity.

This study also demonstrates that the EAST1 gene of porcine ETEC strains is slightly more divergent from the EAST1 gene of EAggEC than is the EAST1 gene of human ETEC strains. The nucleotide sequence upstream from the EAST1 gene of $K88^+$ ETEC strains, shown in Fig. 3, was also slightly divergent from those of the human-colonizing ETEC (37), DAEC (39), and EAggEC strains; the upstream sequences of the human-colonizing ETEC, DAEC, and EAggEC strains were identical to each other. This difference in the upstream region may contribute to the host specificity of the expression (or activity) of EAST1.

The STI gene of bovine ETEC strains has been shown to be on a transposon (33). The EAST1 gene is more widely distributed among different categories of diarrhea-associated *E. coli*, human- and animal-colonizing ETEC strains (this study; 37), EAggEC strains (28, 29), and DAEC strains (39). This, together with that fact that a human-colonizing (CFA/I^+) ETEC strain possesses multiple copies of the EAST1 gene on the CFA/I-encoding plasmid and chromosome (37), suggests that the EAST1 gene may be on a transposon.

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