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Characterization of Atypical Enteropathogenic *Escherichia coli* Strains Harboring the *astA* Gene That Were Associated with a Waterborne Outbreak of Diarrhea in Japan

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The virulence traits of the *Escherichia coli* strain associated with a waterborne diarrhea outbreak were examined. Forty-one of 75 students (ages 12 to 15) in Akita Prefecture, Japan, showed clinical symptoms. Seven *E. coli* Ouk:K-:H45 isolates were isolated from the patients as the causative agent of this outbreak. One isolate (EC-3605) showed the presence of *E. coli* attaching-and-effacing (*eaeA*) and enteroaggregative *E. coli* heat-stable enterotoxin-1 (*astA*) genes and the absence of Shiga toxin (*stx1* and *stx2*) genes. A polymorphic enteropathogenic *E. coli* (EPEC) adherence factor plasmid was detected in EC-3605 with a major structural gene deletion and a regulatory gene frameshift mutation, revealing that EC-3605 represents an atypical EPEC strain harboring the *astA* gene. The role that atypical EPEC strains harboring the *astA* gene play in human disease is unclear. Our results, along with those of others, present a possibility that these strains comprise a distinct category of diarrheagenic *E. coli* and that *astA* affects the age distribution of atypical-EPEC infection.

Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhea, particularly in children <2 years old (23), and is a cause of sporadic diarrhea, primarily in developed countries (31). Several outbreaks of diarrhea due to EPEC have been reported (3, 42, 45). EPEC causes characteristic attaching-and-effacing (A/E) lesions, which can be observed by intestinal biopsy in both human patients (39) and animal models (27). A/E is characterized by loss of microvilli, intimate adherence of bacteria between epithelial cell membranes (34, 41), and cytoskeletal changes, such as actin polymerization directly beneath the adherent bacteria (20). EPEC possesses a 35-kb chromosomal pathogenicity island called the locus of enterocyte effacement (LEE), which contains genes required for production of A/E lesions (26). The LEE includes the *E. coli* A/E (*eaeA*) gene that encodes intimin, a 94-kDa outer membrane protein (15, 16); a type III secretion system (13); and at least three secretion proteins: EspA (19), EspB (5), and EspD (22). In the prototype EPEC strain E2348/69, the LEE is inserted at the 82-min position in the *E. coli* chromosomal gene encoding the tRNA for selenocysteine (*selC*) (25), while in Shiga toxin-producing *E. coli* (STEC) O26:H^{-}, the LEE is inserted in the *pheU* locus (94-min position), which encodes the tRNA for phenylalanine (37). In addition to the LEE, typical EPEC (18) strains possess a 60-MDa plasmid called the EPEC adherence factor (EAF) plasmid (29). The EAF plasmid has been shown to harbor a 14-gene bundle-forming pilus (*bfp*) operon that encodes a type IV pilus called the bundle-forming pilus (BFP) (38) and a subset of three genes called the plasmid-encoded regulator genes, *perA*, *perB*, and *perC*, which activate the *bfp* operon and chromosomal *eaeA* expression (7). The presence of the EAF plasmid in EPEC strains can be detected by the EAF probe, an ~1-kb DNA fragment derived from pMAR 2, the 60-MDa plasmid of EPEC strain E2348/69 (28). EPEC strains that do not possess the EAF plasmid are called atypical EPEC, and there is some debate as to whether these strains are true pathogens (18).

Recently, a food-borne outbreak of diarrhea involving >100 adult patients was reported in Minnesota (9). The implicated organism was *E. coli* O39:NM, which hybridized with the probes for *eaeA* and other genes in the LEE but did not hybridize with the EAF probe (9). *E. coli* O39:NM also hybridized with the DNA probe for the enteroaggregative *E. coli* (EAggEC) heat-stable enterotoxin 1 (EAST-1) gene (*astA*) (9). EAST-1 is a genetically distinct toxin structurally related to heat-stable enterotoxin I (ST I) of enterotoxigenic *E. coli* (ETEC) (35), but little is known about its significance in the pathogenic mechanism of EAggEC. Hedberg et al. pointed out in their report (9) that this *E. coli* O39:NM strain should therefore be categorized as an atypical EPEC strain that harbors the *astA* gene. Few reports, however, are available on the role that atypical EPEC strains harboring the *astA* gene play in human diseases.

We isolated *E. coli* strains as the etiological agents from patients with diarrhea in a waterborne outbreak involving junior high school students and teachers and characterized the virulence traits of a representative isolate. We describe in this report the virulence traits of the outbreak-associated *E. coli* strain, which should be categorized as an atypical EPEC strain.

MATERIALS AND METHODS

Description of the diarrheal outbreak and bacterial strains. On 30 May 2000, an outbreak of diarrhea occurred in Akita Prefecture, Japan, among students and teachers attending a fieldwork program held at a farm. Of the 75 attendees, 41 students (ages 12 to 15 years) showed clinical symptoms, including diarrhea (85% of the patients), fever (34%; 37.0 to 38.9°C), nausea (37%), vomiting (15%), and abdominal pain (90%). A marked difference in the attack rate was noted between students who drank tap water (86.1%) at the pasture and those who only washed their hands with tap water (26.3%). The tap water was provided

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TABLE 1. Primers used for examination of virulence traits of EC-3605

Designation	Location	Sequence (5′ to 3′)	Target gene	Accession no.	Amplicon size (bp)	Reference
V1	213–230	AGT TAA TGT GGT GGC GAA				
V5	1013–1029	GAC TCT TCC ATC TGC CG	stx_1	M19473	817	21
V3	289–306	TTC GGT ATC CTA TTC CCG				
V4	745–762	TCT CTG GTC ATT GTA TTA	stx_2	X07865	474	21
EA-1	1846–1865	AAA CAG GTG AAA CTG TTG CC				
EA-2	2280–2299	CTC TGC AGA TTA ACC TCT GC	*eaeA*	M58154	454	45
EAF1	546–567	CAG GGT AAA AGA AAG ATG ATA A				
EAF25	922–942	TAT GGG GAC CAT GTA TTA TCA	EAF	X76137	397	6
EP-1	120–140	AAT GGT GCT TGC GCT TGC TGC				
EP-2	423–443	GCC GCT TTA TCC AAC CTG GTA	*bfpA*	Z12295	324	8
bfpAJYS	99–120	TCT TGC TTT GAT TGA ATC TGC A				
bfpAJYAS	273–295	GTA AAA TCG TTG AGT CCA ATC CA	*bfpA*	Z12295	197	This study
EAST-1S	63–82	GCC ATC AAC ACA GTA TAT CC				
EAST-1AS	149–168	GAG TGA CGG CTT TGT AGT CC	*astA*	L11241	106	45
AggRks1	1307–1327	GTA TAC ACA AAA GAA GGA AGC				
AggRkas2	1541–1560	ACA GAA TCG TCA GCA TCA GC	*aggR*	Z32523	254	33

from a brook, without chlorination, as an animal water supply. Of the 36 patients examined for diarrheagenic pathogens, 9 were positive for *E.coli* harboring the *eaeA* gene (A/E *E. coli* [AEEC]), which was the only pathogen isolated from the patients. The serogroup of two of the nine AEEC isolates was O8, while the remaining seven AEEC isolates could not be serogrouped using a commercially available serum kit (11). The tap water collected on 2 June was positive for the O untypeable AEEC. The eight O untypeable AEEC isolates (seven from patients and one from the tap water) displayed identical *Xba*I pulsed-field gel electrophoresis (45) patterns, indicating that the O untypeable AEEC strains were a single clone and were the causative agent of the diarrheal outbreak. Strain EC-3605, one of the seven AEEC patient isolates, was serotyped as Ouk:K$^-$:H45 by Flemming Scheutz at the International *Escherichia* and *Klebsiella* Centre, Statens Serum Institute, Copenhagen, Denmark.

EC-3605 isolated from a patient with diarrhea was employed for characterization of the virulence traits. EPEC E2348/69, EAggEC 17-2, and the pJPN16 plasmid in *E. coli* HB101 were kindly provided by James B. Kaper, University of Maryland School of Medicine, Baltimore, Md. STEC O157:H7 EDL-931 was a kind gift from Yasuo Kudoh, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan.

PCR conditions. PCR was performed, unless otherwise stated, in a 20-μl reaction mixture containing 100 μM (each) deoxynucleoside triphosphate, 2 mM $MgCl_2$, 0.5 μM (each) primer, 2 μl of 10-fold-concentrated polymerase reaction buffer, and a 2-μl aliquot of the boiled bacterial suspension. The mixtures were subjected to 20 cycles of amplification in a Gene Amp PCR System 2400 (Perkin-Elmer, Applied Biosystems Division, Norwalk, Conn.). The parameters for the amplification cycle were 1 cycle of preheating for 30 s at 94°C, followed by 20 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 30 s at 72°C. After the last cycle, the reaction tubes were incubated for 2 min at 72°C. The amplified products were analyzed by electrophoresis using a 2.0% agarose gel.

Examination of virulence genes by PCR. EC-3605 was examined by PCR with the primers listed in Table 1 for the presence of the following virulence genes: Shiga toxin genes (*stx1* and *stx2*) (12), *eaeA* (15), the EAF gene (29), *bfpA* (38), *astA* (35), and *aggR* (transcriptional activator for EAggEC aggregative adherence fimbria I expression) (30). The bfpAJY primers were designed within a putative conserved region, which was identified by comparing the *bfpA* sequences from EPEC E2348/69 (GenBank accession no. Z12295) and EPEC O128ab:H2 strain 20 (accession no. AF119170).

Southern blot analysis of plasmid DNA of EC-3605. Plasmid DNA was extracted from EC-3605 by the method of Kado and Liu (17) and electrophoresed in a 0.7% agarose gel. Using standard techniques, the plasmid DNA was denatured in the gel, transferred to a nylon membrane, and cross-linked by UV irradiation (24). The EAF gene probe was a 1-kb *Sal*I-*Bam*HI fragment (1) prepared from the pJPN16 plasmid (14). The DNA probe for the *bfpA* gene was PCR amplified from EPEC E2348/69 with the primers *bfpA* ORF S (5′-AGG AAA ACA GTT TTT ATG GTT TCT-3′) and *bfpA* ORF AS (5′-GGC GTA TTA TGT AGA TTA CTT CAT-3′) to produce a 612-bp fragment including the *bfpA* open reading frame (ORF). PCR was performed as described above except that 25 amplification cycles were performed and the total reaction volume was 50 μl. The 612-bp *bfpA* probe was purified with a SUPREC-02 column (Takara Shuzo Co., Shiga, Japan) according to the manufacturer's instructions. Probe labeling, hybridization under high-stringency conditions, and signal detection were performed using the AlkPhos Direct Gene image kit (Amersham Pharmacia Biotech, Piscataway, N.J.) according to the manufacturer's instructions.

Cloning and sequencing of $bfpA_{3605}$. The ~60-MDa plasmid p3605 was extracted from EC-3605 by the method of Kado and Liu (17) and digested with *Sal*I and *Eco*RI according to the manufacturer's instructions. The resultant DNA fragments were cloned into pBluescript SK II by using a T4 DNA ligase ligation kit (Ligation Pack; Nippon Gene Co., Toyama, Japan). The host, *E. coli* strain JM109, was transformed with the recombinant plasmids by the standard $CaCl_2$ method (24) using a transformation kit (Nippon Gene Co.). The resultant *E. coli* JM 109 strains harboring $bfpA_{3605}$ were identified by a standard color test based on α-complementation (24) in combination with PCR with bfpAJY primers under the conditions described above. The recombinant plasmid harboring $bfpA_{3605}$ was designated pJY3605. The DNA sequence of $bfpA_{3605}$ in pJY3605 was determined by the Sanger dideoxynucleotide chain termination method with an ABI model 377 sequencer. A 312-bp DNA fragment containing $bfpA_{3605}$ was amplified from EC-3605 by PCR under the conditions described above with the primers bfpA3605 ORF S (5′-TTC CGT GAC CTA TTA ATA CGG-3′) and bfpA3605 ORF AS (5′-CTT CCC CGA GCA TGT TGG-3′), purified with the SUPREC-02 column, and sequenced directly using the same primers in order to verify the DNA sequence data of $bfpA_{3605}$ from pJY3605.

Examination of *bfp* and *per* genes in EC-3605 by PCR. The *bfpB*, *bfpD-F*, *bfpI-L*, *perA*, *perB*, and *perC* genes were examined by PCR with the primers listed in Table 2. For detection of the *bfp* genes, the parameters for the amplification cycle were 1 cycle of preheating for 30 s at 94°C, followed by 20 cycles of denaturation for 30 s at 94°C, annealing for 60 s at 55°C, and extension for 60 s at 72°C. After the last cycle, the reaction tubes were incubated for 2 min at 72°C. For detection of the *per* genes, PCR was performed as described above.

Sequencing of $perA_{3605}$. An 877-bp DNA fragment containing the *perA* gene of EC-3605, $perA_{3605}$, was amplified from EC-3605 by PCR with the primers PerA Seq S (5′-ACA AAC GCG CAT GAA GGT GGT-3′; positions 33 to 53; Z48561) and PerA Seq AS (5′-ATA AGA TTT TAA ATA TCT CTA ACA-3′; positions 886 to 909; Z48561) using proofreading Pylobest DNA polymerase (Takara Shuzo Co.), purified using the SUPREC-02 column, and sequenced directly using the same primers.

Insertion site of LEE in EC-3605. The insertion site of the LEE in the chromosome of EC-3605 was examined by PCR with primers K260 and K261 for the intact *selC* (tRNA for selenocysteine) locus, K913 and K914 for the intact *pheU* (tRNA for phenylalanine) locus, K255 and K260 for the right junction of the LEE inserted into the *selC* locus, and K295 and K296 for the left junction of the LEE inserted into the *selC* locus (37) (Table 2). PCR was performed as described above.

DNA sequence analysis. DNA sequence data were analyzed with DNASIS software (Hitachi Software Engineering Co.).

Nucleotide sequence accession numbers. The nucleotide sequences of the $perA_{3605}$ and $bfpA_{3605}$ genes obtained in this study were deposited in the GenBank database under accession numbers AY212287 and AY212288, respectively.

TABLE 2. Primers used for examination of the *per* and *bfp* genes and insertion site of the LEE

Designation	Location	Sequence (5′ to 3′)	Target gene	Accession no.	Amplicon size (bp)	Reference
PerAS	523–542	TGT CAT CCT TAG TGC TTC AT				
PerAAS	857–876	GGC AAT GTT CCT TGT GTA AT	*perA*	Z48561	354	45
PerB1	958–978	GTA GTT TTT GAT GGA TGT ATG				
PerB2	1267–1286	TCA CCG CCT CCT TCC ATC GT	*perB*	Z48561	329	2
PerC1	1419–1439	GAC GAG CTG CAG ATG CCT TGG				
PerC2	1575–1595	GCC CCA TTT TCT TAT ATG CCT	*perC*	Z48561	177	2
BfpB1	2947–2966	CGC CAG AAG CCT TGA GAT CA				
BfpB2	1726–1744	GAA CAG TGC AAC AGG CGG A	*bfpB*	Z68186	1,241	2
BfpD	5190–5210	CTT ATC AGG CTG CTC GTA TAG				
BfpF	7862–7881	CAA GCT CAC GTG CAT CCA TC	*bfpD-F*	Z68186	2,692	2
BfpI	9550–9571	TCA CTG ATT GAA GCG TCA TTA				
BfpL	11282–11301	GCT CGT CCG ACA GAA TAC TC	*bfpI-L*	Z68186	1,752	2
K260	7328–7351	GAG CGA ATA TTC CGA TAT CTG GTT				
K261	7833–7854	CCT GCA AAT AAA CAC GGC GCA T	*selC*	AE000443	527	37
K913	13–32	CAT CGG CTG GCG GAA GAT AT				
K914	303–320	CGC TTA AAT CGT GGC GTC	*pheU*	S67565	308	37
K255	NA[c]	GGT TGA GTC GAT TGA TCT CTG G				
K260	315–338	GAG CGA ATA TTC CGA TAT CTG GTT	RJ[a]	AF031371	418	37
K295	459–478	CGC CGA TTT TTC TTA GCC CA				
K296	NA	CAT TCT GAA ACA AAC TGC TC	LJ[b]	AF031372	418	37

[a] RJ, right junction of LEE inserted into *selC*.
[b] LJ, left junction of LEE inserted into *selC*.
[c] NA, not available.

RESULTS

Virulence genes of EC-3605. The results of the PCR examination of EC-3605 for *E. coli* virulence-associated genes were positive for *eaeA* and *astA* and negative for the EAF gene, *aggR*, *stx1*, and *stx2*. The presence of the *bfpA* gene in EC-3605 was determined by PCR with two sets of primers. The results using the primers described by Gunzberg et al. (8), EP-1 and EP-2, were negative, while the bfpAJY primers gave an expected 197-bp fragment (Fig. 1), suggesting that EC-3605 harbors a *bfpA* homologue. Southern blot hybridization analysis of the ~60-MDa plasmid of EC-3605, p3605, for the presence of the EAF locus and a *bfpA* homologue using the EAF gene and *bfpA* probes confirmed that p3605 represents the EAF plasmid harboring the *bfpA* homologue, $bfpA_{3605}$ (data not shown). EC-3605 was negative for the *invE* gene of enteroinvasive *E. coli* and for both the heat-stable and heat-labile enterotoxin genes of ETEC (data not shown).

Characterization of the EC-3605 *bfp* and *per* genes. To characterize $bfpA_{3605}$, we cloned it into pJY3605 and determined its DNA sequence. pJY3605 harbors an ~2.3-kb *Eco*RI-*Sal*I fragment that originated from p3605, and an ~1-kb region flanking the *Eco*RI site of the 2.3-kb fragment was sequenced. A 234-bp ORF was identified in the 2.3-kb fragment. As shown in Fig. 2, the DNA sequences of the 234-bp ORF and the 5′ 234-bp region of $bfpA_{E2348/69}$ are 94.9% identical, indicating that this 234-bp ORF represents $bfpA_{3605}$ (AY212288). The DNA sequence downstream of the $bfpA_{3605}$ ORF, however, varied significantly from the corresponding region of $bfpA_{E2348/69}$, suggesting that a deletion occurred in the 3′ end of the EC-3605 *bfpA.* Furthermore, PCR using primers for *bfpB*, *bfpD-F*, and *bfp I-L* yielded no amplicons of the expected sizes from EC-3605, while PCR products of the expected sizes were amplified from E2348/69 (Fig. 3A). These results indicated that a deletion of the entire *bfp* operon of EC-3605 occurred, suggesting that it is unable to produce BFP, as described by Bortolini et al. (2). In fact, by performing Western blotting with polyclonal antisera raised against E2348/69 BFP, we determined that EC-3605 failed to produce BFP (data not shown). We next characterized the three genes constituting the *per* operon, *perA*, *perB*, and *perC*, in EC-3605. As shown in Fig. 3B, EC-3605 was positive for *perA*, *perB*, and *perC* by PCR. However, sequencing of the *perA* gene of EC-3605, $perA_{3605}$ (AY212287), revealed a frameshift mutation caused by a single base deletion at position 325 corresponding to the *perA* gene of E2348/69, $perA_{E2348/69}$ (Z48561), which results in a truncation of $perA_{3605}$ to a 357-bp ORF compared to the 825-bp ORF of $perA_{E2348/69}$ (data not shown). These results indicated that the *per* operon of EC-3605 is unable to activate the virulence gene promoter, as described by Okeke et al. (32).

Insertion site of the LEE in EC-3605. To characterize the evolutionary lineage of EC-3605, we examined the insertion site of the LEE in the chromosomal DNA of EC-3605. Figure 4 schematically presents the region surrounding the LEE in the EC-3605 chromosome. Amplicons of the expected size, 418 bp, were obtained with primers K295-K296 and K255-260 from EC-3605, indicating that the LEE is inserted within the *selC* locus in the EC-3605 chromosome, as well as in the E2348/69 chromosome (25). Although the data are not shown, interruption of the *selC* locus was confirmed in both EC-3605 and E2348/69 by PCR with primers K260 and K261. The *pheU* locus is interrupted by an unknown insert in EC-3605, which was evidenced by a negative result in PCR with primers K913 and K914, while the *pheU* locus is intact in E2348/69, because an amplicon of the expected size, 308 bp, was obtained with primers K913 and K914 (data not shown). These results indicate that both EC-3605 and E2348/69 belong to the same cluster, EPEC 1.

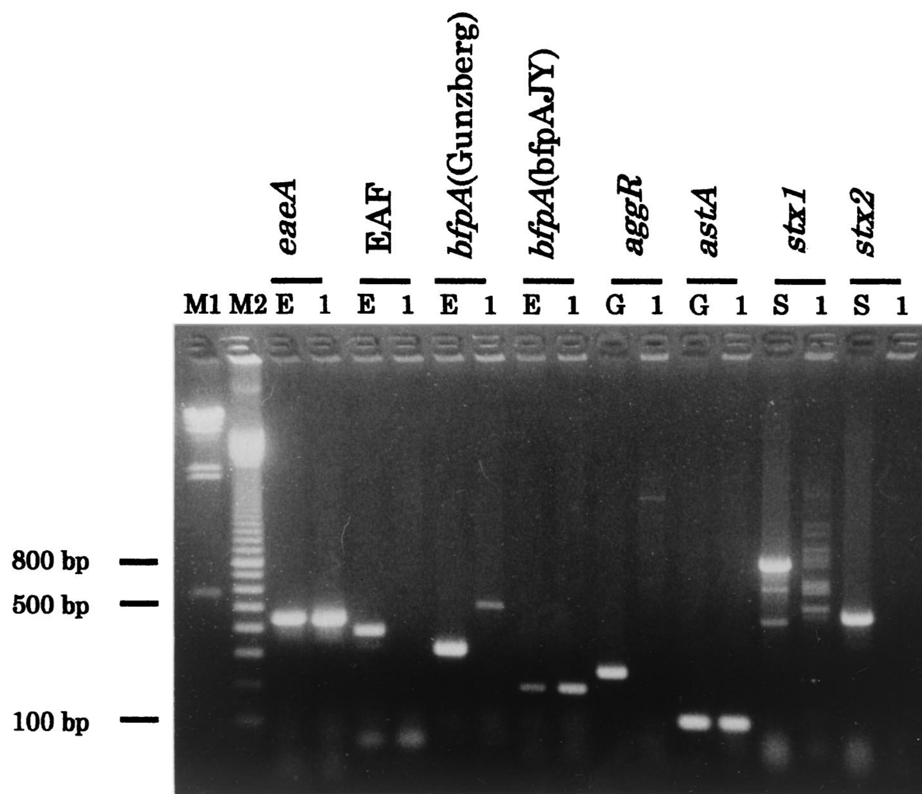

FIG. 1. Agarose gel electrophoresis of the PCR products of *E. coli* virulence genes showing virulence traits of EC-3605. Lanes: M1, lambda/*Hin*dIII molecular size marker; M2, 100-bp-ladder molecular size marker; 1, EC-3605; E, EPEC E2348/69; G, EAggEC 17-2; S, STEC O157: H7 EDL-931.

DISCUSSION

In this study, we characterized the virulence traits of *E. coli* isolated as an etiological agent in a waterborne diarrhea outbreak in Japan. The *E. coli* strain, EC-3605, that was isolated from patients during this outbreak was shown by PCR to be positive for the *eaeA* gene and by Southern hybridization to harbor the EAF plasmid. However, PCR indicated that this strain was negative for the EAF plasmid. This discrepancy suggests that the EAF locus of p3605 shows a low sequence homology, at least in the primer annealing sites, with pMAR2 from E2348/69. According to the consensus definition achieved at the Second International Symposium on EPEC (18), a typical EPEC strain produces the A/E lesion without *stx* genes and possesses the EAF plasmid, while an atypical EPEC strain lacks the EAF plasmid. Therefore, EC-3605 apparently represents a typical EPEC strain, although its serotype, Ouk:K-:H45, does not fit into any of the known EPEC serotypes. In addition to these virulence genes, EC-3605 harbors the *astA* gene, a putative heat-stable enterotoxin gene originally identified in the EAggEC strain 17-2 (35).

The importance of the EAF plasmid in typical EPEC pathogenicity is attributable to the functions of its *bfp* and *per* operons. The *perA* gene encodes a protein belonging to the AraC family of transcriptional activators, and the *perB* and *perC* genes encode proteins that enhance *perA* gene expression by an unknown mechanism (7). The *per* operon has been shown to increase expression of both the *eaeA* (7) and *bfpA* genes (40). Hicks et al. (10) recently showed, using in vitro organ culture with human pediatric small intestinal biopsy tissue, that BFP is involved in the formation of complex three-dimensional EPEC colonies on the intestinal epithelial tissue via bacterium-bacterium interactions, which results in the maintenance and stabilization of EPEC microcolonies. Stone et al. (38) demonstrated that 14 ORFs, those for *bfpA*, *-G*, *-B*, *-C*, *-U*, *-D*, *-R*, *-F*, *-P*, *-H*, *-I*, *-J*, *-K*, and *-L*, are sufficient to reconstitute BFP with the aid of artificial promoters, which indicates that these 14 genes are necessary for BFP biosynthesis. Recently, Bortolini et al. (2) reported that several EPEC strains harbored a truncated *bfpA* gene and lacked the other *bfp* operon genes, resulting in a lack of BFP production. The conflicting results regarding the presence of the EAF plasmid in EC-3605 observed in PCR and Southern blot hybridization suggested a sequence polymorphism between the EAF loci of p3605 and pMAR2. The conflicting results regarding the presence of the *bfpA* gene in EC-3605 were also observed in PCR with Gunzberg primers and bfpAJY primers, suggesting that the 3′ end of $bfpA_{3605}$ is deleted, as described by Bortolini et al. (2), or that the nucleotide sequence of the EP-2 annealing site in $bfpA_{3605}$ has low sequence homology with that in *bfpA* of the prototype EPEC E2348/69, $bfpA_{E2348/69}$. In fact, we demonstrated that the entire *bfp* operon is deleted in EC-3605, and Western blot analysis confirmed that the EC-3605 *bfp* operon cannot produce BFP. Additionally, we identified a mutating frameshift that leads to truncation of the *perA* gene in EC-3605. This trunca-

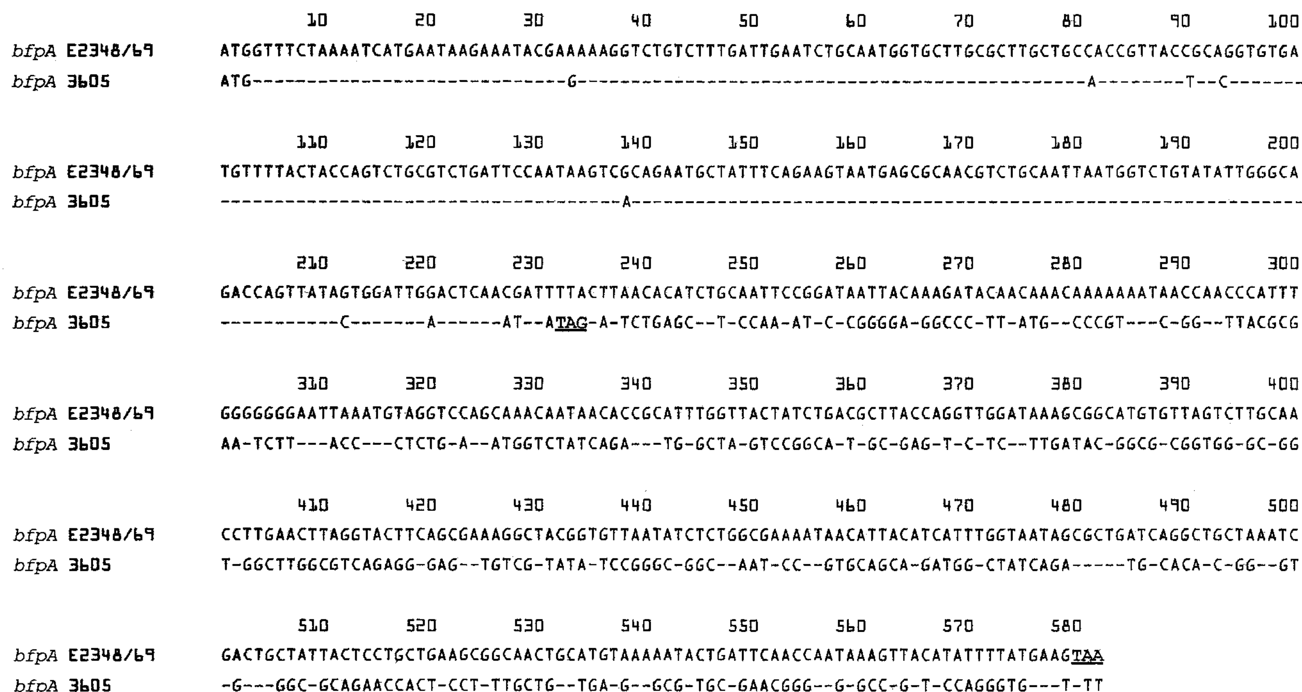

FIG. 2. Alignment of the *bfpA* nucleotide sequences of EPEC E2348/69 and EC-3605. The dashes represent consensus nucleotides. The stop codons are underlined.

tion could result in *per* operon inactivation, as described by Okeke et al. (32), in a minority of EPEC strains, including O119:H2, a subset of O128:H2, and O142:H6. Our present results indicate that even though EC-3605 harbors the EAF plasmid, the $bfpA_{3605}$ and $perA_{3605}$ genes are unable to function properly because of a structural gene deletion and a *perA* frameshift mutation, respectively, revealing that EC-3605 should functionally represent an atypical EPEC strain. It was reported previously (45) that the atypical EPEC O55:NM was an etiological agent of an infantile-diarrhea outbreak. Taken with our present results, this indicates that neither BFP nor the *perA* gene is essential for EPEC to cause diarrhea in humans. Diagnosis of typical EPEC strains depends on detection of *eaeA* and the EAF plasmid by DNA probe or PCR (31). Our

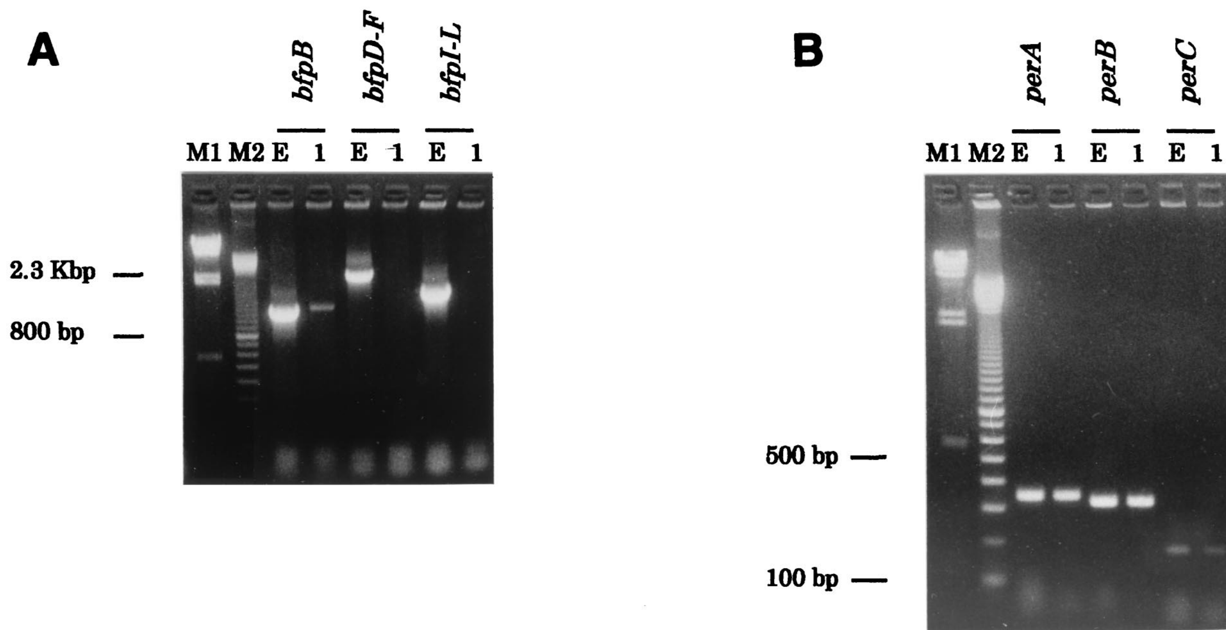

FIG. 3. Agarose gel electrophoresis of the PCR products of genes comprising the *bfp* operon (A) and the *per* operon (B). Lanes: M1, lambda/*Hin*dIII molecular mass marker; M2, 100-bp-ladder molecular size marker; E, EPEC E2348/69; 1, EC-3605.

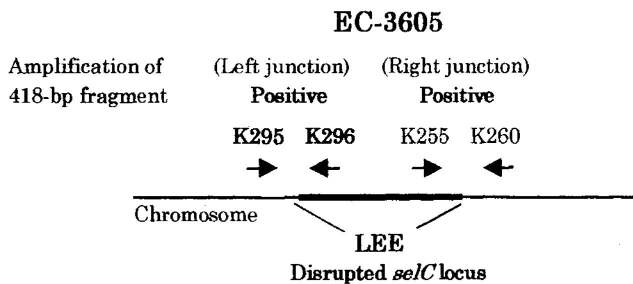

FIG. 4. Schematic representation of the region surrounding the LEE in the EC-3605 chromosome. The PCR primers listed in Table 2 are indicated by arrows. The diagram is not to scale.

present results, together with those of Bortolini et al. (2), reveal that some of the strains diagnosed to be typical EPEC strains by EAF probe harbor a nonfunctioning *bfp* operon. These strains should be categorized instead as atypical EPEC strains because such strains are unable to produce BFP. Such strains cannot be distinguished from "true" typical EPEC strains possessing functioning *bfp* and *per* operons by hybridization analysis using an EAF probe. A reliable and conventional method for identifying such strains needs to be developed.

EPEC infection is primarily a disease of infants <2 years old (23). An infantile-diarrhea outbreak caused by the atypical EPEC strain O55:NM (45) was also recently reported. However, a few food-borne diarrhea outbreaks involving adults caused by atypical EPEC strains have been reported. In one recent outbreak involving >100 adults, the implicated pathogen was *E. coli* O39:NM (9). Although the serotype O39:NM does not fit into any of the known EPEC serotypes, this strain was positive for *eaeA* and other genes within the LEE but negative for the EAF plasmid (9), which is representative of an atypical EPEC strain. Another diarrhea outbreak involving >600 people, including students, teachers, and auxiliary personnel, was also reported (42). The implicated pathogen in this outbreak was *E. coli* O111, which was *eaeA* positive but EAF plasmid negative (31). Interestingly, both of the outbreak-associated *E. coli* strains, O39:NM and O111, were *astA* positive. We have shown in this study that EC-3605 is also *astA* positive. The *astA* gene was first identified in EAggEC as a structural gene that encodes a distinct low-molecular-weight putative enterotoxin (35). Recently, the *astA* gene has been detected not only in EAggEC but also in EPEC, atypical EPEC, ETEC, STEC, and enteroinvasive *E. coli* strains (4, 36, 44). Although the significance of the *astA* gene in *E. coli* pathogenesis remains unclear, our findings, along with those of Hedberg et al. (9) and Viljanen et al. (42), suggest the possibility that the presence of the *astA* gene affects the age distribution of atypical-EPEC infection. Whether atypical EPEC strains harboring the *astA* gene are more frequently isolated from adults needs further elucidation.

It has recently been reported that chromosomal LEE insertion sites vary according to the evolutionary lineage of LEE-harboring *E. coli* strains, including EPEC and STEC. In the clusters of strains designated EPEC 1, which is composed of serotypes O127:H6 and O55:H6, and STEC 1, which is composed of serotype O157:H7, the LEE is inserted downstream of the *selC* locus (43). In contrast, in EPEC 2, which is composed of serotypes O128:H2, O111:NM, and O111:H2, and STEC 2, which is composed of serotypes O111:NM, O111:H8, O111:H11, O26:NM, and O26:H11, the LEE is inserted into the *pheU* locus (37). Our present data indicate that in EC-3605, the LEE is inserted into the *selC* locus. These observations indicate that EC-3605 belongs to the EPEC 1 cluster, just as EPEC E2348/69 does.

Certain serotypic and genotypic features of EC-3605 are quite similar to those of *E. coli* O39:NM, which is the etiological agent of food-borne diarrhea outbreaks. Neither of these strains fits into any of the known EPEC serotypes, they have virulence traits characteristic of atypical EPEC, and they harbor the *astA* gene. The Finnish outbreak-associated *E. coli* O111 strain also has the same virulence traits as *E. coli* O39:NM, but it belongs in the classical EPEC O serogroup (42). Interestingly, Hedberg et al. (9) also pointed out that *E. coli* O39:NM may be regarded as an atypical ETEC strain because it produces an adhesin, intimin, in combination with the heat-stable enterotoxin EAST-1. The significance of atypical EPEC strains that harbor the *astA* gene, whether they belong to the known EPEC serotypes or not, is unclear, but our results, along with those of Hedberg et al. (9) and Viljanen et al. (42), raise the possibility that these *E. coli* strains comprise a distinct category of diarrheagenic *E. coli*.

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