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Lineage and Host Source Are Both Correlated with Levels of Shiga Toxin 2 Production by *Escherichia coli* O157:H7 Strains▿†

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***Escherichia coli* O157:H7 strains fall into three major genetic lineages that differ in their distribution among humans and cattle. Several recent studies have reported differences in the expression of virulence factors between *E. coli* O157:H7 strains from these two host species. In this study, we wished to determine if important virulence-associated "mobile genetic elements" such as Shiga toxin 2 (Stx2)-encoding prophage are lineage restricted or are host source related and acquired independently of the pathogen genotype. DNA sequencing of the stx_2 flanking region from a lineage II (LII) strain, EC970520, revealed that the transcriptional activator gene Q in LI strain EDL933 (upstream of stx_2) is replaced by a *pphA* (serine/threonine phosphatase) homologue and an altered Q gene in this and all other LII strains tested. In addition, nearly all LI strains carried stx_2, whereas all LII strains carried variant stx_{2c} and 4 of 14 LI/II strains had copies of both stx_2 and variant stx_{2c}. Real-time PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA) demonstrated that LI and LI/II strains produce significantly more stx_2 mRNA and Stx2 than LII strains. However, among LI strains significantly more Stx2 is also produced by strains from humans than from cattle. Therefore, lineage-associated differences among *E. coli* O157:H7 strains such as prophage content, toxin type, and toxin expression may contribute to host isolation bias. However, the level of Stx2 production alone may also play an important role in the within-lineage association of *E. coli* O157:H7 strains with human clinical disease.**

Escherichia coli O157:H7 is associated with outbreaks and sporadic cases of hemorrhagic colitis and the hemolytic-uremic syndrome (HUS) in humans (24). Since its isolation in 1982, this organism has become one of the most important food- and waterborne zoonotic pathogens in the world (16, 28). Shiga toxin 1 (Stx1) and Stx2 are two of the most important virulence factors produced by this pathogen and are encoded by lambda-like prophages integrated into the bacterial chromosome.

It is well recognized that *E. coli* O157:H7 has a bovine reservoir and that the organism is well adapted for life in the ruminant gastrointestinal tract (6, 7, 14, 32). In contrast to humans, cattle colonized by *E. coli* O157:H7 are asymptomatic.

Several recent studies have reported differences in the phenotype and genotype of *E. coli* O157:H7 strains from humans and cattle. Baker et al. (2) found that *E. coli* O157:H7 strains isolated from healthy cattle were less virulent in gnotobiotic piglets compared with strains of the pathogen isolated from human disease outbreaks and that the amount of Stx2 but not Stx1 produced by *E. coli* O157:H7 strains was correlated with a reduction in piglet survival and signs of central nervous system disease. LeJeune et al. (22) reported that Q-gene allelic variation (upstream of the prophage stx_2 region) and Stx2 production differed between *E. coli* O157:H7 isolates from cattle and humans. Besser et al. (3) also reported a greater diversity of Stx-encoding bacteriophage insertion sites among *E. coli* O157:H7 isolates from cattle than those from humans. It has also been reported that *E. coli* O157:H7 strains vary markedly in the levels of locus of enterocyte effacement (LEE) (16, 28) secreted proteins and that a high-level secretion phenotype is more prevalent among strains associated with human disease than strains shed by healthy cattle (30, 31). Bono et al. (5) recently identified five polymorphisms in a 1,627-bp segment of the intimin receptor (*tir*) and found that alleles of two *tir* polymorphisms, *tir* 255 T>A and repeat region 1-repeat unit 3 (RR1-RU3; presence or absence), had significantly different distributions among human and bovine *E. coli* O157:H7 strains.

Within *E. coli* O157:H7 populations, significant genomic diversity is evident using high-resolution methods, such as octamer-based genome scanning (OBGS) (17), whole-genome PCR scanning (WGPS) (27), DNA microarray (25, 39, 40, 42), subtractive suppressive hybridization (34), single nucleotide polymorphism analysis (41), and genome nucleotide sequencing. Comparisons of genome sequences of *E. coli* O157:H7 strains with the laboratory strain K-12 have revealed that prophages are associated with a significant amount of the divergence among *E. coli* O157:H7 strains (26). In addition, Asadulghani et al. (1) have recently reported that even defective prophages in the *E. coli* O157:H7 genome are capable of lateral gene transfer and that recombination of DNA segments occurs among prophages present in the genome of this pathogen.

Kim et al. (17) used OBGS to show that *E. coli* O157:H7

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TABLE 1. The primers used in this study, their sequences, gene targets, and origin

Name	Sequence	Target gene	Reference
Non-RT-PCR primers			
restx1	GTGGTATAACTGCTGTCCGTTGTC	stx_2	This study
restx2	GAATACTGGACCAGTCGCTGGAATC	stx_2	This study
oli320b	GGTCACTGGTTCGAATCCAGTAC	stx_2 and its variants	9
oli321	GGGATCCTGAATTGTGACACAGATTACACTTGTTAC	stx_2 and its variants	9
595	AAGAAGATGTTTATGGCGGT	stx_2	22
Q933	CGGAGGGGATTGTTGAAGGC	Q	22
P2	CGACGACGAGAGGAGCAGAA	*ninG*	This study
P4	GACGGAATCGACGACCTGAG	Q′	This study
RT-PCR primers			
gapA-F	TGGCTCCGCTGGCTAAAGTTATCA	*gapA*	This study
gapA-R	AGTCTTTGTGAGACGGGCCATCAA	*gapA*	This study
gapA-p	6-FAM-ATCGAAGGTCTGATGACCACCGTTCA-IABFQ[a]	*gapA*	This study
F386	CCATGACAACGGACAGCA	stx_2	This study
R479	GATGAAACCAGTGAGTGACGA	stx_2	This study
P431	6-FAM-CGCTGGAACGTTCCGGAATGCAAATCA-IABFQ	stx_2	This study

[a] IABFQ, Iowa Black FQ (Integrated DNA Technologies).

strains can be divided into two major lineages and that these lineages are nonrandomly distributed among human and bovine hosts. *E. coli* O157:H7 lineage I (LI) strains are isolated from both humans and cattle at approximately equal frequencies while LII isolates are proportionally much more commonly isolated from cattle. In a recent comparative genomic hybridization study (42), we identified a third major cluster of *E. coli* O157:H7 strains, which we designated LI/II, with genetic characteristics intermediate between LI and LII. Furthermore, in the study 11 different genomic regions were found to be dominant in LI strains whereas the LI/II strains possessed eight of these LI-dominant loci. Several differences in virulence-associated loci were noted between LI and LII strains, including divergence within S-loop 69 (also called Sakai prophage 5 or SP5), which encodes Stx2. In addition, Dowd and Ishizaki (10) have used oligonucleotide miniarrays to compare the expression of a set of 610 genes between three LI and three LII strains, noting differential expression of stx_2 when strains were grown under anaerobic conditions. This group has also recently reported that LI strains produce significantly more Stx2 than LII strains (11). Collectively, these studies suggest that *E. coli* O157:H7 lineages are genetically distinct and that lineage-specific genetic differences may be responsible for the phenotypic differences observed between human and bovine isolates of the pathogen.

In this study, we wished to determine if the differences observed between bovine and human strains in stx_2-encoding prophages and stx_2 expression could be best explained by host origin and/or the lineage type of the *E. coli* O157:H7 strain.

MATERIALS AND METHODS

Bacterial strains. One hundred seventeen *E. coli* O157:H7 isolates used in this study were obtained from a variety of human and bovine sources across a broad span of time from different geographic origins (see Table S1 in the supplemental material). Lineage I strains included 25 bovine, 3 environmental, and 29 human isolates; lineage I/II strains included 6 bovine and 8 human isolates; lineage II strains included 29 bovine and 15 human isolates and two isolates of unknown source.

PCR analysis. Primers from previously published papers and those designed in this study are listed in Table 1. Long-template PCR was carried out in a total reaction volume of 25 μl, with each deoxynucleoside triphosphate (dNTP) at 0.2 mM, each primer at 0.25 μM, Advantage 2 PCR buffer at 1×, Advantage 2 polymerase at 0.6 U, and 100 ng of genomic DNA. The reaction mixture was denatured at 95°C for 1 min before being cycled 5 times for 30 s at 95°C, followed by a 10-min extension at 72°C. The reaction mixture was subsequently cycled 25 to 30 times with a 30-s, 95°C denaturation and a 10-min, 68°C extension before being subjected to a final 10-min extension at 72°C. Long-template PCR products were visualized using 0.60% agarose gels containing 0.5 μg/ml of ethidium bromide (EB).

Amplification of stx_2 variants with the oli320b-oli321 primer pair was carried out in a reaction mixture volume of 50 μl, with each deoxynucleoside triphosphate (dNTP; Invitrogen) at 0.2 mM, each primer at 1 μM, Qiagen PCR buffer containing $MgCl_2$ at 1×, and *Taq* polymerase (Qiagen) at 5 U. Then 200 ng of template DNA was heated at 95°C for 3 min before being cycled 30 times with a 30-s step at 94°C, a 30-s annealing step at 55°C, and a 3-min extension at 72°C before being subjected to a final 5-min extension step at 72°C.

Restriction fragment length polymorphism (RFLP) analysis of stx_2 variants. Genotyping of stx_2 variants was conducted as described by De Baets et al. (9) using restriction enzyme digests of oli320b-oli321 amplicons. The four restriction enzymes PvuII (P), HaeIII (H), HincII (I), and AccI (A) were used in separate digestions in a total reaction volume of 20 μl containing 17 μl PCR product, 2 μl of 10× reaction buffer, and 1 μl of each restriction enzyme (Invitrogen) followed by an incubation at 37°C for 3 h. The enzyme digests were subjected to electrophoresis in 2.0% agarose containing 0.5 μg/ml ethidium bromide, and the resulting PHIA patterns were photographed and scored as described previously (9).

Inverse PCR. Five micrograms of bacterial genomic DNA was digested with the appropriate restriction enzymes (BamHI, EcoRI, HindIII, and SalI), which were selected based on the analysis of the genomic island O island 45 (OI#45) of the published genomic sequence of *E. coli* O157:H7 strains EDL933 and Sakai (16, 28). After restriction, the DNA was purified with phenol-chloroform-isoamyl alcohol (25:24:1), precipitated with ethanol, and dissolved in 10 μl of Tris-EDTA (TE; 10/1; pH 8.0) buffer. After ligation of the restricted DNA by addition of ligase and ligation buffer (Invitrogen) overnight at 4°C, 1 μl of the ligation product was used as template for PCR amplification performed as described for long-template PCR.

Cloning and sequencing of inverse PCR products. The inverse PCR products were cloned using the pCRII-Topo cloning system (Invitrogen) or the Expand PCR cloning kit (Roche) depending on the size of the PCR product. The inserts from various strains were used for further subcloning or direct sequencing. DNA sequencing was performed at the DNA sequencing center of the University of Calgary, Alberta, Canada.

Each sequence was divided into a segment based on the EcoRI site.

Quantification of Stx2 production. The amount of Stx2 produced by each strain was quantified as previously described (43), with the following modification: cells were lysed by incubation with 0.5 mg/ml polymyxin B (Sigma) at 37°C for 60 min, rather than 1.5 mg/ml for 5 min. The values of three independent experiments were used to determine the average toxin production for each strain.

RNA isolation. An overnight culture at 37°C was diluted in new 10-ml LB broth and cultured at 37°C until it reached an optical density at 600 nm (OD_{600})

TABLE 2. Inverse PCR investigation using EcoRI-digested sequences of the stx_2 flanking region of *E. coli* O157:H7

Strain group	Length (kb)	Pattern
Lineage I (n = 7)	4.1	A
Lineage I (n = 1)	5.9	B
Lineage II (n = 10)	4.9	C
Lineage II (n = 1; strain R834)	6.2	D
Lineage I/II (n = 4)	4.1/4.9	E

of 0.6. One milliliter of this culture was transferred to an RNase-free 1.5-ml tube and spun at 13,000 rpm, after which the supernatant was discarded. The bacterial pellet was dissolved in 1 ml fresh TRIzol (Invitrogen) and incubated for 5 min at room temperature. Afterward, 200 μl of chloroform was added and the suspension was incubated for an additional 5 min at room temperature. The upper phase was subsequently transferred to a fresh RNase-free 1.5-ml tube, and 500 μl of isopropanol was added prior to a 15-min incubation at room temperature. Nucleic acids were collected by centrifugation and washed with 1 ml of 70% ethanol and then dissolved in 50 μl of RNase-free water. The sample was next treated with Turbo DNase (Ambion) to digest the DNA. RNA was precipitated with 1/10 volume of sodium acetate (3 M, pH 5.2), 1 μl of glycogen (Sigma), and 2 volumes of 100% ethanol at −20°C overnight. The isolated RNA was dissolved in a final volume of 50 μl RNase-free water.

cDNA synthesis. Reverse transcription (RT) was performed in a reaction mixture containing 10 μl of RNA (0.5 μg/μl), 2 μl of random hexamer primer (20 pmol/μl; Invitrogen), 2.5 μl of dNTPs (10 mM; Invitrogen), 5 μl of 5× RT buffer (Invitrogen), 2 μl of dichlorodiphenyltrichloroethane (0.1 M; Invitrogen), and 20 U RNase inhibitor (GE Life Sciences). The mixture was incubated for 10 min at room temperature. Following incubation, 400 U RT Superscript II (Invitrogen) was added to the mixture and incubated in the following stages: 10 min at 25°C, 50 min at 45°C, 15 min at 70°C, and 2 min at 99°C. The second-strand synthesis contained 10 nmol dNTP, 10× Klenow buffer (Invitrogen), and 1 U Klenow polymerase. The reaction mixture was incubated for 30 min at 37°C, followed by the addition of 2 ng RNase A (Sigma). A subsequent incubation of 30 min at 37°C was performed, and the cDNA was purified from solution by use of a Microcon column (Millipore) in 10 mM Tris-HCl (pH 7.5).

Detection of *stx*$_2$ or *stx*$_{2c}$ gene expression by RT-PCR using dually labeled probes. Real-time PCR (RT-PCR) was performed using a Rotorgene 6000 (Corbett Life Science) in a volume of 25 μl containing the following: 12.5 μl of 2× buffer (Platinum SuperMix UDG; Invitrogen), 100 ng of cDNA, and 10 nmol of each primer and probe. As in Table 1, the probes were conjugated with fluorescent reporter dyes 6-carboxyfluorescein (FAM) at the 5′ end and the quencher dye Iowa Black FQ at the 3′ end (Integrated DNA Technologies Inc.). The *gapA* gene of *E. coli* was measured as an internal standard to normalize values between RNA samples as previously described (12). For the establishment of a standard curve, standard gene concentrations were required. Three different concentrations of each of the following were prepared: *gapA* (100 ng/μl, 10 ng/μl, and 1 ng/μl) and the stx_2 gene (100 ng/μl, 10 ng/μl, and 1 ng/μl). For each unknown sample, 3 replicates at the same concentration (100 ng/μl) were tested in the same experiment. Reactions were performed with the following cycling conditions: holding at 95°C for 3 min and 30 cycles of 95°C for 10 s followed by 60°C for 45 s. Acquisition was set to the FAM/Sybr channel, and the gain was set to 10.

Statistical analyses. Student's two-tailed, unpaired t test assuming equal variance was used to analyze Stx2 production data from enzyme-linked immunosorbent assay (ELISA) experiments and mRNA copy number from RT-PCR experiments. Dixon's Q test was used at the 80% confidence limit to remove outliers in the replicate ELISA data prior to further analysis (8).

Nucleotide sequence accession numbers. The nucleotide sequences have been deposited in GenBank and have the accession numbers EU999145, EU999146, EU999147, EU999148, EU999149, EU999150, EU999151, EU999152, EU999153, and EU999154.

RESULTS

Comparison of the *stx*$_2$ gene flanking regions in LI, LI/II, and LII *E. coli* O157:H7 strains. As shown in Table 2, five distinct patterns were obtained following inverse PCR with genomic DNA from 20 strains ligated following EcoRI digestion. Seven LI strains shared the same pattern as EDL933 and Sakai and generated a PCR band of 4.1 kb (pattern A), with one LI strain producing an amplicon of 5.9 kb (pattern B); 10 LII strains gave a 4.9-kb PCR band (pattern C), with one LII strain producing an amplicon of 6.2 kb (pattern D); four lineage I/II strains generated two amplicons, one of 4.1 kb and one of 4.9 kb (pattern E), suggesting that there were two copies of stx_2 in these genomes.

Nucleotide sequence analysis of the stx_2 (or stx_{2c}) 5′ flanking segment and the 3′ flanking region surrounding the EcoRI site was carried out. This analysis showed that the 4.1-kb and 4.9-kb amplicons of pattern E had 99% nucleotide sequence identity to the amplicons of LI-associated pattern A and predominantly LII-associated pattern C, respectively (data not shown). The 5′ stx_2 flanking sequences of all LII strains shared 99% nucleotide sequence identity with each other. As shown in Fig. 1, LII strains (patterns C and D) shared the same gene organization in the 3.3-kb 5′ stx_2 flanking region, which differs from that found in LI strains EDL933 and Sakai (pattern A). In OI#45 of EDL933, the Q gene (38), which encodes a 144-amino-acid-residue antiterminator protein, is located upstream of stx_2A and is thought to act as a transcriptional activator. In LII strains (patterns C and pattern D), the Q gene is replaced with the *pphA* gene (ORF223), which is identical to the gene encoding diadenosine tetraphosphatase and is related to other serine/threonine protein phosphatases (19) (Fig. 1). ORF162 between *pphA* and stx_2A in LII strains showed very low (22%) identity to the LI Q protein at the amino acid sequence level and no identity with Q of 933W at the nucleotide sequence level; despite this low level of identity, it has been designated Q′ (35). The small open reading frame (ORF) Z1460/ECs1204 (in LI strains EDL933 and Sakai, respectively) was not present in any of the LII strain sequences examined. When the LII (pattern C) 3.3-kb 5′ stx_2 flanking region was BLASTed against the NCBI database, 99% sequence identity to sequences from *E. coli* O157:H7 strain Thai-12 (19) (accession no. AB168110.1), phage 2851 in O157:H7 (36) (accession no. AJ605767), and *E. coli* O157:H7 carrying stx_2 *vhdA* and stx_2 *vhdB* genes (15) (accession no. AB071845.1) was noted. The BLAST results also showed more than 99% sequence identity to the shotgun sequences from *E. coli* O157:H7 strains EC4113 (accession no. NZ_ABHP01000075) and EC4115 (accession no. NZ_ABHN01000014).

Three of the four sequenced LII strains exhibiting pattern C had the same gene organization in their 3′ stx_2 flanking region, which differed from LI strains (Fig. 1). Furthermore, when the 3′ stx_2 flanking sequences of these strains were aligned with the stx_2 flanking sequence of other *E. coli* strains from serotypes O26:H11, O145:H$^-$, and *E. coli* O157:H$^-$ (37), high identity (>97% similarity) and the same gene organization were observed among them (Table 3). However, only very low similarity (<60%) was observed between LI and LII strains in the 3′ stx_2 flanking sequence (Fig. 1).

As shown in Fig. 1, ORF107, which is identical to gene B1160 in the K-12 genome (4), is located between stx_2B and Z1466/ECs1207 (a 645-amino-acid protein found in both EDL933 and Sakai). In LII strain R834, ORF107 and two genes, *orfB* and *orfA* from IS*629* (which is located downstream of ECs1207 in the Sakai genome [16, 28]), were inserted between stx_2B and Z1466/ECs1207, which corresponds to inverse

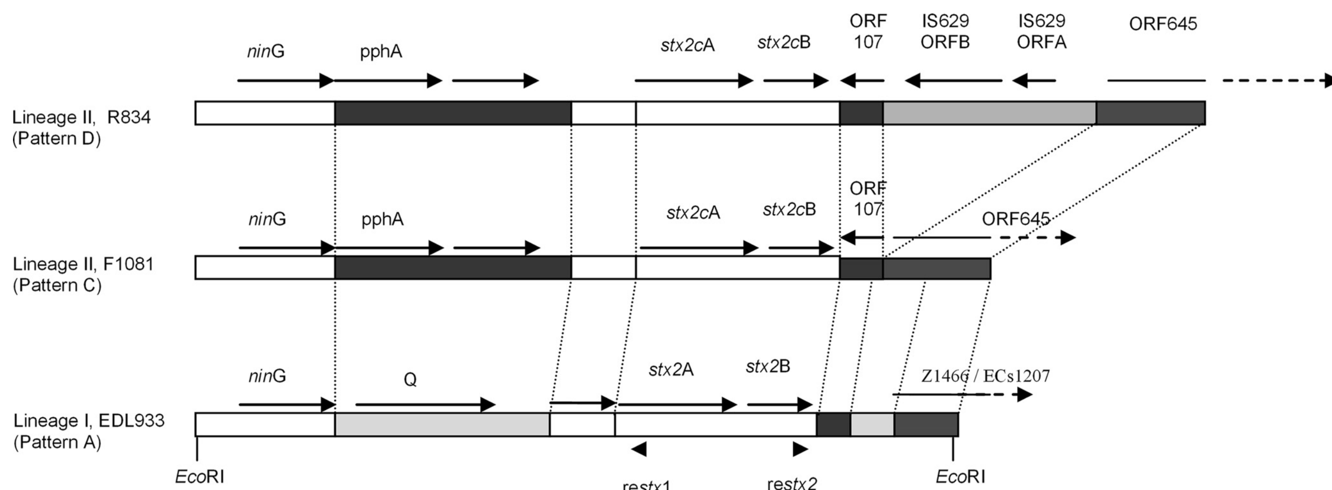

FIG. 1. Schematic representation of stx_2 gene-containing EcoRI fragments in lineage I (EDL933, pattern A) and lineage II (R834, pattern D, and F1081, pattern C) strains. Aligned boxes bearing the same shading show regions of high homology. Solid arrows indicate ORFs and their expression direction in the fragment. The dashed arrows indicate incomplete ORFs and their expression direction. STP, serine/threonine protein phosphatase homologue; Q, transcriptional activator antiterminator Q; Q′, a protein bearing low homology to Q. restx1 and restx2 are two primers used for inverse PCR.

PCR pattern D in Table 2 and was the only LII strain found to contain this gene arrangement.

Inverse PCR showed that LI/II strain R1388 carried two copies of the Stx2 gene, one with pattern A and another with pattern C. DNA sequence analysis confirmed that one has a flanking region identical to that of LI (pattern A) strains EDL933 and Sakai and the other has a flanking region identical to that of the LII (pattern C) strains (data not shown).

To investigate genetic differences in the stx_2 flanking region in a larger set of strains from all three lineages, two primer sets were used to amplify specific targets in the stx_2 gene flanking region. The primer set Q933-595 was used to target the flanking region upstream of stx_2 in LI strain EDL933 and included the Q antiterminator gene (38). The primer set P2-P4 targeted the region from the lambda *ninG* gene upstream of stx_{2c}. Results of assays with these two primer sets on DNA from a total of 117 *E. coli* O157:H7 strains (56 LI, 14 LI/II, and 47 LII strains) are presented in Table 4. All LI strains generated an amplicon of expected size with the primer set Q933-595, while LII strains did not. Forty-six of 47 LII strains (98%) produced an amplicon using the P2-P4 primer set, while no LI strains did. Four of 14 LI/II strains were PCR positive with both primer pairs, while the other 10 were positive only with primer set Q933-595.

Analysis of the stx_2 (stx_{2c}) gene in LI, LI/II, and LII strains by RFLP. The coding sequence of the variant stx_{2c} toxin of the *E. coli* O157:H7 reference strain E32511 differs from that of the prototypical Stx2 toxin found in bacteriophage 933W by three amino acid replacements in the B subunit, whereas the A subunits in the two toxins are identical (33). A comparison of the predicted amino acid sequence of Stx2 from LII strain LS68 to that of Stx2c present in the prototypical strain E32511 revealed 100% identity in the B subunit and one amino acid difference in the A subunit of LII strain LS68.

RFLP analysis of the oli320b-oli321 PCR product using the restriction enzymes PHIA distinguished among the stx_2 gene variants from each lineage. As shown in Table 5, the PHIA pattern from LI strains was 1-1-1-1, consistent with stx_2; the digestion pattern for lineage II strains was 1-2-2-1, consistent with stx_{2c}; and two lineage I/II strains analyzed produced a PHIA pattern of 1-1 + 2-1 + 2-1, consistent with possession of both the stx_{2c} and stx_2 genes.

TABLE 3. DNA sequence comparison of the 3′ stx_2 flanking region in 11 Shiga toxin-containing *E. coli* strains, encompassing O157:H7, O26:H11, and O145:H$^-$ serotypes, to LII strain EC970520

Strain	Sequence accession no.	Start nucleotide	End nucleotide	Similarity (%)	Reference
O157:H7 LII strain EC970520	EU999153	1	1629		This study
O157:H7 LII strain F1081	EU999151	1	1629	99.9	This study
O157:H7 LII strain LS68	EU999152	1	1629	99.9	This study
O157:H7 LI/II strain R1388	EU999145	1	1629	99.9	This study
O157:H$^-$ LI/II strain E32511	AJ251452	137	1765	99.8	37
O26:H11 strain 1448/97	AJ250954	137	1765	97.7	37
O26:H11 strain ED-147	AJ251483	137	1766	97.8	37
O145:H$^-$ strain 3985/96	AJ251520	137	1766	97.8	37
O145:H$^-$ strain 4865/96	AJ271063	137	1767	97.6	37
O157:H7 LI strain EDL933	933W	22568	23819	59.7	28
O157:H7 LI strain Sakai	VT2_sakai	22059	23310	59.7	16

TABLE 4. Genotyping of 117 lineage I, I/II, and II *E. coli* O157:H7 strains using the primer pairs Q933-595 and P2-P4

Lineage(s)	Source	*n*	No. positive by primer set:	
			Q933-595	P2-P4
I	Bovine	25	25	0
	Environment	3	3	0
	Human	28	28	0
II	Bovine	29	0	28
	Human	16	0	16
	Unknown	2	0	16
I/II	Bovine	6	6	2
	Human	8	8	2

TABLE 5. PCR-RFLP typing of stx_2 variants in lineage I, I/II, and II *E. coli* O157:H7 strains using PvuII, HaeIII, HincII, and AccI (PHIA) restriction enzymes

Lineage(s) (no. of strains)	Pattern according to RFLP analysis of PCR product[a]				Stx2 variant type
	PvuII (P)	HaeIII (H)	HincII (I)	AccI (A)	
Lineage I (n = 7)	1	1	1	1	Stx2
Lineage II (n = 9)	1	2	2	1	Stx2c
Lineage I/II (n = 6)	1	1	1	1	Stx2
Lineage I/II (n = 2)	1	2	2	1	Stx2 + Stx2c

[a] 1 and 2 are specific RFLP patterns described by De Baets et al. (9).

Stx2 production by LI, LI/II, and LII strains. Stx production was measured using an ELISA for 27 LI, 12 LII, and 14 LI/II *E. coli* O157:H7 strains (Fig. 2). LI strains containing stx_2 produced significantly more Stx2 than the Stx2 and/or Stx2c produced by LI/II strains (P = 7.93E−03). LII strains produced significantly less Stx2 and/or Stx2c than LI and LI/II strains (P = 1.43E−08 and P = 2.16E−07, respectively). Stx2 production among LI strains was more variable than among either LI/II or LII strains, with EDL933 producing very high levels of toxin and F1299 producing very low levels of toxin. RT-PCR (Fig. 3) also showed that LI and LI/II strains produced significantly higher levels of stx_2 and/or stx_{2c} mRNA than LII strains (P = 8.83E−03 and P = 8.74E−04, respectively) and that LI strains produced significantly higher levels of stx_2 and/or stx_{2c} mRNA than LI/II strains (P = 7.11E−03).

We also compared Stx2 production using an ELISA among LI and LI/II strains isolated from humans and cattle (Fig. 2). Among LI strains, those of human origin produced more of this toxin than those of bovine origin (the means were 179.33 ng/ml and 122.89 ng/ml, respectively) and the difference was statistically significant (P = 2.50E−02). RT-PCR also showed that human strains produced significantly higher levels of stx_2 mRNA than bovine strains within LI (Fig. 3) (P = 8.08E−02); however, no significant difference was observed in Stx2 production between bovine and human strains within LI/II or LII using either ELISA or RT-PCR.

DISCUSSION

In this study, we investigated Stx2 production by *E. coli* O157:H7 strains and found that LI and LI/II strains produce significantly larger amounts of Stx2 than LII strains independently of host origin. However, LI human strains also produced more Stx than LI bovine strains, in agreement with the observations made by other researchers who have studied *E. coli* O157:H7 strains without consideration of lineage or other genotype (22).

Although the toxin mRNA expression levels differed from the amount of toxin measured in the culture supernatant by ELISA, strains that produced smaller amounts of mRNA tended to produce lower ELISA readings as well. The variation observed is possibly due to differences in mRNA half-life, translation efficiency, amount of cell-associated Stx, the amount of toxin-specific antibody in the ELISA, or differences in experimental conditions used for each of the assays. However, the differences between mRNA and toxin expression levels do not affect the conclusions drawn from these data. While Stx2 production by Stx-producing *E. coli* (STEC) has been shown to be associated with more-severe clinical disease (13), it is also clear that among *E. coli* O157:H7 strains, low-level Stx2c-producing strains and strains which produce Stx1 only, as well as LII Stx2c-producing strains, have all been isolated from human patients with clinical disease and therefore should not be considered nonpathogenic.

It was previously reported that basal production of Stx by HUS-associated STEC exceeded that of bovine-associated STEC (29). In addition, following mitomycin C treatment, Stx2 production by HUS-associated STEC was significantly greater than that by bovine-associated STEC. In a separate study, Dowd and Williams (11) recently reported that LI strains produce significantly more Stx2 than LII strains; however, within-lineage production of Stx2 between strains isolated from humans or cattle was not reported nor was Stx2 production by LI/II strains compared with that of LI and LII strains. A recent study by Ziebell et al. also showed on average more Stx2 production among LI strains than strains of LII and found that LII strains of phage type (PT) 23 and PT67 produced less Stx2 than strains of other LII PTs (43). Other studies have also compared Stx production following treatment of cultures with mitomycin C and ciprofloxacin. These agents were found to increase levels of Stx production and exaggerate differences among lineage groups; however, the relevance of studies using phage-inducing agents for *in vivo* toxin production is not clear.

It has been demonstrated that phage induction results in dramatic increases of Stx production involving two mechanisms. First, there is induction of the Stx-converting phage, which brings about an increase in toxin production due to a concomitant multiplication of toxin gene copies. Second, there is the influence of a phage-encoded regulatory factor, recently characterized as the Q transcription activator protein (23). Our results from RT-PCR assays showed that more stx_2/stx_{2c} mRNA is produced by LI and LI/II strains than stx_{2c} mRNA by LII strains. The detailed study of the stx_2/stx_{2c} flanking region carried out here showed that LI and LI/II strains carry a different phage Q gene from the Q′ gene found in LII strains. Recent experiments indicate that phage induction may also contribute to the significant difference in Stx2 production observed among LI, LI/II, and LII strains (data not shown).

It has been reported that a bovine isolate, Thai-12, carries an

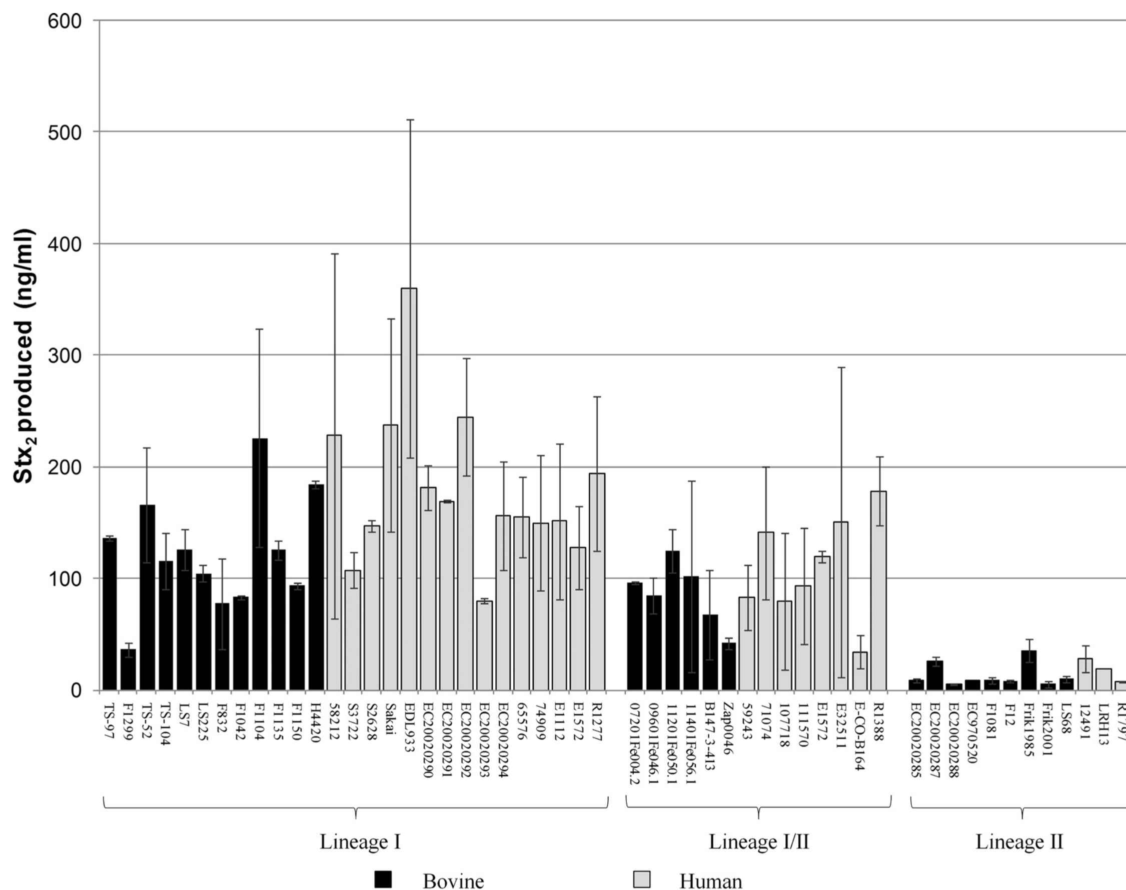

FIG. 2. Comparison of Stx2 production among *E. coli* O157:H7 LI, LI/II, and LII by using an ELISA. Mean values indicate the average values of three experimental replicates, with outliers removed using Dixon's Q test at an 80% confidence limit, and error bars represent sample standard deviations. Human strains are represented as gray bars and bovine strains as black bars.

stx_{2c} gene but does not produce Stx, and it has been postulated that modification of the q-stx_2 region by homologous recombination may have been responsible for the lack of expression of its stx_{2c} gene (18, 19). Alignment of the 5′ stx_{2c} flanking region of LII strain sequences from the present study showed that all had more than 99% sequence identity to Thai-12 (data not shown). Therefore, LII strains may have lower expression of Stx2c through a mechanism related to that of Thai-12 (19). However, while toxin production measured in this study was low for LII strains, it was nevertheless readily detectable using ELISA and RT-PCR assays. It is therefore possible that the methods used to measure toxin production by the Thai-12 strain were less sensitive than those employed in this study for LII strains; however, distal (*trans*) factors may also be involved in the regulation of toxin production/phage induction. The precise reason for the lack of toxin production by Thai-12 requires further study.

In this study, we also identified the Q′ gene as a genetic marker specific for the presence of the stx_{2c}-producing phage which could be used in conjunction with the Q gene-specific primers developed by LeJeune et al. (22) to identify phage carrying either or both stx_2 and stx_{2c}.

It is well known that Stx2-encoding prophages can integrate into the chromosome of other *E. coli* strains. Recently, Asadulghani et al. (1) have reported that not only functional but also defective prophage DNA can be transferred to other *E. coli* strains. They have also shown that recombination of DNA segments between different Stx-encoding prophages occurs in the genome. Based on this information, one would expect to see considerable variation in prophage content and structure in the *E. coli* O157:H7 genome independent of host bacterial genotype. However, the results of this and other studies suggest that prophage gene content and organization and the phenotypic traits associated with them, such as toxin production, are relatively stable and characteristic of members of specific *E. coli* O157:H7 genetic lineages. This suggests that selective forces are acting to maintain the prophage content in these lineages and that significant change in these prophages must provide a selective advantage for the bacterium in order for them to be a driving force in the evolution of new genotypes of this human pathogen.

RFLP and DNA sequencing data showed that all of the LII strains examined carried the Stx2c gene. A previous study reported that while stx_{2c} was the only stx_2 variant associated with HUS, the frequency of HUS associated with STEC strains harboring the stx_{2c} genotype is significantly lower than that of

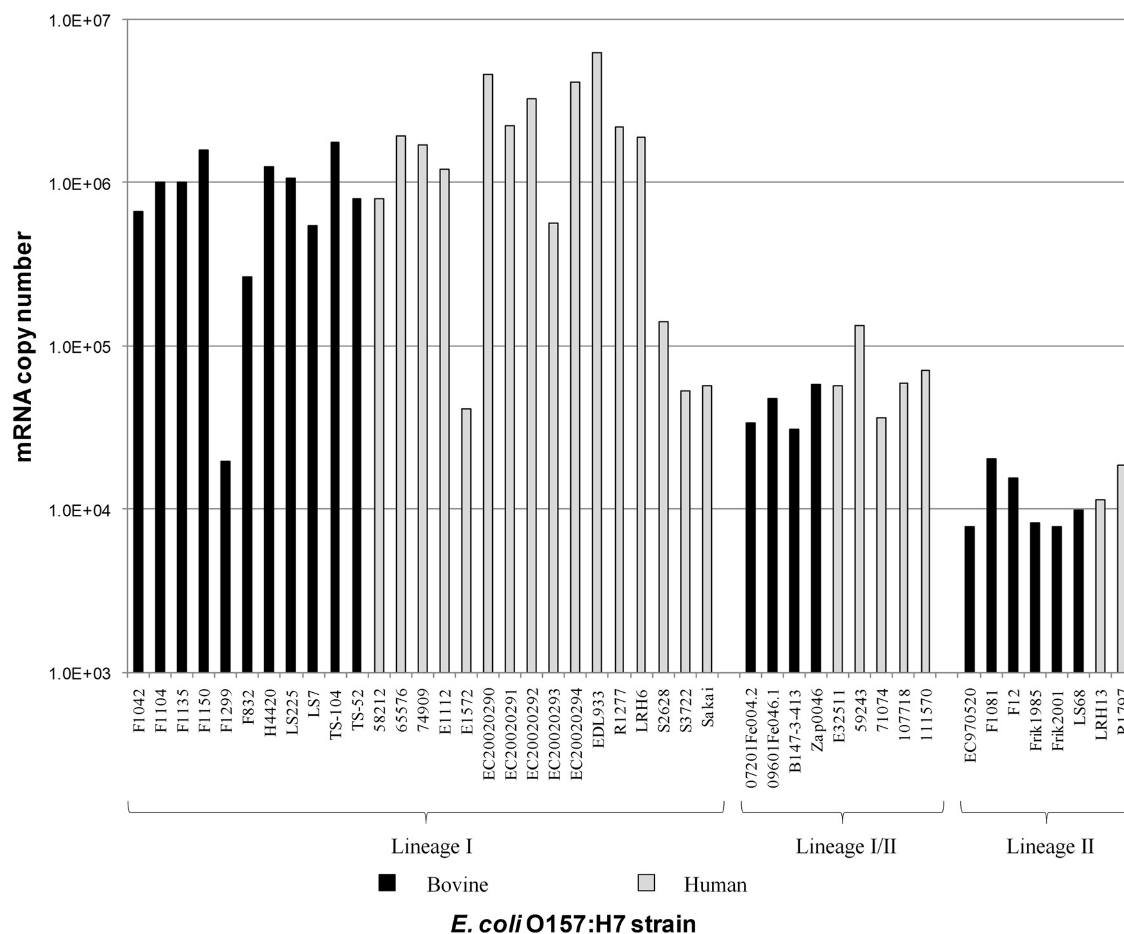

FIG. 3. Comparison of stx_2 mRNA production among *E. coli* O157:H7 LI, LI/II, and LII strains by using RT-PCR. Human strains are represented as gray bars and bovine strains as black bars.

STEC with the stx_2 genotype (13). We noted that there is considerable variation in the levels of toxin production among *E. coli* O157:H7 strains, and stx_{2c}-bearing LII strains produced the smallest amount of toxin on average, with higher levels of toxin produced by stx_2-containing LI and stx_2- and stx_{2c}-containing LI/II strains. It has previously been reported that the majority of toxin produced by clade 8 (LI/II) (21) strains containing both stx_2 and stx_{2c} is Stx2 (20). It has also been shown that both LI and LI/II strains are more likely to be associated with human illness than LII strains (43). Therefore, the amount of Stx2 toxin produced is likely more important than whether a strain contains only stx_2 or both stx_2 and stx_{2c} in determining the frequency of association of these lineage types with human illness.

The reason for the difference in Stx2 production among strains within LI was not clear given the high level of similarity in the toxin promoter sequences found within this genotype. This suggests that other factors that affect phage induction, toxin expression, or translation exist elsewhere in the phage or genome. At present, it is not known if high toxin producers cause more frequent and/or more overt human illness or if toxin production is in some way enhanced during the course of illness. Additionally, it is not currently known whether the levels of toxin production observed *in vitro* correspond to the amount of toxin produced *in vivo*. It is also possible that other genetic elements contribute to the virulence of these strains and that these elements are only genetically linked to high toxin production. LI and LI/II strains are more frequently associated with human disease than LII strains but do not universally exhibit high toxin production. This suggests that both relatively fixed lineage-specific factors and adaptation may play a role in disease associations and is clearly an area in need of further investigation.

In summary, based on the data from inverse PCR and DNA sequencing, we found the organization of genes flanking the stx_{2c} gene of LII strains differed from that of LI strains. The 5′ stx_2 flanking region of LII strains contains a *pphA* gene (serine/threonine protein phosphatase homologue) integrated in the upstream portion of a gene with very low similarity to the transcriptional activator antiterminator Q of LI strains. The Q and Q′ genes appear to be useful molecular markers to distinguish among Stx2- and Stx2c-encoding phages. PCR-RFLP investigations of Stx2 gene variants showed that all LII strains carried only the Stx2c gene and LI strains carried the Stx2 gene; four LI/II strains carried both stx_{2c} and stx_2, while the majority contained only stx_2. Both ELISA and RT-PCR revealed that LII strains produce significantly less Stx2 than LI and LI/II strains. Finally, among *E. coli* O157:H7 LI strains,

strains of human origin produced significantly more Stx2 than strains of bovine origin, suggesting that high-level Stx2/Stx2c-producing *E. coli* O157:H7 strains are likely more virulent (human disease associated) than strains which produce lower levels of Stx2.

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