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Presence of Activatable Shiga Toxin Genotype (stx_{2d}) in Shiga Toxigenic *Escherichia coli* from Livestock Sources

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Stx2d is a recently described Shiga toxin whose cytotoxicity is activated 10- to 1,000-fold by the elastase present in mouse or human intestinal mucus. We examined Shiga toxigenic *Escherichia coli* (STEC) strains isolated from food and livestock sources for the presence of activatable stx_{2d}. The stx_2 operons of STEC were first analyzed by PCR-restriction fragment length polymorphism (RFLP) analysis and categorized as stx_2, $stx_{2c\ vha}$, $stx_{2c\ vhb}$, or $stx_{2d\ EH250}$. Subsequently, the $stx_{2c\ vha}$ and $stx_{2c\ vhb}$ operons were screened for the absence of a *Pst*I site in the stx_{2A} subunit gene, a restriction site polymorphism which is a predictive indicator for the stx_{2d} (activatable) genotype. Twelve STEC isolates carrying putative stx_{2d} operons were identified, and nucleotide sequencing was used to confirm the identification of these operons as stx_{2d}. The complete nucleotide sequences of seven representative stx_{2d} operons were determined. Shiga toxin expression in stx_{2d} isolates was confirmed by immunoblotting. stx_{2d} isolates were induced for the production of bacteriophages carrying *stx*. Two isolates were able to produce bacteriophages ϕ1662a and ϕ1720a carrying the stx_{2d} operons. RFLP analysis of bacteriophage genomic DNA revealed that ϕ1662a and ϕ1720a were highly related to each other; however, the DNA sequences of these two stx_{2d} operons were distinct. The STEC strains carrying these operons were isolated from retail ground beef. Surveillance for STEC strains expressing activatable Stx2d Shiga toxin among clinical cases may indicate the significance of this toxin subtype to human health.

Shiga toxigenic *Escherichia coli* (STEC) isolates are important food-borne pathogens that exist as commensal bacteria of ruminant animals. Shiga toxins (Stxs) comprise an A subunit that carries the toxic function and a B-subunit pentamer that binds the toxin to the eukaryotic cell receptor (for a review, see reference 22). Studies have indicated that Stx type 2 (Stx2), encoded by the stx_2 operon, has an epidemiological relationship with the severe human disease conditions hemolytic-uremic syndrome and hemorrhagic colitis. In addition, toxins Stx2 and Stx2c, encoded by different genotypic subtypes of stx_2, have also been associated with a greater severity of human disease. In contrast, the more recently described stx_2 genotype, $stx_{2d\ EH250}$ (26), is apparently of lesser clinical significance.

The nomenclature designating the Stx2d EH250 subtype is complicated by the prior use of Stx2d to designate activatable Stx2 (16, 18). Both Stx2d and Stx2d EH250 possess Ser291 and Glu297 residues in the Stx2A subunit toxin-coding region; however, only Stx2d is activatable. While STEC isolates carrying $stx_{2d\ EH250}$ are commonly found, particularly from sheep (8), very few reports describe activatable stx_{2d} genotype in STEC.

Activatable stx_{2d} was originally detected in STEC strain B2F1. Two activatable operons, stx_{2d1} and stx_{2d2}, have been identified in the B2F1 (17). Prior to their designation as stx_{2d1} and stx_{2d2}, these operons were designated $stx_{2c\ vha}$ and $stx_{2c\ vhb}$, respectively, due to the restriction fragment length polymorphisms (RFLPs) present in the stx_{2B} genes. However, it is now clear that stx_{2d1} and stx_{2d2} are not synonymous with the stx_2 subtypes $stx_{2c\ vha}$ and $stx_{2c\ vhb}$, respectively; rather, stx_{2d1} and stx_{2d2} are subsets of $stx_{2c\ vha}$ and $stx_{2c\ vhb}$, respectively, which are further defined by additional mutations in the stx_{2A} subunit gene encoding the activatable toxin phenotype.

STEC strain B2F1 is extremely virulent in an orally infected streptomycin-treated mouse model, and the Stx2d toxins produced by this strain have increased cytotoxicities for Vero cells after incubation with mouse or human intestinal mucus (17). Stx2dA subunits possess two amino acid substitutions relative to the sequence of classical Stx2, Ser291 and Glu297. These residues are present in the A2 peptide and are believed to be associated with the property of activation. Ser291 and Glu297 contribute to a recognition motif that allows intestinal mucus or elastase purified from intestinal mucus to cleave the A2 peptide between Thr295 and Gly296, decreasing the length of the A2 peptide by two residues (16, 19). The modified A2 peptide is then able to interact with the Stx2B subunit pentamer, producing the activatable phenotype (19). Interestingly, the stx_{2d} operons described by Piérard et al. (26) ($stx_{2d\ EH250}$) also possess Ser291 and Glu297; however, these residues are adjacent to several other amino acid substitutions, which apparently mitigate the property of activation by intestinal mucus. Hence, Stx2d EH250 toxins are not activatable (A. R. Melton-Celsa and A. D. O'Brien, personal communication).

The aims of this study were (i) to examine the distribution of stx_2 subtypes in livestock ruminant animals, (ii) determine if activatable stx_{2d} operons were present, (iii) characterize stx_{2d} isolates and operons, and (iv) determine if stx_{2d} operons were borne on bacteriophages. We have demonstrated the presence of the activatable stx_{2d} genotype in food and livestock sources and have shown by nucleotide sequencing that this stx_2 subtype may be clearly distinguished from the $stx_{2d\ EH250}$ subtype. We have further demonstrated that some stx_{2d} operons are carried

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Stx2d1      1 MKCILFKWVLCLLLGFSSVSYSREFTIDFSTQQSYVSSLNTIRTEISTPLEHISQGTTSV
EC782       1 ...........................................................
EC1720a     1 ..........................................S................
EC173b      1 ..........................................S................
EC152a      1 .....L....................................S................
EC604a      1 .....L....................................S................
EC1586      1 ..........................................S................
EC1871a     1 ..........................................S................
Stx2        1 ..........................................S................

Stx2d1     61 SVINHTPPGSYFAVDIRGLDVYQARFDHLRLIIEQNNLYVAGFVNTATNTFYRFSDFTHI
EC782      61 ...........................................................
EC1720a    61 ...........................................................A..
EC173b     61 ...........................................................
EC152a     61 ...........................................................
EC604a     61 ...........................................................
EC1586     61 ...........................................................
EC1871a    61 ...........................................................
Stx2       61 ...........................................................

Stx2d1    121 SVPGVTTVSMTTDSSYTTLQRVAALERSGMQISRHSLVSSYLALMEFSGNTMTRDASRAV
EC782     121 ...........................................................
EC1720a   121 ...........................................................
EC173b    121 ...........................................................
EC152a    121 ...........................................................
EC604a    121 ...........................................................
EC1586    121 ...........................................................
EC1871a   121 ...........................................................
Stx2      121 ...........................................................

Stx2d1    181 LRFVTVTAEALRFRQIQREFRQALSETAPVYTMTPGDVDLTLNWGRISNVLPEYRGEDGV
EC782     181 ...........................................................
EC1720a   181 ...........................................................
EC173b    181 ...........................................................
EC152a    181 ...........................................................
EC604a    181 ...........................................................
EC1586    181 ...........................................................
EC1871a   181 ......................V....................................
Stx2      181 ...........................................................

Stx2d1    241 RVGRISFNNISAILGTVAVILNCHHQGARSVRAVNEESQPECQITGDRPVIKINNTLWES
EC782     241 ...........................................................
EC1720a   241 ...........................................................
EC173b    241 ...........................................................
EC152a    241 ...........................................................
EC604a    241 ...........................................................
EC1586    241 ...........................................................
EC1871a   241 ..................................D........................
Stx2      241 ...........................................................

Stx2d1    301 NTAAAFLNRKSQSLYTTGE
EC782     301 .................
EC1720a   301 .................
EC173b    301 .................
EC152a    301 .................
EC604a    301 .................
EC1586    301 .................
EC1871a   301 .................
Stx2      301 ............F.....K
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FIG. 1. Predicted amino acid sequences of the stx_{2d} A-subunit genes from isolates identified in this study aligned with the published sequences of Stx2d1 from STEC isolate B2F1 (11, 17) and Stx2 (GenBank accession no. AB035143). Dots indicate residues identical to those in Stx2d1. Residues Ser291 and Glu297, which distinguish the Stx2d1A-subunit carboxy terminus from that of Stx2A, are boldface and underlined.

by inducible bacteriophages that may be propagated in *E. coli* K-12.

MATERIALS AND METHODS

Isolation of STEC. STEC strains were isolated from food and livestock sources following enrichment broth culture, *stx*-specific PCR, and hydrophobic grid membrane filtration (20). The virulence determinants of each isolate were determined by a multiplex PCR for stx_1, stx_2, *eae*, and *ehxA* (24). Isolates carrying stx_{2d} were kindly serotyped by Roger Johnson, Health Canada, Guelph, Ontario, Canada.

***stx*$_2$ characterization and subtyping.** STEC isolates that carried stx_2 were further characterized by PCR-RFLP analysis to determine the presence of stx_2 subtypes. Primers GK5 and GK6 (30) were used to amplify the stx_{2B} subunit gene. Amplicons were digested with *Fok*I and *Hae*III to discriminate stx_2 from stx_{2c} subtype genes. Use of restriction enzymes *Nci*I and *Rsa*I for PCR-RFLP analysis enabled classification of stx_{2c} genes as $stx_{2c\ vha}$, $stx_{2c\ vhb}$, or $stx_{2d\ EH250}$ (26). Isolates encoding $stx_{2c\ vha}$ and $stx_{2c\ vhb}$ were further examined to identify genes of this type which did not possess a *Pst*I site in the 3′ region of the stx_{2A} subunit gene. $stx_{2c\ vha}$ and $stx_{2c\ vhb}$ operons without *Pst*I sites were sequenced in the 3′ region of the stx_{2A} subunit gene. Putative stx_{2d} genes showed identity to the sequence of activatable stx_{2d1} carried by O91:H21 strain B2F1 (11, 16).

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Stx2d1   MKKMFMAVLFALVSVNAMAADCAKGKIEFSKYNENDTFTVKVAGKEYWTSRWNLQPLLQSAQLTGMTVTIKSSTCESGSGFAEVQFNND
EC782    ................................................................................................
EC1871a  ................................................................................................
EC1720a  ................................................................................................
EC173b   ................................................................................................
EC152a   ..............................................................................A.............
EC604a   ..............................................................................A.............
EC1586   ..........................................D.....................................A.............
stx2     ..........A.....................D.......D.....................................................
```

FIG. 2. Predicted amino acid sequences of the stx_{2d} B-subunit genes from isolates identified in this study aligned with the published sequences of Stx2d1 from STEC isolate B2F1 (11, 17) and Stx2 (GenBank accession no. AB035143). Dots indicate residues identical to those of Stx2d1. Residues Asn16 and Asp24, which distinguish the Stx2d1B subunit from that of Stx2B, are boldface and underlined.

DNA sequencing. Complete stx_{2d} operons were amplified with the primers 5′-GATGGCGGTCCATTATC-3′ (25) and 5′-ACTGAATTGTGACACAGAT TA-3′ (A. R. Melton-Celsa, personal communication). Both strands of each stx_{2d} operon amplicon were completely sequenced. Sequencing was performed with a Big Dye Cycle Sequencing kit following the instructions of the manufacturer and an ABI 377 fluorescent sequencer (Applied Biosystems, Foster City, Calif.) at the Australian Genome Research Facility, University of Queensland, Brisbane, Australia. Nucleotide sequence alignments were performed by the using BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (1). The identities of any two sequences were compared by the using the BLAST 2 SEQUENCES program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) (34). The predicted amino acid alignments presented in Fig. 1 and 2 were prepared by using the CLUSTALW program (version 1.8) at the Baylor College of Medicine Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and the BOXSHADE program (version 3.21; http://www.ch.embnet.org/software /BOX_form.html).

Immunoblot detection of Stx expression from isolates with activatable stx_{2d}. Expression of stx_{2d} operons was assayed by immunoblotting (2). Briefly, STEC isolates were patch inoculated onto nitrocellulose membranes placed on an agar plate surface. The production of Stx was determined by using rabbit anti-Stx antibodies reactive with all major Stx types and variants. Stx antisera were kindly provided by Roger Johnson, Health Canada.

Isolation and characterization of stx_{2d} bacteriophages. stx_{2d} isolates were treated with mitomycin C (1 μg/ml) to induce the bacteriophage lytic cycle and production of phage. Lysed culture supernatants were filtered through a 0.45-μm-pore-size filter to ensure a cell-free lysate. The bacteriophage titers in the cell-free lysates were determined by serial dilution and propagation on *E. coli* Q358 (*supE hsdR* $\phi 80^r$ *recA*$^+$) (13). Single phage plaques were picked from appropriately diluted indicator plates, resuspended in 100 μl of sterile H_2O, and confirmed to carry stx_2 by PCR of the plaque supernatant. Several purified single plaques from each isolate were confirmed to carry stx_{2d} by PCR with primers GK5 and GK6, followed by RFLP analysis of the PCR amplicons.

Nucleotide sequence accession numbers. The sequences of the complete stx_{2d} operons from strains EC152a, EC173b, EC604a, EC782, EC1586, EC1720a, and EC1871 have been submitted to GenBank and given the accession numbers AF500187, AF500190, AF500192, AF500193, AF500188, AF500189, and AF500191, respectively.

RESULTS

***stx_2* genotypes among STEC isolates from livestock sources.** STEC isolates (n = 311) possessing stx_2 were isolated from a variety of food and livestock sources. Each isolate was then characterized to designate a specific stx_2 subtype (Table 1). The dominant stx_2 subtype found in STEC strains of ovine origin was $stx_{2d\ EH250}$, first described by Piérard et al. (26). A total of 79 of 103 (77%) isolates from lamb meat had the $stx_{2d\ EH250}$ genotype, while 30 of 38 (79%) isolates from sheep carcasses and feces were $stx_{2d\ EH250}$. In comparison, classical stx_2 was dominant among STEC strains isolated from beef cattle (26 of 51; 51%) and dairy cattle (33 of 56; 59%) sources. STEC strains isolated from ground beef samples showed greater heterogeneity in stx_2 genotypes, in which stx_2 (29%), $stx_{2d\ EH250}$ (21%), $stx_{2c\ vha}$ (11%), and $stx_{2c\ vhb}$ (11%) were characterized.

Presence of stx_{2d} in STEC. Isolates possessing stx_2 were further characterized for the possession of putative stx_{2d} operons. By PCR-RFLP analysis and preliminary sequencing of the DNA, 12 STEC isolates were found to possess stx_2 operons, with the predicted amino acid sequences of the stx_{2A} subunit

TABLE 1. Genetic subtypes of stx_2 present in STEC isolates of different animal or meat origins

stx_2 subtype	% STEC isolates from different sources possessing alternative stx_2 genetic subtypes[a]				
	Lamb meat (n = 103)	Sheep (carcasses and feces) (n = 38)	Ground beef (n = 63)	Beef cattle (carcasses and feces) (n = 51)	Dairy cattle sources (n = 56)
stx_2	2	3	29	51	59
stx_2 and $stx_{2c\ vha}$	0	0	0	14	4
stx_2 and $stx_{2c\ vhb}$	0	0	3	2	11
stx_2 and $stx_{2d\ EH250}$	3	0	0	0	0
$stx_{2c\ vha}$	1	8	11	22	5
$stx_{2c\ vhb}$	2	0	11	4	7
$stx_{2d\ EH250}$	77	79	21	6	2
$stx_{2c\ vha}$ and $stx_{2c\ vhb}$	1	0	0	2	2
$stx_{2d\ EH250}$ and $stx_{2c\ vha}$	1	0	0	0	0
$stx_{2d\ EH250}$ and $stx_{2c\ vhb}$	1	0	0	0	0
Negative[b]	8	0	16	0	11
Other[c]	5	11	10	0	0

[a] Percentages are rounded to the nearest integer value.
[b] Primers GK5 and GK6 did not amplify the correct amplicon from the stx_{2B} gene.
[c] Primers GK5 and GK6 amplified amplicons of the correct size from the stx_{2B} gene; however, the amplicons did not digest in a manner consistent with the anticipated RFLP scheme.

TABLE 2. Characterization of STEC isolates with the stx_{2d} genotype

Isolate no.	Serotype	Isolate source	Virulence factor genotype[a]			stx_{2B} subtype[b]	Phage recovered
			stx	*eae*	*ehxA*		
152a	O?:H29	Beef cattle feces	2	−[d]	−	2	Non-*stx*
173b	O174:H21	Beef cattle feces	2	−	−	vhb	Non-*stx*
604a	O2:H29	Dairy farm	2	−	−	2	Non-*stx*
782	O?:NM[c]	Beef cattle feces	2	−	−	vha	Non *stx*
1564b	O1:H20	Ground beef	2	−	+	vhb	
1585	O174:H21	Dairy farm	2	−	−	vhb	Non-*stx*
1586	O174:H8	Ground beef	1 and 2	−	+	2	stx_1
1662a	O174:H21	Ground beef	2	−	−	vha	stx_{2d}
1720a	O174:H21	Ground beef	2	−	−	vha	stx_{2d}
1871a	O?:H11	Dairy farm	2	−	+	vhb	Non-*stx*
1995a	O174:H8	Ground beef	1 and 2	−	+	vhb	stx_1
2062a	O8:H19	Lamb meat	2	−	−	vhb	

[a] STEC isolates were screened for the virulence factors *stx* (Stx genes stx_1 and stx_2), *eae* (*E. coli* attaching-and-effacing gene), and *ehxA* (enterohemolysin gene) by multiplex PCR (see Materials and Methods).
[b] The genetic subtype of the toxin B subunit gene for each stx_{2d} operon was classified by RFLP analysis and nucleotide sequencing (see Materials and Methods).
[c] NM, nonmotile.
[d] −, negative.

showing carboxy-terminal modifications specific for stx_{2d}. Seven putative stx_{2d} operons from isolates that represented the diversity of isolates by original source of isolation and the $stx_{2c\ vha}$, $stx_{2c\ vhb}$, or non-$stx_{2c\ vha}$ and $stx_{2c\ vhb}$ classification were completely sequenced. The DNA sequences of the stx_{2d} operons from isolates EC152a and EC604a were identical. The DNA sequences of the other five isolates were each unique and conformed with the PCR-RFLP analysis characterization for each stx_{2d} operon. Isolates identified as carrying stx_{2d} are listed in Table 2.

Comparison of the predicted amino acid sequences of the Stx2A subunit with the Stx2d sequence from O91:H21 strain B2F1 (11, 17) indicated that all the stx_{2d} operons encoded Stx2A with Phe291Ser and Lys297Glu amino acid substitutions, characteristic of activatable Stx2d (17) (Fig. 1). Comparison of the predicted amino acid sequences of the B subunits also showed Asp16Asn and Asp24Ala substitutions characteristic of Stx2dB subunits. The one exception was EC1586, in which Asp24 was conserved in the Stx2dB subunit (Fig. 2). The stx_{2dB} subunit genes of isolates EC152a, EC604a, and EC1586 did not conform to the $stx_{2c\ vha}$ or $stx_{2c\ vhb}$ RFLP designation that has been typical of other stx_{2d} operons.

The nucleotide sequence of the stx_{2d} operon from EC782 was identical to that of stx_{2d1} (11, 16). The identical nucleotide sequences of the stx_{2d} operons from EC152a and EC604a differed by one nucleotide from the sequence of stx_2-NV206 (3). Pairwise comparison and alignments of the stx_{2A} and stx_{2B} genes from EC173b, EC1586, EC1720a, and EC1871a with homologs from the activatable operons stx_{2d1}, stx_{2d2}, and stx_2-NV206 (3, 11, 16) showed 98 to 100% identities and indicated that the new stx_{2d} operons comprised mosaic sequences.

***stx*$_{2d}$ bacteriophage characterization.** For each of the 12 stx_{2d}-containing isolates identified, the bacteriophage lytic cycle was induced with mitomycin C. EC1564b and EC2062 cultures did not lyse in response to mitomycin C induction, and their culture supernatants did not show plaques on indicator plates. Phage plaques were identified from the induced culture lysates of all stx_{2d}-carrying isolates except EC1564b and EC2062a. The phages from EC152a, EC173b, EC604a, EC782, EC1585, and EC1871a did not carry *stx* operons, while those from EC1586 and EC1995 were characterized as carrying stx_1. The stx_2 phages ϕ1662 and ϕ1720a were induced from EC1662 and EC1720a, respectively. PCR-RFLP analysis specific for the stx_{2B} subunit genes of ϕ1662 and ϕ1720a showed that the stx_2 operons of these phages corresponded to the stx_{2d} operons sequenced from their respective STEC host isolates. RFLP analysis of ϕ1662 and ϕ1720a genomic DNA with the *Ava*I restriction enzyme indicated that these two phages were related; however, digestion of the DNA samples was complicated

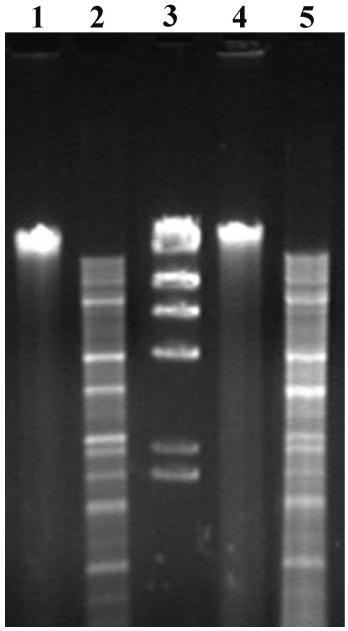

FIG. 3. RFLP analysis of stx_{2d} bacteriophages. Bacteriophage genomic DNA was isolated, digested with *Ava*I, and electrophoresed. Lanes: 1, undigested ϕ1720a; 2, ϕ1720a; 4, undigested ϕ1662a; and 5, ϕ1662a. Lane 3, DNA size markers of 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb.

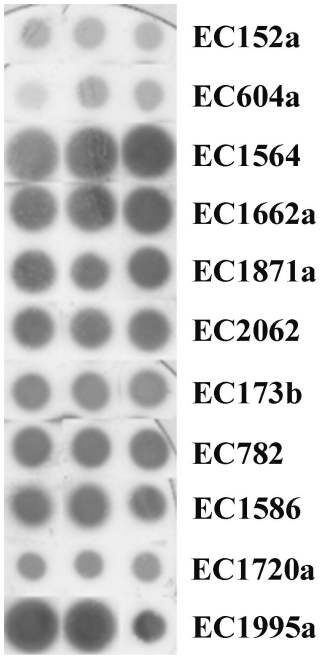

FIG. 4. Immunoblot analysis of Stx expression from stx_{2d}-containing isolates. Equivalent inocula of stx_{2d}-containing isolates were grown on nitrocellulose membranes placed on tryptic soy agar plates and grown overnight. The Stx expressed by bacterial colonies was captured on a capture membrane precoated with rabbit anti-*stx* antibodies. The capture membrane was probed with alkaline phosphatase-labeled rabbit anti-mouse immunoglobulin G. Triplicate inocula were made for each isolate, as indicated.

by contaminating nuclease activated during enzyme incubation (Fig. 3).

Stx expression from stx_{2d} strains. Eleven stx_{2d}-containing isolates were examined for expression of Stx by immunoblotting (Fig. 4). Isolates EC1586 and EC1995a carried stx_1 genes, in addition to stx_{2d}, such that Stx expression from these isolates is the cumulative expression of both stx_1 and stx_{2d}. All other isolates examined carried only stx_{2d}. Stx expression determined by the diameter and intensity of the zone of staining was variable between isolates. Of particular note were the different levels of Stx2d expression by EC1662a and EC1720a, isolates that were both serotype O174:H21, whose stx_{2B} subunit genes had vha RFLP patterns, and that produced phages stx_{2d} possessing similar genome RFLP profiles.

DISCUSSION

In Australia, STEC strains that do not possess the locus of enterocyte effacement are the major causes of hemolytic-uremic syndrome and bloody diarrhea (9). We have begun to characterize the stx_2 genotypes of STEC strains isolated from food-producing animal sources to determine if these stx_2 operons are characterized by specific properties. STEC strains possess an array of stx_2 genotypic variants (26, 32, 36); however, the roles of particular genotypic variants in human pathogenesis are not clear. We determined that STEC strains isolated from sheep or sheep meat sources most frequently carry $stx_{2d\ EH250}$, while STEC strains isolated from cattle sources most frequently carry stx_2 or stx_{2c} variant $stx_{2c\ vha}$ or $stx_{2c\ vhb}$. While cattle have been known to be a significant reservoir of STEC isolates, recent studies have also shown that $stx_{2d\ EH250}$ is routinely associated with ovine STEC isolates (8) (K. S. Gobius et al., unpublished data). The predominance of $stx_{2d\ EH250}$ among ovine STEC isolates compared with its prevalence among bovine STEC isolates suggests that particular stx_2 subtypes may be associated with specific ruminant species.

Using screening by PCR-RFLP analysis, we were able to identify activatable stx_{2d} variants in STEC isolates that were predominantly from bovine sources. Partial sequencing of the stx_2 operons from 12 putative stx_{2d} isolates further confirmed that the operons were consistent with the presence of stx_{2d}. Further nucleotide sequencing of seven complete stx_2 operons confirmed that they encoded the activatable stx_{2d} genotype.

Each STEC isolate carrying activatable stx_{2d} was recovered from a separate sampling location or on independent sampling dates, ensuring that the isolates were not clonal representatives of the same strain. The nucleotide sequence polymorphisms of the stx_{2d} operons present in all isolates (except EC152a and EC604a, for which the stx_{2d} operons were identical; data not shown) support the independent origins of these isolates. Similarly, the diversity of serotypes among the strains carrying stx_{2d} also supports the conclusion that the isolates are not related. While four of the isolates were serotype O174:H21, the stx_{2d} operons carried by each of the isolates were differentiated by RFLP analysis and/or nucleotide sequencing.

All the serotypes that carried stx_{2d} in this study have previously been isolated from livestock sources in other countries, including Argentina, Brazil, Canada, France, Germany, New Zealand, and the United Kingdom (2, 4, 6, 7, 23, 28, 29, 31, 39); however, none of these serotypes have previously been associated with stx_{2d}. Isolates of serotypes O1:H20, O2:H29, O174:H8, and O174:H21, which have been identified to carry stx_{2d}, have previously been isolated from healthy or symptomatic humans in a variety of countries (14, 15, 27, 33).

Bertin et al. (3) recently described a novel stx_2 subtype, stx_2-NV206, present in STEC isolates of serotype O6:H10 isolated from a healthy cow. stx_2-NV206 operons also possess Ser291 and Glu297; however, the stx_{2B} gene of this subtype does not have restriction sites characteristic of $stx_{2c\ vha}$ or $stx_{2c\ vhb}$ (which have previously been associated with activatable stx_{2d} operons). In our study, the identical stx_{2d} operons carried by isolates EC152a and EC604a differed by a single nucleotide from the sequence of stx_2-NV206. Similarly, the sequence of stx_{2d} from EC782 was identical to that of stx_{2d1} from B2F1, originally isolated in North America. The occurrence of identical or nearly identical activatable stx_{2d} operons in bovine STEC isolates from different global locations is intriguing. Their presence in the geographically separated locations of Australia, France, and North America suggests both conservation of these stx_2 variants and the possibility of their global dissemination.

In contrast to the conserved stx_{2d} variant operons present in isolates EC152a, EC604a, and EC782, we also identified stx_{2d} operons whose sequences did not show complete identity to those of previously described stx_{2d} operons. The stx_{2d} operons from EC173b, EC1586, EC1720a, and EC1871a showed nucleotide sequence polymorphisms in mosaic blocks of sequence. The mosaic structures of *stx* bacteriophage genomes, gained

through bacteriophage genome shuffling, have been understood for some time (10, 37). However, recombination in *stx* operons is more difficult to detect by sequence analysis if recombination occurs between identical sequences. Our data provide evidence that alternative activatable stx_{2d} operons have arisen through recombination of independent stx_2 sequences. Such stx_2 operon recombination may account for the increasing number of stx_2 variants that continue to be detected and described. Recombination in stx_2 operons, coupled with bacteriophage lambda recombination and mosaicism, may create a rich genetic pool of *stx* phages capable of affecting horizontal gene transfer within the broad population of members of the family *Enterobacteriaceae*.

stx genes are bacteriophage borne or are associated with defective prophages (21). The stx_{2d1} operon from strain B2F1 has been shown to be carried by an inducible bacteriophage that formed small turbid plaques on *E. coli* K-12 strain DH5α (35). We were able to induce and propagate stx_{2d} bacteriophages from only 2 of 12 stx_{2d}-positive STEC isolates. Notwithstanding the limited number of propagating stx_{2d} phages detected in our study, by infection of an *E. coli* K-12 indicator strain, we have confirmed that stx_{2d} operons are potentially transferred by bacteriophage induction and infection of new enterobacterial hosts. In contrast to the work of Teel et al. (35), we did not detect *stx* phages by induction from strain EC782, which was shown to possess an stx_{2d} operon identical to stx_{2d1}. Since the stx_{2d} bacteriophages were propagated on an *E. coli* K-12 strain, it is possible that other stx_{2d} phages may have been induced from additional STEC isolates but that such a phage(s) was unable to propagate on the specific indicator strain used or readily formed lysogens (unable to be detected by plaque formation). James et al. (12) found that the *stx* phage ϕ24B::Kan was capable of infecting only a minority of wild *E. coli* strains tested. However, of the strains able to be infected, the majority were susceptible to lysogeny by this phage. Alternative explanations for the detection of a small number of stx_{2d} phages following induction in our study may be (i) that the stx_{2d} operons in these isolates are carried on prophage remnants no longer capable of lytic induction or (ii) stx_{2d} phages were induced from these STEC isolates and rapidly formed Q358 lysogens so that detection by plaque formation was not possible.

The two stx_{2d} phages (ϕ1662a and ϕ1720a) successfully induced from isolates EC1662a and EC1720a were related when they compared by using phage genome-specific RFLP analysis. The apparent nuclease contamination of phage DNA may be similar to that observed with plasmid DNA isolated from various STEC strains (5). Given that both phages were induced from isolates of the same O174:H21 serotype and that the stx_{2d} operons showed RFLP patterns characteristic of the $stx_{2c\ vha}$ genotype, it was surprising that the level of Stx2d expression by EC1720a appeared to be less than that by EC1662a. Recently, Wagner et al. (38) have shown that stx_2 transcription is regulated by the late promoter $p_{R'}$ of lambda phages, such that expression of Stx2 is coordinated with induction of the phage lytic cycle. Therefore, we conclude that transcription from $p_{R'}$ in ϕ1662a may be greater than that from $p_{R'}$ in ϕ1720a, leading to a higher level of Stx2d expression by EC1662a.

In conclusion, we have identified the activatable stx_{2d} genotype in STEC strains isolated from food-producing livestock sources. The STEC isolates carrying stx_{2d} consisted of multiple serotypes, and the nucleotide sequences of the stx_{2d} operons varied. In at least two isolates stx_{2d} was carried by inducible *stx* phages that could infect and propagate on an *E. coli* K-12 strain, demonstrating the potential for the horizontal transfer of stx_{2d}. It has been suggested previously that STEC strains involved in human infection, which carry stx_{2d}, may be more virulent due to mucus activation of the toxin. The increased virulence of the Stx2d toxin may compensate for the absence of other virulence attributes such as expression of the attaching-and-effacing gene (*eae*) commonly found in other enterohemorrhagic *E. coli* strains (17). Therefore, surveillance for STEC strains expressing the activatable Stx Stx2d in human illness may indicate the significance of this toxin subtype to human health.

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