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Greater Diversity of Shiga Toxin-Encoding Bacteriophage Insertion Sites among *Escherichia coli* O157:H7 Isolates from Cattle than in Those from Humans▽

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***Escherichia coli* O157:H7, a zoonotic human pathogen for which domestic cattle are a reservoir host, produces a Shiga toxin(s) (Stx) encoded by bacteriophages. Chromosomal insertion sites of these bacteriophages define three principal genotypes (clusters 1 to 3) among clinical isolates of *E. coli* O157:H7. Stx-encoding bacteriophage insertion site genotypes of 282 clinical and 80 bovine isolates were evaluated. A total of 268 (95.0%) of the clinical isolates, but only 41 (51.3%) of the bovine isolates, belonged to cluster 1, 2, or 3 ($P < 0.001$). Thirteen additional genotypes were identified in isolates from both cattle and humans (four genotypes), from only cattle (seven genotypes), or from only humans (two genotypes). Two other markers previously associated with isolates from cattle or with clinical isolates showed similar associations with genotype groups within bovine isolates; the *tir* allele sp-1 and the Q_{933W} allele were under- and overrepresented, respectively, among cluster 1 to 3 genotypes. Stx-encoding bacteriophage insertion site typing demonstrated that there is broad genetic diversity of *E. coli* O157:H7 in the bovine reservoir and that numerous genotypes are significantly underrepresented among clinical isolates, consistent with the possibility that there is reduced virulence or transmissibility to humans of some bovine *E. coli* O157:H7 genotypes.**

Escherichia coli O157:H7 is an important food- and waterborne zoonotic pathogen that expresses two cardinal virulence factors: the ability to produce one or more Shiga toxins (Stx) encoded by genes located in lambdoid bacteriophages (8, 33) and the ability to attach intimately to epithelial cells via expression of a pathogenicity island termed the locus of enterocyte effacement (LEE) (31).

Feng et al. described a plausible series of evolutionary events leading to the evolution of the dominant non-sorbitol-fermenting, β-glucuronidase-negative *E. coli* O157:H7 clade from a nontoxigenic progenitor, *E. coli* O55:H7 (5). Shaikh and Tarr (27) assayed clinical isolates of this dominant *E. coli* O157:H7 clade for the Stx-encoding bacteriophage insertion sites defined in the strains that have been sequenced (7, 24). Three principal groups of isolates sharing Stx bacteriophage insertion site genotypes were identified: isolates with an Stx2-encoding bacteriophage inserted at a location other than *wrbA* and with *yehV* occupied by a centrally truncated bacteriophage (cluster 1), isolates with an Stx2-encoding bacteriophage inserted into *wrbA* and with *yehV* occupied by a truncated bacteriophage as in cluster 1 (cluster 2), and isolates with a complete Stx1-encoding bacteriophage inserted into *yehV* and with an Stx2-encoding bacteriophage inserted into *wrbA* (cluster 3).

Association of the genetic diversity within the sorbitol-negative, β-glucuronidase-negative *E. coli* O157:H7 clade with prophage insertions has been demonstrated previously. Kim et al. (14) identified two major lineages within this clade using octamer-based genomic scanning and also found prophage sequences in some of the polymorphic bands detected by this method. Ohnishi et al. (22) used whole-genome PCR scanning and concluded that variation among bacteriophages is a major factor in generating genomic diversity within the O157 lineage. Comparative genomic microarray hybridization demonstrated that prophage or prophage-related elements accounted for >85% of the variably present genes in 12 *E. coli* isolates representing the O157:H7 evolutionary lineage (34).

Cattle are a major reservoir of *E. coli* O157:H7, but colonized animals typically exhibit no disease (6). *E. coli* O157 is ubiquitous on cattle farms, and the prevalence of cattle shedding this agent frequently is greater than 10% and can approach 100% (6). The high prevalence of this agent in cattle contrasts with the comparative rarity of human infection, despite the reportedly low infectious dose (13, 28, 32).

In this study, we characterized the bacteriophage insertion site genotypes of *E. coli* O157:H7 isolates from cattle and from humans and found increased genotype diversity

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TABLE 1. Year and state or province of origin for isolates used in this study

Year of isolation	Host	Study(ies)	No. of isolates obtained at the following locations:											
			WA	ID	OR	MT	WY	MO	KS	NE	TX	PA	AB	Total
1984	Human	1	1	0	0	0	0	0	0	0	0	0	0	1
1986	Human	1	3	0	0	0	0	0	0	0	0	0	0	3
1987	Human	1	4	0	0	0	0	0	0	0	0	0	0	4
1991	Bovine	V[a]	10	0	0	0	0	0	0	0	0	0	0	10
1993	Bovine	V	5	1	0	0	0	0	0	0	0	0	1	7
1994	Bovine	V	2	0	1	0	0	0	0	0	0	0	0	3
1995	Bovine	V	5	3	3	0	0	0	0	1	0	0	0	12
1996	Bovine	V	4	0	0	0	0	0	0	1	1	1	1	8
1997	Human	2	18	0	0	0	0	0	0	0	0	0	0	18
	Bovine	V	1	1	0	0	0	0	0	0	0	0	0	2
1998	Human	2, 3, 4	16	1	1	6	0	0	0	0	0	0	0	24
	Bovine	V	2	0	0	0	0	0	1	0	0	0	1	4
1999	Human	2, 3, 4	38	1	1	25	1	0	0	0	0	0	0	66
	Bovine	V	6	0	0	0	0	0	0	0	0	0	0	6
2000	Human	2, 3	42	1	1	0	0	0	0	0	0	0	0	44
	Bovine	V	7	0	0	0	0	0	0	0	0	0	0	7
2001	Human	3	27	1	1	0	0	0	0	0	0	0	0	29
	Bovine	V	8	1	0	0	0	0	0	0	0	0	1	10
2002	Human	2, 5	16	0	2	0	0	0	0	0	0	0	0	18
	Bovine	V	1	1	0	0	0	0	0	0	0	0	0	2
2003	Human	2, 5	18	0	2	0	0	3	0	0	0	0	0	23
2004	Human	2, 5	29	7	1	0	0	3	0	0	0	0	0	40
	Bovine	V	0	0	0	0	0	0	0	0	0	0	9	9
2005	Human	2, 5	5	0	0	0	0	7	0	0	0	0	0	12
Total	Human	All	217	11	9	31	1	13	0	0	0	0	0	282
	Bovine	V	51	7	4	0	0	0	1	2	1	1	13	80

[a] V indicates that isolates were obtained in various cattle studies at Washington State University.

among bovine isolates and differential representation of genotypes in these host species.

MATERIALS AND METHODS

Bacterial isolates. Eighty *E. coli* O157:H7 isolates from cattle were randomly selected from isolates obtained during various Washington State University surveys between 1991 and 2004 (Table 1). All studies involving animals were performed under appropriate animal use guidelines with the approval of the WSU Institutional Animal Care and Use Committee. The random selection was stratified so that the group included 20 isolates each from four time periods (1991 to 1994, 1995 and 1996, 1997 to 2000, and 2001 to 2004), limited to single isolates from individual farms within each period. An additional set of stx_1-negative, stx_2-positive bovine isolates was selected to search for an unusual genotype, as described below.

Clinical *E. coli* O157:H7 isolates were obtained from five different studies (Table 1). Study 1 isolates were obtained from Seattle area patients of all ages (30). Study 2 isolates originated from children up to 10 years old in a four-state region (4, 12). Study 3 isolates originated from patients up to 21 years old at the Seattle Children's Hospital and Regional Medical Center (CHRMC) (16). Study 4 isolates originated from Montana patients of all ages (12). Study 5 isolates originated from CHRMC patients up to 21 years old. Only a single isolate from each recognized cluster of infections was included in this analysis to maximize the independence of the isolates. Outcomes (hemolytic-uremic syndrome [HUS] versus no HUS) were available for 248 patients, 51 of whom developed HUS. Each of these studies was performed in accordance with the human experimentation guidelines of the U.S. Department of Health and Human Services and with approval from one or more of the Institutional Review Boards of the CHRMC, the University of Washington, and Washington University.

The control strains used in this study included *E. coli* O157:H7 strains 87-14 (cluster 1) and 86-24 (cluster 2) (30), and EDL933 (= ATCC 43895; ATCC, Manassas, VA) (cluster 3), and *E. coli* K-12 strain INVαF′ (Invitrogen Corp., Carlsbad, CA).

Bovine isolate characterization. Bovine isolates were tested for sorbitol fermentation and β-glucuronidase expression as described previously (18). O157 antigen expression was detected by latex agglutination (*E. coli* PRO O157; PL.070HD; Hardy Diagnostics, Santa Maria, CA). Multiplex PCR was used to detect *eae*, stx_1, and stx_2 in all bovine isolates (23).

For multilocus sequence typing, seven loci (*aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh*, and *uidA*) were sequenced and allele profiles were used to assign a sequence type

TABLE 2. Nucleotide sequences of primers used for PCR analyses in this study

Primer	Sequence (5′–3′)	Target	Reference(s)
A	AAGTGGCGTTGCTTTGTGAT	*yehV* insertion site region	27
B	AACAGATGTGTGGTGAGTGTCTG		
A	AAGTGGCGTTGCTTTGTGAT	Bacteriophage-*yehV* right junction	27
E	GATGCACAATAGGCACTACGC		
F	CACCGGAAGGACAATTCATC	Bacteriophage-*yehV* left junction	27
B	AACAGATGTGTGGTGAGTGTCTG		
C	AGGAAGGTACGCATTTGACC	*wrbA* insertion site region	27
D	CGAATCGCTACGGAATAGAGA		
C	AGGAAGGTACGCATTTGACC	Bacteriophage-*wrbA* right junction	27
G	ATCGTTCGCAAGAATCACAA		
H	CCGACCTTTGTACGGATGTAA	Bacteriophage-*wrbA* left junction	27
D	CGAATCGCTACGGAATAGAGA		
A	AAGTGGCGTTGCTTTGTGAT	Variant bacteriophage-*yehV* right junction	27; this study
E2	CAGGGAATCAATCGCAGTTT		
595	CCGAAGAAAAACCCAGTAACAG	stx_2-antiterminator *Q* gene junction	33, 17
Q_{933}	CGGAGGGGATTGTTGAAGGC		
TirF	TCATGTTCAGCCGTTTATCG	*tir*	This study
TirR	AAGCTCAAGAGTTGCCCATC		
A	AAGTGGCGTTGCTTTGTGAT	Bacteriophage-*yehV* right junction (~8 kb)	27; this study
R1	GCAGATGATGAGCGTGATTG		
FimH460F	CTTATGGCGGCGTGTTATCTA	FimH mannose-binding pocket flanking N135K	Shaikh et al., submitted
FimH460R	GCCAGTAGGCACTACCACATC		
stx2c-F919	CTGAACAGAAAGTCACAGTYTTTA	stx_{2c}-specific region	This study
stx2c-R1100	GGCCACTTTTACTGTGAATGTATC		
O-F163	ATGCGCAAGACATACGGATTC	Stx2c bacteriophage *O* gene	This study
O-R695	TGCACAAACGCCCTGACATA		
Q-F69	GGGCGCATGGGTTTATTCA	Stx2c bacteriophage *Q* gene	This study
Q-R389	ACTTCCCGTCGGCAGGTTG		
mdh-F41	AGGCGCTTGCACTACTGTTA	*mdh*	This study
mdh-R875	AGCGCGTTCTGTTCAAATG		

(ST) to each bovine isolate (11, 25) using protocols available on the STEC Center website (http://www.shigatox.net/mlst).

Stx1 and Stx2 expression was detected by a gold-labeled immunosorbent assay (DuoPath Verotoxins, EMD Inc., Darmstadt, Germany) with broth culture supernatants, using carbadox (25 ng/ml; Sigma-Aldrich, St. Louis, MO) to induce Stx production. The verocytotoxicity of bovine isolates was confirmed by direct microscopic observation of Vero cell monolayers in 96-well plates 24 and 48 h after addition of 1:10 dilutions of filter-sterilized (0.45 μm; Millipore, Billerica, MA) overnight carbadox-induced broth culture supernatants in minimal essential medium (MEM) containing 5% fetal bovine serum (FBS). Total Stx production by bovine isolates was quantified by an enzyme immunoassay (Premier EHEC assay; Meridian Bioscience, Cincinnati, OH) with overnight carbadox-induced broth culture supernatants. *E. coli* DH5α (*stx* negative) was used as a negative control and *E. coli* O157:H7 strain EDL933 was used as a positive control for these Stx assays.

Stx-encoding bacteriophage insertion sites. Study isolates were interrogated for Stx genes, for Stx-encoding bacteriophage-chromosome junctions, and for intact bacteriophage insertion site regions by PCR (27) (Table 2). The presence of intact chromosomal insertion site regions (*yehV* and *wrbA*) was confirmed in all isolates with negative reactions for Stx-encoding bacteriophage-chromosome junctions. Selected bovine isolates with variant patterns of bacteriophage insertion in *yehV* (right junction negative and left junction positive) were subjected to amplification of a longer segment of the variant right junction by long PCR (Platinum *Taq* High Fidelity DNA polymerase [Invitrogen]; 35 cycles of 94°C for 30 s, 58°C for 30 s, and 68°C for 5 min). Cloning (TA cloning kit; Invitrogen) and partial DNA sequencing (Amplicon Express, Pullman, WA) of the resulting product allowed us to design a new reverse primer (E2) (Table 2) specific for the variant right *yehV*-bacteriophage junction. An additional group of 128 stx_2-positive, stx_1-negative bovine isolates was subjected to PCR analysis of Stx-encoding bacteriophage insertion sites to screen for the cluster 2 genotype, which occurs in a minority of human clinical isolates (27) and was absent from the initial group of 80 randomly selected bovine isolates.

Detection of additional genetic markers in bovine isolates. A multiplex PCR was designed to specifically detect the Stx2c-encoding bacteriophage (29). In the multiplex reaction we used 0.5 μl of 10 μM primers stx2c-F, stx2c-R, O-F, O-R, Q-F, and Q-R (Table 2) to amplify stx_{2c} and the *O* and *Q* genes of the Stx2c-encoding bacteriophage, as well as primers to amplify the malate dehydrogenase (*mdh*) gene as a positive PCR control. Each 25-μl reaction mixture contained 2.5 μl of 10× buffer II (Applied Biosystems, Foster City, CA), 2.5 μl of 2 mM deoxynucleoside triphosphates, 2.0 μl of 25 mM $MgCl_2$, the six Stx2c-encoding bacteriophage-specific primers listed above, 1 μl of each of the two *mdh* primers, 0.3 μl of 1.5 U AmpliTaq Gold (Applied Biosystems), 1 μl of a 25-ng/μl genomic DNA template, and 11.7 μl of double-distilled H_2O. The amplification cycle included an initial denaturation step (94°C for 10 min), 35 amplification cycles

TABLE 3. Genotyping data, isolate sources, and further characterization of bovine isolates of *E. coli* O157:H7

Genotype[a]	Genotyping data			No. (%) of isolates from:		Additional markers (bovine isolates only)			
	stx[b]	*yehV*[c]	*wrbA*[d]	Cattle	Clinical sources	*fim* (N135K)[e]	Q_{933W}-*stx*$_2$[f]	*stx*$_{2c}$[g]	*tir* sp-1 (D78E)[h]
Cluster 1	2	Occupied	Intact	8 (10)	85 (30.1)	N (5)	7	1	D (7), E (1)
Cluster 2	2	Occupied	Occupied	0 (0)	7 (2.5)				
Cluster 3	1 + 2	Occupied	Occupied	33 (41.3)	176 (62.4)	K (5)	33	1	D (32), E (1)
Genotype 4	2	Intact	Occupied	1 (1.3)	5 (1.8)	K (1)	1	0	D (1)
Genotype 5	2	Variant-R	Intact	9 (11.3)	4 (1.4)	N (5)	1	8	D (3), E (6)
Genotype 6	1 + 2	Variant-R	Intact	15 (18.8)	2 (0.7)	N (5)	10	13	D (10), E (5)
Genotype 7	1 + 2	Variant-R	Occupied	5 (6.3)	1 (0.3)	K (5)	5	0	D (5)
Genotype 8	1	Occupied	Intact	0 (0)	1 (0.3)				
Genotype 9	None	Intact	Intact	0 (0)	1 (0.3)				
Genotype 10	None	Occupied	Intact	1 (1.3)	0 (0)	N (1)	0	0	D (1)
Genotype 11	2	Intact	Intact	2 (2.5)	0 (0)	N (2)	1	0	D (1)
Genotype 12	2	Variant-R	Occupied	1 (1.3)	0 (0)	K (1)	1	0	D (1)
Genotype 13	1	Variant-R	Intact	1 (1.3)	0 (0)	K (1)	1	0	D (1)
Genotype 14	1	Variant-R	Occupied	2 (2.5)	0 (0)	K (2)	0	0	D (2)
Genotype 15	1 + 2	Intact	Intact	1 (1.3)	0 (0)	N (1)	1	1	D (1)
Genotype 16	1 + 2	Occupied	Intact	1 (1.3)	0 (0)	N (1)	1	0	D (1)

[a] Stx-encoding bacteriophage insertion site genotypes. The cluster genotypes are genotypes identified in multiple clinical isolates in a previous study (27). The genotypes are based on patterns of Stx genes and Stx-encoding bacteriophage insertion sites described in this study.

[b] PCR gene detection of *stx*$_1$ (1) and/or *stx*$_2$ (2) (23).

[c] "Occupied" indicates bilateral detection of the *yehV*–Stx-1-encoding bacteriophage junctions (with the A-E and B-F primer pairs, respectively [Table 2]). "Intact" indicates detection of neither junction. "Variant-R" indicates that the left junction between the bacteriophage and the chromosome was detected but the right junction was not detected. Most *yehV*-Variant-R isolates share a different right junction sequence, as described in Results.

[d] "Occupied" indicates bilateral detection of the *wrbA*–Stx-2-encoding bacteriophage junctions (with the C-G and D-H primer pairs [Table 2]). "Intact" indicates detection of neither junction.

[e] N and K indicate the status of the N135K polymorphism (N. Shaikh et al., submitted for publication) in the FimH mannose-binding pocket in bovine isolates. All isolates were tested for genotype groups containing less than six isolates; five isolates were randomly selected for testing from each larger genotype group.

[f] The numbers indicate the numbers of bovine isolates that were PCR positive for the *Q*-*stx*$_2$ junction of phage 933W.

[g] The numbers indicate the numbers of bovine isolates positive as determined by multiplex PCR for the Stx2c-encoding bacteriophage.

[h] The numbers in parentheses indicate the numbers of bovine isolates in which the alleles of the D78E (T238A) single-nucleotide polymorphism in *tir* were detected.

(92°C for 1 min, 55°C for 1 min, and 72°C for 30 s), and a final extension step (72°C for 5 min). PCR products were visualized on ethidium bromide-stained 1.5% agarose gels by illumination with UV light. The presence of the Stx2c-encoding bacteriophage was inferred in isolates that produced the following four amplicons: *mdh* (835 bp), *stx*$_{2c}$ (182 bp), *O* (533 bp), and *Q* (321 bp).

The sp-1 polymorphism (D78E) in *tir* was detected by a real-time PCR TaqMan assay (J. L. Bono, J. E. Keen, M. L. Clawson, L. M. Durso, M. P. Heaton, and W. W. Laegreid, Abstr. 104th Gen. Meet. Am. Soc. Microbiol., abstr. C-388, 2005) or by sequencing a *tir* PCR product (Table 2). The junction between the antiterminator *Q* of bacteriophage 933W and *stx*$_2$ was detected by PCR (Table 2) (17). An N135K polymorphism in *fimH* was detected by sequencing PCR products (Table 2) (N. Shaikh, N. J. Holt, J. R. Johnson, and P. I. Tarr, submitted for publication).

Adherence to MDBK cells was quantified as described by Monteville et al. (21). A three-color immunofluorescence analysis of the *E. coli* O157:H7 interaction with MDBK cells was performed using strains transformed with a green fluorescent protein (GFP) gene-containing plasmid, pMEK91 (20). MDBK cells (5×10^4 cells/well) were cultured on glass coverslips for 18 h at 37°C in a humidified incubator containing 5% CO_2. The cells were infected with a 10:l dilution of a log-phase culture of GFP-transformed *E. coli* in 1 ml of Tris (10 mM, pH 7.4)-buffered minimal essential medium supplemented with 1% (vol/vol) FBS. Infected cells were incubated (3 h), rinsed with MEM, and reincubated in MEM containing FBS (4 h). Cells were washed three times with phosphate-buffered saline, fixed (2% paraformaldehyde), and permeabilized (0.1% Triton X-100). Actin was stained using tetramethylrhodamine isothiocyanate-labeled phalloidin (0.2 μg/ml; Sigma, St. Louis, MO). Coverslips were mounted using Vectashield (Vector Laboratories Inc., Burlingame, CA) with 4′,6-diamidino-2-phenylindole (DAPI) and were visualized using epifluorescence (Nikon Eclipse TE2000). Images were captured with the MetaMorph version 5 imaging software and were formatted using Adobe Photoshop 3.0.4.

Statistical analysis. The distribution of Stx-encoding bacteriophage insertion site genotypes in human and cattle isolates and the associations between the *fimH*, *tir* sp-1, Q_{933}, and *stx*$_{2c}$ markers and the Stx-encoding bacteriophage insertion site genotypes in isolates from cattle were evaluated by χ^2 tests (SigmaStat; SPSS, Inc., Chicago, IL). Differences in Stx production and differences in adherence of selected *E. coli* O157:H7 isolates to MDBK cells were evaluated by one-way analysis of variance (SigmaStat), and where differences were detected, pairwise multiple comparisons were performed using the Tukey test.

RESULTS

Identification and characterization of bovine isolates. All bovine isolates expressed the O157 antigen, were positive for *eae* and *tir*, and fermented lactose but not sorbitol; none expressed β-glucuronidase. All but one isolate were positive for *stx*$_1$ or *stx*$_2$ or both (Table 3). Multilocus sequence typing assigned 79 of the 80 isolates to ST-66, the most common ST of the β-glucuronidase-negative, non-sorbitol-fermenting clade of *E. coli* O157:H7 in the United States, consistent with their membership in this clade. The one remaining isolate was assigned to ST-350, which differs from ST-66 by a single nucleotide in *fadD*.

Stx-encoding bacteriophage insertion sites. Clinical isolates of *E. coli* O157:H7 (Table 1) were analyzed to determine their Stx-encoding bacteriophage insertion genotypes. Clusters 1 to 3, as defined previously, accounted for 268 (95%) of the 282 isolates (Table 3). Six additional, less frequent genotypes were identified among the clinical isolates. Genotype 4 ($n = 5$; 1.8%) was characterized by the presence of *stx*$_2$, the absence of *stx*$_1$, an intact *yehV* site, and a bacteriophage-occupied *wrbA* site; genotype 5 ($n = 4$; 1.4%) was characterized by the presence of *stx*$_2$, the absence of *stx*$_1$, amplification of the left but not the right *yehV*-bacteriophage junction (designated *yehV*-Variant-R), and an intact *wrbA* site; genotype 6 ($n = 2$; 0.7%) was

characterized by the presence of both stx_1 and stx_2, *yehV*-Variant-R, and an intact *wrbA* site; genotype 7 ($n = 1$; 0.4%) was characterized by the presence of both stx_1 and stx_2, *yehV*-Variant-R, and a bacteriophage-occupied *wrbA* site; genotype 8 ($n = 1$; 0.4%) was characterized by the presence of stx_1, the absence of stx_2, a bacteriophage-occupied *yehV* site, and an intact *wrbA* site; and genotype 9 ($n = 1$; 0.4%) was characterized by the absence of stx_1 and stx_2 and intact *yehV* and *wrbA* sites (Table 3).

In contrast, the cluster 1 to 3 genotypes were found much less frequently among the bovine isolates ($n = 41$; 51.3%) (Table 3). The distribution of clusters 1 to 3 and "other" genotypes (all remaining genotypes) differed significantly in the clinical and bovine isolates ($\chi^2 = 98.2$, 3 df, $P < 0.001$; including no more than a single isolate per farm with the same genotype). One bovine isolate had genotype 4. Genotypes 5 ($n = 9$; 11.3%), 6 ($n = 15$; 18.8%), and 7 ($n = 5$; 6.3%) were each overrepresented among cattle isolates compared to the clinical isolates (in each case, $\chi^2 > 9.8$, 1 df, and $P < 0.01$). The remaining nine bovine isolates had seven additional Stx-encoding bacteriophage insertion site genotypes that were not detected among the clinical isolates. These genotypes, designated genotypes 10 to 16, each were found in only one or two strains; the specific results are shown in Table 3. Because the cluster 2 genotype was not detected among the randomly chosen set of 80 bovine isolates, 124 additional bovine isolates previously determined to have the appropriate stx_1-negative, stx_2-positive profile were screened for the cluster 2 genotype, and four of these strains (3.2%) had cluster 2 genotypes, confirming the presence of cluster 2 isolates in the bovine reservoir, albeit at a low frequency.

The *yehV*-Variant-R bacteriophage junction was characterized further in view of the relatively high frequency of genotypes with this reaction, particularly among bovine isolates. Considering the possibility that the region complementary to primer E (Table 2) had been deleted or disrupted, we attempted to amplify a larger segment of the right bacteriophage-*yehV* junction using *yehV* primer A (Table 2) paired with a primer designed to amplify approximately 7.5 kb of the right *yehV*-stx_1 bacteriophage junction. This long PCR amplified a 7.5-kb product from the template of the positive control strain (EDL933; cluster 3); in contrast, it amplified 8-kb products from the templates of two *yehV*-Variant-R isolates (genotype groups 5 and 6). This PCR product was cloned, and a partial 5′ sequence was obtained. The initial 362 bp of this sequence was 100% identical to the *yehV*-stx_1 prophage junction of enterohemorrhagic *E. coli* Sakai, but the next 738 bp was most similar (>96% identity) to *E. coli* O157:H7 strain Sakai genes ECs2153 and ECs2154, located adjacent to another cryptic prophage in the Sakai genome (GenBank accession no. EF081034). This sequence was used to design a new reverse primer, E2 (Table 1), which, combined with *yehV* forward primer A, specifically amplified the *yehV*-Variant-R junction sequence from 23 of 30 (76.7%) bovine *yehV*-Variant-R isolates. All bovine isolates in which *yehV* sites were intact ($n = 13$) or occupied ($n = 10$) were negative as determined by this PCR.

Stx and LEE effector expression by bovine *E. coli* O157:H7 isolates. All bovine isolates in which one or more *stx* genes were detected produced verocytotoxigenic culture supernatants; the single *stx*-negative isolate (genotype 10) was nontoxic. The Stx expression detected by the gold-labeled immunosorbent assay agreed exactly with the *stx* detected by PCR for 76 of the 80 bovine isolates. The four exceptions included one genotype 5 isolate and two genotype 6 isolates that did not express detectable Stx2 and one genotype 16 isolate that did not express detectable Stx1. Five randomly selected bovine isolates from genotype groups with six or more isolates and all isolates from genotype groups with fewer members were evaluated for Stx production by an enzyme immunoassay (Table 4); significant differences in Stx production in different genotype groups were detected ($P = 0.019$, as determined by one-way analysis of variance). The Tukey multiple-comparison procedure indicated that the cluster 3 isolates tested produced significantly more Stx than the genotype 5 isolates tested produced; no other statistically significant differences in Stx production in different genotype groups were identified.

TABLE 4. Stx production by and cytoadherence of *E. coli* O157:H7 genotypes isolated from cattle

Genotype	Stx production[a]		Cytoadherence[b]	
	No. of isolates	Mean optical density (SD)	No. of isolates	Mean adherence (SD)
Cluster 1	5	1.61 (0.88)	2	39.7 (31.7)
Cluster 3	5	1.73 (0.93)	2	9.8 (7.7)
Genotype 4	1	1.57		ND[c]
Genotype 5	5	0.24 (0.27)	3	27.7 (19.6)
Genotype 6	5	0.57 (0.54)	3	55.3 (82.7)
Genotype 7	5	1.53 (1.02)	2	11.3 (6.4)
Genotype 8	1	0.08		ND
Genotype 9	2	1.87 (0.10)		ND
Genotype 10	1	1.66		ND
Genotype 11	1	0.18		ND
Genotype 12	2	0.27 (0.04)		ND
Genotype 13	1	0.31		ND
Genotype 14	1	1.00		ND

[a] Stx production as measured by an enzyme immunoassay using overnight broth cultures of *E. coli* O157:H7 isolates with bacteriophage induction by carbadox (25 ng/ml).
[b] Cytoadherence to MDBK cells. The results are fold increases in the number of adherent CFU per well compared to the *E. coli* INVαF′ control strain.
[c] ND, not determined.

Randomly selected bovine isolates with the cluster 1 or cluster 3 genotype or genotype 5, 6, or 7 all exhibited comparable quantitative adherence to MDBK cells (Table 4). Similarly, randomly selected bovine isolates with the cluster 1, cluster 2, or cluster 3 genotype or genotype 5 or 6 all produced qualitatively similar adherent *E. coli* O157:H7 microcolonies and focal actin accumulation on MDBK cells (Fig. 1).

Geographic and temporal distributions of *E. coli* O157:H7 genotypes. The bovine isolates originated from 69 different cattle farms in seven states in the United States and one Canadian province (Table 1). Neither secular nor geographic trends were observed in the distribution of Stx-encoding bacteriophage insertion site genotypes (data not shown). Twenty-five of the bovine isolates were obtained from predominantly beef breeds and 55 were obtained from dairy breeds, and there were not significant differences in genotype distribution between the breeds. Eight farms contributed more than one study isolate. Multiple isolates obtained during different time peri-

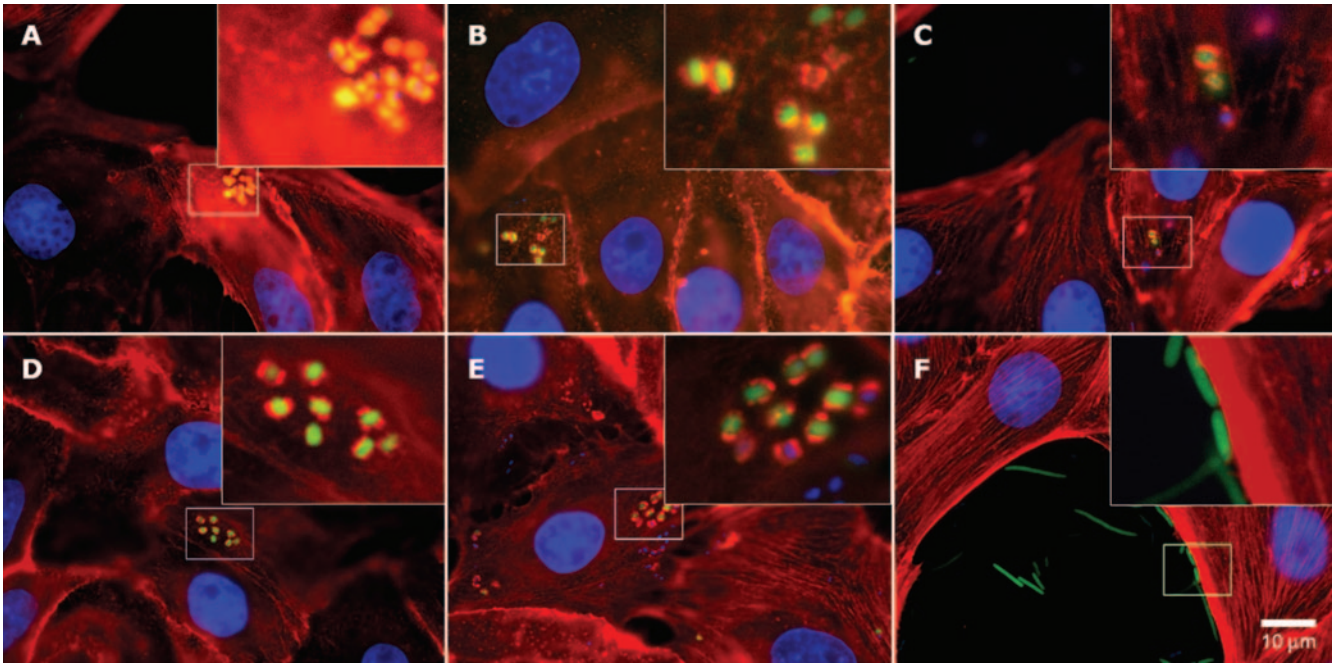

FIG. 1. Actin polymerization associated with *E. coli* O157:H7 microcolonies adhering to MDBK cells: three-color immunofluorescence analysis of *E. coli* O157:H7 interaction with MDBK cells. Blue, DAPI staining of nuclear DNA; red, tetramethylrhodamine isothiocyanate-conjugated phalloidin staining of actin; green, GFP plasmid-transformed *E. coli* cells. (A) *E. coli* O157:H7 strain EDL933 (cluster 3; positive control); (B) *E. coli* O157:H7 strain 86-24 (cluster 2; positive control); (C) *E. coli* O157:H7 bovine isolate (cluster 1); (D) *E. coli* O157:H7 genotype 5 bovine isolate; (E) *E. coli* O157:H7 genotype 6 bovine isolate; (F) *E. coli* K-12 strain InvαF′ (negative control).

ods from single farms tended to share Stx-encoding bacteriophage insertion site genotypes; six farms contributed isolates with the same genotype on two (four farms) or three (two farms) occasions separated by 1 to 8 years. The genotypes repeatedly isolated from these single farms included cluster 1 (one farm) and 3 (three farms) genotypes and genotypes 5 and 11 (one farm each). Two isolates obtained 2 years apart from a seventh farm had genotype 6, while a third isolate from that farm obtained 4 years later was a cluster 3 strain. Finally, two isolates obtained 2 years apart on the eighth farm had the cluster 3 genotype and genotype 6.

Human isolates were recovered from patients in six states in the United States in two time periods, 1984 to 1987 and 1997 to 2005. Neither secular nor geographic trends were observed in the distribution of the Stx-encoding bacteriophage insertion site genotypes (data not shown).

Stx2c bacteriophage. The Stx2c-encoding bacteriophage, as inferred by PCR detection of stx_{2c} and specific *O* and *Q* gene sequences, was detected in 24 of the 80 bovine isolates, including cluster 1 and 3 genotype and genotype 5, 6, and 15 isolates (Table 3). stx_{2c} occurred less frequently among bovine isolates with cluster 1 and 3 genotypes (2 of 41 isolates) than in bovine isolates with other genotypes (22 of 39 isolates; $\chi^2 = 22.9$, 1 df, $P < 0.001$) (Table 3). In the context of human disease, while *E. coli* O157:H7 with genes encoding Stx2c is unusual, it may be associated with more severe illness (12).

***fimH* mannose binding pocket polymorphism.** Representative bovine isolates (all isolates in genotype groups with less than five isolates and five randomly chosen isolates each for all other genotype groups) were examined for the presence of a single-nucleotide polymorphism in *fimH* resulting in an N135K amino acid sequence change in the mature FimH protein. Fourteen isolates with *wrbA* occupied by bacteriophage all carried the K allele, while 20 of 21 isolates with *wrbA* intact carried the N allele ($\chi^2 = 27.3$, 1 df, $P < 0.001$) (Table 3). The segment of *wrbA* amplified by primers C and D (Table 2) in the single *wrbA*-intact, K allele-positive isolate was identical to the segment of the same region in *E. coli* K-12 *wrbA*.

Q_{933W} and *tir* polymorphic markers. The bacteriophage Q_{933W}-stx_2 junction was detected by PCR in 40 of 41 cattle isolates with the cluster 1 or 3 genotype but in only 21 of 39 isolates having other genotypes ($\chi^2 = 18.7$, 1 df, $P < 0.001$). The T238A (D78E) *tir* polymorphism designated sp-1 was detected in only 2 of 41 cattle isolates with the cluster 1 or 3 genotype but in 11 of 38 isolates with other genotypes, particularly genotypes 5 (6 of 9 isolates) and 6 (5 of 15 isolates) ($\chi^2 = 10.7$, 1 df, $P < 0.001$).

DISCUSSION

Our typing system revealed a diversity of Stx-encoding bacteriophage insertion site genotypes among *E. coli* O157:H7 from the bovine reservoir that was considerably broader than that expected on the basis of analysis of human isolates. Some of the genotypes detected among isolates from both species were markedly overrepresented in clinical isolates (clusters 1 and 3) or bovine isolates (genotypes 5 to 7). All genotypes represented by two or more clinical isolates (cluster 1 to 3 genotypes and genotypes 4 to 6) were identified in isolates from cattle (although discovery of a bovine cluster 2 strain

required screening of additional isolates), consistent with the hypothesis that cattle are the reservoir for *E. coli* O157:H7 resulting in human disease. Most (10/13) of the genotype groups identified for the first time in this study had only one or two members, indicating that identification of additional diversity is likely to occur when more isolates, particularly isolates from the bovine reservoir, are analyzed.

We considered the possibility that lysogenization and excision events in the short term, such as during a single human or animal gastrointestinal passage, could result in changes in the Stx-encoding bacteriophage insertion site genotypes of *E. coli* O157:H7 characterized in this study. However, the available evidence generally does not support this possibility. First, previous studies of non-sorbitol-fermenting, β-glucuronidase-negative *E. coli* O157:H7 suggested that the acquisition of Stx-encoding phages was a key event in the evolution of this pathogenic clade from precursor strains and thus predated the divergence detected on the basis of these genotypes within the clade (5, 34). Consistent with this view, we demonstrated here the correlation between *wrbA* site occupancy and *fimH* (a nonprophage gene) single-nucleotide polymorphism and also the correlation between stx_{2c} carriage and genotypes 5 and 6 (defined by insertion sites of other phages). Both of these correlations are inconsistent with highly dynamic phage movement. Second, we observed stability of cluster 1 and cluster 3 genotypes among multiple isolates from several farms over multiple years, which is inconsistent with the possibility that the phage exposure in bovine environments results in rapid conversion to "bovine" phage insertion site genotypes. More generally, pulsed-field gel electrophoresis profiles, which would be expected to be highly sensitive to genetic changes at the scale of bacteriophage insertions or excisions, have been empirically observed to be well conserved among epidemiologically related isolates of β-glucuronidase-negative *E. coli* O157:H7 (1, 2, 32). In contrast, in a different lineage of enterohemorrhagic *E. coli* O157, sorbitol-fermenting *E. coli* O157:NM, the pulsed-field gel electrophoresis patterns are less stable because of rapid bacteriophage recombination activity (3, 19). It will be important to specifically evaluate the stability of diverse Stx-encoding bacteriophage insertion site genotypes in future studies.

The heterogeneity of bovine *E. coli* O157:H7 genotypes reported here has implications for pathophysiology, evolutionary genetics, and food safety. First, two key virulence factors, the production of Stx and the ability to colonize the colonic surface through the functions of LEE-encoded products, are believed to be central to the pathophysiology of *E. coli* O157:H7-induced disease. Most isolates, including those with genotypes underrepresented among clinical isolates, nevertheless possessed both of these factors. Thus, it is possible either that these isolates differ in the level of expression of Stx or LEE-encoded products (as was demonstrated here with reduced Stx production for genotype 5 compared to the cluster 3 genotype) or that they lack another, currently unrecognized virulence factor(s). Therefore, identification of the genetic basis underlying the nonrandom distribution of these genotypes could expand our understanding of the pathophysiology of this important human pathogen. This could apply even more broadly to Stx-producing *E. coli* isolates belonging to other serotypes. Of the more than 250 serotypes of Stx-producing *E. coli* detected in the bovine reservoir, fewer than 100 have been associated with human illnesses (9, 10), and only a few serotypes cause most human infections. It is possible that some of the same genetic differences that account for the distribution of nonclinical genotypes of *E. coli* O157:H7 also account for the distribution of nonclinical Stx-producing *E. coli* serotypes.

Second, these findings shed additional light on the recent evolution of *E. coli* O157:H7. At any given time, the vast majority of *E. coli* O157:H7 isolates reside in the animal reservoirs and not in humans, but studies of the evolution of *E. coli* O157:H7 have focused on clinical isolates. Shaikh and Tarr proposed an evolutionary lineage in which evolving *E. coli* O157:H7 sequentially acquired Stx-encoding bacteriophages in specific chromosomal locations (27). It is possible that some of the genotypes identified here represent additional steps in this lineage. For example, genotype 9 has the *fimH* sequence, *yehV* and *wrbA* occupancy, and Stx profile expected of an immediate ancestor of the cluster 1 genotype. However, most of the new genotypes reported here appear to represent evolutionary offshoots within the β-glucuronidase-negative *E. coli* O157:H7 clade. For example, genotype 8 could represent a single excision of the Stx2-encoding bacteriophages from a cluster 3 *E. coli* O157:H7 strain. Additional genetic information is required in order to place such offshoots into a plausible phylogeny.

Third, these findings should prompt additional epidemiologic studies of *E. coli* O157:H7 in the animal reservoir, in the environment, and in contaminated food products in order to determine whether the cluster 1 to 3 genotypes differ from other genotypes in terms of the distribution among potential sources of human infection. It is also possible that regulatory attention should be focused more on the subset of *E. coli* O157:H7 genotypes overrepresented among clinical isolates.

Kim et al. (14) first reported differential distribution of *E. coli* O157:H7 lineages among isolates from cattle and humans that was consistent with differences in human infectivity or pathogenicity in certain isolates. These investigators used octamer-based scanning to identify two *E. coli* O157:H7 lineages, one of which more frequently contained human clinical isolates and the other of which more frequently contained bovine isolates. Interestingly, much of the variation seen with octamer-based scanning was associated with bacteriophage-related sequences. The results of subsequent investigations of a larger and more diverse panel of isolates using simplified techniques supported the hypothesis that there is nonrandom distribution of these lineages in cattle and human hosts but that the degree is considerably lower than that originally proposed (15, 35). The correlation, if any, between the lineages reported in these previous studies and the genotypes reported here is not known.

Roe et al. (26) reported differences between human clinical and bovine isolates in the proportion of cells expressing LEE-encoded EspA filaments, with human isolates having higher proportions of filament-expressing cells. The possibility that EspA expression varies between genotypes found in both bovine and clinical isolates (cluster 1 to 3 genotypes and genotype 4) and genotypes found only in bovine isolates has not been evaluated to date. However, data for in vitro cytoadherence and actin accumulation (Fig. 1 and Table 4) do not indicate that there are large intrinsic differences between these genotype groups in terms of LEE expression.

Our Stx-encoding bacteriophage insertion site data correlate with two other markers previously reported to be nonrandomly

distributed among *E. coli* O157:H7 strains from cattle and humans. The stx_2-Q_{933W} antiterminator gene junction was more common in human isolates than in cattle isolates (17). The D78E *tir* polymorphism was more common in cattle isolates than in human isolates (Bono et al., Abstr. 104th Gen. Meet. Am. Soc. Microbiol.). Here, we show that these markers are nonrandomly distributed among *E. coli* O157:H7 genotypes in our set of cattle isolates and that their distribution is consistent with the frequency at which these genotypes occur in human disease isolates. Q_{933W} occurred in all but one bovine cluster 1 to 3 genotype or genotype 4 isolate but significantly less frequently in isolates with other genotypes. The *tir* polymorphism sp-1 was significantly less common in bovine cluster 1 to 3 genotype or genotype 4 isolates than in isolates with other genotypes. These observations suggest that genotypes with diverse potentials for human disease distinguishable by their Stx-encoding bacteriophage insertion site genotypes coexist in bovine populations.

In summary, the diversity within the non-sorbitol-fermenting, β-glucuronidase-negative *E. coli* O157:H7 clade is underestimated by evaluation of clinical isolates alone. This diversity is more fully demonstrated by animal (bovine) isolates, and it appears that no single linear phylogeny could encompass this diversity. Studies of broader panels of isolates from cattle and perhaps other animal species are necessary to confirm the distribution of *E. coli* O157:H7 Stx-encoding bacteriophage insertion site genotypes in cattle and to understand the basis for their different occurrence in clinical isolates.

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ERRATUM

Greater Diversity of Shiga Toxin-Encoding Bacteriophage Insertion Sites among *Escherichia coli* O157:H7 Isolates from Cattle than in Those from Humans

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Volume 73, no. 3, p. 671–679, 2007. Page 675, Table 4: "Genotype 8" should read "Genotype 10," "Genotype 9" should read "Genotype 11," "Genotype 10" should read "Genotype 12," "Genotype 11" should read "Genotype 13," "Genotype 12" should read "Genotype 14," "Genotype 13" should read "Genotype 15," and "Genotype 14" should read "Genotype 16."