

Genotypic Characterization and Prevalence of Virulence Factors among Canadian *Escherichia coli* O157:H7 Strains^{∇†}

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In this study, the association between genotypic and selected phenotypic characteristics was examined in a collection of Canadian *Escherichia coli* O157:H7 strains isolated from humans and cattle in the provinces of Alberta, Ontario, Saskatchewan, and Quebec. In a subset of 69 strains selected on the basis of specific phage types (PTs), a strong correlation between the lineage-specific polymorphism assay (LSPA6) genotype and PT was observed with all strains of PTs 4, 14, 21, 31, 33, and 87 belonging to the LSPA6 lineage I (LSPA6-LI) genotype, while those of PTs 23, 45, 67, and 74 belonged to LSPA6 lineage II (LSPA6-LII) genotypes. This correlation was maintained when additional strains of each PT were tested. *E. coli* O157:H7 strains with the LSPA6-LI genotype were much more common in the collection than were the LSPA6-LII or lineage I/II (LSPA6-LI/II)-related genotypes (82.6, 11.2, and 5.8%, respectively). Of the strains tested, proportionately more LSPA6-LI than LSPA6-LII genotype strains were isolated from humans (52.7% versus 19.7%) than from cattle (47.8% versus 80.2%). In addition, 96.7% of the LSPA6-LII strains carried the *stx*_{2c} variant gene, while only 50.0% of LSPA6-LI/II and 2.7% of LSPA6-LI strains carried this gene. LSPA6-LII strains were also significantly more likely to possess the colicin D gene, *cda* (50.8% versus 23.2%), and have combined resistance to streptomycin, sulfisoxazole, and tetracycline (72.1% versus 0.9%) than were LSPA6-LI strains. The LSPA6 genotype- and PT-related characteristics identified may be important markers of specific ecotypes of *E. coli* O157:H7 that have unique epidemiological and virulence characteristics.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157:H7 is the leading cause of hemorrhagic colitis and hemolytic-uremic syndrome (HUS) throughout the world (16, 23, 25). Cattle colonized by *E. coli* O157:H7 are thought to be the primary reservoir of this bacterium, and its transmission to humans frequently results from the ingestion of contaminated food and water (16, 23, 35).

Results of multiple studies suggest that *E. coli* O157:H7 strains may differ in their association with human disease. An increasing body of evidence has shown that strains can differ in the type and level of expression of virulence factors (3, 28, 29, 47, 48). Similarly, in vivo testing of strains in the gnotobiotic pig model has shown that human isolates caused more severe symptoms than cattle isolates, suggesting that cattle-derived strains may differ in their virulence with respect to those isolated from humans (3). High-resolution genotyping studies on *E. coli* O157:H7 strains from the United States and Australia using octamer-based genome scanning (OBGS) first demonstrated that the *E. coli* O157:H7 clonal complex has diverged through two primary lineages, designated lineage I and lineage

II, and that these two lineages differ in their frequency of association with human disease (28, 29, 54). Subsequent studies using a more efficient multiplex PCR assay based on OBGS, the lineage specific polymorphism assay (LSPA6), among a collection of 1,400 *E. coli* O157:H7 strains from the United States, showed that although lineage I (LSPA6 genotype 111111) was the most common genotype isolated from humans or cattle, it was proportionately overrepresented among human isolates. In contrast, lineage II (LSPA6 genotype 222222) occurred at a significantly higher frequency among bovine strains than among human strains. The authors of these studies suggest that *E. coli* O157 lineage II strains may be less virulent for humans than lineage I strains (54). Recent studies which demonstrate that the genomic island and prophage content of lineage I and II strains differ support this contention (5, 30, 55). Further, the levels of expression of certain genes, including some of those associated with virulence, such as *stx*₂, also appear to differ between strains of the two lineages (30). Taken together, these multiple lines of evidence suggest that genotypic differences between the two lineages underlie apparent phenotypic differences in virulence and host ecology.

The purpose of the present study was to examine whether the genotypic differences between *E. coli* O157:H7 OBGS/LSPA lineages are correlated with phenotypic differences that may help to explain known differences in their epidemiology/host ecology. Strains isolated from human and cattle sources were genotyped using OBGS and LSPA-6 and were characterized for a range of phenotypic characteristics, including phage

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TABLE 1. *E. coli* O157:H7 strains characterized in this study

Strain	Set no.	PT	LSPA6 genotype ^a	Lineage ^b	Origin	Strain	Set no.	PT	LSPA6 genotype ^a	Lineage ^b	Origin
ECI-1382	1	1	111111	I	Bovine	ECI-327	1	14	111111	I	Human
ECI-665	1	1	111111	I	Bovine	ECI-351	1	14	111111	I	Human
ECI-282	1	1	111111	I	Human	ECI-377	1	14	111111	I	Human
1557	2	1	111111	I	Bovine	ECI-485	1	14	111111	I	Human
00301Fe036.1	2	1	111111	I	Bovine	ECI-529	1	14	111111	I	Human
0106-1	2	1	111111	I	Bovine	ECI-543	1	14	111111	I	Human
07101Fe031.2	2	1	111111	I	Bovine	ECI-853	1	14	111111	I	Human
07101Fe036.01	2	1	111111	I	Bovine	R1832/99-1	1	14	111111	I	Human
A533	2	1	111111	I	Bovine	09301Fe058.1	2	14	111111	I	Bovine
bb4-1	2	1	111111	I	Bovine	10801Fe022.2	2	14	111111	I	Bovine
c199-2-472	2	1	111111	I	Bovine	ECI-871	2	14	111111	I	Human
ec20010334	2	1	111111	I	Bovine	ER5769	2	14	111111	I	Human
F156DB	2	1	111111	I	Bovine	KPCA-4-1	1	21	111111	I	Bovine
ECI-243	2	1	111111	I	Human	ECI-247	2	21	111111	I	Human
ECI-882	2	1	211111	I/II	Human	ECI-508	2	21	111111	I	Human
ER1728	2	1	222121	II	Human	ER3199-2	2	21	111111	I	Human
LRC.319	2	1	111111	I	Human	ER6931	2	21	111111	I	Human
M01MD3000	2	1	111111	I	Human	ECI-1433	1	23	222222	II	Bovine
278F1	1	2	211111	I/II	Human	ECI-633	1	23	222222	II	Bovine
C69F1	1	2	211111	I/II	Bovine	03601Fe029.2	2	23	222222	II	Bovine
ECI-497	1	2	211111	I/II	Human	05301Fe029.2	2	23	222222	II	Bovine
ECI-504	1	2	211111	I/II	Human	05401Fe004.2	2	23	222222	II	Bovine
09601Fe010.1	2	2	211111	I/II	Bovine	06801Fe023.1	2	23	222222	II	Bovine
B147-3-413	2	2	211111	I/II	Bovine	06901Fe002.1	2	23	222222	II	Bovine
ER1941	2	2	211111	I/II	Human	07501Fe007.1	2	23	222222	II	Bovine
ER5767	2	2	211111	I/II	Human	09701Fe007.1	2	23	222222	II	Bovine
ECI-577	1	4	111111	I	Bovine	104P1(IMS)	2	23	222222	II	Bovine
ECI-846	1	4	111111	I	Human	113-X-9A	2	23	222222	II	Bovine
R82F2(P)	1	4	111111	I	Human	666-X-5-1-R2	2	23	222222	II	Bovine
E622-1	2	4	111111	I	Bovine	666-C-5D	2	23	222222	II	Bovine
ER2966	2	4	111111	I	Human	CG29-1	2	23	222222	II	Bovine
H112-2	2	4	111111	I	Human	ECI-1435	2	23	222222	II	Bovine
LRC.E318N	2	4	111111	I	Human	ECI-564	2	23	222222	II	Bovine
EC01-296	1	8	111111	I	Bovine	ECI-672	2	23	222222	II	Bovine
99-4367	1	8	111111	I	Human	ECI-684	2	23	222222	II	Bovine
00102Fe040.2	2	8	211111	I/II	Bovine	F12 DIRECT	2	23	222222	II	Bovine
02601Fe041.1	2	8	111111	I	Bovine	F203 DB	2	23	222222	II	Bovine
02701Fe070.1	2	8	211111	I/II	Bovine	666-H-4-1	2	23	222222	II	Human
03301Fe011.2	2	8	111111	I	Bovine	ECI-306	2	23	222222	II	Human
04601Fe023.1	2	8	111111	I	Bovine	ECI-886	2	23	222222	II	Human
07501Fe027.1	2	8	111111	I	Bovine	ER3192	2	23	222222	II	Human
09401Fe052.2	2	8	111111	I	Bovine	ER6554	2	23	222222	II	Human
09501Fe030.2	2	8	211111	I/II	Bovine	0077P1	1	31	111111	I	Bovine
09701Fe053.1	2	8	111111	I	Bovine	ECI-579	1	31	111111	I	Bovine
3D-1-1	2	8	111111	I	Bovine	ECI-212	1	31	111111	I	Human
ECI-2128	2	8	111111	I	Bovine	ECI-310	1	31	111111	I	Human
EC20040003	2	8	111111	I	Human	ECI-880	1	31	111111	I	Human
ER3762	2	8	111111	I	Human	10001Fe065.2	2	31	111111	I	Bovine
ER4073	2	8	111111	I	Human	11701Fe002.1	2	31	111111	I	Bovine
ER4102-1	2	8	111111	I	Human	ER3312	2	31	111111	I	Human
ER4104	2	8	111111	I	Human	ER5019	2	31	111111	I	Human
ER5533	2	8	111111	I	Human	ECI-653	1	32	111111	I	Bovine
ER5850	2	8	111111	I	Human	ECI-291	1	32	111111	I	Human
ER6066	2	8	111111	I	Human	ER2933	2	32	111111	I	Human
M01MD3094	2	8	111111	I	Human	ER5697	2	32	211111	I/II	Human
EC01-1243	1	14	111111	I	Bovine	I92P1	1	33	111111	I	Bovine
EC01-129	1	14	111111	I	Bovine	EC01-1228	1	33	111111	I	Bovine
EC01-142	1	14	111111	I	Bovine	ECI-221	1	33	111111	I	Human
ECI-1330	1	14	111111	I	Bovine	ECI-881	2	33	111111	I	Human
ECI-1375	1	14	111111	I	Bovine	ER6321	2	33	111111	I	Human
ECI-1396	1	14	111111	I	Bovine	ER6423	2	33	111111	I	Human
ECI-1479	1	14	111111	I	Bovine	ECI-600	1	34	111111	I	Bovine
ECI-563	1	14	111111	I	Bovine	99-4408	1	34	111111	I	Human
ECI-589	1	14	111111	I	Bovine	29-X-13C-R3	2	34	232222	II	Bovine
ECI-606	1	14	111111	I	Bovine	ec19920027	2	34	212222	II	Bovine
ECI-624	1	14	111111	I	Bovine	ECI-2152	2	34	222213	II	Bovine
ECI-642	1	14	111111	I	Bovine	eci-2153	2	34	212232	II	Bovine
ECI-657	1	14	111111	I	Bovine	ECI-270	2	34	111111	I	Human
ECI-681	1	14	111111	I	Bovine	ER5707	2	34	111111	I	Human
271F1	1	14	111111	I	Human	ECI-357	1	45	222222	II	Human
279f1	1	14	111111	I	Human	01301Fe007.1	2	45	222222	II	Bovine
98-2724	1	14	111111	I	Human	07901Fe003.2	2	45	222222	II	Bovine
98-5734	1	14	111111	I	Human	CG16-2A	2	45	222222	II	Bovine
99-4285	1	14	111111	I	Human	CG9-1	2	45	222222	II	Bovine
99-4360	1	14	111111	I	Human	E275	2	45	222222	II	Bovine
ECI-219	1	14	111111	I	Human	E294	2	45	232212	II	Bovine
ECI-250	1	14	111111	I	Human	IE252	2	45	222222	II	Bovine
ECI-278	1	14	111111	I	Human	IE292	2	45	222222	II	Bovine
ECI-309	1	14	111111	I	Human	IE297	2	45	222222	II	Bovine
ECI-320	1	14	111111	I	Human	RECDE291	2	45	222222	II	Bovine

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TABLE 1—Continued

Strain	Set no.	PT	LSPA6 genotype ^a	Lineage ^b	Origin	Strain	Set no.	PT	LSPA6 genotype ^a	Lineage ^b	Origin
ECI-509	2	45	222222	II	Human	07101FE041.2	2	74	232212	II	Bovine
ECI-240	1	54	222213	II	Human	C55-2B	2	74	232212	II	Bovine
02001Fe052.1	2	54	222213	II	Bovine	EC01-1097	2	74	222222	II	Bovine
K114D1	1	67	222222	II	Bovine	F136 DB	2	74	222212	II	Bovine
08401Fe026.1	2	67	222223	II	Bovine	ECI-242	2	74	212222	II	Human
08401Fe054.1	2	67	222223	II	Bovine	ER4511	2	74	232212	II	Human
114-X-3B-R1	2	67	222222	II	Bovine	EC01-1139	1	82	222222	II	Bovine
29-C-3A	2	67	222222	II	Bovine	ECI-603	1	87	111111	I	Bovine
K124A-K-1	2	67	222222	II	Bovine	herd 166DB	1	87	111111	I	Bovine
K124D1	2	67	222222	II	Bovine	ECI-558	1	87	111111	I	Human
ECI-331	2	67	222222	II	Human	CO-9283	2	87	111111	I	Bovine
F136	1	74	222212	II	Bovine	ECI-1258	2	87	111111	I	Bovine
ECI-241	1	74	222212	II	Human	F1084	2	87	111111	I	Bovine
06501FE02.1	2	74	232212	II	Bovine						

^a LSPA6 genotype (54).^b Predicted OBGS lineage derived from LSPA6 genotype result (28).

type (PT), type and level of Stx production, colicin genes, and antimicrobial resistance (AMR), in order to examine any potential associations between lineage and phenotype. The results show a strong correlation between lineage and PT and a biased distribution of *stx* genotype, colicin content, and AMR. Our findings provide further evidence for the differentiation of physiological, ecological, and virulence characteristics between the two primary lineages of *E. coli* O157:H7 and suggest that these characteristics may be useful epidemiological markers.

MATERIALS AND METHODS

Bacterial strains. Initially, 69 *E. coli* O157:H7 strains (set 1), comprising 37 strains from humans and 32 strains from cattle, were selected from the culture collection of the Laboratory for Food-Borne Zoonoses based on their PT (Table 1). These strains had been isolated from the provinces of Alberta, Ontario, Saskatchewan, and Quebec. A total of 43 additional human strains and 75 additional bovine strains were subsequently included to examine certain trends that became evident after initial analysis of set 1. Altogether, 187 test strains comprising 80 human and 107 bovine sources were selected (Table 1). In addition, 10 *E. coli* O157:H7 reference strains of known OBGS and LSPA6 type (28) were used in the study as positive controls in the LSPA6 and OBGS studies (Table 2). All strains were stored at -70°C in brain heart infusion broth (Difco) with 15% glycerol. When required, the strains were grown in 1 ml of brain heart infusion broth overnight at 37°C .

Phage typing and virulence gene profiling. PTs were determined for all of the strains as described previously (1, 26). All of the strains were tested by PCR to determine their Shiga toxin (*stx*) type, the presence of the EHEC hemolysin (*ehxA*) and the attaching and effacing genes (*eaeA*) (40, 57). Set 1 strains were *stx*₂ subtyped (4). Since the method used for *stx*₂ subtyping does not differentiate between *stx*_{2c}, *stx*_{2da}, and *stx*_{2v(521)} (57), all strains were tested by an *stx*_{2c}-specific PCR developed by Rolgaard and coworkers that successfully detected 97% of

*Stx*_{2c}-producing O157:H7 strains (B. Rolgaard, unpublished data). The primer sequences were designed to specifically target genes upstream of the *stx* gene, namely, the *q* and *ileZ* genes encoded by the Stx phages, 933w (*stx*₂) and Nil2 (*stx*_{2c}). The primer sequences were as follows: the *q*_{933w} forward primer, 5'-TGC CGA CGG GAA GTT GAC-3'; the *q*_{933w} forward primer, 5'-GGC GGC AAA TAA CTA TGA GG-3'; and the *ileZ* reverse primer, 5'-AGC GAC CTG GCG ATT ATG AG-3'. Briefly, each 25- μl reaction mixture contained 5 μl of cell template, 1 \times PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂, and 0.01% [wt/vol] gelatin; Applied Biosystems, Foster City, CA), 200 μM concentrations of each deoxynucleoside triphosphate (Applied Biosystems), each primer at a concentration of 0.5 μM , and 0.1 U of AmpliTaq (Applied Biosystems)/ μl . Amplification was conducted at 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 47.0°C for 60 s, and 72°C for 90 s. Products of 478 and 886 bp were produced for the O157:H7 strains containing *stx*_{2c} and *stx*₂, respectively.

The presence of the colicin genes K, V, B, and M was determined on set 1 strains by using previously described PCR assays (21, 50). Colicins Ia/Ib and E2/E3/E6/E7 genes were detected in set 1 strains by PCR using previously described primers (50) with the following conditions: each 25- μl PCR mixture contained 1 μl of cell template, 1 \times Gold buffer (Applied Biosystems) 1 μM concentrations of each primer, 0.2 mM concentration of each deoxynucleotide triphosphate (Applied Biosystems), 1 mM MgCl₂, and 0.036 U of AmpliTaq Gold (Applied Biosystems). Thermal cycling conditions were the same as in the published report (50) except that the initial denaturation step was at 94°C for 10 min. The presence of the colicin D gene, *cda*, was determined on all test strains by PCR, as previously described (21).

LSPA6 and OBGS typing. All strains were typed by the LSPA6 method as two reactions rather than the one multiplex reaction as originally described (54). The LSPA6 primer sequences used in the present study are listed in Table 3. One multiplex reaction contained *folD*, Z5935, *rhsB*, and *arp* primers and the second multiplex reaction contained *yhc* and *rtcB* primers. Lysates for PCRs were prepared by boiling washed cells (44). Amplification reactions contained 4 μl of the cell lysate combined with 16 μl of the mastermix that consisted of 1 \times PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 1.5 mM MgCl₂ and 0.01% [wt/vol] gelatin), 250 μM concentrations of each deoxynucleoside triphosphate, each primer at a concentration of 0.5 μM , and 0.05 U of AmpliTaq (Applied Biosystems)/ μl . Amplification was conducted at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 52.2°C for 30 s, and 72°C for 45 s. Aliquots (5 μl) of the amplified products were analyzed by standard submarine gel electrophoresis using a 3% Metaphor agarose gel (Cambrex, Rockland, ME) stained with ethidium bromide, and the bands visualized with UV transillumination. Images of the amplicon gel profiles were captured by using a Gel Doc 100 (Bio-Rad, Mississauga, Ontario, Canada) and analyzed in Bionumerics (version 3.5; Applied Maths, Austin, TX).

LSPA6 alleles and genotypes were defined by using a binary character table that was generated in Bionumerics and exported to Microsoft Excel. Alleles shared with the lineage I control strain were designated 1, and those shared with the lineage II control strain were designated 2. Unique alleles were designated 3, and a zero character was assigned if no band was present. To generate the genotype classification, the alleles were placed in the following order: *folD*, Z5935 gene, *yhcG*, *rtcB*, *rhsB*, and *arp-iclR*.

Thirty-five strains from set 1 were also tested by traditional OBGS, using the primer pairs KOCT22A/KOCT6C, KOCT21/KOCT13C, KOCT19/6C, KOCT3B/KOCT4C, and KOCT14/KOCT1C. The OBGS reference strains were included in each gel. Binary files were created in Microsoft Excel from printed

TABLE 2. *E. coli* O157:H7 LSPA6 reference strains

Strain	LSPA6 ^a	Origin	PT	Lineage ^b
FRIK920	222222	Bovine	23	II
FRIK1985	222223	Bovine	45	II
FRIK1990	222222	Bovine	54	II
FRIK1999	222222	Bovine	23	II
FRIK2001	222213	Bovine	54	II
93-001	111111	Human	14	I
FRIK523	111111	Human	34	I
FDA516	111111	Human	21	I
FDA518	111111	Human	21	I
FDA520	111111	Human	1	I
MC1061 (K-12)				

^a LSPA6 (lineage specific polymorphism assay) genotype (54).^b Predicted OBGS lineage derived from LSPA6 genotype result (28).

TABLE 3. LSPA6 PCR primer sequences

Primer	Sequence (5'-3')	Predicted product length (bp)	Source or reference
<i>folD</i> -F	TAC GTA GGT CGA AGG G	161 or 170	54
<i>folD</i> -R	CCA GAT TTA CAA CGC C		
Z5935-F	GTG TTC CCG GTA TTT G	133 or 142	54
Z5935-R	CTC ACT GGC GTA ACC T		
<i>yhcG</i> -F	CTC TGC AAA AAA CTT ACG CC	142 or 219	54
<i>yhcG</i> -R	AAC GGA AAG TAA ACG GAC G		This study
<i>rbsB</i> -F	AGT TTA ATG TTC TTG CCA GCC	209 or 218	54
<i>rbsB</i> -R	ATT CAC CGC TTT TTC GCC		
<i>rtcB</i> -F	GCG CCA GAT CGA TAA AGT AAG	270 or 279	54
<i>rtcB</i> -R	GCC GTT GTA AAC GTG ATA AAG		
<i>arp-iclR</i> -F	GCT CAA TCT CAT AAT GCA GCC	279 or 288	54
<i>arp-iclR</i> -R	GCG ATG GTG ATT AAA GCG G		This study

copies of the images produced by an Alden 9315CTP photographic quality thermal printer (Alden Electronics, Westborough, MA). The files were generated from the presence or absence of bands between 200 and 1,500 bp. The lineage designation was obtained by comparison of the binary strings of each sample to the binary string of the OBGS reference strains.

All strains that were LSPA6 genotype 111111 were classified as lineage I. Most other allele combinations corresponded to OBGS lineage II. LSPA6 genotype 211111 was originally considered a lineage II strain (54), but recent reports indicate that LSPA6 genotype 211111 strains have characteristics of both lineage I (LSPA6 111111) and lineage II strains (52, 55). Accordingly, LSPA6 genotype 211111 was designated as lineage I/II and was not considered a lineage II LSPA6 genotype in the strain comparisons for the present study.

Shiga toxin production. The levels of Stx₂ produced by a subset of strains were quantified by using a Stx-enzyme-linked immunosorbent assay (Stx-ELISA) with monoclonal antibodies that recognize Stx₂, Stx_{2c}, Stx_{2d}, and Stx_{2e} variants (2). Quantification was achieved by generation of a standard curve with known concentrations of purified Stx₂. The results were recorded as picograms of toxin per 10⁸ CFU. Cultures were prepared with or without induction by mitomycin C as previously described (46) except that 1 ml of the cells were lysed by incubation at 37°C for 5 min after the addition of a warm solution of polymyxin B (Sigma) to a concentration of 1.5 mg/ml rather than by sonication.

Antimicrobial resistance testing. Antimicrobial resistance was determined as previously described (42) using the following antibiotics and their respective concentrations: amikacin (16 µg/ml), ampicillin (32 µg/ml), carbadox (30 µg/ml), cefotaxime (64 µg/ml), cefoxitin (32 µg/ml), ceftiofur (8 µg/ml), ceftriaxone (8 µg/ml), cephalothin (32 µg/ml), chloramphenicol (32 µg/ml), ciprofloxacin (0.125, µg/ml), cotrimoxazole (80 µg/ml: sulfisoxazole and trimethoprim at 76 and 4 µg/ml, respectively), florfenicol (16 µg/ml), gentamicin (16 µg/ml), kanamycin (64 µg/ml), nalidixic acid (32 µg/ml), nitrofurantoin (64 µg/ml), spectinomycin (64 µg/ml), streptomycin (64 µg/ml), sulfisoxazole (512 µg/ml), tetracycline (16 µg/ml), tobramycin (8 µg/ml), and trimethoprim (16 µg/ml). These antibiotics were chosen since they are or have been used therapeutically in veterinary or human medicine or as growth promoters in animal agriculture and may provide a selective advantage to resistant strains. Quality control strains included American Type Culture Collection (ATCC) strains *Pseudomonas aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *E. coli* ATCC 35218 (36, 37), as well as *E. coli* strain R1022, a multiresistant bovine isolate.

Plasmid profiling. Plasmid DNA was isolated by the method of Crosa and Falkow (10), except that the strains were grown overnight on veal infusion yeast extract agar, scraped off the surface of the agar with a sterile toothpick, and suspended in lysis buffer (pH 12.4). The plasmid DNA was subjected to electrophoresis in a horizontal 0.7% agarose gel in Tris-acetate buffer and stained with ethidium bromide, and the bands were visualized by UV transillumination. Images were captured by using a Gel Doc 100 (Bio-Rad). Plasmids from PT 31 and PT 33 strains of *E. coli* O157:H7 appeared to be degraded by nucleases. For these strains, the addition of 50 µM thiourea (Sigma) to the running buffer was used to protect against degradation (43).

Statistical analysis. All pairwise associations between LSPA6 genotypes, PTs, the presence of *stx*_{2c} and *cda* genes, and AMR were tested by using the Fisher exact test (<http://www.matforsk.no/ola/fisher.htm>). (For a comprehensive listing of *P* values, see Tables S1, S2, and S3 in the supplemental material.)

RESULTS AND DISCUSSIONS

Distribution of LSPA6 and OBGS types. In order to examine the frequency of specific LSPA6 types in the collection of *E. coli* O157:H7 strains, an initial subset of 69 strains (set 1, Table 1) was selected based on the approximate proportions of each PT in our collection (Table 1). PT 14 was the most common PT in the collection, which is consistent with what has previously been reported for Canada (11). The other 16 PTs included: 1, 2, 4, 8, 21, 23, 31, 32, 33, 34, 45, 54, 67, 74, 82, and 87. Among the 69 strains, five LSPA6 genotypes were identified: 57 (82.6%) were of LSPA6 genotype 111111 (LSPA6-LI), while the remaining 12 (17.4%) strains were of other LSPA6 genotypes. These included four (5.8%) LSPA6 genotype 211111 (LSPA6-LI/II) strains, five (7.2%) LSPA6 genotype 222222 strains, two (2.9%) genotype 222212 strains, and one (1.4%) genotype 222213 strain. LSPA6 and OBGS typing results from the 35 strains tested by both methods agreed with respect to lineage designation, with the exception of three strains that could not be typed by the OBGS method but which were resolved by LSPA6 typing (data not shown).

Association between LSPA6 type, PT, and source of isolation. A strong association between PT and LSPA6 genotype was observed in the 69 strains from set 1 (Table 1). However, certain PTs had very few representatives, and additional strains of the same PTs were selected at random from the larger collection of *E. coli* O157:H7 strains to further examine this association. In this expanded set of strains, PTs 4, 14, 21, 31, 33, and 87 were all genotype LSPA6 111111 (LSPA6-LI), while three PTs (45, 67, and 74) had minor differences in LSPA6 genotypes but remained within lineage II (e.g., PT 67 had six strains of LSPA6 genotype 222222 and two of LSPA6 genotype 222223) (Fig. 1). Two PTs (PT 8 and PT 32) had strains of LSPA6 111111 and 211111. Only 2 of 17 PTs (PT 1 and PT 34) had strains from both LSPA6-LI and LSPA6-LII genotypes. To the best of our knowledge, this is the first time that an association has been demonstrated between PT and LSPA6 genotype.

Within this expanded set of strains, the LSPA6-LI genotype made up 73.8% (59 of 80) and 49.5% (53 of 107) of *E. coli* O157:H7 strains isolated from human and bovine sources, respectively (Fig. 2). The relative overrepresentation of LSPA6-LI among human isolates was also observed by Yang et al. (54) in their study of a large collection of bovine and human isolates. Nine LSPA6-LI/II (211111) genotype strains were of human origin and five were of bovine origin. LSPA6-LII genotypes represented 15.0% (12 of 80) of the isolates from humans (222222, 222212, 222213, 232212, 212222, and 222121) and 45.8% (49 of 107) of the isolates from cattle (222222, 222223, 222212, 222213, 232212, 212222, 212232, 222121, 232222, and 232212). The bovine source bias among LSPA6-LII genotypes was highly significant (Fisher exact test; $P = 8.047 \times 10^{-6}$). Certain lineage II-related PTs such as PT 23 also had a significant (Fisher exact test; $P = 1.681 \times 10^{-2}$) bovine host isolation bias. The strong association between PT

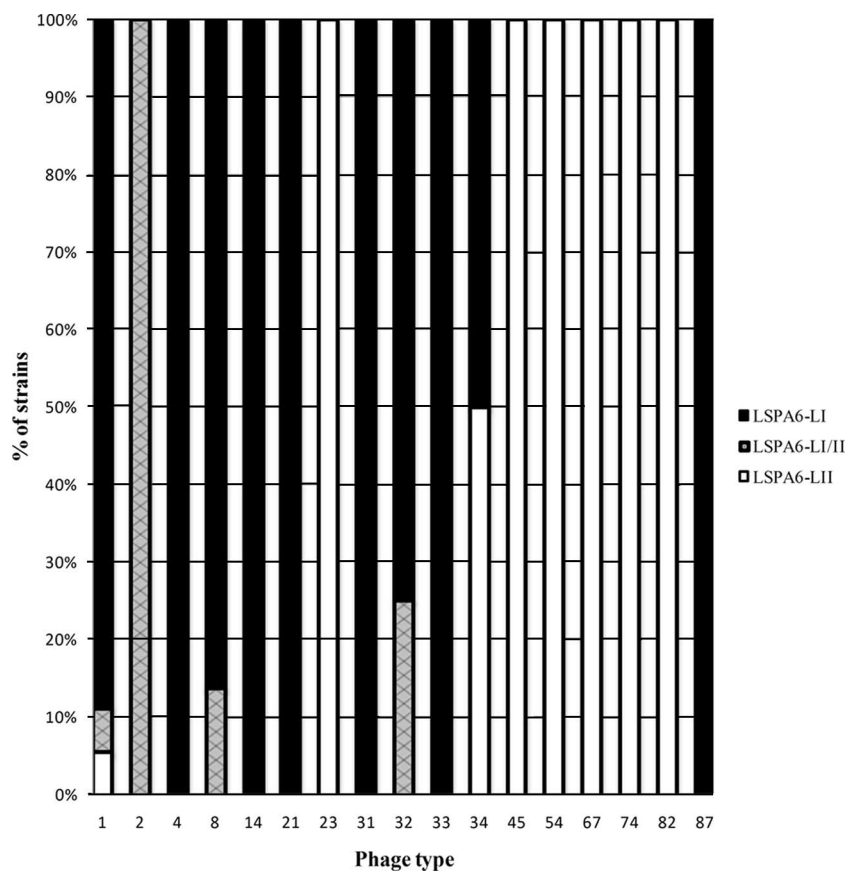


FIG. 1. Relationship between PT and LSPA6 genotype for the 187 *E. coli* O157:H7 strains in Table 1.

and LSPA6 genotype suggests that some PT/LSPA6 genotype combinations are stable, although further investigation will be required to explore whether this association is widespread.

Association between LSPA6 genotype, PT, Stx₂ genotype, and toxin production. Considerable variation was found among LSPA6 genotypes in *stx* gene content and *stx*₂ genotype. *stx*₁ and *stx*₂ were identified in 79% of strains tested, *stx*₂ alone was present in 17% of the strains, *stx*₁ alone was present in 3% of the strains, and one strain did not contain either toxin-producing gene. The *stx*_{2c} variant was detected by the *stx*_{2c} specific PCR in 59 of 61 LSPA6-LII strains (96.7%). An additional band of approximately 660 bp was noted for all strains regardless of the *stx*₂ content; however, the nature and significance of this band is not known (data not shown). The remaining two strains contained only the *stx*₁ gene. In contrast, the *stx*_{2c} variant was only detected in two of the 112 LSPA6-LI strains (1.8%) and seven of 14 LSPA6-LI/II strains (50%) (Table 4). The two LSPA6-LI strains contained both *stx*_{2c} and *stx*₂. The difference in the carriage rates of *stx*_{2c} between LSPA6-LII strains and LSPA6-LI genotype strains was found to be statistically significant (Fisher exact test; $P \geq 3.089 \times 10^{-41}$). Although Stx_{2c} has been reported to have the same level of toxicity as Stx₂ in mice (32), the risk of developing HUS after infection with *stx*_{2c}-containing STEC has been reported to be significantly lower than with STEC possessing the *stx*₂ toxin genotype (14). This is also in agreement with other studies that

report that most HUS cases were associated with *E. coli* strains carrying *stx*₂ rather than other *stx*₂ variant genes (6, 49).

Although it is possible that these Stx₂ variants differ in toxicity for humans, it has been suggested that Stx_{2c}-producing strains simply produce less Stx (7, 12, 38). To determine whether this was the case, Stx₂ production by eight Stx₂-producing strains (seven LSPA6-LI strains and one LSPA6-LI/II strain) and nine Stx_{2c}-producing strains (all LSPA6-LII) was quantified by an Stx-ELISA that reacts with all Stx₂ variants. Figure 3 shows the amount of toxin produced by selected strains from LSPA6-LI, -LII, and -LI/II before and after induction with mitomycin C. For five of the nine LSPA6-LII Stx_{2c}-producing strains, the amounts of toxin produced without induction with mitomycin C were very low compared to other LSPA6-LII strains and the Stx₂-producing LSPA6-LI and LI/II strains. Since the low-toxin-producing strains were from PTs 23 and 67 strains, an additional five PT 23 and five PT 67 strains were tested by the Stx-ELISA. These strains also produced very low levels of toxin and showed similar increases on induction with mitomycin C. Therefore, low production of Stx_{2c} may be more strongly associated with these PTs rather than strictly with the LSPA6 genotype. Production of low levels of Stx_{2c} by O157:H7 strains of PT 23 and 67 may explain the infrequent association of these PTs with human disease, and further studies should be performed to examine this relationship.

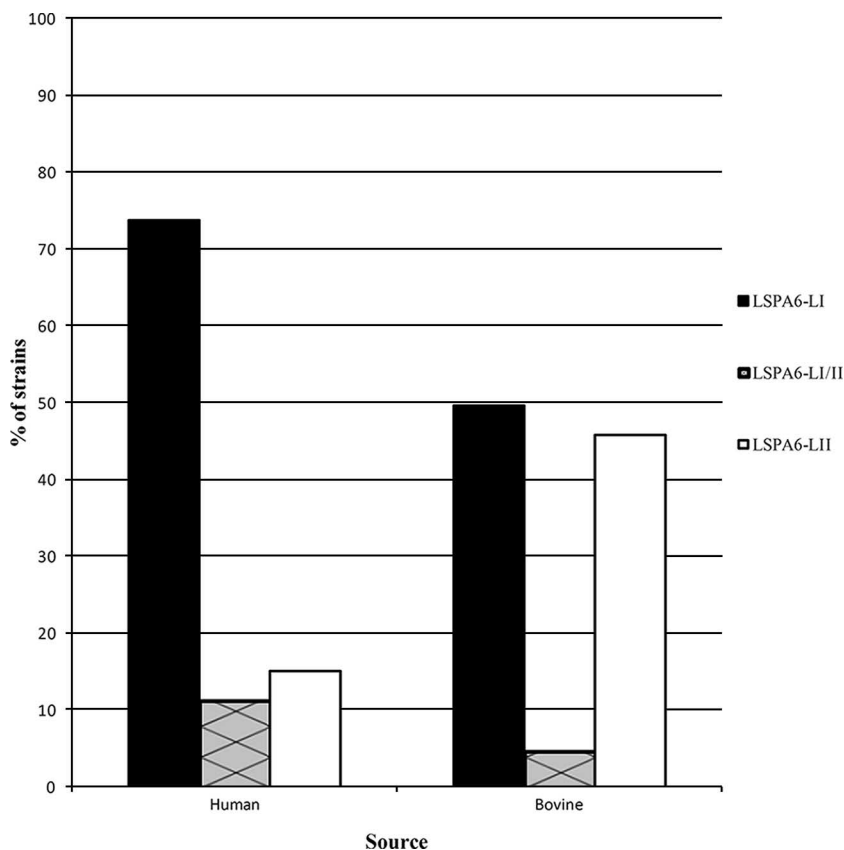


FIG. 2. Relationship between source and LSPA6 genotype for the 187 *E. coli* O157:H7 strains in Table 1.

Association between plasmid profiles and LSPA6 genotype. Thirteen different plasmid profiles were observed among the *E. coli* O157:H7 strains in set 1 (data not shown). Nine plasmids ranging in size from 70 to 2.2 MDa were identified in set

1 strains. All strains possessed the large 60-MDa pO157 virulence plasmid and were PCR positive for the enterohemolysin gene (*hlyA*). The two predominant profiles among these strains were the presence of pO157 alone and pO157 combined with

TABLE 4. Differences in the presence of *cda*, *stx_{2c}*, and streptomycin, sulfisoxazole, and tetracycline antimicrobial resistance among LSPA6 genotypes

Genotype ^a	Origin	n ^b	Presence of ^c :								
			<i>stx_{2c}</i>			<i>cda</i>			Streptomycin, sulfisoxazole, and tetracycline resistance		
			No. positive	% Positive	SS	No. positive	% Positive	SS	No. positive	% Positive	SS
LSPA6-LI	Bovine	53	1	1.9	a	15	28.3	a, c	1*	1.9	a
	Human	59	1	1.7	a	11	18.6	a	0*	0	a
	Total	112	2	1.8	a	26	23.2	a	1*	0.9	a
LSPA6-LI/II	Bovine	5	4	80	b, c	1	20	a, b	0	0	a, b
	Human	9	3	33.3	b	0	0	a	0	0	a
	Total	14	7	50	b	1	7.1	a	0	0	a
LSPA6-LII	Bovine	49	48	98	c	27	55.1	b	36†	73.5	c
	Human	12	11	91.7	b, c	4	33.3	a, b	8†	66.7	b, c
	Total	61	59	96.7	c	31	50.8	b, c	44†	72.1	c

^a LSPA6-LI refers to LSPA6 genotype 111111, LSPA6-LI/II refers to LSPA6 genotype 211111, and LSPA6-LII refers to all lineage II-related LSPA6 genotypes.

^b n, Number of samples examined.

^c Statistical significance (SS) was evaluated at the *P* < 0.01 level. Test groups of strains with the same letter designation did not have a statistically significant difference in carriage rate for the specific trait (Fisher exact test, *P* < 0.01), e.g., bovine LSPA6-LI and human LSPA6-LI do not have a significant difference in the presence of *cda*, whereas bovine LSPA6-LI and bovine LSPA6-LII do. For the *P* values associated with the various pairwise comparisons, please refer to Tables S1, S2, and S3 in the supplemental material. *, one bovine and five human LSPA6-LI strains were resistant to single antibiotics or other combinations of antibiotics other than streptomycin, sulfisoxazole, and tetracycline; †, all LSPA6-LII strains expressing AMR were resistant to streptomycin, sulfisoxazole, and tetracycline.

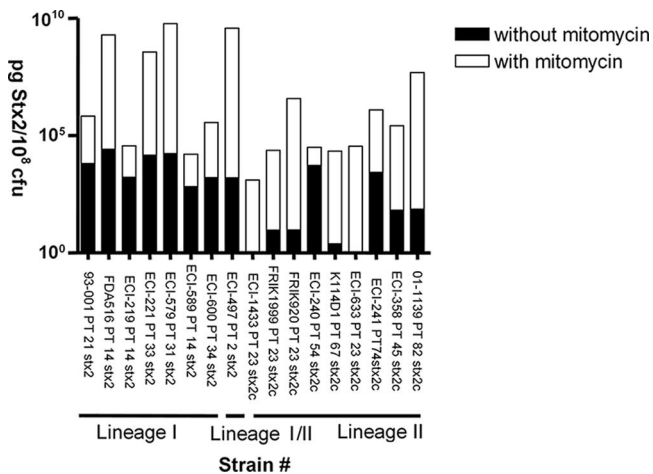


FIG. 3. Stx₂ or Stx_{2c} production by selected *E. coli* O157:H7 strains.

a 2.2-MDa plasmid. These two profiles were observed in 56.9 and 15.3% of strains, respectively. The common occurrence of the 2.2-MDa plasmid is in agreement with another study that found this plasmid in 57% of O157 strains (51). In the present study, only PTs 21, 31, 32, and 33 contained the 2.2-MDa plasmid, and there appeared to be no bias in distribution among human and nonhuman strains. Previously, a 2.2-MDa

plasmid, designated p4821, was characterized by Haarmann et al. (18) and found to be similar to the antibiotic resistance plasmid of *Salmonella enterica* serovar Typhimurium strains. There did not appear to be any significant association between specific plasmid profiles and LSPA6 genotype or isolate source.

Association between colicins and LSPA6 genotype. *E. coli* O157:H7 strains positive for the colicin D gene *cda* were found in 26 of 112 (23.2%) LSPA6-LI strains, 1 of 14 (7.1%) LSPA6-LI/II strains, and 31 of 61 (50.8%) LSPA6-LII strains (Table 4). The difference in *cda* carriage rates between LSPA6-LI and LII strains was highly significant (Fisher exact test; $P = 3.427 \times 10^{-4}$). Interestingly, the *cda* gene was observed in only three out of the eight LSPA6-LII-associated PTs (PTs 23, 45, and 67) (Fig. 4), and carriage of *cda* was largely confined to certain LSPA6-LI PTs (PTs 1, 4, and 8) as well. Therefore, it appears that possession of *cda* may be more strongly associated with PT than with the LSPA6 genotype.

Other colicins were found at a much lower frequency among *E. coli* O157:H7 strains; e.g., one of 69 (1.4%) strains had colicins B and M and two strains (2.8%) had colicin Ia/Ib (data not shown). The higher prevalence of *cda* among the *E. coli* O157:H7 strains is in agreement with the results of previous reports (15, 51). As expected, all strains that contained the 4.6-Mda plasmid were found by PCR to possess the *cda* gene (8, 21).

Colicins may offer a selective advantage to their host (45). In

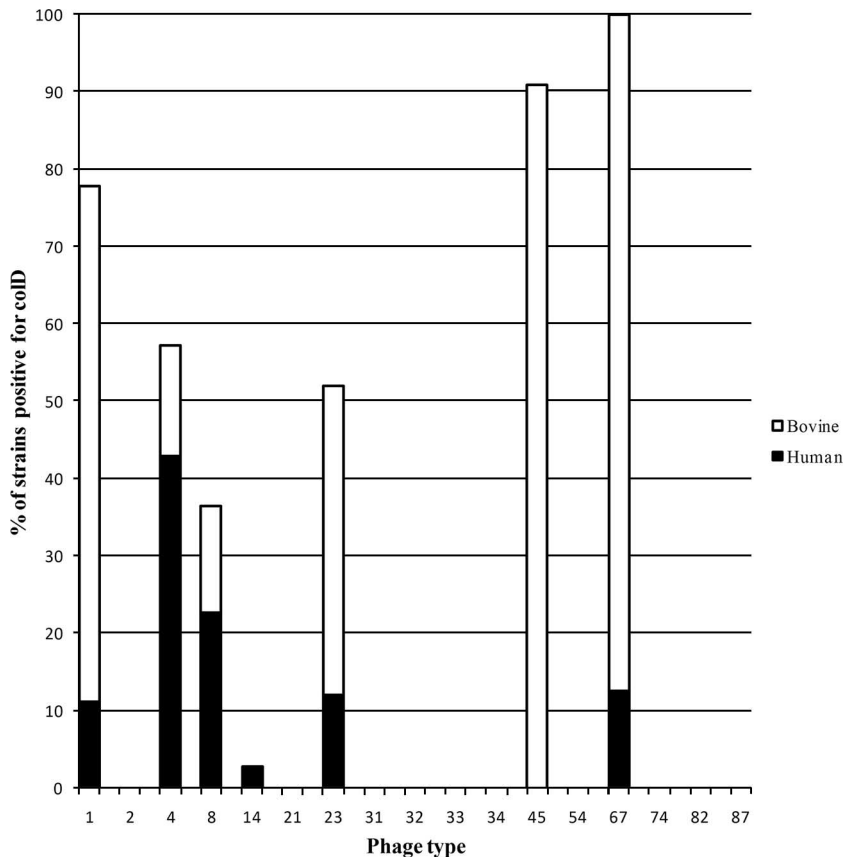


FIG. 4. Relationship between PT and presence of *cda* for the 187 *E. coli* O157:H7 strains in Table 1.

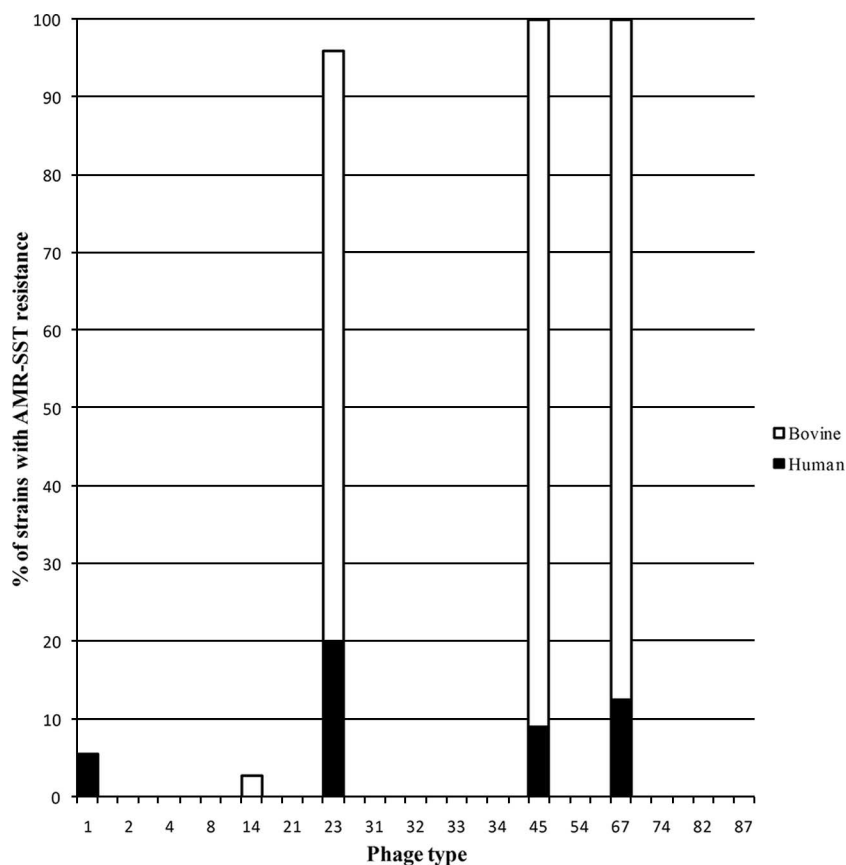


FIG. 5. Relationship between PT and combined antimicrobial resistance to streptomycin, sulfisoxazole, and tetracycline for the 187 *E. coli* O157:H7 strains in Table 1.

one study, pColD157 was identified in the majority of *E. coli* O157:H7 strains associated with human disease (8). Another study found that pColD157 was less prevalent in recent *E. coli* O157:H7 isolates than those obtained several years previously (21). Although interesting, the significance of a higher frequency of *cda* in lineage II strains observed in the present study is unknown. Since *cda* is not widespread among *E. coli* O157:H7 strains, it does not appear to be essential, or perhaps its function (or that of genetically linked genes) has been replaced by some other attribute of the organism.

Association between AMR and LSPA6 genotype. Only 7.2% (5 of 69) of the *E. coli* O157:H7 strains (set 1) selected from the collection based on PT frequency displayed resistance to one or more of the antibiotics tested. All of the resistant strains from this initial set of strains had combined resistance to streptomycin, sulfisoxazole, and tetracycline (AMR-SST) and one AMR-SST bovine strain was also resistant to spectinomycin. Other studies have also found that resistance to streptomycin, sulfisoxazole, and tetracyclines is common in *E. coli* O157 strains (27, 34, 53, 56). Only 1 of the 37 human strains from set 1 displayed resistance to the antimicrobials tested, whereas 4 of the 32 (12.5%) bovine strains showed AMR (data not shown). Four of the five *E. coli* O157:H7 strains with AMR were of LSPA6-LII genotypes and also of PTs 23, 45, and 67.

To determine whether any association exists between AMR and LSPA6 genotype and/or PT, additional strains from each

PT were tested for AMR (Table 1). The results for all strains indicated that within LSPA6-LII strains, 44 of 61 (72.1%) were AMR-SST positive (Table 4) compared to only 1 of 112 (0.9%) of the LSAP6-LI strains (Fisher exact test; $P = 7.536 \times 10^{-26}$). For strains of LSPA6-LII-associated PTs, 24/25 PT 23, 11/11 PT 45, and 8/8 PT 67 strains were AMR-SST, whereas strains of the LSPA6-LII-associated PTs, PT 54 and PT 74, had no AMR. Based on these data it would seem that AMR-SST is largely confined not only to LSPA6-LII strains but also to specific PTs within this group (Fig. 5). Although it can be argued that the strains in the collection may not be representative of these PTs from other geographical regions, Mora et al. (34) have also recently reported an association between PT 23 *E. coli* O157:H7 strains and AMR-SST in Spain (34). It is possible that AMR may play a role in the survival and persistence of LSPA6-LII *E. coli* O157:H7 strains in the bovine reservoir.

At 34.5%, the AMR prevalence in bovine strains in the present study is similar to the rate observed in the United States (33) but lower than the 65% AMR rate reported for *E. coli* O157 strains from cattle in feedlots in Saskatchewan, Canada (53), and the 53% AMR rate of *E. coli* O157 strains from cattle in Spain (34). However, comparisons between studies should consider differences in study design, strain selection, geographic region, and scope of the study.

Conclusions. In our comparison of characteristics of LSPA6 genotypes and PTs of *E. coli* O157:H7 strains isolated from Canadian sources, the majority of strains were genotype LSPA6-LI, and smaller proportions belonged to the LSPA6-LI/II and LSPA6-LII genotypes. One of the key findings was that PTs 23, 45, 54, 67, and 74 were genotype LSPA6-LII specific. As has previously been observed in O157:H7 strains from the United States, LSPA6-LII genotypes were significantly more likely to be isolated from cattle than from humans.

Certain genes, such as *eaeA*, were conserved across all lineages and PTs (data not shown), whereas the *stx_{2c}* variant toxin was strongly associated with LSPA6-LII strains. In addition, LSPA6-LII strains of PTs 23 and 67 produced lower levels of toxin than strains from other PTs and LSPA6 genotypes within lineage I and lineage II. *E. coli* O157:H7 strains of LSPA6-LII were also significantly more likely to possess *cda* and have AMR than LSPA6-LI and LSPA6-LI/II strains. Collectively, our results imply that the two lineages of *E. coli* O157:H7 indeed have many unique physiological and ecological characteristics that could influence their propensity to cause disease or their ability to propagate in bovine production environments. Recent studies on *E. coli* genomes have highlighted the importance of genomic island content in differentiating among various *E. coli* serotypes and pathotypes (9, 20, 39, 41). Specific sets of these large genetic elements presumably explain the phenotypic differences among these *E. coli* groups in host adaptation, pathogenesis, virulence, epidemiology, and ecology (19, 24). Generation of new genotypes of *E. coli* O157:H7 is therefore likely to be driven by the acquisition, rearrangement, and loss of these genetic elements. However, it is interesting that despite the apparent instability of these elements there is also evidence of stable gene linkage (e.g., as parts of functional units such as the LEE type III secretion system or genes of related function such as multiple-antibiotic resistance) (17, 31). The apparent stability within *E. coli* groups of certain of these mobile multigene elements suggests that they have been retained through the selection for one or more phenotypic traits encoded by these genes. In addition, certain of these elements may act to exclude the uptake of other mobile genetic elements through mechanisms such as plasmid incompatibility, changes in phage receptors, and phage-related immunity to superinfection with other bacteriophages (13, 22) and thereby contribute to genotypic and phenotypic stability of the organism. Linkages of LSPA6 typing markers to genes encoding phenotypic characteristics observed in the present study appear to be indirect. It is important to note that the markers targeted by the LSPA6 assay were selected because they are expected to be selectively neutral (54). The differential association of the various phenotypic traits that were tested in the present study (colicin production, AMR, toxin production, and PT) with neutral lineage-specific markers would suggest that certain combinations of these traits arose early in the divergence of the two lineages and that these combinations have remained stable over time. It is possible that geographical or niche isolation may also have played a role in maintaining relatively fixed constellations of genes in certain populations of *E. coli* O157:H7 and helped create some of the lineage-specific phenotypes observed in the present study.

Further study of *E. coli* O157:H7 strains from other geographical regions is needed to determine whether the associ-

ations between LSPA6 genotype, PT, and other phenotypic characteristics observed in the present study are widely applicable.

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