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Characterization of *Escherichia coli* O157:H7 Strains Isolated from Supershedding Cattle

Terrance M. Arthur,[a] Rafiq Ahmed,[b] Margo Chase-Topping,[c] Norasak Kalchayanand,[a] John W. Schmidt,[a] James L. Bono[a]

U.S. Department of Agriculture, Agricultural Research Service, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, Nebraska, USA[a]; Public Health Agency of Canada, Canadian Science Centre for Human and Animal Health, Winnipeg, Manitoba, Canada[b]; Centre for Immunity, Infection and Evolution, Ashworth Laboratories, University of Edinburgh, Edinburgh, Scotland[c]

Previous reports have indicated that a small proportion of cattle shedding high levels of *Escherichia coli* O157:H7 is the main source for transmission of this organism between animals. Cattle achieving a fecal shedding status of 10^4 CFU of *E. coli* O157:H7/gram or greater are now referred to as supershedders. The aim of this study was to investigate the contribution of *E. coli* O157:H7 strain type to supershedding and to determine if supershedding was restricted to a specific set of *E. coli* O157:H7 strains. Fecal swabs ($n = 5,086$) were collected from cattle at feedlots or during harvest. Supershedders constituted 2.0% of the bovine population tested. Supershedder isolates were characterized by pulsed-field gel electrophoresis (PFGE), phage typing, lineage-specific polymorphism assay (LSPA), Stx-associated bacteriophage insertion (SBI) site determination, and variant analysis of Shiga toxin, *tir*, and antiterminator *Q* genes. Isolates representing 52 unique PFGE patterns, 19 phage types, and 12 SBI clusters were obtained from supershedding cattle, indicating that there is no clustering to *E. coli* O157:H7 genotypes responsible for supershedding. While being isolated directly from cattle, this strain set tended to have higher frequencies of traits associated with human clinical isolates than previously collected bovine isolates with respect to lineage and *tir* allele, but not for SBI cluster and *Q* type. We conclude that no exclusive genotype was identified that was common to all supershedder isolates.

Escherichia coli O157:H7 is an important food-borne pathogen that poses a serious public health concern and financial burden. Cattle are the principal animal reservoir of *E. coli* O157:H7, and while the rumen has been shown to harbor this pathogen on occasion, it is found more frequently in the distal portion of the bovine gastrointestinal (GI) tract, with the rectoanal junction (RAJ) identified as the predominant colonization site (1, 2). Once colonized, an animal can shed various concentrations of *E. coli* O157:H7 organisms in the feces. Among colonized individuals, a small proportion will excrete *E. coli* O157:H7 at levels of $\geq 10^4$ CFU/g of feces. These animals have been referred to as supershedders and are reported to be responsible for increased transmission of *E. coli* O157:H7 within cattle production environments as well as having significant effects on contamination of the cattle hide and carcass (3–6).

Matthews et al. (7) presented data suggesting that 20% of the *E. coli* O157:H7 infections in cattle on Scottish farms were responsible for 80% of the transmission of the organism between animals. Another study showed that 9% of the animals shedding *E. coli* O157:H7 at harvest produced over 96% of the total *E. coli* O157:H7 fecal load for the group (6). Conversely, feedlot cattle that did not shed *E. coli* O157:H7 over the course of study were five times more likely to be housed in a pen that did not contain a supershedder (8). In addition, Arthur et al. (3) have shown that 95% of feedlot pens containing at least one supershedder had *E. coli* O157 prevalence rates on cattle hides exceeding 80%, whereas only 29% of pens without a supershedder exceeded 80% hide prevalence. Therefore, it is critical to identify and minimize or eliminate supershedders in the cattle population in order to reduce *E. coli* O157:H7 transmission and ultimately beef carcass contamination for enhancing food safety.

The supershedding phenomenon can be broken down into three potential components: (i) the phylogenetic lineage or strain-specific characteristics of the *E. coli* O157:H7 strain being shed, (ii) the community composition of the microbiota inhabiting the rectoanal junction, and (iii) the host genotype and phenotype, including the innate and adaptive immune response of the host. Defining the role that each of these determinants plays in achieving supershedding status is critical in the development of technologies to mitigate high-level shedding and reduce *E. coli* O157:H7 transmission within cattle populations.

The aim of this study was to investigate the contribution of *E. coli* O157:H7 strain type to supershedding and to determine if supershedding was restricted to a specific set of O157:H7 strains. While data regarding *E. coli* O157:H7 strains recovered from supershedders are limited, previous studies have identified phage type 21/28 (PT21/28) as being associated with supershedding in cattle on farms in Scotland (4, 9). The study reported herein was designed to isolate *E. coli* O157:H7 strains from cattle in the United States shedding at levels of $\geq 10^4$ CFU/swab and characterize the strains to identify supershedder-specific traits.

MATERIALS AND METHODS

Animals and sample collection. Fecal samples were collected from approximately 3,500 cattle at slaughter in five high-capacity commercial processing plants and 1,500 cattle in 18 commercial feedlots in the summer months of 2009 and 2010. The feedlots and processing plants were located in four of the eight Beef Industry Food Safety Council (BIFSCo) regions of the United States. The BIFSCo regions were number 3, southwest (Arizona, New Mexico, and Texas); number 5, upper Midwest (Nebraska, North Dakota, South Dakota, Minnesota, and Wisconsin); num-

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Address correspondence to Terrance M. Arthur, Terrance.Arthur@ars.usda.gov.

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ber 6, central (Iowa, Kansas, and Missouri); and number 7, southeast (Oklahoma, Arkansas, Louisiana, North Carolina, South Carolina, Florida, Alabama, Mississippi, Georgia, and Tennessee). Samples were collected at beef processing plants after animals were stunned, exsanguinated, and shackled. At feedlots, animals were restrained in squeeze chutes for sampling. Each sample represented an individual animal, and no animals were sampled more than once. Swab samples were collected by swabbing the RAJ as described by Rice et al. (10) and then placing the swabs in 15-ml conical tubes containing 4 ml of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD). Tubes were stored on ice for shipment to the laboratory.

Laboratory methods. (i) Isolation of *E. coli* O157:H7. Upon arrival at the lab, the sample tubes were vortexed vigorously for 30 s, and then 50-μl aliquots were plated for enumeration of *E. coli* O157:H7 organisms using a spiral plater. Samples were plated onto CHROMagar O157 with 5 mg/liter of novobiocin and 2.5 mg/liter of potassium tellurite as described previously (11). When detected, counts of O157:H7 were reported as CFU/swab. Animals were classified as supershedders when counts were $\geq 10^4$ CFU/swab. This threshold is slightly more stringent than that put forward by Chase-Topping et al. (4), as the mean fecal mass accumulated by swabbing the rectoanal junction of cattle is less than 1 g. Cobbold et al. (8) previously determined the amount of fecal mass collected per swab to average 0.242 g. Up to 20 presumptive *E. coli* O157:H7 colonies were picked for confirmation by multiplex PCR. The multiplex PCR was used to confirm that each *E. coli* isolate harbored genes for the O157 antigen, H7 flagella, γ-intimin, and at least one of the Shiga toxins as previously described (12). Isolates were stored in 15% glycerol at −70°C.

(ii) Determination of diversity within a supershedder sample. In order to determine how many isolates should be analyzed to be representative of a sample, multiple isolates ($n = 1{,}061$) from 98 samples were analyzed by pulsed-field gel electrophoresis (PFGE) and phage typing to determine within sample diversity. Thirty-three samples had ≥20 isolates per sample analyzed by PFGE, including one sample in which 40 isolates were analyzed. Another 13 samples had PFGE analysis for ≥10 isolates per sample. An additional 52 samples had 4 isolates/sample analyzed by PFGE. Phage typing was conducted for 5 isolates per sample from 20 samples.

(iii) PFGE. *E. coli* O157:H7 isolate fingerprints generated and analyzed in this study were based on PFGE separation of XbaI-digested genomic DNA, as currently used by members of PulseNet (13) and previously described (14). *Salmonella enterica* serotype Braenderup strain H9812 was used as a control and for standardization of gels (15). Position tolerance setting used 1.5% optimization and 1.5% band tolerance. Genotypes were defined strictly as isolates that grouped together and had indistinguishable (100% Dice similarity) banding patterns.

(iv) Determination of phage type and *stx* type. Phage types were determined for all of the strains as described previously (16). Phage typing was conducted at the Canadian National Microbiological Laboratory. Shiga toxin typing was conducted using PCR-based typing methods described previously (17).

(v) LSPA. The lineage-specific polymorphism assay (LSPA) was carried out as previously reported (18), with the modifications described by Hartzell et al. (19). Reference strains for lineage I (FRIK 523 and FDA 520) and lineage II (FRIK 920 and FRIK 1990) were generously provided by Andrew Benson at the University of Nebraska—Lincoln.

(vi) *tir* single-nucleotide polymorphism (SNP) genotyping. *E. coli* O157:H7 isolates were genotyped for either the *tir* 255 T>A allele by real-time PCR genotyping as described previously (20). Each reaction mixture consisted of TaqMan Universal PCR Master Mix (2×) (Applied Biosystems), 0.5 ng of genomic DNA, 1× assay mix (0.9 μM concentration of each primer and 0.2 μM concentration of each fluorescent probe), and molecular-grade water to a final volume of 25 μl. Amplification and detection were carried out in optical-grade 96-well plates, sealed with optical film in a Chromo4 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The reactions were cycled at 50°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with optical reading taken after the extension step. Opticon 3.0 application software (Bio-Rad Laboratories) was used to determine the *tir* allele for each strain, with the threshold set to three times the standard deviation of the mean fluorescence for the negative controls.

(vii) SBI genotyping. Stx-encoding bacteriophage insertion (SBI) genotypes were analyzed by uniplex PCR assays to detect the presence or absence of stx_1 and stx_2 genes (21–23), and phage integration, full, partial, or lack thereof, into either *yehV* (Stx1-encoding bacteriophage) or *wrbA* (Stx2-encoding bacteriophage) (24, 25). The presence or absence of PCR amplicons was determined via gel electrophoresis. Resultant PCR products were coded for six characters (0 for absence and 1 for presence) for the following concatenated genotypes: stx_1, stx_2, *yehV*-left and Stx1-encoding bacteriophage junction, *yehV*-right and Stx1-encoding bacteriophage junction, *wrbA*-left and Stx2-encoding bacteriophage junction, and *wrbA*-right and Stx2-encoding bacteriophage junction.

(viii) Detection of Q_{933} and Q_{21} alleles. The bacteriophage antiterminator gene alleles (Q_{933} and Q_{21}) were detected by PCR as described previously (26).

Statistical methods. (i) Confidence intervals (CIs). For the overall prevalence, the 95% exact binomial confidence limits were calculated using StatXact, version 8.0 (Cytel Software Corp., Cambridge, MA).

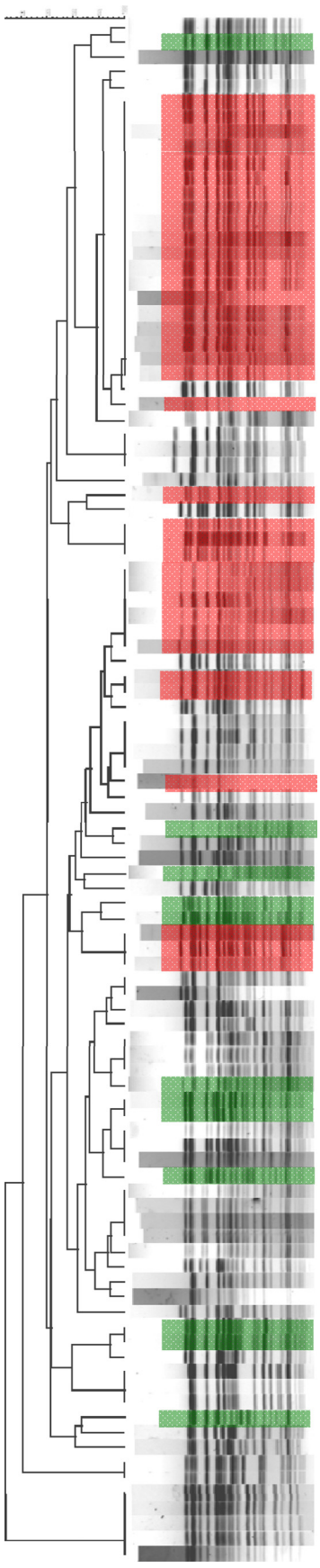
(ii) Univariate strain associations. The level of concordance between typing methods was calculated using the adjusted Wallace coefficient (AW) (27). The AW provides a directional measurement of clustering concordance between methods, i.e., if the results of one typing method can predict the results of another method. All calculations were done using the freely available online tool Comparing Partitions located at http://darwin.phyloviz.net/ComparingPartitions/.

(iii) Multivariate strain associations. In order to look for patterns among all of the different typing methods, the data were analyzed using nonmetric multidimensional scaling (NMS) using PC-ORD software, version 6.03 (MjM software Design, Gleneden Beach, OR). NMS is a nonparametric ordination technique well suited to data that are nonnormal or on arbitrary or discontinuous scales (28). The advantage of NMS is that it avoids the assumption of linear relationships among variables. It uses the ranked distances, which linearizes the relationships between variables (28). A main matrix including all the typing methods except PFGE was created. An additional second matrix was created with variables that may be of interest: human (were any of the PFGE types detected found in humans), CFU count (4 categories: 10^4 to 10^5, 10^5 to 10^6, 10^6 to 10^7, and 10^7 to 10^8), and location (processing plant and feedlot).

The final matrix consisted of 38 variables (typing methods) and 102 events (*E. coli* O157 strains). NMS was used with a Euclidian distance measure after standardizing each variable by division by its standard deviation. The dimensionality of the data set was determined by plotting an inverse measure of fit ("stress") to the number of dimensions. Optimal dimensionality was based on the number of dimensions with the lowest stress. A two-dimensional solution was shown to be optimal. Five hundred iterations were used for each NMS run, using random starting coordinates. Several NMS runs were performed for each analysis to ensure that the solution was stable and represented a configuration with the best possible fit. Multiresponse permutation procedure (MRPP) analysis was performed to test the hypothesis of no difference between groups (human, CFU count, and location) of interest.

RESULTS AND DISCUSSION

The focus of this work was to investigate the *E. coli* O157:H7 strain contribution made to supershedding by characterizing strains isolated from supershedding cattle. This characterization was aimed at identifying strain-specific traits that would be necessary for attaining supershedder levels in the lower GI tract of cattle. To address this issue, it was deemed a high priority to collect samples from a diverse group of cattle, as previous studies have reported dominant *E. coli* O157:H7 genotypes within individual feedlots



(29, 30). This priority led to the collection of a majority of the samples from cattle at harvest. Sample collection at beef processing facilities enabled a broad survey from many more cattle production sources than would have been feasible to obtain by traveling to individual feedlots. Through this sampling design, samples from 5,086 cattle were analyzed for supershedding of *E. coli* O157:H7. Overall, supershedders were identified as 2.01% (n = 102; 95% CI = 1.64 to 2.43%) of the bovine population tested. The maximum shedding level observed was 4×10^7 CFU/swab.

Prior to isolate characterization, it was necessary to determine how many *E. coli* O157:H7 strain types were present in a sample. Previous studies have identified variation in the diversity of *E. coli* O157:H7 PFGE types within a given bovine fecal sample (31–34). Vali et al., Jacob et al., and Renter et al. (31, 33, 34) found averages of 1.3, 1.6, and 1.8 PFGE types within bovine fecal samples, respectively. A similar study concluded that a few samples had multiple PFGE types, but the vast majority of isolates shed by a given animal comprised indistinguishable PFGE patterns, indicating a dominant strain type within a sample (32). However, all four studies cited here used fecal pat sampling, either alone or in combination with rectal palpation sampling, not fecal swabs as conducted for the current study. In addition, these studies used immunomagnetic separation of enriched samples, not direct plating for high-concentration samples as was used in this study.

In order to determine how many isolates from a given sample would need to be evaluated to be representative of a supershedder swab sample, the typical strain diversity was determined using PFGE and phage typing. Multiple isolates (n = 1,061) were characterized from 98 supershedder samples, and in every case, all isolates originating from a sample were found to be the same strain by PFGE analysis (data not shown). A subset of isolates (5 isolates from each of 20 samples) was subjected to phage typing, producing the same result that within a sample the isolates were indistinguishable. Based on these results, it was decided to select one isolate from each of the 102 supershedder samples for evaluation using all seven typing methods.

Univariate strain associations. Among the 102 supershedder isolates, PFGE analysis identified 52 unique genotypes (Fig. 1; Table 1). The two most common PFGE genotypes contained 19 and 6 isolates. All other genotypes were comprised of 5 or fewer isolates. All 52 unique PFGE patterns were screened against the PulseNet database for *E. coli* O157:H7 isolates obtained from human illness cases in the United States. Interestingly, when compared to the top 10 genotypes from the PulseNet database, the two largest genotype clusters among the supershedder isolates were found to match the two most common human illness genotypes (Fig. 1). Matches also were identified for the 3rd through 5th and 7th through 9th most common PulseNet genotypes, but not for the 6th and 10th. Thirty-four supershedder genotypes matched PulseNet genotypes not in the top 10, and 10 supershedder PFGE types did not match any genotypes in the PulseNet database (Fig. 1).

FIG 1 PFGE analysis of *E. coli* O157:H7 supershedder isolates. The PFGE dendrogram of XbaI-digested genomic DNA was generated from 102 supershedder strains. Restriction digest patterns shaded red indicate PFGE patterns that matched the top 10 human disease patterns from the CDC PulseNet database. Patterns shaded green did not match any human disease patterns from the CDC PulseNet database. Unshaded patterns matched patterns in the CDC PulseNet database but not in the top 10.

TABLE 1 Distribution of lineage, PFGE, and phage types for *E. coli* O157:H7 supershedder strains

PFGE type[a]	2	4	8	14	23	32	34	49	54	74	85	14a	14c	14d	Atypical 1	Atypical 2	Atypical 3	Atypical 4	Atypical 5	Total
1		1[b]																		1
2		1																		1
3		1																		1
4		1																		1
5		1																		1
6		19																		19
7		1																		1
8		1																		1
9		1																		1
10														3						3
11													1							1
12	1																			1
13									1											1
14								3												3
15						2						2	1	1						6
16														1						1
17																	2			2
18														1						1
19				1								2		1						4
20						1														1
21												1								1
22												1								1
23										1										1
24														1						1
25												1								1
26														1						1
27														1						1
28									1											1
29									1											1
30		3																		3
31		1													1					2
32										1										1
33												1								1
34										3										3
35							1													1
36										2										2
37									1	2										3
38										1										1
39										4										4
40										1										1
41																			1	1
42									1											1
43							1													1
44										1										1
45			2																	2
46											1									1
47																3				3
48									1											1
49				1																1
50																		1		1
51					2															2
52			5																	5
Total	**1**	**31**	**7**	**2**	**2**	**3**	**2**	**3**	**6**	**16**	**1**	**8**	**2**	**10**	**1**	**3**	**2**	**1**	**1**	**102**

[a] PFGE type numbering is arbitrary and based on dendrogram shown in Fig. 1.
[b] Values represent the count of supershedder strains matching designated PFGE and phage types. Lineages are color coded as follows: lineage I, blue; lineage I/II, orange; and lineage II, green.

When comparing *E. coli* O157:H7 PFGE genotypes for strains isolated from ground beef or human clinical cases, Lanier et al. (35) similarly reported that the two most frequently isolated ground beef genotypes matched the top two isolated human genotypes. Likewise, Arthur et al. (36) determined that the two most common PFGE patterns for *E. coli* O157:H7 strains obtained from cattle hide samples at harvest also matched the top two PulseNet genotypes.

In the United States, PFGE analysis of *E. coli* O157:H7 strains is the most commonly utilized method for strain tracking and analysis and is the current "gold standard" for outbreak investigation. Other countries do not rely as heavily on PFGE for strain analysis and instead use other methods such as phage typing. The supershedder strains were classified into 19 different phage types (Table 1). There was good predictive power of the PFGE-based genotype clusters for the phage type (AW, 0.653 [95% CI, 0.603 to 0.703]).

The converse was not so (AW, 0.448 [95% CI, 0.294 to 0.603]) (Table 2). PT4 consisted of the largest number of strains ($n = 31$ [30%]), including those from the largest PFGE genotype, which also was the top human illness genotype in the United States. In Scotland, phage typing was utilized in a national study to identify various O157:H7 strains associated with cattle production and human illness. It was determined that the phage type most prevalent in the cattle population (PT21/28) also was the most common type isolated from human illness cases (37). Therefore, in both Scotland and the United States, the phage types found most frequently in supershedders also were most frequently isolated in human illness cases. Similarly, Mora et al. (38) determined that the most frequently found phage types (PT2 and PT8) among human isolates were also the most frequently found among bovine isolates in Spain.

Not only was PT21/28 the most common strain type isolated from human and bovine samples collected in Scotland, but also a significant association was observed between PT21/28 and supershedding individuals within the cattle population (9, 39). No strains of PT21/28 were isolated in the current study. This is of interest, as over 70% of the phage types identified in the Scottish study (37), including 4 of the 5 most prevalent, were found in the current study, with the notable exception of their most prevalent type, PT21/28. Scotland has been shown to have higher rates of human *E. coli* O157:H7 cases than North American countries, where PT21/28 does not appear to be as prevalent (37).

While PFGE and phage typing are useful tracking tools for bacterial strains, they do not provide much information regarding the relatedness of strains (40). In order to determine relatedness of the *E. coli* O157:H7 supershedder strains, the lineage-specific polymorphism assay (LSPA) developed by Yang et al. (18) was utilized. The supershedder strains were spread among the three lineages approximately equally, with lineage I/II (36%) being the most frequently identified lineage among the supershedder strains and lineage I (29%) the least (Fig. 2; Tables 1 and 3). PFGE types were not found to contain strains from multiple lineages. However, similar to the findings of Ziebell et al. (41), certain phage types did contain strains from multiple lineages (Table 1). Each PFGE cluster was composed almost exclusively of isolates of the same lineage (AW, 0.947 [95% CI, 0.926 to 0.968]), indicating a good predictive power of the PFGE-based genotypes for the lineage (Table 2). The converse was not so (AW, 0.211 [95% CI, 0.114 to 0.308]).

The LSPA was originally designed to discriminate between two lineages (I and II) of *E. coli* O157:H7, originally identified through octamer-based genome scanning (42). Lineage I had been associated with strains that cause the majority of human illnesses, as opposed to lineage II. The third major lineage (I/II) shares characteristics of both previous lineages (43), but it has been shown to be a cause of human illness at frequencies similar to those of lineage I strains. It is interesting that all three lineages have been isolated from cattle at high rates previously (18, 44, 45), but only rarely are lineage II strains found to cause human disease.

Both Abu-Ali et al. and Lowe et al. (46, 47) reported associations between *E. coli* O157:H7 lineage, with respect to likelihood of causing human disease, and bovine colonization potential. Lowe et al. (47) determined the hierarchy of *E. coli* O157:H7 colonization to be lineage I > intermediate lineages > lineage II, assigning strains least likely to cause human disease with the least bovine colonization potential. Abu-Ali et al. (46) concluded that

TABLE 2 Adjusted Wallace coefficients and respective 95% confidence intervals for different typing methods used in this study

Typing method	AW (95% CI) with indicated typing method							
	stx$_2$ type	*stx*$_1$	Lineage	Q allele	SBI	*tir* allele	PFGE	Phage type
stx$_2$ type		0.145 (0.037–0.252)	0.531 (0.397–0.665)	1.00 (1.00–1.00)	0.268 (0.169–0.367)	0.586 (0.457–0.715)	0.104 (0.044–0.165)	0.114 (0.050–0.179)
stx$_1$	0.077 (0.021–0.134)		0.236 (0.135–0.337)	0.077 (0.021–0.134)	0.259 (0.162–0.356)	0.046 (0.00–0.223)	0.088 (0.016–0.160)	0.100 (0.021–0.180)
Lineage	0.568 (0.475–0.662)	0.472 (0.320–0.624)		0.568 (0.475–0.662)	0.493 (0.367–0.619)	0.789 (0.647–0.931)	0.211 (0.114–0.308)	0.239 (0.132–0.347)
Q allele	1.00 (1.00–1.00)	0.145 (0.037–0.252)	0.531 (0.397–0.665)		0.268 (0.169–0.367)	0.568 (0.457–0.715)	0.104 (0.044–0.165)	0.114 (0.050–0.179)
SBI	0.531 (0.452–0.609)	0.960 (0.898–1.00)	0.913 (0.815–1.00)	0.531 (0.452–0.609)		0.794 (0.643–0.946)	0.259 (0.136–0.381)	0.337 (0.203–0.471)
tir allele	0.222 (0.104–0.341)	0.033 (0.00–0.109)	0.280 (0.208–0.351)	0.222 (0.104–0.341)	0.152 (0.066–0.238)		0.072 (0.011–0.134)	0.113 (0.026–0.200)
PFGE	0.502 (0.423–0.580)	0.793 (0.661–0.926)	0.947 (0.926–0.968)	0.502 (0.423–0.580)	0.626 (0.464–0.789)	0.916 (0.882–0.949)		0.653 (0.603–0.703)
Phage type	0.377 (0.276–0.479)	0.617 (0.381–0.853)	0.737 (0.559–0.914)	0.377 (0.276–0.479)	0.560 (0.372–0.749)	0.983 (0.961–1.00)	0.448 (0.294–0.603)	

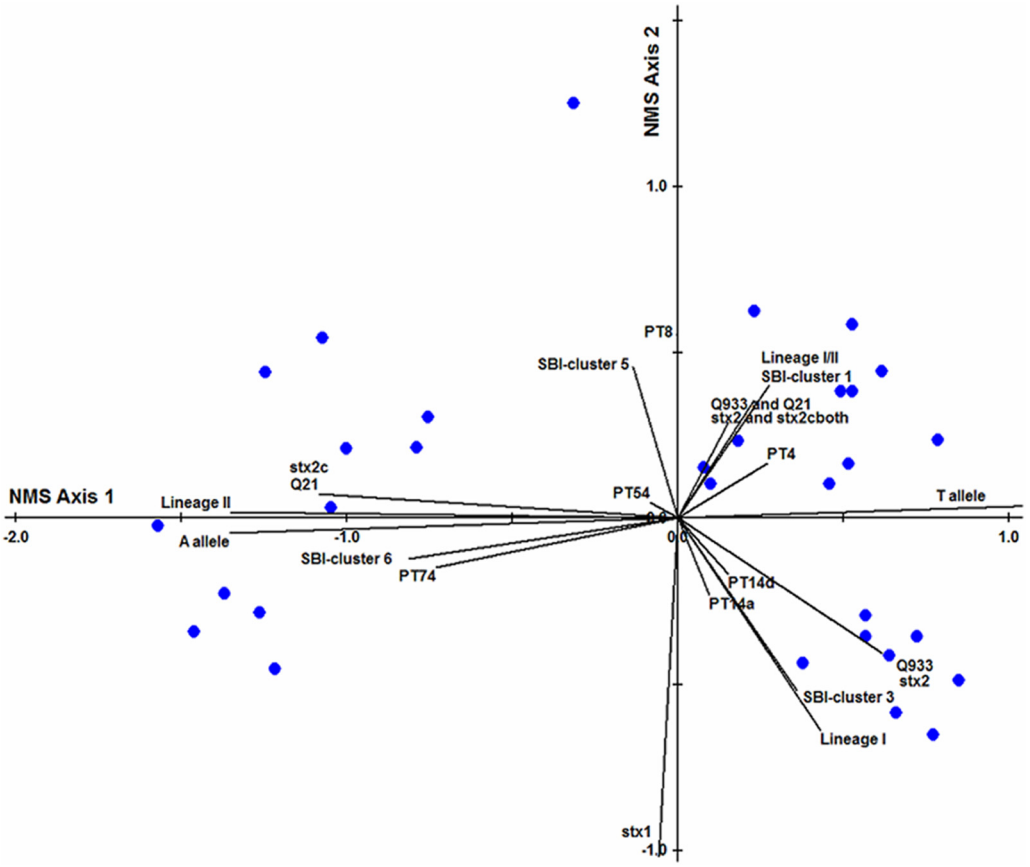


FIG 2 NMS ordination of cattle typing data. Typing variables correlated with NMS axis 1 and NMS axis 2 are shown as vectors radiating from the centroid of points; the direction indicates positive and negative correlation along the axis. The final NMS solution was two dimensional and explained 85.9% (cumulative $r^2 = 0.859$; axis 1 $r^2 = 0.638$; axis 2 $r^2 = 0.221$) of the variation in typing methods and also explained more variation than expected by chance (Monte Carlo test, $P = 0.004$). Final stress for the two-dimensional solution was 5.94, with no real risk of drawing false inferences (28). Final instability was 0, with 70 iterations. Number of iterations is the number of steps that NMS performed to find the final solution (28).

lineage I/II strains, previously found to be more likely to cause severe human disease outcomes such as hemolytic-uremic syndrome, have an enhanced ability to attach to bovine epithelial cells compared to other *E. coli* O157:H7 lineages. The supershedder lineage distribution in the current study did not demonstrate a decreased colonization potential for lineage II strains compared to either lineage I or lineage I/II individually, but as a whole, human lineage strains were isolated twice as often as nonhuman from supershedder samples.

Other typing methods have been used to discriminate between *E. coli* O157:H7 isolates based on their propensity to cause disease in humans. One of these methods utilizes a base change (either an A or T allele) at position 255 in the sequence of the *tir* gene (20). The *E. coli* O157:H7 supershedder strains harbored the T allele more often (71%) than the A allele (29%) (Table 3). The presence of the T allele at position 255 was observed previously to be associated with human disease, as it was identified in more than 99% of strains isolated from human illness cases, but it has been identified in only 55% of strains collected from bovine fecal samples (20).

There was a good concordance between lineage and *tir* SNP genotype (AW, 0.789 [95% CI, 0.606 to 0.972]) (Table 2; Fig. 2). All strains that were determined to be in lineages I and I/II by LSPA typing were found to carry the T allele at position 255 of the *tir* gene. Of the 35 strains identified as lineage II, most (30 of 35 [86%]) carried the A allele, while the remaining 14% carried the T allele, indicating that they may be more likely to be associated with human disease than other lineage II strains (20) (Table 3).

While this is one of the first studies to investigate the contribution of *tir* type to supershedding, previous studies have implicated *tir* in colonization of the bovine rectoanal junction. Sheng et al. (48) have shown that *E. coli* O157:H7 strains lacking *tir* were less efficient in colonizing the rectoanal junction of cattle than were wild-type strains. The *tir* type, however, does not appear to impact colonization, as experiments investigating the effects on bovine intestinal colonization using strains harboring different alleles at position 255 of the *tir* gene found no significant difference in fecal shedding of the pathogen (unpublished data).

TABLE 3 *tir* alleles distributed by LSPA genotype

tir allele	No. of strains of lineage:			Total
	I	I/II	II	
T	30	37	5	72
A			30	30
Total	30	37	35	102

TABLE 4 SBI clusters and stx_2 content

SBI clusters	No. of strains with stx_2 genotype(s):				Total
	2a	2a, 2c	2c	None	
1 and 3	36	17	4		57
5 and 6		1	32		33
Other	6	1	3	2	12
Total	42	19	39	2	102

TABLE 5 stx_2 type distribution by stx_1, lineage, and *Q* allele

stx_2 type(s)	No. of strains								
	stx_1		Lineage			*Q* allele			
	Present	Absent	I	I/II	II	Q_{933}	Q_{21}	Both	Neither
2a	28	14	30	12		42			
2a, 2c		19		19				19	
2c	22	17		6	33		39		
None	2				2				2
Total	52	50	30	37	35	42	39	19	2

Another *E. coli* O157:H7 typing scheme characterizes the insertion sites for bacteriophages encoding genes for Shiga toxin production. This method also has been utilized to identify variations in human versus bovine strain representation among the *E. coli* O157:H7 population, but it utilizes more resulting clades than typing based on the *tir* alleles (25). Stx-associated bacteriophage insertion (SBI) genotyping grouped the supershedder strains into 12 clusters. Clinical genotype clusters 1 and 3 contained 57 (55.9%) supershedder strains (Table 4). Clusters 5 and 6, previously associated with bovine isolates (25), contained the majority of the remaining supershedder strains ($n = 33$ [32.4%]). Besser et al. (25) previously reported that 95% of clinical strains were classified as clusters 1 to 3, but these cluster genotypes were found much less frequently (53%) in bovine isolates, similar to the distribution of the supershedder strains observed in the current study.

The determination of SBI for a particular strain is dependent on where the *stx* genes are inserted, but also on which *stx* genes are present. The supershedder strains were found to carry either stx_{2a} or stx_{2c} more frequently than they were found to carry the combination of stx_{2a} and stx_{2c} (Table 4). The Shiga toxin 1 gene was carried by half (51%) of the supershedder strains (Table 5). None of the supershedder isolates were found to harbor the collective set of stx_{2a}, stx_{2c}, and stx_1 genes (Table 5). The Shiga toxin 2 genes also were associated by lineage (Table 5). All lineage I strains harbored stx_{2a}. All lineage II strains harbored stx_{2c}, with the exception of two strains that carried only Shiga toxin 1 genes. Lineage I/II strains were found to carry stx_{2a} alone, stx_{2c} alone, or both variants.

The final characterization of the supershedder strains also focused on genes associated with Shiga toxin production. It was determined that 59.8% of the supershedder isolates harbored Q_{933} either alone (41.2%) or in combination with Q_{21} (18.6%) (Table 5). The Q_{933} and Q_{21} variants were found to be exactly correlated to the presence or absence of stx_{2a} and stx_{2c}, respectively (AW, 1.00 [95% CI, 1.00 to 1.00] [Table 2]). LeJeune et al. (26) initially determined that the antiterminator *Q* gene, which controls expression of the Shiga toxin gene, had variants nonrandomly distributed between *E. coli* O157:H7 populations from cattle and humans. In that study (26), the *Q* gene of bacteriophage 933W (Q_{933}) was detected in 90% of clinical isolates, while it was detected in only 44.5% of bovine isolates, with the Q_{21} variant being dominant in the cattle population (26). Taylor et al. (49) also found a higher proportion of Q_{933}-harboring strains among human clinical isolates (87.5%) than among bovine isolates (54.3%).

The *Q* gene can be indirectly associated with effects on bovine intestinal colonization through Shiga toxin expression. Previous studies have determined that Stx2 enhances *E. coli* O157:H7 colonization of cattle intestinal tissues (50) and that *E. coli* O157:H7 strains harboring the Q_{933} variant of the antiterminator gene produced significantly higher levels of Shiga toxin 2 than strains with the Q_{21} variant or strains harboring both Q_{933} and Q_{21} (26, 49, 51). The current results indicate that Q_{933} does not impact supershedding, implying that increased Stx2 expression also does not affect supershedding. As Shiga toxin expression was not measured in this study, further study is required for confirmation.

Multivariate strain associations. The results of the NMS models are shown in a joint plot (Fig. 2) for all of the genetic variables tested and in a two-dimensional ordination graph (Fig. 3) of the distance between sample units based on the association with genotypes previously isolated from human clinical cases. The NMS identified clusters within the data associated with different typing methods (Fig. 2). The cluster in the left upper and lower quadrants is characterized by strains with *tir*A, Q_{21}, lineage II, and stx_{2c}. In the right upper and lower quadrants, strains are characterized by the presence of *tir*T, with strains in the upper right quadrant characterized by lineage I/II, stx_{2a} and stx_{2c}, and Q_{21} and Q_{933} (Fig. 2). In the lower right quadrant, strains are characterized by lineage I, stx_{2a}, and Q_{933} (Fig. 2). Interestingly, the supershedder NMS is very similar to the ordination plot produced previously (52) using a composite of bovine, food, and human clinical *E. coli* O157:H7 isolates obtained in the Netherlands. However, in that study, Franz et al. (52) found the bovine isolates to be biased toward the stx_{2c}, *tir*A, and Q_{21} genotypes. The stx_{2a}, *tir*T, and Q_{933} genotypes were dominated by human clinical isolates (52).

The supershedder strain set described here covered the spectrum of genotypes from those that were frequently found to cause human illness to those that had not been associated with human disease. Strains that were found to be identical to the top 10 human PFGE types were clustered in the right portion of the graph (Fig. 3). The differences between the strains associated with the human top 10 and strains that caused human disease but were not in the top 10 and those not found in humans were statistically significant (MRPP, $P < 0.001$). The strains that had PFGE patterns similar to the human top 10 PFGE patterns were primarily characterized by stx_{2a} or stx_{2a} and stx_{2c}, *tir*T, lineage I or I/II, and Q_{933} or Q_{933} and Q_{21} positivity. There was no difference between strains not found in the top 10 and those not found in humans (MRPP, $P > 0.05$).

This study was designed to investigate the requirements of supershedding at the *E. coli* O157:H7 strain level. Therefore, samples were screened for supershedders, and those samples that harbored *E. coli* O157:H7 organisms below supershedding levels were discarded. Not having a "nonsupershedder" control group is a detriment to the current results, but it was decided when designing the project that strains obtained at levels below supershedding levels could not be definitively assigned to a nonsupershedder category.

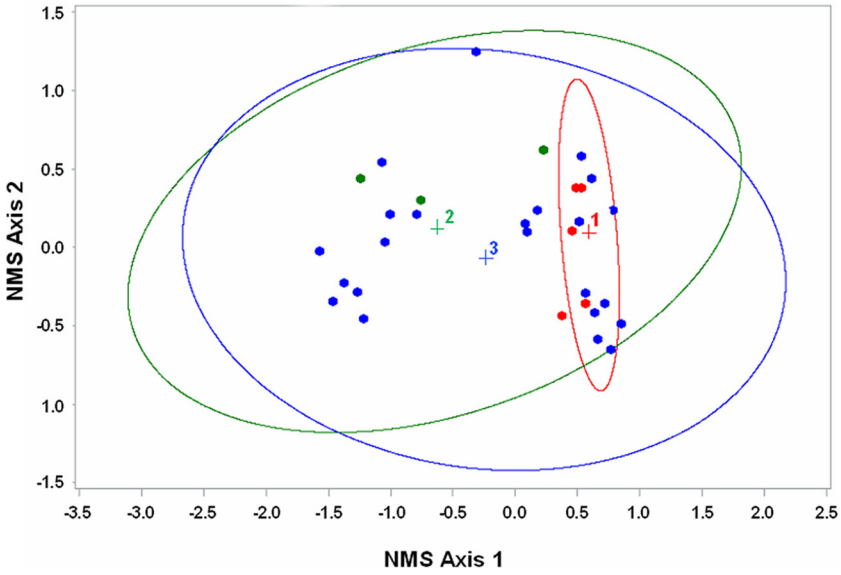


FIG 3 NMS ordination of cattle typing data. Shown is a two-dimensional ordination graph of the distance between sample units. Ovals indicate 95% confidence ellipses for designated groups within the data. 1, PFGE patterns that matched the top 10 human disease patterns from the CDC PulseNet database; 2, patterns that did not match any human disease patterns from the CDC PulseNet database; 3, patterns that matched patterns in the CDC PulseNet database but not in the top 10. MRPP: 1 versus 2, $P < 0.001$; 2 versus 3, $P = 0.192$; 1 versus 3, $P < 0.001$.

As supershedding is a periodic event, one could isolate supershedder strains at levels less than 10^4 CFU during the increase toward or decrease from a shedding event, thus blurring the desired distinction of supershedder-specific traits. Thus, it was decided to investigate the supershedder population for indications of clustering within a particular trait.

As this study did not identify any strict requirements necessary for strains to become supershedders, a future study is needed to determine if certain strains are more likely to attain supershedder levels. To answer this question, one would need to obtain *E. coli* O157:H7 strains shed at any level, high or low, to determine if certain strains are more commonly associated with high shedding levels. As the data set herein contains only isolates from high shedding events, additional experiments will be needed to address this question.

In the current strain set, the supershedder type most commonly isolated was the same type as that which caused the most human disease, and overall, the supershedder strain set appeared to have a slight bias for characteristics (lineage and *tir* allele) associated with *E. coli* O157:H7 strains found to cause human disease. It remains to be determined if the increased disease burden of certain strains is a function of increased virulence potential or simply population dynamics with these strains comprising the largest fraction of the total O157:H7 population. An increased colonization potential, especially colonization leading to supershedding, would greatly enhance the competitive fitness within the *E. coli* O157:H7 population. A recent study (53) determined that *E. coli* O157:H7 strains found to be persistent and predominant in a dairy farm environment were able to utilize a larger repertoire of carbon sources than less commonly isolated O157:H7 strains. Additional studies will be required to determine if there is any connection with supershedding strain types and human illness-associated strains.

This study addressed the question of supershedder requirements via various macrolevel (lineage, PFGE, phage type, and SBI) and microlevel (*Q* type, *stx* type, and *tir* allele) analyses. While no specific supershedding traits were identified through these analyses, there remains the possibility that evaluation of these strains with other methods (e.g., whole-genome sequencing or microarray analysis) may have identified commonalities not seen here. Relatedness on a phenotypic basis by increased expression of various virulence or attachment factors that are independent of the macrogenotypic traits identified herein would have been missed. As stated above, increased Shiga toxin 2 expression has been associated with increased colonization in cattle (47, 50). Also, heterogeneity in expression levels from attachment and virulence genes found on the pO157 plasmid and the locus of enterocyte effacement has been observed among strains of *E. coli* O157:H7 (54, 55). Hence, future studies will be required to investigate if supershedding can be linked to differential protein expression patterns associated with virulence factors of enterohemorrhagic *E. coli*.

Conclusions. Through a diverse sampling scheme, isolates representing 52 unique PFGE patterns, 19 phage types, and 12 SBI clusters were obtained from supershedding cattle, indicating that there is no clustering by *E. coli* O157:H7 genotypes responsible for supershedding. *E. coli* O157:H7 strains of phage type 21/28, previously associated with bovine supershedding in Scotland (9), are not a frequent source of supershedding in the United States. The supershedder strains characterized herein tended to have higher frequencies of traits associated with human clinical isolates than previously collected bovine isolates with respect to lineage and *tir* allele, but not for SBI cluster or *Q* type (18, 20, 25, 49). We conclude that no exclusive genotype was identified that was common to all supershedder isolates.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

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