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FOOD MICROBIOLOGY

Shiga Toxin-Producing Serogroup O91 *Escherichia coli* Strains Isolated from Food and Environmental Samples

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ABSTRACT Shiga toxin-producing *Escherichia coli* (STEC) strains of the O91:H21 serotype have caused severe infections, including hemolytic-uremic syndrome. Strains of the O91 serogroup have been isolated from food, animals, and the environment worldwide but are not well characterized. We used a microarray and other molecular assays to examine 49 serogroup O91 strains (environmental, food, and clinical strains) for their virulence potential and phylogenetic relationships. Most of the isolates were identified to be strains of the O91:H21 and O91:H14 serotypes, with a few O91:H10 strains and one O91:H9 strain being identified. None of the strains had the *eae* gene, which codes for the intimin adherence protein, and many did not have some of the genetic markers that are common in other STEC strains. The genetic profiles of the strains within each serotype were similar but differed greatly between strains of different serotypes. The genetic profiles of the O91:H21 strains that we tested were identical or nearly identical to those of the clinical O91:H21 strains that have caused severe diseases. Multilocus sequence typing and clustered regularly interspaced short palindromic repeat analyses showed that the O91:H21 strains clustered within the STEC 1 clonal group but the other O91 serotype strains were phylogenetically diverse.

IMPORTANCE This study showed that food and environmental O91:H21 strains have similar genotypic profiles and Shiga toxin subtypes and are phylogenetically related to the O91:H21 strains that have caused hemolytic-uremic syndrome, suggesting that these strains may also have the potential to cause severe illness.

KEYWORDS STEC, O91, virulence, diversity

Shiga toxin (Stx) is the main virulence factor of Shiga toxin-producing *Escherichia coli* (STEC). There are two main Stx types, designated Stx1 and Stx2, and each has many subtypes. However, STEC pathogenesis is complex, and Stx alone, without an adherence factor, is deemed to be insufficient to cause severe diseases like hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) (1, 2). The most common STEC adherence factor is the *eae*-encoded intimin adhesin, which resides on the locus of enterocyte effacement (LEE) pathogenicity island. However, some STEC strains, such as those of serotypes O91:H21, O113:H21, and others, lack *eae* but have still caused severe disease (3–5) and so are postulated to have other means for adherence. Genes such as *saa*, which codes for STEC agglutinating adhesin (6), and *sab*, which codes for an outer membrane autotransporter protein that enhances biofilm formation (7), have been found in LEE-negative STEC strains and are thought to play a role in adherence.

STEC serotype O91:H21 strains are LEE negative but have caused HUS (8, 9). The

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Stx2d subtype, which is activated by elastase in the intestinal mucus to become 10- to 1,000-fold more cytotoxic, was also discovered in strain B2F1, an STEC strain of the O91:H21 serotype (10). STEC strains that produce the Stx2d subtype have been shown to be significantly associated with the ability to cause severe illness, such as HC and HUS (11). Various studies on STEC prevalence have found serogroup O91 strains in clinical samples from Finland (12), the Netherlands (13), Germany (9, 14), and Switzerland (15). In one German study, O91 strains were the fourth most common STEC serogroup found in patient samples (16). Surveys of nonclinical sources have shown that STEC serogroup O91 strains are also present in foods. These strains have been isolated from raw milk and cheeses in France (17) and Switzerland (18), from minced beef in France (19), and from cattle feces in Argentina (20), and they are the second most common STEC serogroup found in various types of meats and dairy products in Germany (16). In the United States, STEC O91 strains have been isolated from ground beef (21), from the production environments of other food animals, and also from fresh produce (22). Most studies that have characterized STEC O91 strains have focused on clinical strains (9), so little is known about food and environmental STEC O91 strains. In this study, we used a PCR microarray and *stx* subtyping PCR to characterize O91 strains isolated from foods and environmental and clinical sources in the United States and in the European Union and compared them to known pathogenic strains to assess their virulence potential. We also used multilocus sequence typing (MLST) and clustered regularly interspaced short palindromic repeat (CRISPR) analyses to examine the phylogenetic relatedness, sequence polymorphisms, and the genetic diversity among these O91 strains.

RESULTS

All 49 strains examined were positive for the serogroup O91 *wzy* (wzy_{O91}) gene, confirming that they are O91 strains. Of these, 23 were positive for the H21 *fliC* ($fliC_{H21}$) gene and 5 were positive for the H10 *fliC* ($fliC_{H10}$) gene and so are O91:H21 (Table 1) and O91:H10 (Table 2) strains, respectively. The remaining 21 strains were molecularly H (flagellar) typed by sequencing, and 20 were identified to be O91:H14 and 1 was identified to be O91:H9 (Table 3). The *fliC* sequences of these strains showed 99 to 100% identity to the respective H type sequences in GenBank (data not shown).

Microarray analysis for 48 STEC genes (Table 4) showed that the following genes were absent from all the O91 strains: *bfpA*, *eae*, *ecf1*, *ecf2*, *espK*, *espM1*, *espN*, *espV*, *etpD*, the H4 *fliC* ($fliC_{H4}$) gene, *nleA*, *terE*, *toxB*, *ureD*, the O113 *wzy* (wzy_{O113}) gene, the O island (OI) 43 (OI-43) open reading frame (ORF) Z1155, the OI-57 ORFs Z2098 and Z2121, and the OI-71 ORF Z6065. Also, the O113 *lpfA* ($lpfA_{O113}$) gene, *wecA*, and wzy_{O91} were present in all the O91 strains, so the results for all these genes were excluded from the tables. The prevalence of the remaining genes among the O91 strains and the *stx* subtypes carried by these strains are shown in Tables 1 to 3. Since most O91 strains within each serotype shared genetic profiles as well as phylogenetic relationships, the results presented below are according to serotype.

O91:H21. Among the 23 O91:H21 strains (Table 1), only 3 carried stx_{1a} and all 3 of these strains also had stx_{2c} and stx_{2d}, including strain TW01674, which was isolated in 1987 from a patient with HC and HUS in Canada. The prototypical O91:H21 strain, B2F1 (TW01393), which has been implicated in HUS, had stx_{2c} and stx_{2d}. Among the food and environmental isolates, six strains had stx_{2a} alone and three other strains had stx_{2d} alone, including CB13035, from the Staten Serum Institute (SSI) in Denmark. Two other strains had stx_{2b} alone, five strains had stx_{2c} and stx_{2d}, three had stx_{2a} and stx_{2d}, and CB13807 did not have any *stx* genes.

All 23 O91:H21 strains had the OI-15 gene *ehaA*, which codes for an enterohemorrhagic *E. coli* (EHEC) autotransporter, and the O26 *lpfA* ($lpfA_{O26}$) fimbrial gene, and 96% (22/23) of the strains had the *iha* and Z4320 genes, which reside on various OIs. Many strains also had genes that are commonly found on the EHEC plasmid. The *ehxA* enterohemolysin gene was found in 87% (20/23) of the strains, and the *espP* serine protease gene was prevalent in 82% (19/23) of the strains, but none had the *katP*

TABLE 1 Characteristics of the O91:H21 strains examined[a]

Strain[b]	Source	ST	CT1	CT2	Presence of the following gene/ORF or allele:																
					stx$_1$	*stx*$_2$	*astA*	*cdt-V*	*ehxA*	*eibG*	*epeA*	*espP*	*iha*	*pagC*	*saa*	*sab*	Z1151	Z1153	Z1156	Z2096	Z2099	Z4320
1-Apr082	Ground beef	89	52	57	−	c, d	−	−	+	−	+	+	+	+	+	+	−	−	−	+	−	+
9-Oct188B	Ground beef	89	52	57	a	c, d	−	−	+	−	+	+	+	+	+	+	−	−	−	+	−	+
CB13035	SSI	89	52	57	−	d	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
TW01393 (B2F1)	Human (HUS)	89	52	57	−	c, d	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
TW01674	Human (HC/HUS)	89	152	57	a	c, d	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
11-Cow453-3	Bovine feces	89	153	57	−	d	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
4-Jun125A	Ground beef	89	153	57	−	a	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
6-MDR0035	Ground beef	89	153	57	−	a	−	−	+	−	+	+	+	+	+	+	−	−	−	+	−	+
M12-56	Spinach	89	153	57	−	a	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
TW05664	Sheep	89	154	57	−	d	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
5-MDR0003	Ground beef	89	156	57	−	a, d	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
10-Oct227	Ground beef	89	157	57	−	a, d	−	+	+	−	+	+	+	+	+	+	−	−	−	−	−	+
12-Imp809A	Beef trim	89	159	57	−	a	−	+	+	−	+	−	+	+	+	+	−	−	−	+	−	+
CB13381	Salad	89	160	57	a	c, d	−	−	−	−	−	+	+	+	−	−	−	−	−	+	−	+
CB14438	Beef	89	161	57	−	b	−	+	+	−	+	+	+	+	+	+	−	−	−	−	−	+
CB15103	Raw milk	89	163	57	−	a	−	+	+	−	+	+	+	+	+	+	−	−	−	−	−	+
CB15152	Beef	89	164	57	−	a, d	−	−	−	−	−	+	+	+	−	−	−	−	−	+	−	+
CB13861	Red deer meat	89	165	191	−	b	+	−	+	+	−	−	+	−	−	+	+	+	+	−	+	+
2-Dec170	Ground beef	89.1	158	57	−	a, c, d	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
3-Jan065	Ground beef	89.2	153	57	−	a	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
CB14856	Raw sausage	89.3	162	57	−	c, d	−	+	+	−	+	+	+	+	+	+	−	−	−	+	−	+
TW00128	Pork	89	155	65	−	c, d	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	+
CB13632	Chicken meat	89	ND	65	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

[a]Abbreviations: ST, sequence type; CT1, CRISPR1 type; CT2, CRISPR2a type; *stx*$_1$, *stx*$_1$ allele; *stx*$_2$, *stx*$_2$ allele; ND, not determined; +, positive; −, negative. In addition to the loci mentioned in the text, all O91:H21 strains examined were positive for *ehaA* and were negative for Z1154.

[b]Strains with the CB designation are from the European Union, strains with the TW designation are from the STEC Center, and all other strains are from various sources in the United States.

TABLE 2 Characteristics of the O91:H10 strains examined[a]

Strain[b]	Source	ST	CT1	CT2	Presence of the following gene/ORF or allele:											
					stx$_2$	*astA*	*ehaA*	*ehxA*	*espP*	*irp2*	*lpfA*$_{O26}$	*pagC*	*saa*	*sab*	*subA*	Z4320
CB14687	EU-RL (reference strain)	140	ND	ND	−	+	−	−	−	+	+	−	−	−	−	+
7-MDR0009B	Ground beef	461	167	192	d	−	+	−	−	−	−	−	−	+	−	−
TW01445	Cow	461	166	38	a	−	+	+	+	−	−	+	+	+	+	+
CB11906	Cheese	461.1	168	38	d	−	+	−	−	−	−	−	−	+	−	−
CB12624	Human feces (HUS)	461.1	169	38	d	−	+	−	−	−	−	−	−	+	−	−

[a]Abbreviations: ST, sequence type; CT1, CRISPR1 type; CT2, CRISPR2a type; *stx*$_2$, *stx*$_2$ allele; ND, not determined; +, positive; −, negative. In addition to the loci mentioned in the text, all O91:H10 strains examined were positive for *iha* and were negative for *cdt-V*, *eibG*, *epeA*, *katP*, and *stx*$_1$.

[b]Strains with the CB designation are from the European Union, strains with the TW designation are from the STEC Center, and all other strains are from various sources in the United States.

catalase peroxidase gene, which is typically found on the same EHEC plasmid. Many LEE-negative STEC strains often have other plasmids that carry putative virulence genes, like *saa* and *sab*, both of which were prevalent in the O91:H21 strains, with 82% (19/23) and 87% (20/23) of the strains carrying *saa* and *sab*, respectively. However, the *subA* subtilase cytotoxin gene (23), which is also common in LEE-negative STEC strains, was not present in any of the O91:H21 strains, nor was the *irp2* gene, which codes for iron-repressible protein 2 (Table 1). Most of the O91:H21 strains had similar genetic profiles. The exception was strain CB13632, isolated from chicken meat, which was very distinct, in that it did not have *stx* or any of the genes tested except *ehaA* (Table 1). Another atypical strain, CB13861, isolated from red deer meat, was the only O91:H21 strain that had the OI-43 genes Z1151, Z1153, and Z1156; the OI-57 gene Z2099; and the *astA* and *eibG* genes, which code for the EAST1 heat-stable enterotoxin 1 and an immunoglobulin binding protein, respectively. With regard to the genes tested on the array, the profile of strain B2F1 (TW01393), the prototypic O91:H21 strain that caused HUS, was identical to that of strain TW01674, which was also implicated in HC and HUS. Furthermore, most of the O91:H21 strains isolated from food and environmental sources had genetic profiles identical or nearly identical to the profiles of these clinical strains that have caused severe diseases.

Clonal analysis indicated that all 23 O91:H21 strains, including the clinical strains, are sequence type (ST) 89 (ST-89) or variants of ST-89. Strain 2-Dec170 (ST-89.1) had nonsynonymous transversions in *aspC* (A → C) and *fadD* (T → A), strain 3-Jan065 (ST-89.2) had a nonsynonymous T → C transition in *icdA*, and strain CB14856 (ST-89.3) had a nonsynonymous A → T transversion, also in *icdA*. Despite these variations, all 23 O91:H21 strains were in the STEC-1 clonal group and clustered together on the phylogenetic tree (Fig. 1).

CRISPR analysis showed that CB13632 did not have a CRISPR1 type (CT1) allele and could not be typed. The other strains showed 68% allelic diversity with 15 different CT1 alleles, of which 13 were seen only once. The two CT1 alleles seen more than once included CT1-52 ($n = 4$) and CT1-153 ($n = 5$). Except for CT1-52, which has been seen previously (24), all the other CT1 alleles were newly identified in this study. The 15 CT1 alleles were built from a pool of 29 spacers, and except for 1 spacer, all the others were 32 bp long. Each allele contained between 8 and 25 spacers (mean ± standard deviation [SD], 17.64 ± 3.16), of which only 2 were not previously identified. Both of these new spacers (spacers 236 and 237) were found in TW00128, a strain isolated from pork.

In contrast, CRISPR2a (CT2) analysis showed only three CT2 alleles, with 87% (20/23) of the strains, including the clinical strains, possessing CT2-57. Of the other three strains, two had CT2-65 and one had CT2-191, which had not been previously identified but was a variant of CT2-65 with one less distal spacer. The CT2 alleles had only 13% diversity so are more conserved than CT1, and they are also significantly shorter (as tested with a nonparametric Mann-Whitney test; $P < 0.001$). Each of the three CT2 alleles contained between two and four spacers (mean ± SD, 2.21 ± 0.6), and all the spacers have been described previously (24).

TABLE 3 Characteristics of the O91:H14 strains examined[a]

Strain[b]	Source	ST	CT1	CT2	Presence of the following gene/ORF or allele:																					
					stx$_1$	*stx*$_2$	*astA*	*cdt-V*	*ehaA*	*ehxA*	*eibG*	*epeA*	*espP*	*iha*	*katP*	*lpfA*$_{O26}$	*pagC*	*saa*	*sab*	*subA*	Z1151	Z1153	Z1154	Z1156	Z2096	Z2099	Z4320
CB11895	Beef	475.1	183	195	−	a	−	−	+	+	−	+	+	+	−	−	−	+	+	+	−	−	−	−	−	−	−
8-Oct097B[c]	Ground beef	815	171	45	a	a, d	−	+	+	+	+	+	+	+	−	+	−	+	+	+	−	−	−	−	+	−	+
CB14259	Lamb meat	815	178	42	a	b	−	−	+	−	+	−	−	+	+	+	−	−	−	−	+	+	+	+	−	+	+
TW00122	Food	815	170	42	a	c	−	−	+	−	+	−	−	+	−	+	−	−	−	+	+	+	+	+	−	+	+
TW05662	Sheep	815	170	42	a	b	−	−	+	−	+	−	−	+	−	+	−	−	−	+	+	+	+	+	−	+	+
TW05663	Sheep	815	170	42	a	b	−	−	+	−	+	−	−	+	−	+	−	−	−	+	+	+	+	+	−	+	+
CB13847	Lamb meat	815.1	177	197	a	b	−	−	+	+	+	−	−	+	−	+	−	−	+	−	+	+	+	+	−	+	−
14-Pork210	Pork skin	815.2	40	42	a	−	−	−	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	−	−	−	+
15-Pork342.1	Pork skin	815.2	172	42	a	−	−	−	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	−	−	−	+
16-Pork456	Pork skin	815.2	40	42	a	−	−	−	+	+	+	+	+	+	+	+	+	−	+	−	−	−	−	+	−	−	+
17-Pork501	Pork skin	815.2	40	42	a	−	−	−	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	−	−	−	+
18-Pork512.2	Pork skin	815.2	40	42	a	−	−	−	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	+	−	−	+
19-JMBO91	Pork skin	815.2	173	42	a	−	−	−	+	−	+	−	−	+	−	+	+	−	−	−	−	−	−	+	−	−	+
CB13595	Human (diarrhea)	815.2	174	196	a	−	−	−	+	+	+	−	−	+	−	+	+	−	+	−	−	−	−	−	−	−	+
CB13807	Bovine feces	815.2	176	194	−	−	−	+	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
CB14569	Minced beef/pork	815.2	179	198	a	−	+	−	+	−	+	−	+	+	−	+	+	−	−	−	+	+	+	+	−	−	+
CB15027	Raw sausage	815.2	182	200	a	−	−	−	+	+	+	−	−	+	−	+	+	−	+	−	−	−	−	−	−	−	+
CB15201	Human (asym)	815.2	180	196	a	−	+	−	+	−	+	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	+
13-PorkP1572	Pork carcass	815.3	40	42	a	−	−	−	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	+	−	−	+
CB13641	Beef	815.4	175	193	a	−	−	−	+	+	+	−	+	+	+	+	+	−	+	−	−	−	−	−	−	−	+
CB14789	Raw sausage	815.5	181	199	a	−	−	−	+	−	+	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	+

[a]Abbreviations: H, flagellar type; ST, sequence type; CT1, CRISPR1 type; CT2, CRISPR2a type; *stx*$_1$, *stx*$_1$ allele; *stx*$_2$, *stx*$_2$ allele; ND, not determined; +, positive; −, negative; asym, asymptomatic.
[b]Strains with the CB designation are from the European Union, strains with the TW designation are from the STEC Center, and all other strains are from various sources in the United States.
[c]Strain 8-Oct097B was typed as O91:H9, but since it possesses ST-815, it is included here with the O91:H14 strains.

TABLE 4 Genes tested on the microarray[a]

Gene (ORF)	Encoded protein or family effector	Genetic source (GenBank accession no.)
stx_1	Shiga toxin 1	Stx phage CP-933V (AE005174)
stx_2	Shiga toxin 2	Stx phage BP-933W (AE005174)
eae (Z5110)	Intimin	LEE (AE005174)
wzy_{O113}	O113 antigen polymerase	*rfb* operon (AF172324)
wzy_{O91}	O91 antigen polymerase	*rfb* operon (AY035396)
$fliC_{H21}$	Flagellin H21	Chromosome (DQ862122)
$fliC_{H10}$	Flagellin H10	Chromosome (AM231654)
$fliC_{H4}$	Flagellin H4	Chromosome (AJ536600)
toxB	Adhesin	EHEC plasmid (NC_007414)
ehxA	Enterohemolysin	EHEC plasmid (NC_007414)
katP	Catalase peroxidase	EHEC plasmid (NC_007414)
espP	Serine protease EspP	EHEC plasmid (NC_007414)
etpD	Type II effector	EHEC plasmid (NC_007414)
subA	Subtilase cytotoxin	aEHEC plasmid (NC_007365)
$lpfA_{O26}$	Major fimbrial subunit of LPFO26	EAEC chromosome (CU928185)
$lpfA_{O113}$	Long polar fimbrial protein	EAEC chromosome (CU928185)
Z2096	Unknown protein encoded within prophage CP-933O	OI-57 (AE005174)
Z2098	Unknown protein encoded within prophage CP-933O	OI-57 (AE005174)
Z2099	Unknown protein encoded within prophage CP-933O	OI-57 (AE005174)
Z2121	Unknown protein encoded within prophage CP-933O	OI-57 (AE005174)
espK	Non-LEE-encoded type III effector	OI-50 (AE005174)
espV	AvrA family effector	OI-44 (AE005174)
espN	Non-LEE-encoded type III effector	OI-50 (AE005174)
Z6065	ORF of unknown function	OI-71 (AE005174)
nleA (Z6024)	Non-LEE-encoded type III effector	OI-71 (AE005174)
espM1	Non-LEE-encoded type III effector	OI-71 (AE005174)
pagC (Z4321)	PagC-like membrane protein	OI-122 (AE005174)
Z4320	ORF of unknown function	OI-122 (AE005174)
ehaA (Z0402)	Autotransporter of EHEC	OI-15 (AE005174)
iha (Z1148)	Iron-regulated gene A homologue adhesin	OI-43 and OI-48 (AE005174)
ureD (Z1142)	Urease-associated protein UreD	OI-43 and OI-48 (AE005174)
terE (Z1176)	Tellurite resistance cluster	OI-43 and OI-48 (AE005174)
Z1151	ORF of unknown function	OI-43 (AE005174)
Z1153	ORF of unknown function	OI-43 (AE005174)
Z1154	Colicin immunity protein	OI-43 (AE005174)
Z1155	Putative membrane protein	OI-43 (AE005174)
Z1156	ORF of unknown function	OI-43 (AE005174)
astA	EAEC heat-stable enterotoxin 1 (EAST1)	EAEC plasmid (HE603111)
ecf1	Enzyme that enhances bacterial membrane structure	EHEC plasmid (NC_007414)
ecf2	Enzyme that enhances bacterial membrane structure	EHEC plasmid (NC_007414)
irp2	Iron-repressible protein 2	High pathogenicity island (CU928185)
saa	STEC autoagglutinating adhesin	aEHEC plasmid (NC_007365)
epeA	Serine protease autotransporter	aEHEC plasmid (NC_007365)
sab	Autotransporter	aEHEC plasmid (NC_007365)
bfpA	Major structural subunit of bundle-forming pilus	pMAR2 plasmid (NC_011603)
cdt-V	Cytolethal distending toxin	Chromosome (AJ508930)
eibG	Immunoglobulin binding protein	aEHEC plasmid (NC_007365)
wecA	Polyisoprenyl-phosphate *N*-acetylhexosamine-1-phosphate transferase	Chromosome (AE005174)

[a]Abbreviations: OI, O island; ORF, open reading frame; LEE, locus for enterocyte effacement; EHEC, enterohemorrhagic *E. coli*; EAEC, enteroaggregative *E. coli*; aEHEC, atypical EHEC (LEE negative).

O91:H10. The five strains identified to be O91:H10 included strain CB12624, which was isolated from the feces of a HUS patient; strain CB14687, a reference strain from the verocytotoxin-producing *Escherichia coli* (VTEC) European Union Reference Laboratory (EU-RL); and three strains isolated from food or animals (Table 2). None of the five strains had stx_1; three, including the HUS patient isolate (CB12624), had stx_{2d} alone; and one had stx_{2a} alone. The EU-RL strain did not have any *stx* gene (Table 2). All five strains had *iha*, and four had *ehaA*, but none of the strains had *katP*. Other EHEC plasmid genes, like *ehxA* and *espP*, and other plasmid genes that are common in LEE-negative STEC strains, like *sab*, *saa*, and *subA*, were found only in strain TW01445 (Table 2). The ground beef (7-MDR0009B), cheese (CB11906), and HUS patient (CB12624) isolates had identical profiles, and all three also had stx_{2d}. The profiles of TW01445 and CB14687,

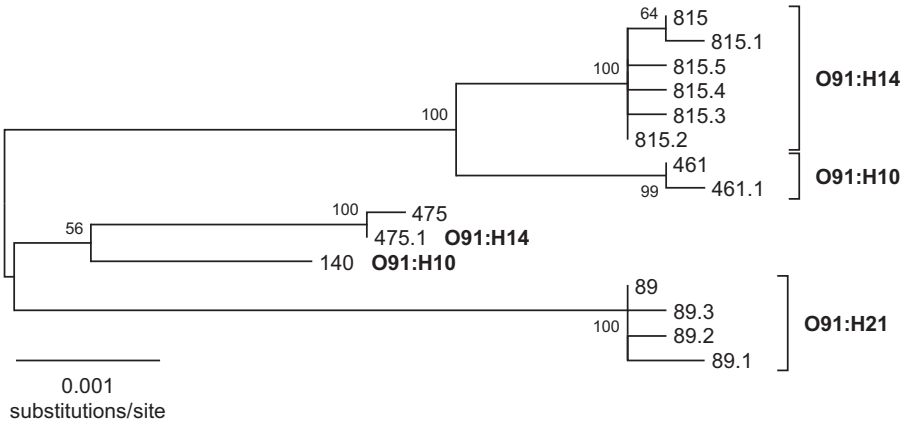

FIG 1 Phylogenetic relationships of O91 sequence types. The neighbor-joining tree was constructed using the Kimura two-parameter distance and 500 bootstrap replications. Sequence type (ST) designations are given at the branch tips.

however, were markedly different from each other and from those of the other three strains.

MLST analysis showed that, except for CB14687, which had ST-140 and belonged to the NT-5 clonal group, the other 4 strains were in the STEC-14 clonal group and had either ST-461 or a variant of ST-461 (ST-461.1) that differed from ST-461 by a single synonymous C → T transition in *lysP*. The ST-140 and ST-461 strains did not cluster together in the phylogenetic tree (Fig. 1).

The EU-RL strain did not have CT1 or CT2 alleles and so could not be typed by CRISPR analysis. Each of the other 4 strains had a different CT1 allele with between 10 and 13 spacers that have been described previously (24), but the spacer combinations observed in these four alleles have not been seen previously. With respect to CT2, three of the four strains had CT2-38, and the last strain had CT2-192, an allele that had not been seen before, but it is a variant of CT2-38 with one less spacer than CT2-38. Consistent with the findings for the O91:H21 strains, the spacers were significantly ($P < 0.05$) shorter in the CT2 alleles (mean ± SD, 3.75 ± 0.5) than in the CT1 alleles (mean ± SD, 11.75 ± 1.5).

O91:H14 and O91:H9. There were 21 O91 strains that could not be H typed by the array but were identified by *fliC* sequencing. Of these, 20 were O91:H14 strains that were isolated mostly from foods but also included CB13595 and CB15201, which were isolated from a patient with diarrhea and an asymptomatic patient, respectively. One additional strain (8-Oct097B) was typed to be O91:H9, and despite having a different H type, it had the same ST as some of the O91:H14 strains and so was grouped accordingly (Table 3). The stx_1 gene was very common in the O91:H14 strains and present in 90% (18/20) of the strains, all of which were the stx_{1a} subtype. In contrast, stx_2 was found in only 30% (6/20) of the strains, with 4 carrying stx_{2b} and 1 each carrying stx_{2a} and stx_{2c}. CB13807 did not have *stx*, and the O91:H9 strain had stx_{1a} and both stx_{2a} and stx_{2d} (Table 3).

All 20 O91:H14 strains had *ehaA* and $lpfA_{O26}$, and the *iha* gene was also common and found in 95% (19/20) of the strains. Genes like Z2096 and *irp2*, however, were not present in any strains, and several genes, like *astA*, *saa*, and *epeA*, were also rare and found in only 5% (1/20) of the strains. Plasmid genes like *ehxA* and *sab* were found in 50% (10/20) of the strains, but *subA* was present in only 4 strains. The genetic profile of the O91:H9 strain (8-Oct097B) was very distinct, in that it had *subA*, *saa*, *irp2*, Z2096 and the *cdt-V* cytolethal distending toxin gene (25). All of these genes were either rare or absent in the O91:H14 strains (Table 3).

Clonal analysis showed that except for CB11895, which was a variant of ST-475 (designated ST-475.1), all the other strains were ST-815 or variants of ST-815, including the O91:H9 strain. ST-475.1 differed from ST-475 by a single synonymous T → A

transversion in *fadD*. ST-475.1 and ST-815 shared the same *clpX* allele but differed from each other by a total of 28 single nucleotide polymorphisms over the other six MLST loci, indicating that these STs likely belong to different clonal groups. Among the remaining members of this group, there was considerable variation within the clonal type, as 5 strains were ST-815 and the other 15 strains possessed one of five different ST-815 variants (Table 3). Variants ST-815.1 (1 strain) and ST-815.2 (11 strains) each differed from ST-815 by a single nonsynonymous mutation: a G → C transversion in *clpX* for ST-815.1 and an A → G transition in *lysP* for ST-815.2. All three remaining ST-815 variants possessed the *lysP* mutation found in ST-815.2, with each variant containing one additional mutation. The ST-815.3 strain (1 strain) had a synonymous G → A transition in *fadD*, the ST-815.4 strain (1 strain) had a synonymous A → C transversion in *aspC*, and the ST-815.5 strain (1 strain) had a nonsynonymous C → A transversion in *clpX*. Thus far, these strains do not appear to be common and have not been assigned to any recognized STEC clonal groups. The O91:H14 strains with ST-815 or ST-815 variants, including the O91:H9 strain, all clustered together, but the ST-475.1 strain clustered apart and was more closely related to the O91:H10 strain with ST-140 (Fig. 1).

Analysis of CRISPR1 showed 15 different CT1 alleles, of which 13 were seen only once. The two CT1 alleles seen more than once included CT1-170 ($n = 3$) and CT1-40 ($n = 5$). Except for CT1-40, all other CT1 alleles were newly identified in this study. Each CT1 allele contained between 5 and 23 spacers (mean ± SD, 10.80 ± 3.88). Except for CT1-183 (CB11895), all the other CT1 alleles, including CT1-171, observed only in the O91:H9 strain, were made up from a common pool of 20 spacers, all of which were previously identified (24). Allele CT1-183 had a peculiar spacer composition with 18 spacers that were not found in the other O91:H14 strains, and 7 of these have never been identified before.

Analysis of CRISPR2a alleles showed less variation, detecting only 10 alleles, 8 of which were seen only once. Of the two other CT2 alleles, CT2-196 was seen twice, both times of which were in human isolates, and CT2-42 was found in 11 strains, mostly from porcine and ovine sources. The O91:H9 strain was the only strain that had CT2-45. Except for CT2-42 and CT2-45, all the other CT2 alleles were newly identified in this study. The CT2 alleles contained 2 to 12 spacers (mean ± SD, 3.76 ± 2.66), and of the 20 different spacers that constituted the CT2 alleles, only 3 were newly identified in this study. As was observed for the O91:H21 and O91:H10 strains, the number of spacers in the CT2 alleles was significantly less ($P < 0.001$) than the number in the CT1 alleles, and the spacers were shorter than those in the CT1 alleles.

DISCUSSION

The 49 STEC O91 strains that we examined mostly comprised O91:H21 and O91:H14 strains, with 5 strains being of the O91:H10 serotype and 1 strain being of the O91:H9 serotype. These strains were isolated from a wide variety of sources, and no association of any particular serotype with a source was observed. At the time of isolation, a few strains were H serotyped by *fliC* sequencing, and the others were tested by PCR for the presence of stx_1, stx_2, and *ehxA* genes. The data that we obtained were consistent with the initial serotype found for those strains, and also, the array and the PCR data were in agreement for the presence of the three genes.

Of the Stx subtypes, Stx1a and, especially, Stx2a, Stx2c, and Stx2d have most often been implicated in HC and HUS (2, 11, 26). The stx_{1a} subtype was not common in O91:H21 strains and was found in only 13% of the strains. However, 95% of the O91:H21strains, including the HUS-associated strains B2F1 and TW01674, had stx_2, and all were stx_{2a}, stx_{2c}, stx_{2d}, or combinations of these subtypes. These results are consistent with data obtained from the analysis of clinical O91 strains (14, 17, 20, 27).

In contrast, 90% of the O91:H14 strains that we tested had stx_{1a}, and for 65% of those strains, it was the only Stx type found. Of the six strains that also had stx_2, most were of the stx_{2b} subtype, which is not often implicated in human illness (26). Analysis of 77 clinical O91:H14 strains showed similar results, in that 89% had stx_1 alone and only 6 strains had both stx_1 and stx_2 (27). None of the five O91:H10 strains that we tested had

stx$_1$, but three had *stx*$_{2d}$ and one strain did not have *stx*. Analysis of O91:H10 strains isolated from food and clinical samples showed that O91:H10 strains can have *stx*$_1$, *stx*$_2$, or both (17, 18, 27), but too few isolates were examined to infer any *stx* subtype prevalence patterns for O91:H10 strains. The lone O91:H9 strain in our study had *stx*$_{1a}$, *stx*$_{2a}$, and *stx*$_{2d}$, and it was isolated from ground beef. STEC O91:H9 strains have not been reported previously.

With respect to the genes tested on the array, the overall genetic profiles of the O91 strains within each serotype were similar but varied greatly from serotype to serotype. One common trait among all the O91 strains was the lack of *eae*. Although a strain of the O91:H40 serotype with *stx*$_2$ and *eae* has been reported (12), *eae* is not common in the O91 strains (9). The *terE* gene, which codes for tellurite resistance, is prevalent in O157:H7 and in some other STEC strains (28) but seems to be rare in LEE-negative STEC strains (29, 30). Bielaszewska et al. (27) examined 100 O91 strains of various serotypes and found that only one O91:H21 strain had *terE*. None of the O91 strains that we tested had *terE*, and therefore, our results are in agreement with the finding that most O91 strains are sensitive to tellurite. The *nleA* gene, which is located on OI-71 and which codes for a non-LEE-encoded type III effector, is common in LEE-positive STEC strains, but it was absent in the O91 strains.

The prevalence of other genes varied depending on the O91 serotype. The chromosomal *cdt* gene, which codes for a cytolethal distending toxin, is common in *E. coli*, which is known to produce at least five Cdt variants (25), of which Cdt-V is common in STEC strains (25, 30). Analysis of clinical O91 strains showed that only O91:H21 strains had *cdt-V*, and it was present in 70% of the strains (27). In our study, 65% of the O91:H21 strains had *cdt-V*, but it was absent in the other serotypes except for one O91:H14 strain, so these findings are consistent with the results from the other studies. Similarly, the Z2099 marker on OI-57 is present in only 1% of generic *E. coli* strains but is prevalent in O157:H7 and in other STEC serogroups that have caused infections and so was thought to be a useful marker for detecting pathogenic STEC strains (31). An analysis of 190 STEC strains confirmed that Z2099 was prevalent in many STEC strains, including three O91 strains (31); however, we found that only one O91:H21 strain and five O91:H14 strains had Z2099, so its prevalence appears to vary among strains of the O91 serogroup.

Many LEE-positive and LEE-negative STEC strains carry the EHEC plasmid, which harbors several STEC-associated genes. The *ehxA* gene for enterohemolysin, a putative virulence factor whose role in pathogenicity remains undetermined, was found to be common in 89% of the STEC strains that often cause infections (32) and in about 60% of the STEC strains isolated from fresh produce (22). The *ehxA* gene was also common in the O91 strains, found in 82% of the O91:H21 strains and 60% of the O91:H14 strains. However, other common EHEC plasmid genes were absent or not consistently present. For example, the *espP* serine protease gene was present in 82% of the O91:H21 strains and in 45% of the O91:H14 strains, but the *katP* catalase peroxidase gene was present in only 40% of the O91:H14 strains and absent in all O91:H21 strains. Another EHEC plasmid gene, *toxB*, which codes for an adhesin, is common in O157:H7 strains and many LEE-positive STEC strains of the O26, O121, and O145 serogroups as well as in enteropathogenic *E. coli* (33). This adhesin is thought to contribute to the adherence properties of O157:H7; however, *toxB* was absent in the O91 strains, suggesting that if these O91 strains had the capacity to adhere, it does not involve the *toxB* adhesin.

Strains of STEC that are LEE negative often have other plasmids that carry many putative virulence factor genes, such as *saa*, *sab*, and *subAB*. All three of these genes are prevalent in O113:H21 strains (29, 30), which are LEE-negative STEC strains that have caused HUS. However, either some of these genes were absent in the O91 strains or their prevalence varied depending on the serotype. For example, *sab* was common and found in 80% and 60% of the O91:H21 and O91:H14 strains, respectively. The *saa* gene was also common in the O91:H21 strains (78%) but was found in only 10% of the O91:H14 strains, and *subAB* was present in 20% of the O91:H14 strains but was absent

in the O91:H21 strains. There are allelic variants of *subAB* among STEC strains (34), and it is uncertain whether the array that we used was able to detect all the *subAB* variants.

No study has examined O91 strains for the prevalence of all these genes that we used on the microarray. However, some studies tested for some of these genes in a few O91 strains. The results that we obtained were mostly consistent with data obtained for two O91:H21 strains isolated from minced meat and raw milk in France (19), four O91:H21 strains isolated from human and bovine sources in Argentina (20), an O91:H21 strain isolated from cattle feces-polluted wastewater in Spain (25), and clinical O91 strains isolated in the European Union (27). From these genotypic studies, we can surmise that the STEC O91 serogroup strains do not have many of the genes that are common in other STEC strains and even differed in the prevalence of genes that are common in other LEE-negative STEC strains, such as serotype O113:H21 strains.

Mellmann et al. (9) examined the clonal relatedness of clinical O91 strains isolated from patients from the European Union with illnesses that ranged from asymptomatic conditions to HUS. Using the Achtman MLST scheme, all 20 O91:H21 strains were found to be ST-442 and most of the 77 O91:H14 strains were ST-33, but there were 5 other O91:H14 strains that each showed a different ST (9). We used the Whittam MLST scheme, but the results were the same as those obtained with the Achtman MLST scheme, in that all the O91:H21 strains were conserved and had ST-89 or variants of ST-89, while the O91:H14 strains were diverse, with the strains being of ST-815 or five different ST-815 variants, and one strain had ST-475.1. To determine if ST-442 obtained by the Achtman MLST scheme and ST-89 obtained by the Whittam MLST scheme described the same clonal group, we examined the draft genome sequence of the B2F1 strain (O91:H21) in the NCBI whole-genome shotgun database (GenBank accession numbers AFDQ01 and AGTI01). Sequence analysis of the MLST loci showed that B2F1 strain was ST-442 by the Achtman scheme and, therefore, is the same as ST-89 of the Whittam scheme. Similarly, draft genome analysis also revealed that ST-33 of the O91:H14 strain of the Achtman MLST scheme was the same as ST-815 of the Whittam MLST scheme that we found in this study. Regardless of the MLST scheme used, the results of these clonal studies are consistent, in that the O91:H21 strains are highly conserved, while the O91:H14 strains are more clonally diverse. This is supported by the findings of CRISPR analysis, where O91:H21 strains showed only three CT2a alleles, of which the most were CT2-57, while 10 different alleles were observed for O91:H14 strains. The O91:H10 strains may also be diverse, but since we examined only five strains, the sample size is too limited to derive any conclusions.

Analysis of 100 clinical O91 strains in correlation to illness symptoms showed that only O91:H21 strains were implicated in severe diseases, with 4/20 strains being isolated from HUS patients, 3/20 being isolated from HC cases, 12/20 being isolated from diarrhea patients, and 1 strain being isolated from a patient that had abdominal cramps (9). The O91:H21 strains that we examined had genetic profiles that were the same or nearly the same as those of the strains that have caused HUS and had the same *stx* subtypes and belonged in the same clonal group as those strains, suggesting that these O91:H21 strains, isolated mostly from foods and the environment, may also have the potential to cause severe disease.

In contrast, of the 63 O91:H14 clinical strains examined (9), only 5 were from HC patients, 51 were from diarrhea cases, 7 were from asymptomatic patients, and none were from HUS patients. Stx2 is more closely implicated in severe illness than Stx1, and since most O91:H14 strains carry only stx_1, it is consistent that this serotype is less often associated with severe diseases. Most of the O91:H14 strains that we examined also had stx_1 alone, and although six strains also had stx_2, they were mostly of the stx_{2b} subtype, which is not often implicated in human disease (26); thus, the findings suggest that these environmental O91:H14 strains do not have the capacity to cause severe illness.

The O91:H10 isolates from ground beef (7-MDR0009B) and cheese (CB11906) had genetic and *stx* profiles identical to those of strain CB12624, isolated from the feces of a HUS patient. The cheese isolate also had an ST similar to that of the HUS-associated strain and the same CT2a as the HUS-associated strain. Some reports have implicated

O91:H10 strains in severe disease. Bonnet et al. (8) isolated an Stx2-positive O91:H10 strain from the stools of an 84-year-old patient with a sporadic case of HUS in France. Pradel et al. (17) characterized two O91:H10 strains isolated from adult HUS patients in France and found the isolates to have stx_{2a} or a unique stx_2 variant. A study of STEC infections in Switzerland from 2000 to 2009 found an O91:H10 strain with stx_2 that was isolated from a 60-year-old HUS patient (15). Although these reports suggest that O91:H10 strains may cause HUS, another study showed that two O91:H10 strains, one with stx_{1a} and the other with stx_{2a} and stx_{2c} (27), were both isolated from patients with only diarrhea and were not associated with severe diseases (9). Variations in human susceptibility could have contributed to the differences in the symptoms reported in these studies. Many STEC virulence genes reside on mobile genetic elements that can be transferred, so strains with the same serotype can have different gene profiles and pathotypes. In this study, for example, CB14687 was an O91:H10 strain but had no *stx* genes and carried genes not found in the other O91:H10 strains. Similarly, CB13632 was an O91:H21 strain but had no *stx* genes or most of the genes found in the other O91:H21 strains. Hence, the use of serotype data to associate a particular STEC serotype with diseases is often inadequate without knowing the specific virulence genes that it carries. At present, the number of O91:H10 strains examined here and in those other studies is very limited, so the potential of O91:H10 strains to cause severe diseases remains uncertain.

In conclusion, we used various molecular tools to characterize 49 O91 strains isolated from the environment, food, and clinical samples in the European Union and in the United States. The O91:H14 strains were phylogenetically diverse, and most carried only stx_{1a}, with only a few strains carrying stx_2 subtypes that are not known to affect humans; hence, these O91:H14 strains do not appear to have the capacity to cause severe diseases. On the other hand, the O91:H21 strains were much more conserved and had profiles identical to or nearly identical to those of the clinical strains that have caused HUS. Furthermore, these strains carried only those *stx* subtypes that are often associated with severe illness and belonged to the same clonal group as the HUS-associated strains, suggesting that these O91:H21 strains from the environment and foods may also have the potential to cause severe disease.

MATERIALS AND METHODS

Bacterial strains. The 49 isolates of the O91 serogroup tested in this study included strains from ground beef, beef trim, pork skin, bovine feces, and spinach in the United States and from various types of meats, salad, cheese, and raw milk in the European Union. The panel also included strains from reference centers in the European Union and from the STEC Center at Michigan State University. Some of the reference strains were isolated from animals, while others were clinical strains from patients, such as strain B2F1 (TW01393), the prototypical O91:H21 strain that has caused HUS and has the mucus-activatable stx_{2d} subtype. These strains are described in Tables 1 to 3.

***stx* subtyping.** To determine the specific *stx* subtypes, strains that had stx_1, stx_2, or both were tested using the PCR protocol described by Scheutz et al. (26). Subtypes stx_{2a}, stx_{2c}, and stx_{2d} share sequence similarities, and primer cross-reactivity has been reported to occur. Hence, strains that carried these subtypes were retested using a 66°C instead of a 62°C annealing temperature, as prescribed by Scheutz el al. (26).

PCR microarray. Strains were tested with a PCR microarray for the presence of 48 STEC virulence genes or genes characteristic of STEC strains. These genes and the proteins that they encode are described in Table 4. The microarray assay protocol and the procedures used here were the same as those used previously in the analysis of STEC O113 strains (30). The microarray tested only for $fliC_{H4}$, $fliC_{H10}$, and $fliC_{H21}$, so strains that did not react with these were genetically serotyped by PCR amplification of the *fliC* flagellin gene (35), followed by sequencing and comparative BLAST analysis with known flagellar (H) type sequences in GenBank.

MLST. The Whittam multilocus sequence typing (MLST) protocol (36) was used in the clonal analysis of the O91 strains. The assay used primers to amplify and sequence internal segments of 7 housekeeping genes (aspartate aminotransferase [*aspC*], caseinolytic protease [*clpX*], acyl coenzyme A synthetase [*fadD*], isocitrate dehydrogenase [*icdA*], lysine permease [*lysP*], malate dehydrogenase [*mdh*], β-D-glucuronidase [*uidA*]). Each unique sequence was given an allele number, and the combinations of alleles from the 7 genes were used to generate an allelic profile or sequence type (ST), which was then compared to those of other *E. coli* strains in the *Ec*MLST database (36). The data were used to construct a phylogenetic neighbor joining tree using the Kimura two-parameter distance and 500 bootstrap replications.

CRISPR. Sequence polymorphisms among the O91 strains were examined using the nomenclature of CRISPR1 and CRISPR2a (24). Respective regions of the clustered regularly interspaced short palindromic repeat (CRISPR) loci were PCR amplified using previously described conditions (37). The amplicons were double-strand sequenced (Eurofins MWG Operon, Courtaboeuf, France), and the CRISPR sequences of the strains were assembled using BioEdit (version 7.1.3.0) software. The CRISPR sequences were analyzed using the CRISPy Python script (38), and alleles not previously described by Yin et al. (24) were assigned a new CRISPR type (CT) numerical designation.

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The use of trade names is necessary to accurately report experimental results. The use of trade names by a USDA author implies no approval of the product to the exclusion of others that may also be suitable; further, the USDA neither guarantees nor warrants the standard of any products mentioned.

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