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Identification of *Escherichia coli* O157:H7 Genomic Regions Conserved in Strains with a Genotype Associated with Human Infection^{∇†}

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Beta-glucuronidase-negative, sorbitol-nonfermenting isolates of Shiga toxin-producing *Escherichia coli* O157 comprise part of a clone complex of related enterohemorrhagic *E. coli* isolates. High-resolution genotyping shows that the O157 populations have diverged into two different lineages that appear to have different ecologies. To identify genomic regions unique to the most common human-associated genotype, suppression subtractive hybridization was used to identify DNA sequences present in two clinical strains representing the human lineage I O157:H7 strains but absent from two bovine-derived lineage II strains. PCR assays were then used to test for the presence of these regions in 10 lineage I strains and 20 lineage II strains. Twelve conserved regions of genomic difference for lineage I (CRD_I) were identified that were each present in at least seven of the lineage I strains but absent in most of the lineage II strains tested. The boundaries of the lineage I conserved regions were further delimited by PCR. Eleven of these CRD_I were associated with *E. coli* Sakai S-loops 14, 16, 69, 72, 78, 82, 83, 91 to 93, 153, and 286, and the final CRD_I was located on the pO157 virulence plasmid. Several potential virulence factors were identified within these regions, including a putative hemolysin-activating protein, an iron transport system, and several possible regulatory genes. Cluster analysis based on lineage I conserved regions showed that the presence/absence of these regions was congruent with the inferred phylogeny of the strains.

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 is a major cause of both large-scale epidemics and sporadic cases of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome in many countries around the world (13, 34, 36, 54, 56). The annual incidence of reported *E. coli* O157:H7 infections in Canada and the United States ranges from 1.7 to 5.3 per 100,000 persons and may be much higher in certain regions (14, 56). Within the United States alone, it has been estimated that approximately 73,000 cases of *E. coli* O157:H7 infection occur annually (33). The most common *E. coli* O157:H7 isolates are motile, non-sorbitol fermenting (SOR⁻), and β-glucuronidase negative (GUD⁻), while a nonmotile SOR⁺ GUD⁺ O157:H⁻ clone has also been isolated in Germany (20) and a nonmotile SOR⁻ GUD⁻ O157:H⁻ clone is commonly isolated in Australia (46).

Population genetic analysis has shown that *E. coli* O157:H7 and O157:H⁻ isolates belong to a geographically disseminated clone complex that acquired virulence genes independently from other EHEC isolates (31, 45, 61, 62). Despite the clonal nature of *E. coli* O157:H7 and O157:H⁻ isolates, significant variability was observed when they were tested by high-resolution genomic typing methods, such as pulsed field gel electrophoresis and octamer-based genome scanning (OBGS) (12,

22, 23, 53), implying that subpopulations are diverging quite rapidly.

OBGS is a large-scale genome comparison method based on pattern analysis of PCR amplification products generated using overrepresented octamers as primers. Recent studies using OBGS suggest that extant populations of O157:H7 isolates have diverged through two primary lineages, lineage I and lineage II, and that these lineages can be detected in geographically unlinked regions, such as the United States and Australia (22, 23). The origin of these two lineages, therefore, appears to predate the geographical spread of *E. coli* O157:H7 and the regional evolution of the SOR⁻ GUD⁻ O157:H⁻ clone commonly isolated in Australia (23). More recently, the lineage-specific polymorphism assay (LSPA-6) was developed, based on six loci that show bias in their allelic distribution between the two lineages. The LSPA-6 is therefore a more efficient alternative for inferring lineage assignments compared to laborious OBGS typing (63). The two methods were demonstrated to generate highly concordant data (63). All lineage I isolates were LSPA-6 genotype 111111 (lineage I allele at each locus), while the majority of lineage II isolates were LSPA-6 genotypes 222222, 211111, and 212111.

In the initial OBGS studies and in the LSPA-6 study, a low proportion of human strains were observed in OBGS lineage II and LSPA-6 genotype 222222, respectively (22, 63). The paucity of OBGS lineage II and LSPA-6 genotype 222222 human isolates led workers to postulate that these *E. coli* O157:H7 isolates may be deficient in their abilities either to be transmitted to humans or to cause clinically significant human infections (22, 63). Several other studies also suggest that there

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TABLE 1. *Escherichia coli* O157:H7 strains included in the study

Strain	LSPA-6 genotype	Source	Country of origin	Phage type	Reference(s)
93-001	111111	Human	United States	14	22, 23
EC19950361	111111	Bovine	Canada	87	
EC20000122	111111	Bovine	Canada	31	
ECI-577	111111	Bovine	Canada	4	
EDL933	111111	Human	United States	21	43
FDA 516	111111	Human	United States	21	22, 23
FDA 518	111111	Human	United States	21	22, 23
FDA 520	111111	Human	United States	1	22, 23
FRIK 523	111111	Human	United States	34	22, 23
Sakai	111111	Human	Japan	32	16
EC19920027	222222	Bovine	Canada	34	
EC19920283	222222	Human	Canada	23	
EC19970520	222222	Bovine	Canada	67	
ECI-1433	222222	Bovine	Canada	23	
ECI-633	222222	Bovine	Canada	23	
ER6554	222222	Human	Canada	23	
FRIK 1990	222222	Bovine	United States	54	22, 23
FRIK 1999	222222	Bovine	United States	23	22, 23
FRIK 920	222222	Bovine	United States	23	22, 23
EC20011139	222222	Bovine	Canada	82	
ECI-882	211111	Human	Canada	1	
278F1	211111	Human	Canada	2	21
Zap0032	211111	Bovine	Scotland	8	32, 47, 48
Zap0054	211111	Bovine	Scotland	32	32, 47, 48
Zap0058	211111	Human	Scotland	87	32, 47, 48
ECI-241	222212	Human	Canada	74	
ER4511	222212	Human	Canada	74	
ECI-240	222213	Human	Canada	54	
FRIK 2001	222213	Bovine	United States	54	22, 23
FRIK 1985	222223	Bovine	United States	45	22, 23

are clear differences in the expression of virulence attributes, such as Shiga toxin and the locus for enterocyte effacement (LEE) proteins, by *E. coli* O157:H7 isolates from humans and from cattle (27, 32, 47, 48). These latter studies, however, did not consider the population structure of *E. coli* O157:H7 (e.g., lineage of descent) as a variable.

In this study, suppression subtractive hybridization (SSH) was used to identify genomic regions present in *E. coli* O157:H7 lineage I (LSPA-6 111111) strains but absent from lineage II (LSPA-6 222222) strains. We show that lineage I strains do indeed share a set of unique genes that are largely absent in lineage II strains. Several of these genes encode proteins that could contribute to virulence characteristics or which are known to regulate expression of virulence genes.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains included in this study are listed in Table 1. OBGS type strains 93-001, FDA 516-520, and FRIK 523-2001 have previously been described by Kim et al. (22). ZAP strains were obtained from David Gally at the University of Edinburgh (32). *Escherichia coli* O157:H7 strains EDL933 (ATCC 700927) and Sakai (ATCC BAA-460) were obtained from American Type Culture Collection (Manassas, VA). The remaining strains were from our culture collection and were isolated from humans or cattle in Canada. LSPA-6 genotyping of these strains was performed as described previously (63).

Preparation of suppression subtractive hybridization DNA libraries. Bacteria were grown overnight in brain heart infusion broth (Difco, Becton Dickinson Microbiology Systems, Sparks, MD) in a 37°C shaker-incubator (200 rpm), and genomic DNA was extracted from harvested cells using the DNeasy tissue kit (QIAGEN, Valencia, CA).

SSH was performed using the Clontech PCR-Select bacterial genome subtraction kit (BD Biosciences, Palo Alto, CA). Advantage polymerase mix (BD Biosciences) was used during amplification steps. Two SSH experiments were per-

formed, the lineage I strain (LSPA-6 genotype 111111) *E. coli* O157:H7 Sakai was subtracted with the bovine-derived lineage II strain FRIK920 (LSPA-6 genotype 222222), and a second human-derived lineage I strain 93-001 (LSPA-6 genotype 111111) was subtracted with bovine-derived lineage II strain EC19970520 (LSPA-6 genotype 222222). In addition to the *RsaI*-digested DNA recommended in the Clontech kit, each SSH experiment was also performed on *AluI*- and *HaeIII*-digested DNA to increase the diversity of DNA fragments obtained. The SSH DNA fragments isolated in these experiments were cloned into the pCR2.1-TOPO plasmid vector, using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and plated on LB agar (Difco) containing 50 µg/ml of ampicillin or kanamycin (Sigma-Aldrich Canada, Oakville, ON, Canada).

Sequence analysis of suppression subtractive hybridization DNA libraries. Cloned DNA inserts were amplified by PCR using the primers M13 Forward, 5'-GTAACGACGCGCCAG-3', and M13 Reverse, 5'-CAGGAAACAGCTA TGAC-3' (Invitrogen). The amplified DNA was purified by passage through superfine Sephadex G-50 (Sigma) packed into a multiscreen 96-well filtration plate (Millipore, Billerica, MA). Sequencing was performed using M13 Forward and M13 Reverse primers with the DYEnamic ET terminator cycle sequencing kit (GE Healthcare, Piscataway, NJ), and sequencing products were purified by passage through superfine Sephadex G-50 plates as before. Sequence analysis was performed on a MegaBACE 500 capillary sequencer (GE Healthcare).

Base calling of sequence trace data was performed using the PHRED algorithm as implemented in the Interphase program (CodonCode Corporation, Dedham, MA). Base-called sequences from both SSH DNA libraries were pooled and analyzed with the SeqMan 5.05 sequence analysis program (DNASTar Inc., Madison, WI). This program removed vector and adaptor sequences and grouped sequences displaying at least 90% sequence homology into "contigs." BlastN (1) searches were then performed on contig consensus sequences using the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) and The Institute for Genomic Research (<http://www.tigr.org/>) BLASTN servers. Sequences that displayed at least 90% sequence identity to published chromosomal and plasmid DNA sequences of the lineage I *E. coli* O157:H7 Sakai strain (16, 29) were included in the final analysis. Genomic regions within the *E. coli* Sakai chromosome and plasmids that contained localized clusters of ORFs represented in the SSH libraries at high frequency were identified. The representation of these regions was significantly higher than the background

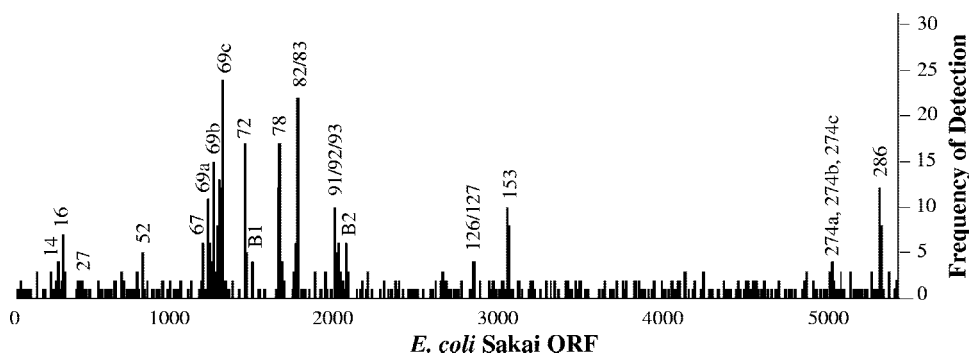


FIG. 1. Frequency of detection of sequences homologous to *Escherichia coli* Sakai (GenBank accession no. BA000007) ORFs within SSH libraries.

frequency across the chromosomal and plasmid sequences, although the frequency at which individual ORFs within these regions were identified varied from 1 to 24 times.

PCR screening. PCR assays were used to determine the distribution of these highly represented genome regions within *E. coli* O157:H7 strains of different LSPA-6 genotypes. Primers for these assays were designed to detect unique DNA sequences from ORFs within each region (see Table S1 in the supplemental material). All primer sequences were queried by BLASTN to ensure that they would not amplify multiple sequences within the *E. coli* Sakai genome. PCR screening reactions were performed against genomic DNA isolated from 10 LSPA-6 genotype 111111 *E. coli* O157:H7 strains, 10 LSPA-6 genotype 222222 strains, and 10 strains of other LSPA-6 genotypes. This strain collection is representative of the diversity within *E. coli* O157:H7 populations but is not representative of the proportions of LSPA-6 genotypes that naturally occur in humans and cattle. Larger numbers of LSPA-6 111111 and 222222 strains were included, as these genotypes were previously reported to display a biased distribution between human and bovine strains (63), and the proportions of human and bovine strains within each major genotype are different than previously described (63). Genome regions that were observed in these initial PCR screening reactions to be conserved in most or all LSPA-6 genotype 111111 strains but rare or absent in LSPA-6 genotype 222222 strains were tested by additional PCR assays against this same strain collection to identify the boundaries of the lineage I-specific regions.

All PCR assays were performed in duplicate in a 20- μ l reaction volume containing 1 \times buffer II (Applied Biosystems, Foster City, CA), 1.5 mM MgCl₂, 0.2 mM each deoxynucleoside triphosphates, 1 U AmpliTaq DNA polymerase (Applied Biosystems), 0.2 μ M primers, and 0.5 ng genomic DNA template. All PCRs included an initial 2-min denaturation step at 94°C, followed by 30 cycles of 1-min denaturation at 94°C, 1-min annealing, and 1-min extension at 72°C, and a final 10-min extension at 72°C. Each PCR was tested against genomic DNA of *E. coli* O157:H7 Sakai strain as a positive control, *E. coli* K12 MC1061 as a negative control, and a no-template blank.

Cluster analysis of the distribution of lineage I-specific regions in *E. coli* O157:H7 strains by UPGMA. Results of the preliminary PCR screening assays were converted into binary data to indicate the presence or absence of a single PCR marker for each of the different lineage I conserved regions within the strains tested. A distance matrix of the binary data was created in PAUP version 4.0b10, and the distance matrix was imported into MEGA 3.1 (26) to generate an unweighted-pair group method using average linkages (UPGMA) dendrogram.

RESULTS AND DISCUSSION

Analysis of SSH-derived sequences. Our experimental approach was to first identify candidate genomic regions that are present in lineage I strains but absent in lineage II strains. Candidate regions were identified using SSH from representative strains, and the candidate regions were then distinguished as strain specific versus lineage specific based on their conservation across a larger, representative strain set. To this end, two SSH experiments were conducted. In one library, the lineage I strain (LSPA-6 genotype 111111) *E. coli* O157:H7

Sakai was subtracted with the bovine-derived lineage II strain FR1K920 (LSPA-6 genotype 222222), and the second library used a human-derived lineage I strain 93-001 (LSPA-6 genotype 111111) subtracted with bovine-derived lineage II strain EC19970520 (LSPA-6 genotype 222222). A total of 1,155 clones prepared from SSH DNA libraries of RsaI-, AluI-, and HaeIII-digested DNA were sequenced. Sequences were assembled into 754 contigs, with each sequence possessing 90% or greater sequence identity to other contributing sequences in the same contig. BlastN search results of the contig consensus sequences revealed that 85.1% of the contigs possessed sequences with greater than 90% sequence identity to one or more of the ORFs present in the lineage I representative strain *E. coli* O157:H7 Sakai chromosome or pO157 and pOSAK1 plasmid sequences (16, 29). This was expected, since *E. coli* Sakai was used as the tester for preparing one of the SSH DNA libraries. The frequency at which sequences possessing 90% or greater sequence identity to each *E. coli* Sakai chromosomal ORF was identified, either partly or wholly, within the SSH libraries was enumerated, and 24 putative regions of genomic difference for lineage I (RD_I) identified on the *E. coli* Sakai chromosome and pO157 and pOSAK1 plasmids were selected for further testing (Fig. 1). These regions consisted of clusters of 1 to 47 ORFs that were each identified in the SSH libraries from 1 to 24 times. The locations of SSH sequences within pO157 and pOSAK1 plasmids are not shown, but plasmid ORFs with 90% or greater sequence identity to SSH library sequences were evenly distributed across pOSAK1 (GenBank accession number NC_002127) and concentrated in the region of a putative reverse transcriptase within pO157 (GenBank accession number AB011549).

Identification of lineage I conserved regions. Having identified candidate regions of the genome that are unique to lineage I strains, the next step was to identify which of the candidate RD_I were conserved across multiple lineage I strains and likewise absent in multiple lineage II strains. While SSH is a versatile tool for identification of sequence differences between bacterial strains, the time required to obtain and analyze the subtracted sequences is substantial, thus limiting the number of strains that can be analyzed. To confirm the lineage-specific distribution of the RD_I, 30 different *E. coli* O157:H7 strains were subsequently tested for the presence of the RD_I by PCR. This confirmatory strain set comprised 10 lineage I

TABLE 2. Results of preliminary screening of ORF clusters identified in SSH libraries

RD ₁	ORF(s) represented in SSH libraries	ORF(s) targeted by primers	No. positive for LSPA-6 genotype				Genotype 11111 conserved?
			111111 (n = 10)	222222 (n = 10)	211111 (n = 5)	Other (n = 5)	
14	ECs0237-ECs0241	ECs0238 and -9	10	0	5	0	Yes
16	ECs0275-ECs0284	ECS0281	10	0	5	0	Yes
27	ECs0377-ECs0379	ECs0377	10	10	5	5	No
52	ECs0759-ECs0760	ECs0760	10	9	5	5	No
67	ECs1114-ECs1121	ECs1120	10	10	5	5	No
69a	ECs1158-ECs1173	ECs1164	8	0	3	0	Yes
69b	ECs1186-ECs1195	ECs1191	7	0	0	0	Yes
69c	ECs1205-ECs1251	ECs1223	10	2	3	0	Yes
72	ECs1377-ECs1393	ECS1382	10	0	4	0	Yes
B1	ECs1425	ECs1425	10	10	5	5	No
78	ECs1576-ECs1600	ECs1588 and -9	8	0	0	0	Yes
82/83	ECs1687-ECs1705	ECs1698	10	0	3	0	Yes
91/92/93	ECs1929-ECs1958	ECs1954	10	0	0	0	Yes
B2	ECs1997-ECs1998	ECs1998	10	10	5	5	No
126/127	ECs2775-ECs2776	ECs2775	10	10	5	5	No
153	ECs2976-ECs2981	ECs2979	10	0	2	0	Yes
274a	ECs4942-ECs4946	ECs4943	3	5	0	3	No
274b	ECs4957-ECs4960	ECs4960	1	4	0	2	No
274c	ECs4998-ECs5005	ECs5000	10	9	5	5	No
286	ECs5242-ECs5252	ECs5250	9	0	4	0	Yes
pO157-RT ^a	pO157, <i>sopB</i> -RT (p36)	Upstream of RT	9	0	0	0	Yes
pO157- <i>katP</i>	pO157, <i>katP</i>	<i>katP</i>	9	10	5	5	No
pO157- <i>etpF</i>	pO157, <i>etpF</i>	<i>etpF</i>	10	10	5	5	No
pOSAK1	pOSAK1	Unknown	5	0	1	0	No

^a RT, putative reverse transcriptase.

strains of LSPA-6 genotype 111111, 10 lineage II strains of LSPA-6 genotype 222222 strains, and 10 lineage II strains having other LSPA-6 genotypes. Each PCR-based RD₁ detection assay was performed using a single PCR assay designed to detect DNA sequences within the 24 RD₁. Twelve of the 24 RD₁ were conserved in at least seven of the LSPA-6 genotype 111111 strains but were found in two or fewer of the genotype 222222 strains (Table 2). Amplicons for each PCR assay corresponded in size to that predicted for *E. coli* Sakai. These conserved RD₁ (CRD₁) were also absent from other LSPA-6 genotypes, except for genotype 211111 strains. Their presence within LSPA-6 genotype 211111 strains was variable.

To determine if the lineage-conserved distribution of these regions was maintained when tested over a larger population of *E. coli* O157:H7 strains, the representative PCR assay from each CRD₁ (except CRD₁ 78) was tested against a larger set of 119 *E. coli* O157:H7 isolates of different LSPA-6 genotypes. Sequences recognized by these assays were present in 81 to 100% of LSPA-6 genotype 111111 strains but were found in ≤7% of LSPA-6 genotype 222222 strains, confirming the earlier screening results for these regions. Complete PCR assay results for the preliminary screening of 30 strains for the presence of the 24 RD₁ regions and the subsequent testing of 119 strains for the presence of the 12 CRD₁ regions are shown in Tables S2 and S3, respectively, of the supplemental material.

Localization of genomic boundaries of CRD₁. While the SSH and PCR screening results suggest 12 CRD₁ exist across the *E. coli* Sakai chromosome and pO157 plasmid, it was not known if the *E. coli* Sakai ORFs identified by SSH accurately reflected the boundaries of these CRD₁ as they exist in lineage I strains. Subsequent PCR screening assays were performed to confirm the boundaries of the CRD₁ by testing for the presence of *E.*

coli Sakai DNA segments flanking the CRD₁ among lineage II strains (Table 3) (16, 29). All but one of the estimated full-length CRD₁ were localized within *E. coli* Sakai S-loops and the corresponding *E. coli* EDL933 O-islands. These *E. coli* O157:H7 chromosomal regions are absent from *E. coli* K-12 and are thought to have arisen by horizontal gene transfer (16). The remaining CRD₁ was located on the pO157 virulence plasmid of *E. coli* O157:H7 (7, 29). Complete PCR results for these assays are shown in Table S2 of the supplemental material. Amplicons from each of these additional PCR assays corresponded in size to that predicted for *E. coli* Sakai.

Relationships between CRD₁ and inferred phylogeny of different *Escherichia coli* O157:H7 LSPA-6 genotypes. To test for congruence between presence/absence of CRD₁ and phylogeny of the strains, cluster analysis was performed on the different strains using a distance matrix developed from comparison of the presence/absence of the CRD₁. The LSPA-6 genotype was then superimposed onto the corresponding neighbor-joining dendrogram (Fig. 2). Strains of LSPA-6 genotypes 111111 and 222222 clustered separately on the basis of the CRD₁ identified in this study. This would be expected based on the definition of the CRD₁, which were selected on the basis of their presence in LSPA-6 111111 strains but not in LSPA-6 222222 strains. LSPA-6 genotype 211111 strains, however, clustered with both of the major groups, a result that is consistent with their variable CRD₁ content. This variability suggests that the *fold2* allele (the only lineage II allele in the 211111 genotype) may have arisen independently in different genomic backgrounds or that the CRD₁ identified in this study emerged differentially in descendants of the original LSPA-6 211111 strains. Given that the *fold2* allele is thought to be the result of a tandem duplication event (23), it seems more plausible that that it is a

TABLE 3. Results of PCR screening to determine boundaries of CRD₁ in *E. coli* O157:H7 strains

CRD ₁	ORF(s) targeted by primers	No. positive for LSPA-6 genotype				LSPA-6 genotype 111111 conserved?
		111111 (n = 10)	222222 (n = 10)	211111 (n = 5)	Other (n = 5)	
14	ECs0234 and -5	10	10	5	5	No
	ECs0238 and -9	10	0	5	0	Yes
	ECs0240	10	0	5	0	Yes
	ECs0243 and -4	10	10	5	5	No
16	ECs0270	10	10	5	5	No
	ECs0271 and -2	10	0	5	0	Yes
	ECs0276	10	0	3	0	Yes
	ECS0281	10	0	5	0	Yes
	ECs0282 and -3	10	10	5	5	No
	ECs0284	10	10	5	5	No
69a	ECs1158	10	10	5	5	No
	ECs1162 and -3	10	0	0	0	Yes
	ECs1164	8	0	3	0	Yes
	ECs1165	9	0	3	0	Yes
	ECs1168	10	1	1	0	Yes
69b	ECs1180 and -2	3	10	3	4	No
	ECs1186 and -8	3	0	0	0	No
	ECs1191	7	0	0	0	Yes
	ECs1192 and -4	10	9	3	3	No
69c	ECs1217 and -8	10	10	5	5	No
	ECs1219	10	2	3	0	Yes
	ECs1223	10	2	3	0	Yes
	ECs1251	10	0	0	0	Yes
	ECs1252	10	10	5	5	No
72	ECs1374 and -6	10	5	0	5	No
	ECs1377	10	0	3	0	Yes
	ECS1382	10	0	4	0	Yes
	ECs1389 and -90	10	0	5	0	Yes
	ECs1394 and -5	10	10	5	5	No
78	ECs1573 and -4	8	10	5	5	No
	ECs1575 and -6	8	10	5	5	No
	ECs1582 and -3	8	3	1	0	Yes
	ECs1584	8	0	1	1	Yes
	ECs1588 and -9	8	0	0	0	Yes
	ECs1590	8	0	1	1	Yes
	ECs1595	8	0	1	1	Yes
	ECs1598	8	0	1	1	Yes
	ECs1599 and -1600	8	0	0	0	Yes
82/83	ECs1687	10	10	5	5	No
	ECs1691	10	0	4	0	Yes
	ECs1698	10	0	3	0	Yes
	ECs1705	10	0	4	0	Yes
	ECs1706	10	10	4	5	No
	ECs1707	9	9	4	5	No
91/92/93	ECs1927	10	10	5	5	No
	ECs1931 and -2	10	0	0	0	Yes
	ECs1937 to -40	10	0	0	0	Yes
	ECs1945	10	2	0	1	No
	ECs1953	10	0	0	0	Yes
	ECs1954	10	0	0	0	Yes
	ECs1957	10	0	0	0	Yes
153	ECs2979	10	0	2	0	Yes
286	ECs5240 and -1	7	10	5	5	No
	ECs5242	9	0	5	0	Yes
	ECs5250	9	0	4	0	Yes
	ECs5252	9	0	5	0	Yes
	ECs5253	10	10	5	5	No
	ECs5254 to -6	10	10	5	5	No
PO157-RT	pO157, <i>sopB</i>	9	10	5	5	No
	pO157, upstream of reverse transcriptase	9	0	0	0	Yes
	pO157, reverse transcriptase	9	0	0	0	Yes
	pO157, p36	9	10	5	5	No

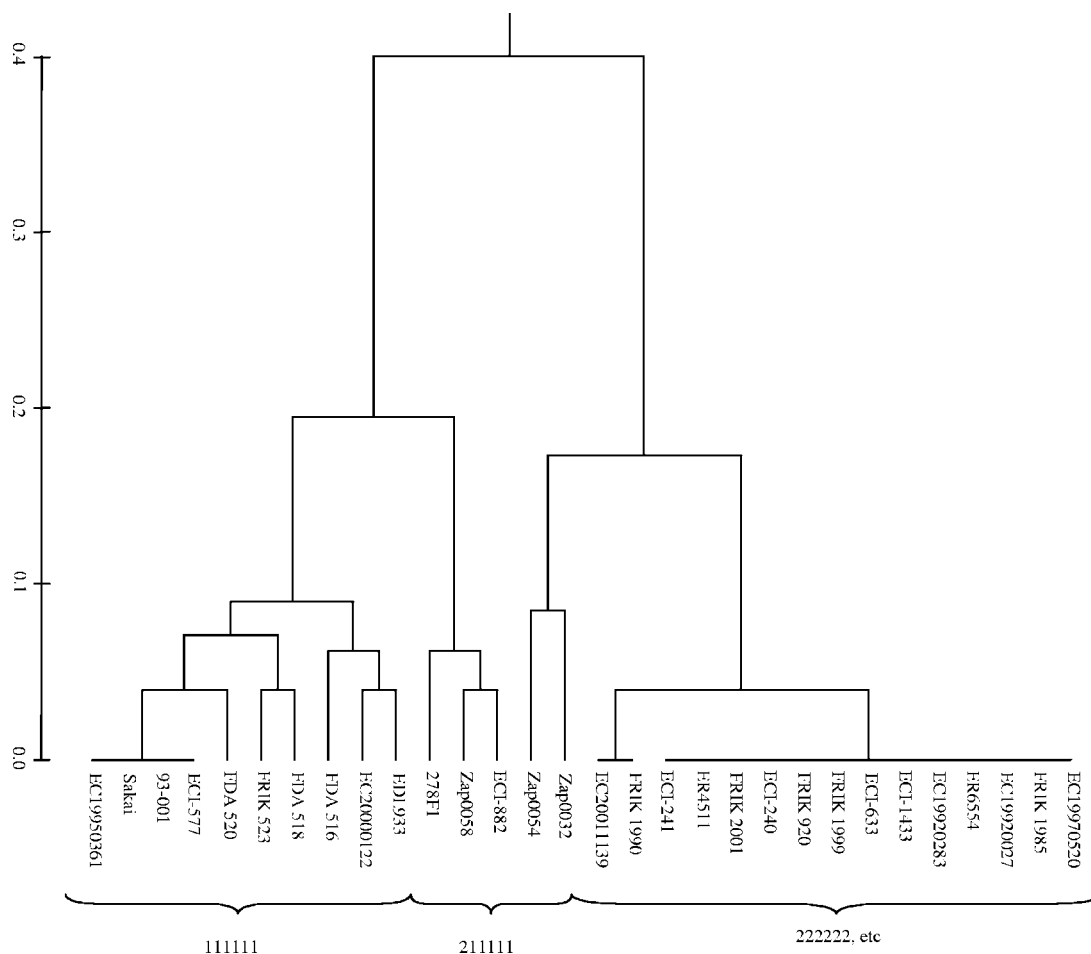


FIG. 2. UPGMA dendrogram displaying relationship between LSPA-6 genotype and the presence of CRD₁ in *Escherichia coli* O157:H7 strains.

polyphyletic allele that arose from a simple mutation or lateral transfer event as opposed to the hypothesis that multiple events resulted in differential CRD₁ distribution within isolates of this genotype. Strains from the remainder of the lineage II LSPA-6 genotypes had profiles that were very similar to genotype 222222 strains.

Functional categorization of genes within CRD₁. The results of the PCR assays indicated that the CRD₁ were strongly associated with LSPA-6 111111 strains and rare or absent in LSPA-6 222222 strains. Because the LSPA-6 genotypes 111111 and 222222 show statistically significant biases in their distribution among strains isolated from human clinical samples and bovine fecal samples (63), it is possible that genes within the CRD₁ could contribute to the apparently different ecological distributions of the LSPA-6 genotypes that have been observed. To identify genes within CRD₁ that could contribute to host specificity, we conducted functional classification of the ORFs present within the CRD₁, based on annotation entries, identification of homologous DNA sequences by BLASTN searches, and Pfam identification (4) of common protein domains and families using hidden Markov models (<http://www.sanger.ac.uk/Software/Pfam/>). The results from this set of experiments are summarized in Table 4.

The most striking characteristic of the ORFs contained within the CRD₁ is their location with respect to mobile genetic elements in *E. coli* Sakai (16). Nine of the 12 CRD₁ were within Sp1, Sp5 (*stx*₂), Sp7, Sp10, and Sp15 prophage and SpLE1 and SpLE5 prophage-like elements (Table 4). One of the CRD₁ was surrounded by Rhs element genes, mobile genetic elements that were originally identified based on their association with *recA*-dependent intrachromosomal recombination (2, 10, 28). Five of the CRD₁ contained or were located in close proximity to transposase genes. One CRD₁ was found on the pO157 virulence plasmid (7, 29). Not surprisingly, a large proportion of the ORFs identified within the CRD₁ were bacteriophage structural and regulatory genes, Rhs genes, and transposases. These elements would be expected to promote genomic recombination and rearrangement and may contribute to bacterial virulence through associated structural and regulatory functions (58, 59).

Lineage I-specific CRD₁ 69 and 153 are located within *E. coli* Sakai Sp5 and Sp15 prophages, respectively (Table 4). These two prophages contain the structural genes for Stx2 and Stx1, respectively. The Sp5 prophage has been shown to be highly variable in both its genomic structure and integration sites (37, 38, 51). The presence of the *stx*₂ gene is strongly

TABLE 4. Summary of potential virulence factors contained within CRD_I in *E. coli* O157:H7 strains

CRD _I	Genetic environment	Potential virulence factor(s) identified within LSPA-6 genotype 111111 CR
14	Rhs elements	None identified
16	Sp1 prophage	None identified
69a	Sp5 prophage	ECs1170, gene for putative protein with DksA/TraR C4-type zinc finger domain (42)
69b	Sp5 prophage	None identified
69c	Sp5 prophage, transposon	ECs1236, gene for putative outer membrane precursor protein with Ail/Lom protein domain (3, 35); ECs1250, gene for putative protein with DksA/TraR C4 type zinc finger domain (42)
72	SpLE1 prophage-like element, transposon	ECs1382, similar to hemolysin-activating protein <i>hecB</i> gene of <i>Neisseria meningitidis</i> (57); ECs1386, similar to immunoglobulin-binding regulator <i>ibrA</i> gene of <i>E. coli</i> ECOR-9 (50); ECs1387, similar to immunoglobulin-binding regulator <i>ibrB</i> gene of <i>E. coli</i> ECOR-9 (50); ECs1388, similar to plasmid-encoded regulator <i>perC</i> gene of EPEC (11); ECs1391, similar to bundle-forming pilus <i>bfpM</i> gene of EPEC EAF plasmid (55)
78	Sp7 prophage	ECs1588, similar to plasmid-encoded regulator <i>perC</i> gene of EPEC (11)
82/83	Transposon	ECs1693, homologue of <i>prxA</i> of a proposed iron transport system in <i>E. coli</i> CFT073 (15); ECs1694, homologue of the <i>modD</i> of a proposed iron transport system in <i>E. coli</i> CFT073 (15); ECs1695, homologue of <i>yc73</i> of proposed iron transport system in <i>E. coli</i> CFT073 (15); ECs1696, homologue of <i>yc73</i> of proposed iron transport system in <i>E. coli</i> CFT073 (15); ECs1697, homologue of <i>fepC</i> of proposed iron transport system in <i>E. coli</i> CFT073 (15); ECs1698, homologue of iron transport permease <i>FecD</i> of <i>E. coli</i> CFT073 (60); ECs1699, homologue of putative ATP-binding protein of ABC transporter in <i>Shigella flexneri</i> 2a strain 301 (18)
91/92/93	Sp10 prophage, transposon	None identified
153	Sp15 prophage	None identified
286	SpLE5 prophage-like element, transposon	ECs5250, required for colonization of calves (9); ECs5252, similar to BamHI control element in <i>Bacillus amyloliquefaciens</i> (6)
pO157-RT ^a	pO157 plasmid	None identified

^a RT, putative reverse transcriptase.

associated with virulence in *E. coli* O157:H7 (5, 16, 24, 41). In a recent study, it was reported that the Q antiterminator gene, which regulates toxin gene expression in *stx*₂ prophage, differs between *E. coli* O157:H7 strains of different OBGS lineages and that these differences are related to toxin production (27). However, the Q antiterminator gene was not among the ORFs identified within the SSH libraries in this study. Only a small region located upstream of the *stx*₁ and Q antiterminator genes in the *Stx1*-converting Sp15 prophage was identified in the SSH libraries. PCR assays confirmed that at least one of these ORFs, ECs2979, was conserved in lineage I strains and absent in lineage II strains.

A cluster of lineage I-specific ORFs within CRD_I 82/83 may represent an iron uptake system. Virulence genes, such as *E. coli* O157:H7 *stx*₁ genes (8), are often iron regulated. The ORFs ECs1693 to ECs1697 are highly homologous to the *prxA-modD-yc73-fepC* gene cluster located on the pyelonephritis and cystitis pathogenicity island of uropathogenic *E. coli* CFT073 (15). This gene cluster was proposed to be involved in iron uptake in *E. coli* CFT073 (64). The *fepC* gene was reported to be present in O157 EHEC isolates and absent from non-O157 isolates, although the OBGS lineage of the O157 EHEC strains tested in this study was not examined (40). This gene is also found in EAEC2 and DAEC2 phylogenetic groups of enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC), respectively, and it has been proposed that these groups might represent hypervirulent isolates of EAEC and DAEC (40). Immediately following the *prxA-modD-yc73-fepC* gene cluster are ECs1698, which is homologous to the Fe(III) dicitrate transport system permease *FecD* protein of *E. coli* CFT073 (60), and ECs1699, which is homologous to a putative

ATP-binding protein of the ABC transporter in *Shigella flexneri* 2a strain 301(18).

The ORF ECs1382 within CRD_I 72 has 34% amino acid similarity at its amino terminus to the gene for the hemolysin activation protein HecB of *Neisseria meningitidis* (57), which directs the translocation of hemolysin A (HlyA) across cytoplasmic and cell membranes (25), and it possesses two copies of a 20-residue repeat found in the *Bordetella pertussis* filamentous hemagglutinin family of adhesins (49). These homologies suggest that ECs1382 might contribute to virulence. In a recent investigation of the distribution of Z1640 (the *E. coli* EDL933 homologue of ECs1382) within different *E. coli* serotypes, intact ECs1382/Z1640 was associated with serotypes that cause hemolytic uremic syndrome and outbreaks of human illness, while fragmented ECs1382/Z1640 was found in nonepidemic human disease-associated strains of serotypes O91:H21 and O113:H21 and animal-associated Shiga toxin-producing *E. coli* serotypes not associated with human disease (52).

A number of ORFs were identified within the SSH libraries that displayed homology to regulatory genes. These genes could affect virulence of *E. coli* O157:H7 by regulating expression of effector genes directly involved in pathogenesis. The genome sequence of *E. coli* O157:H7 Sakai contains five genes homologous to the plasmid-encoded LEE regulatory protein PerC, which is produced by certain enteropathogenic *E. coli* (EPEC) strains (16, 17, 39, 44). Two of these *perC* homologue (*pch*) genes, ECs1388 of CRD_I 72 and ECs1588 of CRD_I 78, encode putative proteins with 25% and 39% sequence identity, respectively, to EPEC PerC. ECs1388 and ECs1588 were identified within the SSH libraries and confirmed by PCR to be present in lineage I strains and absent from lineage II strains.

While other *pch* genes have been demonstrated to modulate expression of LEE transcription units in *E. coli* O157:H7 (17), deletion or overexpression of ECs1388 or ECs1588 has not, and their function, if any, in gene regulation remains to be determined (44).

Other potential regulatory genes identified within the SSH libraries include ECs1386 and ECs1387 of CRD_I 72, which display 80 and 85% identity to immunoglobulin-binding regulator genes *ibrA* and *ibrB* of *E. coli* ECOR-9. These genes regulate production of *Escherichia coli* immunoglobulin-binding (*eib*) genes in this strain (50). The ORF ECs5252 of CRD_I 286 encodes a putative transcriptional regulator with homology to the BamHI control element of *Bacillus amyloliquefaciens* (6). Finally, the ORFs ECs1170 of CRD_I 69a and ECs1250 of CRD_I 69c encode putative proteins with a pfam01258:Zn-dskA_traR domain (30). The *dskA* gene of *E. coli* regulates rRNA transcription (19, 42).

Several additional ORFs were identified within the CRD_I with the potential to act as virulence factors. The ORF ECs1236 of CRD_I 69c encodes a putative outer membrane precursor protein with a Pfam06316:Ail_Lom domain (30). Proteins with this domain include the Ail protein of *Yersinia enterocolitica*, which contributes to invasion of cultured cell lines (35), and the Lom bacteriophage protein, which has been shown to confer serum resistance (3). *E. coli* O157:H7, however, is not invasive, and so the role of this invasin-like protein is unclear. The amino terminus of ORF ECs1391 of CRD_I 72 displayed high sequence homology to the bundle-forming pilus (BfpM) protein of EPEC (55). This gene is not required for formation of bundle-forming pili in EPEC and no other *bfp* homologues exist in the *E. coli* O157:H7 genome, and so it is difficult to postulate what its role might be (55). The ECs5250 of CRD_I 286 was shown to be required for colonization of calves (9), but its function is unknown. The CRD_I pO157-RT includes a putative reverse transcriptase on the pO157 virulence plasmid of *E. coli* O157:H7 (7, 29), but the function of the putative reverse transcriptase in *E. coli* O157:H7 is also unknown. Lastly, several hypothetical protein genes were also identified on prophage or prophage-like element-associated CRD_I. Again, the effects of these genes on bacterial virulence, if any, are unknown.

The paucity of human clinical isolates within *E. coli* O157:H7 LSPA-6 genotype 222222 and some subsets of OBGs lineage II suggests but does not prove that these strains lack virulence factors present in other *E. coli* O157:H7 lineages (22, 63). The identification of potential virulence factors in LSPA-6 111111 strains that are lacking in LSPA-6 222222 strains supports this hypothesis, but there is no direct evidence that these regions are involved in pathogenicity. Further functional and epidemiological analyses of genes within the CRD_I are essential to elucidate the impact that genome evolution has had on the virulence characteristics of populations emerging within the O157:H7 clonal complex.

CRD_I support the hypothesis that lineage I may be the ancestral state of contemporary O157:H7. Based on previous studies, it has been proposed that lineage I isolates represent the ancestral state of O157:H7, whereas lineage II isolates are derived (22, 63). This conclusion was based largely upon findings that several lineage I-specific genome segments or alleles are shared with K-12 and other *E. coli* strains (63). Our find-

ings with CRD_I are consistent with this hypothesis. As discussed above, many of the CRD_I can be found in other *E. coli* strains, including distantly related K-12 and UPEC strains. The simplest explanation for their absence in lineage II strains is that they were lost during the divergence of lineage II populations.

Many of the ORFs associated with the CRD_I regions were found in close proximity to bacteriophage, transposon, and Rhs element genes. Given the presence of numerous copies of these elements in *E. coli* O157:H7 genomes (16, 43), it seems highly likely that genome evolution occurred rapidly by movement of these elements or by recombination events occurring within or near these elements. Genomic diversity in human strains of *E. coli* Sakai was previously shown by comparative genomic hybridization and whole-genome PCR scanning to be strongly associated with the presence of bacteriophage (37, 38).

In a recent publication by Wick et al. (62), a microarray was used to study the conservation of *E. coli* O157:H7 genes in strains representing intermediates in the proposed evolution of *E. coli* O157:H7 and *E. coli* O55:H7. The microarray results indicated that the majority of the ORFs identified in this study within CRD_I 16, 69a, 69b, 69c, 72, 78, 91/92/93, 153, and 286 were acquired recently by *E. coli* O157:H7 SOR⁻ GUD⁻ strains. All of these CRD_I were located on bacteriophage, suggesting that these differences were a result of bacteriophage excision or recombination.

In conclusion, several CRD_I that are conserved in LSPA-6 genotype 111111 strains but are not found in genotype 222222 strains were identified, supporting the existence of two genomic lineages of *E. coli* O157:H7 strains. These CRD_I contain a number of potential virulence factors, including a putative hemolysin activation protein, a possible iron transport system, and several regulatory genes. These potential virulence factors warrant further study to determine their contribution to the pathogenicity of *E. coli* O157:H7 strains.

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