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Genetic Diversity and Virulence Potential of Shiga Toxin-Producing *Escherichia coli* O113:H21 Strains Isolated from Clinical, Environmental, and Food Sources

Peter C. H. Feng,[a] Sabine Delannoy,[b] David W. Lacher,[c] Luis Fernando dos Santos,[d] Lothar Beutin,[e] Patrick Fach,[b] Marta Rivas,[f] Elizabeth L. Hartland,[g] Adrienne W. Paton,[h] Beatriz E. C. Guth[d]

Division of Microbiology, Food and Drug Administration, College Park, Maryland, USA[a]; Laboratory for Food Safety, French Agency for Food, Environmental, and Occupational Health and Safety, Maisons-Alfort, France[b]; Division of Molecular Biology, Food and Drug Administration, Laurel, Maryland, USA[c]; Department of Microbiology, Immunology and Parasitology, Universidade Federal de São Paulo, São Paulo, Brazil[d]; National Reference Laboratory for *Escherichia coli*, Federal Institute for Risk Assessment, Berlin, Germany[e]; Servicio Fisiopatogenia, Instituto Nacional de Enfermedades Infecciosas-ANLIS "Dr. Carlos G. Malbrán," Buenos Aires, Argentina[f]; Department of Microbiology and Immunology, University of Melbourne at the Peter Doherty Institute for Infection and Immunity, Melbourne, Australia[g]; Research Centre for Infectious Diseases, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, Australia[h]

Shiga toxin-producing *Escherichia coli* strains of serotype O113:H21 have caused severe human diseases, but they are unusual in that they do not produce adherence factors coded by the locus of enterocyte effacement. Here, a PCR microarray was used to characterize 65 O113:H21 strains isolated from the environment, food, and clinical infections from various countries. In comparison to the pathogenic strains that were implicated in hemolytic-uremic syndrome in Australia, there were no clear differences between the pathogens and the environmental strains with respect to the 41 genetic markers tested. Furthermore, all of the strains carried only Shiga toxin subtypes associated with human infections, suggesting that the environmental strains have the potential to cause disease. Most of the O113:H21 strains were closely related and belonged in the same clonal group (ST-223), but CRISPR analysis showed a great degree of genetic diversity among the O113:H21 strains.

Shiga toxin-producing *Escherichia coli* (STEC) represents a large, diverse group of bacteria characterized by the production of Shiga toxins (Stx). There are two main Stx types, designated Stx1 and Stx2 and within each are many subtypes. There are hundreds of known STEC serotypes that can produce any of the Stx types or combination of subtypes. However, the production of Stx alone is deemed to be insufficient to cause severe human illness. Also, some Stx subtypes are produced mostly by environmental or animal strains and have not affected humans, so, not all STEC strains appear to be human pathogens (1, 2). In contrast, enterohemorrhagic *E. coli* (EHEC) is a pathogenic subset of STEC strains that carry other virulence factors. Most notable of these is the intimin protein that enables EHEC to attach to epithelial cells. Intimin is encoded by the *eae* gene that resides on a pathogenicity island called locus of enterocyte effacement (LEE). The presence of *eae* and stx_2 is a reliable predictor that the STEC strain may cause severe illness such as hemorrhagic colitis (HC) or hemolytic-uremic syndrome (HUS) (3). Well-known EHEC serotypes such as O157:H7, O26:H11, and O111:H8 all have *eae* and have caused severe disease. However, there are LEE-negative EHEC strains, such as O113:H21, that do not have *eae* but were first implicated in HUS in 1983 (4) and also caused a cluster of HUS cases in Australia in 1998 (5, 6). Since O113:H21 strains are *eae* negative, they are postulated to have other binding factors and virulence genes (7). Analysis of the HUS-associated O113:H21 strains from Australia identified the STEC agglutinating adhesin (Saa) as a possible adherence factor (8). These strains also carry the *subAB* genes that code for subtilase cytotoxin (9), *sab* that codes for an outer membrane, autotransporter protein that enhances biofilm formation (10), and *ehxA* that encodes enterohemolysin. Although these genes are commonly found in O113:H21 strains, their precise role in the pathogenicity of LEE-negative EHEC strains has not been fully determined.

Serotype O113:H21 strains are prevalent in the environment and have been isolated from ground beef (11) and from other foods and animals in various countries (12, 13). Several O113:H21 strains have also been isolated from fresh spinach in the United States and found to possess traits similar to those of the pathogenic O113:H21 strains (14). In most of these studies, however, the strains were not fully characterized so, the virulence potential of these environmental O113:H21 strains was only speculative. Also, a study looked at diversity in O113:H4 strains and showed them to be distinct from O113:H21 strains (15), but the genetic diversity among O113:H21 strains has not been examined. In this study, we used a PCR microarray and *stx* subtyping PCR to examine O113:H21 strains isolated from various sources and countries and compared them to the HUS-associated strains from Australia, to determine whether the environmental strains may also be of health risk. We also used multilocus sequence typing (MLST) to examine phylogenetic relatedness and clustered regularly interspaced short palindromic repeat (CRISPR) to look for sequence polymorphisms and genetic diversity among the O113:H21 strains.

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Address correspondence to Peter C. H. Feng, peter.feng@fda.hhs.gov.
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MATERIALS AND METHODS

Bacterial strains. A panel of 65 O113:H21 strains was used in the present study: 12 strains from Argentina, mostly from ground beef and cattle but also included a few strains isolated from HUS and diarrhea cases; 32 mostly bovine strains from Brazil, but these included a few from goat and meats; 3 strains from France, with one strain from ground beef, one that was originally isolated in Canada, and one from an unknown source; and 11 strains from Germany, that included isolates from cattle manure, clinical strains from patients with abdominal cramps, diarrhea, and HC, and a strain from a meat sample suspected of having caused HUS. Also, three of the German strains had originated elsewhere, with one strain from dog feces in the United Kingdom, one from cattle feces in Norway, and one from an HC case in Australia. There were five strains from the United States, with three strains isolated from fresh spinach and two others obtained from the STEC Center at Michigan State University. One of these was originally isolated from a HUS patient in Canada (strain TW01391 or CL-3), and another was from a diarrhea patient in Thailand (strain TW02918 or DEC16a). Lastly, two O113:H21 strains (98NK2 and EH41) that were implicated in HUS in Australia were included for comparison and reference. The metadata for all of these strains are shown in Table 1.

All of the isolates had been serotyped at the time of isolation and also identified as *eae*-negative STEC of the O113:H21 serotype. Some isolates had also been tested for *saa*, *subAB*, *ehxA*, and *sab* genes, but since various PCR assays were used, it was uncertain whether the results were comparable due to differences in primer specificities. However, these genes are also on the array, which served to verify the presence of these genes in the strains.

PCR microarray. The 65 O113:H21 strains were tested for the presence of 41 virulence or characteristic genetic markers. These genes and the proteins they encode are described in Table 2. The primers and probes used for the detection of stx_1, stx_2, *eae*, wzx_{O113} (i.e., the O113 strain *wzx* gene), $fliC_{H21}$, *bfpA*, *ehxA*, *katP*, *espP*, *etpD*, *toxB*, *saa*, *subA*, *nleA*, *astA*, *irp2*, $lpfA_{O113}$, $lpfA_{O26}$, *iha*, *terE*, *ureD*, *Z2098*, *Z2099*, *Z2121*, *pagC*, *ent*, *nleB*, *nleE*, *efa1*, and *efa2* were described previously (16–19). Primers for the detection of *ehaA*, *epeA*, *sab*, *cdt-V*, *Z2096*, *Z4318*, *Z4320*, *Z4322*, *Z4325*, *Z4327*, and *Z4331* were designed for the present study. The *wecA* gene, which is part of the *wec* cluster that codes for the synthesis of the enterobacterial common antigen, was used as a reference marker for *E. coli* (20).

High-throughput real-time PCR (rtPCR) amplifications with FAM- or HEX-labeled TaqMan probes was performed using a LightCycler1536 (Roche, Meylan, France) as described previously (21). High-throughput rtPCR amplification of the O island 122 (OI-122) open reading frame (ORF) genes was done with a BioMark rtPCR system (Fluidigm, San Francisco, CA) using the EvaGreen DNA binding dye (Biotium, Inc., Hayward, CA) as described previously (20). Amplicons were examined by melting-curve analyses.

Stx subtyping. Strains that had stx_1, stx_2, or both were tested by PCR to determine the specific *stx* subtypes. Subtypes stx_{2a}, stx_{2c}, and stx_{2d} share sequence similarities, so primer cross-reactivity can occur. Hence, strains found to carry two or all three of these subtypes were retested using a 66°C annealing temperature instead of 62°C. All of the laboratories used the subtyping PCR protocol described by Scheutz et al. (22) to ensure that the results were comparable.

MLST. Clonal analysis was performed on most strains, except for those from Brazil, where almost all 32 strains were from bovine sources and, since some of these had nearly identical pulsed-field gel electrophoresis profiles (23), only eight strains that showed greater profile differences were examined. The Whittam MLST protocol, described elsewhere (http://www.shigatox.net/ecmlst/cgi-bin/index), used primers to amplify and sequence internal segments of seven housekeeping genes (aspartate aminotransferase [*aspC*], caseinolytic protease [*clpX*], acyl coenzyme A synthetase [*fadD*], isocitrate dehydrogenase [*icdA*], lysine permease [*lysP*], malate dehydrogenase [*mdh*], and β-D-glucuronidase [*uidA*]). Each unique sequence is given an allele number, and the combinations of alleles from the seven genes are used to obtain an allelic profile or sequence type (ST), which is then compared to those of other *E. coli* strains in the *Ec*MLST database (24).

CRISPR. Sequence polymorphisms in the strains were examined using the nomenclature of CRISPR1 and CRISPR2a (25). Respective regions of the CRISPR loci were PCR amplified using conditions that are described previously (21). Amplicons were double strand sequenced (Eurofins MWG Operon, Courtaboeuf, France), and the CRISPR sequences of the strains were assembled using BioEdit v7.1.3.0. The method and R-script developed by Yin et al. (25) was used to assign the allele numbers and the sequence types for each O113:H21 strain. Briefly, each unique spacer and repeats were assigned a number and a letter, respectively. Each unique spacer combination within a CRISPR locus defined a CRISPR allele. Alleles not previously described by Yin et al. (25) were assigned a new numerical designation. Each unique CRISPR1 and -2a combination was assigned a CRISPR type (CT).

Nucleotide sequence accession numbers. CRISPR1 and CRISPR2 locus sequences have been deposited in GenBank under accession numbers KJ500180 to KJ500244 and KJ500245 to KJ500309, respectively.

RESULTS

PCR microarray. The array data obtained for the O113 and H21 specific genes and the stx_1 and stx_2 genes were consistent with the serological and/or PCR data obtained previously. The genes *eae*, *katP*, *etpD*, *toxB*, *nleA*, *bfpA*, *irp2*, *ureD*, and $lpfA_{O26}$, the OI-57 ORFs *Z2098*, *Z2099*, and *Z2121*, and the OI-122 ORFs *Z4322*, *Z4325*, *Z4326*, *Z4327*, *Z4328*, *Z4329*, *Z4331*, *Z4332*, and *Z4333* were absent from all of the strains and so are not included in Table 1. Similarly, the genes wzx_{O113}, $fliC_{H21}$, $lpfA_{O113}$, and *ehaA* were present in all of the strains and were therefore also excluded from Table 1. The distributions and the patterns of the 16 remaining genes among the 65 strains tested are shown in Table 1. The O113:H21 strains implicated in illness or isolated from humans are listed at the top of the table, while the bottom of the table lists the environmental strains.

Excluding the genes present in all strains, the most prevalent gene among the O113:H21 strains was *sab*, and this gene was present in 59/65 (90%) strains. Other common genes were *espP* and *iha*, found in 56/65 (86%) strains and *ehxA* and the OI-122 genes *Z4320* and *Z4321* found in 51/65 (78%) strains, followed by *saa* (49/65 [75%]), *subA* (48/65 [73%]), and *epeA* (47/65 [72%]) (Table 1). With a few exceptions, the *ehxA*, *saa*, *subA*, *epeA*, *Z4320*, and *Z4321* genes were mostly absent from strains that had stx_1, and *cdt-V* was not detected in any stx_1-positive strain. In contrast, *astA* was detected only in bovine strains from Brazil that had stx_1 and was found in 8/14 (57%) stx_1-positive strains. There were several genes that were rarely found in the O113:H21 strains. The OI-122 ORF *Z4318* gene was only found in one spinach isolate from the United States, and *terE* was detected in only 5/65 (7%) strains, mostly bovine isolates from Brazil and Germany. The OI-57 ORF *Z2096* was also found in only five strains: three were from Australia and have been implicated in severe illnesses, and the other two were bovine isolates (Table 1). Lastly, strain Ec41/03 from Brazil only had *stx* and the *iha* gene, and strain CB8531 that was originally isolated from dog feces in the United Kingdom did not have any of the 16 genes listed in Table 1, including the *stx* genes.

Stx subtyping. There were four strains that did not have any *stx* gene. No strain carried stx_1 alone, and of the 14 strains that carried both stx_1 and stx_2, 12 were bovine isolates from Brazil. All of the stx_1-positive strains had the stx_{1a} subtype (Table 1), and the stx_2 subtypes found in these strains included 8/14 (57%) strains with

TABLE 1 Characteristics of O113:H21 strains examined

Isolate	*stx* type(s)		Virulence gene														MLST	CRISPR	Country	Source
	stx_1	stx_2	*astA*	*cdt-V*	*ehxA*	*epeA*	*espP*	*iha*	*saa*	*sab*	*subA*	*terE*	*Z2096*	*Z4318*	*Z4320*	*Z4321*				
Clinical																				
EH41	–	c	–	–	+	+	+	+	+	+	+	–	+	–	+	+	820	9	Australia	HUS
98NK2	–	a	–	–	+	+	+	+	+	+	+	–	+	–	+	+	820	13	Australia	HUS
1108/01	–	a, c	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	27	Argentina	HUS
889/06	–	a	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	25	Argentina	HUS
370/02	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	223v	47	Argentina	Diarrhea
CB8578	–	a	–	+	+	+	+	–	+	+	+	–	–	–	+	+	223	8	Germany	HC
CB7267	–	a, d	–	–	+	–	+	–	+	+	+	–	+	–	+	+	820	1	Germany (Australia)	HC
CB7612	–	d	–	–	–	–	+	+	–	–	–	+	–	–	+	+	223	30	Germany	Diarrhea
CB2125	–	–	–	–	+	–	+	+	–	+	–	–	–	–	+	+	846	28	Germany	Diarrhea
CB7960	a	a, d	–	–	+	–	–	+	–	+	–	–	–	–	–	–	846	6	Germany	Human
CB7561	–	a, d	–	+	+	+	+	–	+	+	+	–	–	–	+	+	223	32	Germany	Human
TW01391	–	a, d	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	49	USA (Canada)	HUS
TW02918	a	c	–	–	+	–	+	+	+	+	–	–	–	–	–	–	223	17	USA (Thailand)	Diarrhea
Environmental																				
FP-054	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	223	33	Argentina	Young steer
FP-120	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	223	44	Argentina	Steer
258/04	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	223	39	Argentina	Hamburger
1112/06	–	a	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	25	Argentina	Hamburger
188/06-28	–	a	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	20	Argentina	Bovine
T842	–	a	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	48	Argentina	Bovine
571/05	–	a, c	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	24	Argentina	Hamburger
997/01	–	a, c	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	26	Argentina	Bovine
226/99	–	c	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	21	Argentina	Hamburger
Ec41/03	a	c	–	–	–	–	–	+	–	–	–	–	–	–	–	–	ND[a]	34	Brazil	Bovine
Ec596/05	a	c, d	–	–	–	–	–	+	–	+	–	+	–	–	–	–	846	40	Brazil	Bovine
397/02	a	c	+	–	–	–	–	+	–	+	–	+	–	–	–	–	ND	50	Brazil	Bovine
Ec182/04	a	d	+	–	–	–	–	+	–	+	–	–	–	–	–	–	ND	3	Brazil	Buffalo
Ec624/05	a	c	+	–	–	–	–	+	–	+	–	–	–	–	–	–	ND	3	Brazil	Bovine
Ec727/05	a	d	+	–	–	–	–	+	–	+	–	–	–	–	–	–	ND	3	Brazil	Bovine
254/2	a	c	+	–	–	–	+	+	–	+	+	–	–	–	–	–	ND	3	Brazil	Bovine
261/1	a	c	+	–	–	–	+	+	–	+	–	–	–	–	–	–	ND	3	Brazil	Bovine
226/1	a	c, d	+	–	–	–	+	+	–	–	–	–	–	–	–	–	846	3	Brazil	Bovine
Ec670/05	a	c	–	–	–	–	+	+	–	+	–	–	–	–	–	–	846	42	Brazil	Bovine
Ec258/01	–	a, c	–	+	–	–	+	+	–	–	–	–	–	–	+	+	ND	5	Brazil	Bovine
Ec719/05	a	a, c	–	–	+	+	+	–	+	+	+	+	–	–	+	+	ND	43	Brazil	Bovine
102MB9	a	c	+	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	2	Brazil	Bovine
Ec62/03	–	–	–	+	+	+	+	+	+	+	+	–	–	–	+	+	ND	35	Brazil	Bovine
Ec472/01	–	a	–	+	+	+	+	+	+	+	+	–	–	–	+	+	ND	37	Brazil	Bovine
Ec678/04	–	a	–	+	+	+	+	+	+	+	+	–	–	–	+	+	ND	27	Brazil	Bovine
Ec684/04	–	a	–	+	+	+	+	+	+	+	+	–	–	–	+	+	ND	27	Brazil	Bovine
Ec689/04	–	a	–	+	+	+	+	+	+	+	+	–	–	–	+	+	ND	27	Brazil	Bovine
Ec254/01	–	a, c, d	–	+	+	+	+	+	+	+	+	–	–	–	+	+	997	12	Brazil	Bovine
Ec585/05	–	–	–	–	+	+	+	+	+	+	+	–	–	–	+	+	223	11	Brazil	Bovine
Ec301/02	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	36	Brazil	Bovine
Ec507/01	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	11	Brazil	Bovine
Gc138	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	45	Brazil	Bovine
Ec858/05	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	41	Brazil	Goat
Ec784	–	a	–	–	+	+	+	+	+	+	+	–	–	–	+	+	997	39	Brazil	Meat
Ec227/01	–	a, c	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	31	Brazil	Bovine
MV1.2/18	–	a, c	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	37	Brazil	Bovine
Ec226/04	–	a, d	–	–	+	+	+	+	+	+	+	–	–	–	+	+	223	19	Brazil	Bovine
Ec648/05	–	a, d	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	38	Brazil	Bovine
Ec503/05	–	a, d	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	11	Brazil	Goat
Ec253/02	–	c	–	–	+	+	+	+	+	+	+	–	–	–	+	+	997	10	Brazil	Bovine
Gc20	–	d	–	–	+	+	+	+	+	+	+	–	–	–	+	+	ND	45	Brazil	Bovine
04-1450	–	d	–	+	+	+	+	+	+	+	+	–	–	–	+	+	ND	18	France (Canada)	Unknown
NV254	–	a, d	–	+	+	+	+	–	+	+	+	–	–	–	+	+	223	16	France	Unknown
07HMPA903	–	a	–	–	+	+	+	–	+	+	+	–	–	–	+	+	223	22	France	Hamburger
CB5250	–	a, d	–	–	+	+	–	–	+	+	–	–	–	–	–	–	223	23	Germany	Meat (HUS)
CB6110	–	a, d	–	+	+	+	+	–	+	+	+	–	+	–	+	+	223	12	Germany	Calf feces
CB9070	–	a, d	–	–	+	+	+	+	+	+	+	–	+	–	+	+	223	29	Germany (Normay)	Heifer feces
CB6699	–	d	–	–	–	–	+	+	–	–	–	+	–	–	+	+	223	4	Germany	Calf feces
CB8531	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	223	7	Germany (UK)	Dog feces
MDP09-27	–	a	–	+	+	+	+	+	+	+	+	–	–	+	+	+	223	14	USA	Spinach
MDP09-47	–	a, d	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	15	USA	Spinach
MDP10-35	–	a, d	–	+	+	+	+	+	+	+	+	–	–	–	+	+	223	46	USA	Spinach

[a] ND, not determined.

TABLE 2 *E. coli* gene targets tested in the PCR microarray assay of O113:H21 strains[a]

Gene (ORF name if chromosomal)	Encoded protein or family effector	Genetic support[b]
ehaA (*Z0402*)	Autotransporter of EHEC	OI-15*
ureD (*Z1142*)	Urease-associated protein UreD	OI-43*, OI-48*
terE (*Z1176*)	Tellurite resistance cluster	OI-43*, OI-48*
iha (*Z1148*)	Iron-regulated gene A homologue adhesin	OI-43*, OI-48*
Z2096	Unknown protein encoded within prophage CP-933O	OI-57*
Z2098	Unknown protein encoded within prophage CP-933O	OI-57*
Z2099	Unknown protein encoded within prophage CP-933O	OI-57*
Z2121	Unknown protein encoded within prophage CP-933O	OI-57*
nleA (*Z6024*)	Non-LEE-encoded type III effector	OI-71*
Z4318	ORF of unknown function	OI-122*
Z4320	ORF of unknown function	OI-122*
pagC (*Z4321*)	PagC-like membrane protein	OI-122*
Z4322	ORF of unknown function	OI-122*
Z4325	ORF of unknown function	OI-122*
ent (*Z4326*)	Ankyrin repeats	OI-122*
Z4327	ORF of unknown function	OI-122*
nleB (*Z4328*)	Non-LEE-encoded type III effector	OI-122*
nleE (*Z4329*)	NleE	OI-122*
Z4331	ORF of unknown function	OI-122*
efa1 (*Z4332*)	EHEC factor for adherence	OI-122*
efa2 (*Z4333*)	EHEC factor for adherence	OI-122*
eae (*Z5110*)	Intimin	LEE*
toxB	Adhesin	EHEC-plasmid†
ehxA	Enterohemolysin	EHEC-plasmid†
katP	Catalase peroxidase	EHEC-plasmid†
espP	Serine protease EspP	EHEC-plasmid†
etpD	Type II effector	EHEC-plasmid†
subA	Subtilase cytotoxin	aEHEC-plasmid‡
astA	EAEC heat-stable enterotoxin 1 (EAST1)	EAEC-plasmid[c]
$lpfA_{O26}$	Major fimbrial subunit of LPFO26	EAEC chromosome§
$lpfA_{O113}$	Long polar fimbrial protein	EAEC chromosome§
irp2	Iron-repressible protein 2	High pathogenicity island§
saa	Saa (STEC autoagglutinating adhesin)	aEHEC-plasmid‡
epeA	Serine protease autotransporter	aEHEC-plasmid‡
sab	Autotransporter	aEHEC-plasmid‡
bfpA	Major structural subunit of bundle-forming pilus	pMAR2 plasmid[d]
cdt-V	Cytolethal distending toxin	Chromosome[e]
stx_1	Shiga toxin 1	Stx phage CP-933V*
stx_2	Shiga toxin 2	Stx phage BP-933W*
wzy_{O113}	O113 antigen polymerase	*rfb* operon[f]
$fliC_{H21}$	Flagellin H21	Chromosome[g]

[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).
[b] *, nomenclature refers to sequence of *E. coli* O157:H7 EDL933 (GenBank accession no. AE005174); †, plasmid pO157 EDL933 (GenBank accession no. NC_007414); ‡, plasmid pO113 (GenBank accession no. NC_007365); §, *E. coli* 55989 (GenBank accession no. CU928145).
[c] Plasmid pHUSEC41 (GenBank accession no. HE603111).
[d] Plasmid pMAR2 (GenBank accession no. NC_011603.1).
[e] *E. coli* 493/89 (GenBank accession no. AJ508930).
[f] *E. coli* 98NK2 (GenBank accession no. AF172324).
[g] *E. coli* O113:H21 (GenBank accession no. DQ862122).

stx_{2c}, 2/14 (14%) strains with stx_{2d}, 3/14 (21%) strains with stx_{2c} and stx_{2d}, and one strain (7%) with stx_{2a} and stx_{2c}. Of the 28 strains that had stx_2 alone, the stx_{2a} subtype was the most common and found in 21/28 (75%) strains, followed by stx_{2d} in 4/28 (14%) strains and stx_{2c} in 3/28 (11%) strains. Of the strains that had multiple stx_2 subtypes, the most common was stx_{2a}-stx_{2d}, found in 12/19 (63%) strains, followed by stx_{2a}-stx_{2c} in 6/19 (31%) strains and one strain that had all three subtypes. No other stx_1 (stx_{1c} or stx_{1d}) or stx_2 (stx_{2b}, stx_{2e}, stx_{2f}, and stx_{2g}) subtypes were observed in any of the O113:H21 strains. The two HUS-associated strains from Australia only had stx_2, and they were either stx_{2a} or stx_{2c}. The same results were obtained from four other HUS-associated Australian O113:H21 strains that we tested (data not shown).

MLST. All of the strains from Argentina had ST-223, except for strain 370/02, which had a variant type of ST-223 (designated ST-223v) that has a −1G frameshift in the *uidA* gene. Of the eight bovine strains from Brazil that were tested, two had ST-223, three had ST-997 (which differs from ST-223 by a single nucleotide polymorphism [SNP] in the *uidA* gene), and three others had ST-846. Among the eleven German strains, two had ST-846, strain

CB7267 had ST-820, and the rest were ST-223, including the two isolates that originated from animal feces in the United Kingdom (CB8531) and Norway (CB9070). All of the strains from France and the United States had ST-223, and the two Australian O113:H21 strains had ST-820 (Table 1).

CRISPR. All isolates were subtyped according to the sequences of the CRISPR1 and -2a loci. The spacer arrangements of CRISPR1 and CRISPR2a loci in the 65 analyzed strains are shown in the supplemental material. The CRISPR1 spacers' repertoire contained 12 different spacers arranged in 31 combinations or alleles (GenBank accession numbers KJ500180 to KJ500244), which resulted in 48% allele diversity (proportion of alleles found in the 65 strains for which the CRISPR1 locus was determined). Among these, 22 alleles were present only once in the 65 strains, 4 were present twice, 1 was found three times, 1 was found five times (7.7% of the isolates), 1 was found seven times (10.8% of the isolates), 1 was found eight times (12.3% of the isolates), and a single allele was found 12 times (18.5% of the isolates). Each allele contained between 3 and 21 spacers (9.97 ± 2.65 [mean ± the standard deviation]), which were typically 32 bp long, but there were three spacers that were 33 bp long. Most of the spacers found had been previously been identified (25); however, four new spacers (numbered 222 to 225) were detected in the present study, though spacer 225 was a simple repeat variant of spacer 76. The order of the spacers was strictly conserved in all but four strains. The direct repeats (DRs) were largely conserved but some had SNPs that led to the definition of two new DRs.

A total of 29 unique spacers arranged in 41 alleles were found in CRISPR2a (GenBank accession numbers KJ500245 to KJ500309). This resulted in 63.1% allele diversity in the 65 strains for which the CRISPR2 locus was determined. The most common allele was found seven times (10.8% of the isolates), while 30 alleles were present only once. Among the others, six alleles were found twice each, two alleles were present three times, one allele was found four times (6.2% of the isolates), and one was found six times (9.2% of the isolates). Within CRISPR2a, the alleles had between five and 21 spacers (13.29 ± 4.55), and the spacers were all 32 bp long. Most of the spacers were previously identified (25); however, four new spacers (numbered 216 to 219) were also identified, with spacers 218 and 219 being single SNP variants of spacer 62. The order of the spacers was strictly conserved in all but two strains, and the DR was largely conserved, but some did contain SNPs resulting in two new repeats being identified.

In total, the CRISPR1 and -2a alleles formed 50 different CTs (77% diversity) among the 65 strains tested, 42 of which were found only once. There were five CTs that were found twice; CT11 was found three times, CT27 was found four times, and CT3, the most prevalent, was found in six strains, all of which were stx_1 positive.

DISCUSSION

Microarray analyses confirmed that all of the isolates are O113:H21 strains and that most carried stx_2 alone, although some also had stx_1. There were four strains that had no *stx* genes, but since the *stx* genes reside on bacteriophages, which can be induced, even during routine culturing (26, 27), it is possible that these strains may have had the ability to produce Stx but had since lost the *stx* phages. There are three known Stx1 subtypes and seven known Stx2 subtypes (22), but only Stx1a, Stx2a, Stx2c, and Stx2d have most often been implicated in human illness (28, 29). These four Stx subtypes were the only ones detected in all of the O113:H21 strains examined.

Some of the O113:H21 strains were previously tested for various markers and the array data were consistent with most of these, but there were also, some discrepancies. The Brazilian and U.S. strains were *sab* negative (14, 30) using the *sab* PCR primers described by Herold et al. (10), but except for three Brazilian strains, all of the other strains were found to be *sab* positive by the array. Also, several Brazilian strains that were previously negative for *epeA*, *cdt-V*, and *iha* (23) were determined to be positive for these genes by the array. These discrepancies are suspected to be due to differences in primer specificities, but they also may indicate sequence heterogeneity within those genes.

The *sab*, *saa*, *epeA*, and *subAB* genes were originally identified in O113:H21 strains (5, 10, 31, 32), so their prevalence in the O113:H21 strains we examined was not unexpected. Although these genes are usually found only in *eae*-negative STEC strains, they may not be present in all strains (33). Our results are consistent in that not all O113:H21 strains carried all four genes.

A few genes were detected in only a limited number of O113:H21 strains. The *terE* gene, which is part of the *ter* cluster that codes for tellurite resistance, was found in only a few O113:H21 isolates. Strains of O157:H7 and some EHEC strains are resistant to tellurite (34), but others, including many *eae*-negative STEC strains, do not have *ter* genes and so are sensitive to tellurite (34, 35). Our finding that only five strains had *terE* is indicative that most O113:H21 strains are also tellurite sensitive. The *astA* gene encodes the EAST1 toxin, which can be found in *Salmonella* but is very prevalent in *E. coli*. Among pathogenic *E. coli* strains, *astA* was found in 86% of enteroaggregative *E. coli* strains and in 88% of EHEC strains (36), but our data showed that *astA* was not common in O113:H21 strains.

The *cdt* gene codes for a cytolethal distending toxin (Cdt) that is produced by many Gram-negative pathogens and *E. coli* is known to produce five Cdt variants (37). A previous study showed that most STEC strains carried *cdt-V*, but the lone O113:H21 strain tested in that study had *cdt-I* (37). We did not have *cdt-I* on the array but found that 37% of the O113:H21 strains had *cdt-V*, suggesting that different O113:H21 strains may produce different Cdt variants. Oddly, *cdt-V* was not found in any stx_1-positive strains, which could be coincidental or perhaps due to some type of phage exclusion. Both the *cdt-V* and the stx_1 genes are coded by lambdoid phages (33, 37) and, although double lysogens that carry both Stx and Cdt phages are known to occur, such strains exhibited variable induction rates and that Cdt phages can be spontaneously released (37).

One of the study objectives was to determine whether any genetic markers were more closely associated with O113:H21 strains that caused severe infections. Previous studies showed that many genomic OI-122-coded genes, especially *nleB* (*Z4328*), were closely associated with EHEC strains (17). Similarly, *Z2098* and *Z2099* from the genomic OI-57 have also been found to be closely linked with typical EHEC strains (18). However, all of these were absent in all of the O113:H21 strains tested, including those from Australia. Hence, no particular gene was found to be closely associated with the pathogenic strains, and no clear patterns of gene presence or absence were apparent between the pathogens from the other countries and the Australian strains or between the environmental and pathogenic strains. For the most part, the environmental strains had very traits similar to those of the pathogens

and, coupled with the finding that all of the environmental strains carried only Stx subtypes that have been linked to diseases, this is strongly suggestive that these environmental strains may also have the potential to cause human disease.

Previous clonal studies using the Whittam MLST system showed that most O113:H21 strains have ST-223 and belong to the STEC-2 clonal group. Consistent with those findings, 70% of the O113:H21 strains that we examined had ST-223, including strain CB8531, which did not have *stx* or any of the 16 genes listed in Table 1. Four other strains, one from Argentina that had ST-223v and three Brazilian bovine strains that had ST-997, are all very closely related to ST-223 and are part of the STEC-2 clonal group.

There were a few strains that had ST-846, which differs from ST-223 by SNPs in *clpX*, *fadD*, *mdh*, and *uidA* genes, so they are distinct and do not belong in the STEC-2 clonal group. Strains of ST-846 are in the NT-5 group that is comprised of a large mix of strains that include both STEC and non-STEC serotypes. It is interesting that ST-846 was only observed in the two German clinical isolates and the three Brazilian bovine isolates but, it is uncertain whether this was merely coincidental or perhaps O113:H21 strains of ST-846 are common in both countries.

The two O113:H21 strains from Australia had ST-820, and so did a German strain (CB7267), but the latter was originally isolated from a HC patient in Australia. We performed MLST on four other HUS- or HC-associated O113:H21 strains from Australia, and these also had ST-820 (data not shown). Comparatively, ST-820 and ST-223 strains differ by a single SNP in *aspC*, so these are very closely related and are within the STEC-2 clonal group. The fact that all of the Australian strains had ST-820 and all are pathogens suggested that perhaps ST-820 may be associated with virulent O113:H21 strains. However, other HUS-associated O113:H21 strains from other countries had ST-223. The fact that ST-820 was observed only in the Australian strains also suggests regional clustering. Evidence of such geographic divergence between the United States and Australia has been reported for O157:H7 strains (38). However, O113:H21 strains with other ST have reportedly been isolated in Australia (15, 39), so it is uncertain whether ST-820 is the prevalent ST in Australia or whether regional ST variation and clustering exists among O113:H21 strains.

MLST results showed that most O113:H21 strains are closely related and belong in the STEC-2 clonal group, but CRISPR analysis showed a high degree of genetic diversity among these strains. CRISPRs are comprised of tandem sequences containing direct repeats of 21 to 47 bp that are separated by spacers of similar sizes. Many of these spacers seem to be derived from foreign DNA, like plasmids and phages, and are thought to confer immunity to subsequent infection by homologous phages and plasmids. Active CRISPRs have higher rates of spacer acquisition, which results in more diversity, and so are more useful in differentiating strains. However, less active CRISPR tends to be evolutionarily conserved and so may be useful as markers to detect clonal populations (25, 40). Studies showed that the CRISPRs in EHEC tend to be fairly well conserved, so a CRISPR-based PCR assay was developed that enabled the detection of eight major EHEC serotypes with 97.5 to 100% specificity (21). In contrast, LEE-negative EHEC strains appear to be genetically diverse. Comparative genomics of nine LEE-negative versus four LEE-positive STEC serotype strains showed broad phylogenetic diversity among the LEE-negative strains, including variations in the types of adherence and virulence factors they carried (33). That study did not include O113:H21 strains, but the results of our CRISPR analysis concurred that there was a lot of genetic diversity in the O113:H21 strains as well. This is consistent with the data of a pulsed-field gel electrophoresis analysis, which showed O113:H21 strains to be diverse genetically since their XbaI profiles shared few similarities (14, 23). These findings suggest that, unlike other EHEC strains, O113:H21 strains seem to have a fairly active CRISPR since it contains considerable sequence polymorphisms.

In conclusion, we used a PCR microarray to characterize 65 O113:H21 strains isolated from the environment, food, and clinical infections from various countries and compared them to the strains isolated from HUS patients in Australia. With respect to the 41 genetic markers tested, there were no clear patterns that distinguished between the pathogens and the environmental strains. Furthermore, all of the strains carried only Stx subtypes associated with human infections, suggesting that the environmental strains may also be a health concern. With few exceptions, all of the O113:H21 strains were closely related and belonged to the same clonal group. Even so, CRISPR analysis showed that there was a great degree of genetic diversity among these strains.

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