Shiga Toxin 1c-Producing *Escherichia coli* Strains: Phenotypic and Genetic Characterization and Association with Human Disease

Alexander W. Friedrich,¹ Julia Borell,¹ Martina Bielaszewska,¹ Angelika Fruth,²
Helmut Tschäpe,² and Helge Karch^{1*}

*Institut für Hygiene, Universitätsklinikum Münster, 48149 Münster,¹ and Robert-Koch Institut,
Bereich Wernigerode, 38855 Wernigerode,² Germany*

Received 21 January 2003/Returned for modification 26 February 2003/Accepted 6 March 2003

The distribution of the *stx*_{1c} allele among Shiga toxin (Stx)-producing *Escherichia coli* (STEC) and the virulence characteristics of *stx*_{1c}-harboring STEC are unknown. In this study, we identified *stx*_{1c} in 76 (54.3%) of 140 *eae*-negative, but in none of 155 *eae*-positive, human STEC isolates ($P < 0.000001$). The 76 *stx*_{1c}-harboring *E. coli* isolates belonged to 22 serotypes, and each produced Stx1c as demonstrated by latex agglutination. Characterization of putative virulence factors demonstrated the presence of the locus of proteolysis activity (LPA) and the high-pathogenicity island in 65.8 and 21.1%, respectively, of the 76 Stx1c-producing *E. coli* isolates. Moreover, all but three of these strains contained *saa*, the gene encoding an STEC autoagglutinating adhesin. The virulence profiles of Stx1c-producing *E. coli* isolates were mostly serotype independent and heterogeneous. This enabled us to subtype the isolates within the same serotype. The individuals infected with Stx1c-producing *E. coli* strains were between 3 months and 72 years old (median age, 23.5 years) and usually had uncomplicated diarrhea or were asymptomatic. We conclude that Stx1c-producing *E. coli* strains represent a significant subset of *eae*-negative human STEC isolates, which belong to various serotypes and frequently possess LPA and *saa* as their putative virulence factors. The phenotypic and molecular characteristics determined in this study allow the subtyping of Stx1c-producing STEC in epidemiological and clinical studies.

Shiga toxins (Stxs) are the major virulence factors of Stx-producing *Escherichia coli* (STEC). STEC strains cause diarrhea and the hemolytic-uremic syndrome (HUS) worldwide (1, 15). Stxs consist of two major types, Stx1 and Stx2 (19). The heterogeneity of the Stx2 group has long been appreciated; it consists of Stx2 and its multiple variants, Stx2c, Stx2d, Stx2e, and Stx2f (26, 33, 35, 38). However, the Stx1 group, especially the toxins from human isolates, now also appears to be a diverse family. We have recently identified an Stx1 variant in STEC isolates of human origin (40) which differed markedly in its amino acid composition from the prototype Stx1 and which was previously found only in STEC isolates from sheep (21). We designated this variant Stx1c and developed *stx*_{1c}-specific PCR and *stxB*₁ restriction fragment length polymorphism strategies for the identification of the *stx*_{1c} allele (40). Using these approaches, we detected *stx*_{1c} in 17.0% of STEC isolates from humans and demonstrated that a prominent feature of *stx*_{1c}-harboring STEC isolates was the lack of the intimin-encoding *eae* gene (40), suggesting the absence of the locus of enterocyte effacement (LEE) from such strains.

Virulence characteristics, especially adherence mechanisms of LEE-negative STEC strains, are poorly understood. Paton et al. (24) identified a novel adhesin termed Saa (for STEC autoagglutinating adhesin), which is encoded on a megaplasmid of LEE-negative STEC strains of serotypes O113:H21, O91:H21, and O48:H21, which have been identified as causes

of outbreaks (23) or sporadic cases of HUS (8, 22). A cloned *saa* gene significantly increases the adherence of an *E. coli* K-12 strain to Hep-2 cells and confers a semilocalized adherence phenotype (24). At the same time, our group identified and characterized a novel pathogenicity island, called the locus of proteolysis activity (LPA), which is inserted in *selC* of LEE-negative *stx*_{2d}-harboring STEC strains (34). In addition to the gene encoding *E. coli* vitamin B₁₂ receptor (BtuB), the LPA contains two genes, *espI* and *iha*, encoding proteins with potential virulence roles (34). *espI* encodes a novel serine protease (EspI) which cleaves swine pepsin A and human apolipoprotein A-I, and *iha* encodes an outer membrane protein that is homologous to Iha (iron-regulated gene A homologue adhesin), identified previously on the tellurite resistance- and adherence-conferring island of *E. coli* O157:H7 (37). Moreover, we have recently demonstrated (14) that some members of *eae*-negative STEC strains of serogroup O128 contain the high-pathogenicity island (HPI) originally identified in pathogenic *Yersinia*. The HPI carries the *irp* (iron-repressible protein) loci involved in the synthesis of the siderophore yersiniabactin, which mediates iron uptake, and the *fyuA* gene encoding the receptor for yersiniabactin and pesticin (14, 36).

Although STEC isolates producing Stx1c represent a subset of human STEC strains (40), the virulence characteristics of such strains are unknown. Therefore, we analyzed Stx1c-producing *E. coli* strains for a spectrum of potential virulence factors, including the pathogenicity islands and new adhesins identified recently in LEE-negative STEC strains and plasmid-encoded genes (enterohemorrhagic *E. coli* [EHEC] *hly*, as well as *espP* and *etpD*) possessed by LEE-positive STEC strains (4, 13, 41). We also serotyped the Stx1c-producing STEC strains

* Corresponding author. Mailing address: Institut für Hygiene, Universitätsklinikum Münster, Robert-Koch-Str. 41, 48149 Münster, Germany. Phone: 49/251-83 55 361. Fax: 49/251-83 55 341. E-mail: hkarch@uni-muenster.de.

and investigated the association between the virulence profiles and serotypes of such strains. Moreover, we determined the distribution of the *stx*_{1c} allele among *eae*-negative and *eae*-positive STEC strains of human origin.

MATERIALS AND METHODS

Bacterial strains. Two hundred and ninety-five STEC isolates (155 *eae* positive and 140 *eae* negative) were investigated in this study. The isolates were recovered at the Institute of Hygiene and Microbiology, University of Würzburg, Würzburg; at the Institute of Hygiene, University Hospital Münster, Münster; and at the Robert Koch Institute, Wernigerode, Germany, during routine diagnostic efforts and epidemiological investigations between 1996 and 2002. The procedures used for the isolation of STEC from stools were described previously (7, 11). Forty-four of the 155 *eae*-positive STEC strains were isolated from patients with HUS, 84 were isolated from patients with diarrhea, and 27 were isolated from asymptomatic carriers. Among the 140 *eae*-negative STEC strains, two originated from HUS patients, 85 originated from patients with diarrhea, and 53 originated from asymptomatic individuals. Thirty-five of the STEC strains investigated in this study were analyzed for *stx*_{1c} in a previous study (40).

Case definition. Patients with diarrhea had three or more watery stools without visible blood per day. HUS was defined as a case of microangiopathic hemolytic anemia (hematocrit, <30% with peripheral evidence of intravascular hemolysis), thrombocytopenia (platelet count, <150,000/mm³), and renal insufficiency (a serum creatinine concentration greater than the upper limit of the normal range for the patient's age) (39). Asymptomatic carriers were apparently healthy individuals without diarrhea.

Phenotypic methods. The STEC isolates were serotyped at the Robert Koch Institute, using antisera against *E. coli* O antigens 1 to 181 and *E. coli* H antigens 1 to 56 and a microtiter method (27) adapted from a standard procedure described by Ørskov and Ørskov (20). Stx1c production was tested using a commercial latex agglutination assay (VTEC-RPLA [verotoxin-producing *E. coli* reverse passive latex agglutination], Denka Seiken Co., Ltd., Tokyo, Japan) performed as described previously (40). Fermentation of sorbitol was detected using sorbitol MacConkey agar plates (17) after overnight incubation. The enterohemolytic phenotype was sought using blood agar plates containing 5% defibrinated and washed human erythrocytes and 10 mM CaCl₂ as described earlier (30, 32).

PCR. PCRs for the detection of putative virulence genes were performed in the iCycler (version 1.259; Bio-Rad, Munich, Germany) or the TGradient 96 cycler (Biometra GmbH, Göttingen, Germany). PCR reagents were purchased from PEQLAB Biotechnologie (Erlangen, Germany), and primers were synthesized by Sigma ARK (Darmstadt, Germany). The 25-μl PCR mixture consisted of 2.5 μl of bacterial suspension (ca. 10⁹ bacteria), 2.5 μl of 10-fold-concentrated polymerase synthesis buffer Y containing 2.0 mM MgCl₂, 5 μl of Enhancer solution, 200 μM (each) deoxynucleoside triphosphate, 15 pmol of each primer, and 0.65 U of *Taq* DNA polymerase. The PCR primers, target sequences, and PCR conditions are listed in Table 1. *E. coli* O157:H7 strain EDL933 (A. D. O'Brien, A. T. Lively, M. E. Chen, S. W. Rothman, and S. B. Formal, Letter, Lancet i:702, 1983) was used as a positive control in PCRs for the detection of *stx*₁, *stx*₂, *eae*, and plasmid genes, including *EHEC-hly*, *espP*, and *etpD*. *E. coli* strains 3115/97 (O128:H2; *stx*_{1c}⁺) (40) and EH250 (ONT:H12; *stx*_{2d}⁺) (26) were used as positive controls in PCRs for the detection of *stx*_{1c} and *stx*_{2d}, respectively. Strain 4797/97 (O91:H⁺), which carries the pathogenicity island LPA containing the *iha*, *espI*, and *btuB* genes (34), and strain 3172/97 (O128:H2) containing the *irp2* and *fjuA* genes located within the HPI (14) were used as positive controls in the respective PCRs. Strain 3937/97 (O91:H⁺; *saa*⁺) from our strain collection was used as a positive control in PCR for the detection of *saa*.

Statistical analysis. The χ^2 test (6) and Epi-Info version 6.04b (Centers for Disease Control and Prevention, Atlanta, Ga., and World Health Organization, Geneva, Switzerland) were used for calculations. *P* values of <0.05 were considered to be significant.

RESULTS

Distribution of *stx*_{1c} among *eae*-positive and *eae*-negative STEC isolates. One hundred and fifty-five *eae*-positive and 140 *eae*-negative STEC isolates that contained members of the *stx*₁ family, as determined by PCR with primers KS7-KS8, and that produced Stx1, as detected by the latex agglutination assay,

were chosen for *stx* subtyping. These strains belonged to a broad spectrum of serotypes (Table 2). Altogether, eight *stx* genotypes were identified (Table 2). The *stx*_{1c} allele was found in none of the 155 *eae*-positive STEC isolates but was present in 76 (54.3%) of the 140 *eae*-negative STEC isolates (*P* < 0.000001). *stx*_{1c} was present either alone or in combination with *stx*_{2d}. Only in a single strain was it associated with *stx*₂ and never with *stx*₁ or *stx*_{2c} (Table 2).

Phenotypic characteristics of *stx*_{1c}-harboring STEC strains.

The strains containing *stx*_{1c} belonged to 22 (Table 3) of the 42 serotypes identified among the 140 *eae*-negative STEC isolates (Table 2). Fifty-three (69.7%) of the 76 *stx*_{1c}-harboring STEC isolates clustered in nine serotypes, including O5:H⁺, O76:H19, O78:H⁺, O113:H4, O128:H2, O146:H21, O174:H8, O178:H⁺, and O181:H16, among which O113:H4 was the most frequent (Table 3). Moreover, two strains were not typeable with the O antisera used and four strains autoagglutinated (Table 3). Within the serogroups to which isolates of different serotypes that were investigated belonged (O91, O113, O128, O146, O174, O178, and O181) (Table 2), the presence of *stx*_{1c} was mostly associated only with specific serotypes (Table 3). The toxin titers detected with the Stx1 latex reagent in culture filtrates of the 76 strains containing *stx*_{1c} ranged from 1:2 to 1:8 (median titer, 1:4). Sixty-five of the 76 Stx1c-producing strains fermented sorbitol. The 11 non-sorbitol-fermenting STEC strains belonged to serotypes O128:H2 (four strains), O174:H8 (two strains), O181:H16 (four strains), and ONT:H2 (one strain) (Table 3). Fifty-nine (77.6%) of the 76 strains displayed the enterohemolytic phenotype.

Potential virulence determinants of Stx1c-producing *E. coli*.

All but three of the 76 Stx1c-producing *E. coli* strains (96.1%) contained the STEC autoagglutinating adhesin (*saa*) gene, mostly (60 strains) in combination with the *EHEC-hly* gene. Fifty (65.8%) of these 76 isolates carried the pathogenicity island LPA, as evidenced by the presence of each of the three genes, *iha*, *espI*, and *btuB*, which were previously shown to be located on the LPA in combination (34). Moreover, 16 strains (21.1%) possessed an HPI. Whereas the LPA was usually (in 38 of the 50 LPA-positive strains) present as a sole pathogenicity island, the HPI was found without an LPA in only four strains. Twelve strains (15.8%) contained the HPI together with an LPA. However, more than one-fourth (28.9%) of the 76 *eae*-negative Stx1c-producing *E. coli* strains possessed neither LPA nor HPI. None of these 76 STEC isolates carried the *espP*, *etpD*, and *efal* genes.

Within the serotypes from which three or more isolates were available, isolates of the same serotype usually displayed more than one combination of virulence characteristics. Identical virulence profiles were observed only in strains of serotypes O5:H⁺ and O78:H⁺ (Table 3).

Clinical symptoms and ages of subjects infected with STEC isolates producing Stx1c.

Forty-three of the 76 individuals from whom the Stx1c-producing *E. coli* strains were isolated had uncomplicated diarrhea, and 32 were asymptomatic. A single patient suffered from HUS, and this patient was infected with the only *stx*_{1c}-harboring strain in this study that also contained *stx*₂ (Tables 2 and 3). The ages of the subjects infected with the Stx1c-producing STEC strains ranged from 3 months to 72 years (median age, 23.5 years).

TABLE 1. PCR primers and conditions used in this study

Primer	Sequence	Target	PCR conditions ^d			PCR product size (bp)	Reference
			Denaturing	Annealing	Extension		
KS7	5'-CCC GGA TCC ATG AAA AAA ACA TTA TTA ATA GC-3'	<i>stxB₁</i> and <i>stxB_{1c}</i> ^b	94°C, 30 s	52°C, 60 s	72°C, 40 s	285	29
KS8	5'-CCC GAA TTC AGC TAT TCT GAG TCA ACG-3'						
Stxlc-1	5'-5'-TTT TCA CAT GTT ACC TTT CCT-3'	<i>stxA_{1c}</i>	94°C, 30 s	51°C, 60 s	72°C, 60 s	498	40
Stxlc-2	5'-5'-CAT AGA AGG AAA CTC ATT AGG-3'						
LP43	5'-ATC CTA TTC CCG GGA GTT TAC G-3'	<i>stxA₂</i> and variants ^c	94°C, 30 s	57°C, 60 s	72°C, 60 s	584	5
LP44	5'-GCG TCA TCG TAT ACA CAG GAG C-3'						
GK3	5'-ATG AAG AAG ATG TTT ATG-3'	<i>stxB₂</i> and <i>stxB_{2c}</i> ^d	94°C, 30 s	52°C, 60 s	72°C, 40 s	260	12
GK4	5'-TCA GTC ATT ATT AAA CTG-3'						
VT2-cm	5'-AAG AAG ATA TTT GTA GCG G-3'	<i>stxB_{2d}</i> ^e	94°C, 30 s	55°C, 60 s	72°C, 60 s	256	26
VT2-f	5'-TAA ACT GCA CTT CAG CAA AT-3'						
SK1	5'-CCC GAA TTC GGC ACA AGC ATA AGC-3'	<i>eae</i>	94°C, 30 s	52°C, 60 s	72°C, 60 s	863	29
SK2	5'-CCC GGA TCC GTC TCG CCA GTA TTC G-3'						
Irp2(FP)	5'-AAG GAT TCG CTG TTA CCG GAC-3'	<i>irp2</i>	94°C, 30 s	60°C, 60 s	72°C, 60 s	280	36
Irp2(RP)	5'-TCG TCG GGC AGC GTT TCT TCT						
FyuA f	5'-TGA TTA ACC CCG CGA CGG GAA-3'	<i>fyuA</i>	94°C, 30 s	63°C, 60 s	72°C, 90 s	880	10
FyuA r	5'-CGC AGT AGG CAC GAT GTT GTA-3'						
Iha-I	5'-CAG TTC AGT TTC GCA TTC ACC-3'	<i>iha</i>	94°C, 30 s	56°C, 60 s	72°C, 90 s	1,305	34
Iha-II	5'-GTA TGG CTC TGA TGC GAT G-3'						
espI-I	5'-ATG GAC AGA GTG GAG ACA G-3'	<i>espI</i>	94°C, 30 s	52°C, 60 s	72°C, 60 s	560	34
espI-II	5'-GCC ACC TTT ATT CTC ACC A-3'						
btuB-I	5'-GCC CCT TCC CAC TGT TTA CT-3'	<i>btuB</i>	94°C, 30 s	55°C, 60 s	72°C, 90 s	1,032	34
btuB-II	5'-GGT ATT GAT TGA TGG AGT GCG-3'						
HlyA1	5'-GGT GCA GCA GAA AAA GTT GTA G-3'	EHEC <i>hlyA</i>	94°C, 30 s	57°C, 60 s	72°C, 90 s	1,550	30
HlyA4	5'-TCT CGC CTG ATA GTG TTT GGT A-3'						
esp-A	5'-AAA CAG CAG GCA CTT GAA CG-3'	<i>espP</i>	94°C, 30 s	56°C, 60 s	72°C, 60 s	1,830	3
esp-B	5'-GGA GTC GTC AGT CAG TAG AT-3'						
D1	5'-CGT CAG GAG GAT GTT CAG-3'	<i>etpD</i>	94°C, 30 s	56°C, 60 s	72°C, 70 s	1,062	31
D13R	5'-CGA CTG CAC CTG TTC CTG ATT A-3'						
SAADF	5'-CGT GAT GAA CAG GCT ATT GC-3'	<i>saa</i>	94°C, 30 s	52°C, 60 s	72°C, 40 s	119	25
SAADR	5'-ATG GAC ATG CCT GTG GCA AC-3'						
E643f	5'-TAT CAG GCC AAT CAA AAC AG-3'	<i>efa-1^f</i>	94°C, 30 s	50°C, 60 s	72°C, 60 s	974	9
E1598r	5'-AGA CAC TGG TAA ATT TCG C-3'						
E5242f	5'-TAA GCG AGC CCT GAT AAG CA-3'	<i>efa-2^f</i>	94°C, 30 s	55°C, 60 s	72°C, 60 s	630	9
E5854r	5'-CGT GTT GCT TGC CTT TGC-3'						
E7044f	5'-TGT CTA ACT GGA TTG TAT GGC-3'	<i>efa-3^f</i>	94°C, 30 s	56°C, 60 s	72°C, 60 s	685	9
E7710r	5'-ATG TTG TTC CCG GCC CAG T-3'						

^a All PCRs included 30 cycles, followed by a final extension of 5 min at 72°C.

^b *stx₁* and *stx_{1c}* were differentiated by restriction analysis with *FspI* and *HhaI* (40).

^c *stx_{2c}*, *stx_{2d}*, and *stx_{2e}*.

^d *stx₂* and *stx_{2c}* were differentiated by restriction analysis with *HaeIII* and *FokI* (28).

^e *stx_{2d}* is defined in this study as an *stx₂* variant amplified with primers VT2-cm and VT2-f (26) and does not refer to the intestinal mucus-activatable Stx2d toxin subtype (encoded by *stx₂* variants that have been classified as *stx_{2c}* [presented at the World Health Organization conference Shiga-Like Toxin Producing *Escherichia coli* with a Special Emphasis on Zoonotic Aspects, Giessen, Germany, (1991)] as defined by Melton-Celsa et al. (18).

^f Regions 113 to 1068, 4812 to 5324, and 6514 to 7180 of the *efal* gene (9) were targeted with the primer pairs E643f-E1598r (*efa-1*), E5242f-E5854r (*efa-2*), and E7044f-E7710r (*efa-3*), respectively.

TABLE 2. *stx* genotyping of *eae*-positive and *eae*-negative *E. coli* strains producing members of the Stx1 family

<i>stx</i> genotype	No. of strains among STEC	
	<i>eae</i> positive ^a	<i>eae</i> negative ^b
<i>stx</i> ₁	90	37
<i>stx</i> _{1c}	0	24
<i>stx</i> ₁ + <i>stx</i> ₂	51	9
<i>stx</i> _{1c} + <i>stx</i> ₂	0	1
<i>stx</i> ₁ + <i>stx</i> _{2c}	11	6
<i>stx</i> ₁ + <i>stx</i> ₂ + <i>stx</i> _{2c}	3	4
<i>stx</i> ₁ + <i>stx</i> _{2d}	0	8
<i>stx</i> _{1c} + <i>stx</i> _{2d}	0	51
Total	155	140

^a Serotypes (numbers of strains, if more than one, are in parentheses): O4:H[−], O25:H[−] (2), O26:H11 (29), O26:H[−] (10), O75:H[−], O84:H2 (3), O84:H[−], O92:H33, O103:H2 (14), O103:H18 (2), O103:H[−] (11), O111:H2 (4), O111:H8 (3), O111:H[−] (11), O118:H[−] (3), O119:H2, O120:H[−], O129:H[−], O145:H25 (3), O145:H[−] (9), O156:H[−], O157:H7 (17), O157:H[−] (23), Orough:H[−], ONT:H[−] (2).

^b Serotypes (numbers of strains, if more than one, are in parentheses): O3:H2, O3:H10, O5:H[−] (5), O8:H19, O8:H[−] (3), O22:H8 (4), O31:H[−], O62:H[−], O68:H4, O75:H8, O76:H19 (8), O78:H[−] (3), O91:H[−] (11), O91:H14 (5), O91:H21 (4), O104:H16, O111:H[−], O112:H2, O112:H[−], O113:H4 (10), O113:H[−], O115:H10 (2), O128:H2 (8), O128:H[−] (3), O136:HNT, O146:H8 (2), O146:H10, O146:H21 (6), O146:H20, O146:H31 (2), O146:H51, O146:H[−] (3), O152:H4, O153:H18 (3), O174:H2 (2), O174:H8 (6), O174:H[−] (2), O176:H[−], O178:H[−] (3), O178:H19 (3), O181:H16 (4), O181:H49, Orough:H2 (2), Orough:H11, Orough:H25, Orough:H[−] (8), Orough:HNT, ONT:H2, ONT:H14 (2), ONT:H21, ONT:H[−], ONT:HNT.

DISCUSSION

In this study, we subtyped *stx* genes in 295 STEC strains isolated from humans during the last 7 years. These strains were selected because of their positive results in PCR with primers KS7 and KS8, which detect a conserved region of both *stx*₁ and *stx*_{1c} B subunit genes. Therefore, these strains do not represent the whole spectrum of the *stx* genotypes present in STEC strains isolated in our laboratories (7). Specifically, all STEC strains containing *stx*₂ or *stx*₂ variants as sole *stx* alleles were not included in the analysis. We could demonstrate that Stx1c-producing *E. coli* strains represent a significant subset of *eae*-negative STEC strains isolated from humans but are absent from *eae*-positive STEC strains. Stx1c-producing *E. coli* strains belonged to a broad spectrum of serotypes. Characterization of these strains revealed that they had some potential virulence determinants in common. Ninety-seven percent of the isolates possessed the *saa* gene encoding the STEC autoagglutinating adhesin, and most of them also produced the EHEC hemolysin, which is an important cytotoxin (30). In addition, 65.8% of the 76 Stx1c-producing *E. coli* isolates possessed a recently identified pathogenicity island called the LPA (34). Moreover, the HPI, which was previously identified in STEC strains of serogroups O26 and O128 (14) and which is common in extraintestinal *E. coli* strains (36), was found in Stx1c-producing *E. coli* strains of six serotypes, demonstrating that it is more widely distributed among STEC strains than was previously shown. Each of the Stx1c-producing *E. coli* strains lacked the *eae* gene encoding intimin, which is typical for STEC O157 strains and non-O157 STEC strains that are associated with bloody diarrhea and HUS (2). However, despite the absence of *eae*, which was used as a marker for the LEE in this study, the majority of the Stx1c-producing *E. coli* strains

possess genes encoding one or two additional putative adhesins identified recently in LEE-negative STEC strains pathogenic for humans, including *Saa* (24) and the *Iha* homologue located within the pathogenicity island LPA (34).

The original identification of *saa* in a LEE-negative O113:H21 STEC strain which caused an HUS cluster in Australia (23), and its discovery in several other LEE-negative STEC strains isolated in sporadic cases of HUS (24) or bloody diarrhea (25), led to the hypothesis that *saa* may be a marker for a subgroup of LEE-negative STEC strains capable of causing severe gastrointestinal or systemic disease in humans (25). However, we demonstrated in this study that *saa* is nearly ubiquitous among STEC strains producing Stx1c, which are mostly associated with mild diarrhea or asymptomatic carriage. This suggests that the presence of *saa* itself cannot be considered to be a predictor that LEE-negative STEC strains will cause a severe disease. Rather, additional virulence characteristics, such as the Stx type or other, as yet unknown virulence loci, may contribute to the ability of LEE-negative STEC strains containing *saa* to cause HUS. Along these lines, it is worth noting that the HUS-associated, *saa*-positive STEC isolates investigated by Paton et al. (24) produced either a toxin closely related to classical Stx2 (23), which has been demonstrated to be a risk factor for HUS development (2, 7), or the mucus-activatable Stx (18), which confers high virulence on STEC strains in a streptomycin-treated-mouse model (18). In contrast, the STEC strains in which *saa* and Stx1c were found were never isolated from patients with HUS in our study. Actually, the only *stx*_{1c}-harboring STEC strain that was isolated from an HUS patient did not possess *saa* and contained *stx*₂ as an additional toxin gene. Also, we did not identify *stx*_{1c} in any of four LEE-negative STEC strains of serotype O91:H21, which has been reported to be associated with HUS (8, 24).

The data reported in this study have important practical implications. Although STEC strains harboring *stx*_{1c}, either alone or in combination with *stx*_{2d}, are frequently detected in stools from patients with diarrhea or asymptomatic carriers, we have not detected such strains among isolates from patients with HUS. Whether it is the *stx*_{1c} allele or the lack of another virulence determinant, such as *eae*, which underlies a milder disease is presently unknown. However, the detection of Stx1c might offer a marker to identify STEC strains that cause milder disease. Because of the inability of the presently available immunoassays to differentiate between Stx1 and Stx1c, there is also a need for the development of an Stx1c-specific commercial immunoassay. Such an assay would provide a simple tool for the identification of Stx1c in primary stool cultures and from single colonies, allowing investigations of the relationship between Stx1c production and the clinical course of infection in prospective studies. Such studies are necessary to verify the association of Stx1c-producing STEC strains with mild disease, as found in the present study and in previous studies (16, 40).

Different combinations of phenotypic and genetic characteristics were observed among the *stx*_{1c}-harboring strains investigated. These combinations enabled us to subtype strains within the same serotype, and they form a basis for the subtyping of such strains in epidemiological studies. Such subtyping, applied during an outbreak, enables identification of the source of the infection and tracing of the spread of an Stx1c-producing outbreak strain throughout the epidemiological chain. Moreover,

TABLE 3. Serotypes and virulence profiles of Stx1c-producing *E. coli* strains

Serotype ^a	Total no. of strains	No. of strains with virulence profile	Presence of virulence factor ^b				No. of strains with <i>stx</i> genotype:		
			LPA	HPI	EHEC <i>hly</i>	<i>saa</i>	<i>stx</i> _{1c}	<i>stx</i> _{1c} + <i>stx</i> _{2d}	<i>stx</i> _{1c} + <i>stx</i> ₂
O113:H4	10	7	+	—	+	+	1	9	0
		2	—	—	+	+			
		1	—	—	—	+			
O128:H2	8	6 (2 ^c)	+	+	+	+	0	8	0
		2 ^c	—	+	+	+			
O128:H [−]	1	1	+	—	+	+	1	0	0
O76:H19	8	7	—	—	+	+	7	1	0
		1	+	—	+	+			
O146:H21	6	4	+	—	+	+	0	6	0
		1	+	—	—	+			
		1	—	—	+	+			
O146:H8	2	1	—	—	+	+	2	0	0
		1	—	—	—	+			
O146:H31	2	2	+	—	+	+	0	2	0
O146:H51	1	1	+	—	+	+	1	0	0
O146:H [−]	2	2	+	—	+	+	1	1	0
O174:H8	6	3	+	—	—	+	2	4	0
		1 ^c	—	—	+	+			
		1 ^c	+	—	+	+			
		1	—	—	—	+			
O174:H [−]	2	2	+	—	—	+	0	2	0
O5:H [−]	5	5	+	—	+	+	0	5	0
O181:H16	4	3 ^c	+	—	+	+	1	3	0
		1 ^c	—	—	+	+			
O78:H [−]	3	3	+	+	+	+	3	0	0
O178:H [−]	3	2	+	—	+	+	0	3	0
		1	—	—	—	+			
O8:H19	1	1	—	—	—	—	1	0	0
O22:H8	1	1	—	—	+	—	0	1	0
O75:H8	1	1	—	+	+	+	0	1	0
O91:H [−]	1	1	—	+	+	+	0	1	0
O112:H2	1	1	+	—	+	+	1	0	0
O136:HNT	1	1	—	—	+	—	0	0	1
O176:H [−]	1	1	+	—	—	+	0	1	0
ONT:H2	1	1 ^c	+	+	+	+	0	1	0
ONT:H21	1	1	—	—	—	+	1	0	0
Orough:H2	2	2	+	+	+	+	0	2	0
Orough:H11	1	1	+	—	—	+	1	0	0
Orough:HNT	1	1	—	—	+	+	1	0	0

^a H[−], nonmotile; HNT, H nontypeable; ONT, O nontypeable; Orough, autoagglutinable.

^b +, presence of the virulence factor; —, absence of the virulence factor.

^c Non-sorbitol-fermenting isolate; all the other strains fermented sorbitol.

subtyping of *stx*_{1c}-harboring STEC strains isolated from sheep, which have been proposed to be the most probable reservoirs of these strains (16, 40), and their comparison with human isolates shed light on this species in the epidemiology of human infections caused by Stx1c-producing STEC strains. Such an approach could also contribute to clarifying the sources of such strains and infection routes.

In conclusion, we have demonstrated the significance of the phenotypic and genetic heterogeneity of Stx1c-producing STEC strains for the subtyping of such strains in epidemiological and clinical studies. Our data also warrant the investigation of the roles of the LPA and Saa in the pathogenesis of infections caused by these strains.

ACKNOWLEDGMENTS

This study was supported by a grant from the Bundesministerium für Bildung und Forschung (BMBF) Project Network of Competence Pathogenomics Alliance “Functional Genomic Research on Enterohaemorrhagic, Enteropathogenic and Enteroggregative *Escherichia*

coli (EHEC, EPEC, EAEC),” Project Group “Schmidt/Karch, Universitätsklinikum Münster” (BD numbers 119523 and 207800) and by a grant from BMBF Verbundprojekt, Forschungsnetzwerk “Emerging Foodborne Pathogens in Germany” no. 01KI 9903.

We thank Phillip I. Tarr (Washington University School of Medicine, St. Louis, Mo.) for critical reading of the manuscript and helpful discussions.

REFERENCES

1. Bielaszewska, M., and H. Karch. 2000. Non-O157:H7 Shiga toxin (verocytotoxin)-producing *Escherichia coli* strains: epidemiological significance and microbiological diagnosis. *World J. Microbiol. Biotechnol.* **16**:711–718.
2. Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, B. J. Johnson, and C. L. Gyles. 1999. Association between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.* **37**:497–503.
3. Brunder, W., H. Schmidt, and H. Karch. 1997. EspP, a novel extracellular serine protease of enterohaemorrhagic *Escherichia coli* O157:H7 cleaves human coagulation factor V. *Mol. Microbiol.* **24**:767–778.
4. Brunder, W., H. Schmidt, M. Frosch, and H. Karch. 1999. The large plasmids of Shiga-toxin-producing *Escherichia coli* (STEC) are highly variable genetic elements. *Microbiology* **145**:1005–1014.
5. Cebula, T. A., W. L. Payne, and P. Feng. 1995. Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their Shiga-like toxin type

- by mismatch amplification mutation assay-multiplex PCR. *J. Clin. Microbiol.* **33**:248–250.
6. Dicker, R. C. 1996. Analyzing and interpreting data, p. 92–131. In M. B. Gregg, R. C. Dicker, and R. A. Goodman (ed.), *Field epidemiology*. Oxford University Press, Inc., New York, N.Y.
 7. Friedrich, A. W., M. Bielaszewska, W.-L. Zhang, M. Pulz, T. Kuczius, A. Ammon, and H. Karch. 2002. *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. *J. Infect. Dis.* **185**:74–84.
 8. Ito, H., A. Terai, H. Kurazono, Y. Takeda, and M. Nishibuchi. 1990. Cloning and nucleotide sequencing of Vero toxin 2 variant genes from *Escherichia coli* O91:H21 isolated from a patient with the hemolytic uremic syndrome. *Microb. Pathog.* **8**:47–60.
 9. Janka, A., M. Bielaszewska, U. Dobrindt, and H. Karch. 2002. Identification and distribution of the enterohemorrhagic *Escherichia coli* factor for adherence (*efa1*) gene in sorbitol-fermenting *Escherichia coli* O157:H⁻. *Int. J. Med. Microbiol.* **292**:207–214.
 10. Johnson, J. R., and A. L. Stell. 2000. Extended virulence genotypes of *Escherichia coli* strains from patients with uresepsis in relation to phylogeny and host compromise. *J. Infect. Dis.* **181**:261–272.
 11. Karch, H., C. Janetzki-Mittmann, S. Aleksic, and M. Datz. 1996. Isolation of enterohemorrhagic *Escherichia coli* O157 strains from patients with hemolytic-uremic syndrome by using immunomagnetic separation, DNA-based methods, and direct culture. *J. Clin. Microbiol.* **34**:516–519.
 12. Karch, H., H.-I. Huppertz, J. Bockemühl, H. Schmidt, A. Schwarzkopf, and R. Lissner. 1997. Shiga toxin-producing *Escherichia coli* infections in Germany. *J. Food Prot.* **11**:1454–1457.
 13. Karch, H., H. Schmidt, and W. Brunder. 1998. Plasmid-encoded determinants of *Escherichia coli* O157:H7, p. 183–194. In J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D.C.
 14. Karch, H., S. Schubert, D. Zhang, W. Zhang, H. Schmidt, T. Ölschlager, and J. Hacker. 1999. A genomic island, termed “high pathogenicity island,” is present in certain non-O157 Shiga toxin-producing *Escherichia coli* clonal lineages. *Infect. Immun.* **67**:5994–6001.
 15. Karch, H., M. Bielaszewska, M. Bitzan, and H. Schmidt. 1999. Epidemiology and diagnosis of Shiga toxin-producing *Escherichia coli* infections. *Diagn. Microbiol. Infect. Dis.* **34**:229–243.
 16. Koch, C., S. Hertwig, R. Lurz, B. Appel, and L. Beutin. 2001. Isolation of a lysogenic bacteriophage carrying the *stx1_{OX3}* gene, which is closely associated with Shiga toxin-producing *Escherichia coli* strains from sheep and humans. *J. Clin. Microbiol.* **39**:3992–3998.
 17. March, S. B., and S. Ratnam. 1986. Sorbitol-MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *J. Clin. Microbiol.* **23**:869–872.
 18. Melton-Celsa, A. R., J. E. Rogers, C. K. Schmitt, S. C. Darnell, and A. D. O'Brien. 1998. Virulence of Shiga toxin-producing *Escherichia coli* (STEC) in orally-infected mice correlates with the type of toxin produced by the infecting strain. *Jpn. J. Med. Sci. Biol.* **51**:108–114.
 19. O'Brien, A. D., V. L. Tesh, A. Donohue-Rolfe, M. P. Jackson, S. Olsnes, K. Sandvig, A. A. Lindberg, and G. T. Keusch. 1992. Shiga toxin: biochemistry, genetics, mode of action, and role in pathogenesis. *Curr. Top. Microbiol. Immunol.* **180**:65–94.
 20. Ørskov, F., and I. Ørskov. 1984. Serotyping of *Escherichia coli*, p. 43–112. In T. Bergan (ed.), *Methods in microbiology*, vol. 1. Academic Press, London, United Kingdom.
 21. Paton, A. W., L. Beutin, and J. C. Paton. 1995. Heterogeneity of the amino-acid sequences of *Escherichia coli* Shiga-like toxin type-I operons. *Gene* **153**:71–74.
 22. Paton, A. W., A. J. Bourne, P. A. Manning, and J. C. Paton. 1995. Comparative toxicity and virulence of *Escherichia coli* clones expressing variant and chimeric Shiga-like toxin type II operons. *Infect. Immun.* **63**:2450–2458.
 23. Paton, A. W., M. C. Woodrow, R. M. Doyle, J. A. Lanser, and J. C. Paton. 1999. Molecular characterization of a Shiga-toxigenic *Escherichia coli* O113: H21 strain lacking *eae* responsible for a cluster of cases of hemolytic uremic syndrome. *J. Clin. Microbiol.* **37**:3357–3361.
 24. Paton, A. W., P. Srimanote, M. C. Woodrow, and J. C. Paton. 2001. Characterization of Saa, a novel autoagglutinating adhesin produced by locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infect. Immun.* **69**:6999–7009.
 25. Paton, A. W., and J. C. Paton. 2002. Direct detection and characterization of Shiga toxigenic *Escherichia coli* by multiplex PCR for *stx1*, *stx2*, *eae*, *exhA*, and *saa*. *J. Clin. Microbiol.* **40**:271–274.
 26. Piérard, D., G. Muyldermas, L. Moriau, D. Stevens, and S. Lauwers. 1998. Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. *J. Clin. Microbiol.* **36**:3317–3322.
 27. Prager, R., A. Fruth, and H. Tschäpe. 2003. Subtyping of pathogenic *Escherichia coli* strains using flagellar (H) antigens: serotyping vs. *fljC* polymorphisms. *Int. J. Med. Microbiol.* **292**:477–486.
 28. Rüssmann, H., H. Schmidt, J. Heesemann, A. Caprioli, and H. Karch. 1994. Variants of Shiga-like toxin II constitute a major toxin component in *Escherichia coli* O157 strains from patients with haemolytic uraemic syndrome. *J. Med. Microbiol.* **40**:338–343.
 29. Schmidt, H., H. Rüssmann, A. Schwarzkopf, S. Aleksic, J. Heesemann, and H. Karch. 1994. Prevalence of attaching and effacing *Escherichia coli* in stool samples from patients and controls. *Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis.* **281**:201–213.
 30. Schmidt, H., L. Beutin, and H. Karch. 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL 933. *Infect. Immun.* **63**:1055–1061.
 31. Schmidt, H., B. Henkel, and H. Karch. 1997. A gene cluster closely related to type II secretion pathway operons of gram-negative bacteria is located on the large plasmid of enterohaemorrhagic *Escherichia coli* O157 strains. *FEMS Microbiol. Lett.* **148**:265–272.
 32. Schmidt, H., C. Geitz, P. I. Tarr, M. Frosch, and H. Karch. 1999. Non-O157 pathogenic Shiga toxin-producing *Escherichia coli*: phenotypic and genetic profiling of virulence traits and evidence for clonality. *J. Infect. Dis.* **179**: 115–123.
 33. Schmidt, H., J. Scheef, S. Morabito, A. Caprioli, L. Wieler, and H. Karch. 2000. A new Shiga toxin variant (Stx2f) from *Escherichia coli* isolated from pigeons. *Appl. Environ. Microbiol.* **66**:1205–1208.
 34. Schmidt, H., W.-L. Zhang, U. Hemmrich, S. Jelacic, W. Brunder, P. I. Tarr, U. Dobrindt, J. Hacker, and H. Karch. 2001. Identification and characterization of a novel genomic island integrated at *selC* in locus of enterocyte effacement-negative, Shiga toxin-producing *Escherichia coli*. *Infect. Immun.* **69**:6863–6873.
 35. Schmitt, C. K., M. L. McKee, and A. D. O'Brien. 1991. Two copies of shiga-like toxin II-related genes common in enterohemorrhagic *Escherichia coli* strains are responsible for the antigenic heterogeneity of the O157:H⁻ strain E32511. *Infect. Immun.* **59**:1065–1073.
 36. Schubert, S., A. Rakin, H. Karch, E. Carniel, and J. Heesemann. 1998. Prevalence of the “high pathogenicity island” of *Yersinia* species among *Escherichia coli* strains that are pathogenic to humans. *Infect. Immun.* **66**: 480–485.
 37. Tarr, P. I., S. S. Bilge, J. C. Vary, S. Jelacic, R. L. Habeeb, T. R. Ward, M. R. Baylor, and T. E. Besser. 2000. Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect. Immun.* **68**:1400–1407.
 38. Weinstein, D. L., M. P. Jackson, J. E. Samuel, R. K. Holmes, and A. D. O'Brien. 1988. Cloning and sequencing of a Shiga-like toxin type II variant from an *Escherichia coli* strain responsible for edema disease of swine. *J. Bacteriol.* **170**:4223–4230.
 39. Wong, C. S., S. Jelacic, R. L. Habeeb, S. L. Watkins, and P. I. Tarr. 2000. The risk of the hemolytic-uremic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *N. Engl. J. Med.* **342**:1930–1936.
 40. Zhang, W., M. Bielaszewska, T. Kuczius, and H. Karch. 2002. Identification, characterization, and distribution of a Shiga toxin 1 gene variant (*stx1c*) in *Escherichia coli* isolated from humans. *J. Clin. Microbiol.* **40**:1441–1446.
 41. Zhang, W.-L., M. Bielaszewska, A. Liesegang, H. Tschäpe, H. Schmidt, M. Bitzan, and H. Karch. 2000. Molecular characteristics and epidemiological significance of Shiga toxin-producing *Escherichia coli* O26. *J. Clin. Microbiol.* **38**:2134–2140.