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Phylogenetic and Molecular Analysis of Food-Borne Shiga Toxin-Producing *Escherichia coli*

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Seventy-five food-associated Shiga toxin-producing *Escherichia coli* (STEC) strains were analyzed by molecular and phylogenetic methods to describe their pathogenic potential. The presence of the locus of proteolysis activity (LPA), the chromosomal pathogenicity island (PAI) PAI I$_{CL3}$, and the autotransporter-encoding gene *sabA* was examined by PCR. Furthermore, the occupation of the chromosomal integration sites of the locus of enterocyte effacement (LEE), *selC*, *pheU*, and *pheV*, as well as the Stx phage integration sites *yehV*, *yecE*, *wrbA*, *z2577*, and *ssrA*, was analyzed. Moreover, the antibiotic resistance phenotypes of all STEC strains were determined. Multilocus sequence typing (MLST) was performed, and sequence types (STs) and sequence type complexes (STCs) were compared with those of 42 hemolytic-uremic syndrome (HUS)-associated enterohemorrhagic *E. coli* (HUSEC) strains. Besides 59 STs and 4 STCs, three larger clusters were defined in this strain collection. Clusters A and C consist mostly of highly pathogenic *eae*-positive HUSEC strains and some related food-borne STEC strains. A member of a new O26 HUS-associated clone and the 2011 outbreak strain *E. coli* O104:H4 were found in cluster A. Cluster B comprises only *eae*-negative food-borne STEC strains as well as mainly *eae*-negative HUSEC strains. Although food-borne strains of cluster B were not clearly associated with disease, serotypes of important pathogens, such as O91:H21 and O113:H21, were in this cluster and closely related to the food-borne strains. Clonal analysis demonstrated eight closely related genetic groups of food-borne STEC and HUSEC strains that shared the same ST and were similar in their virulence gene composition. These groups should be considered with respect to their potential for human infection.

Shiga toxin-producing *Escherichia coli* (STEC) strains can cause serious human infections ranging from mild diarrhea to life-threatening hemolytic-uremic syndrome (HUS) (1, 2). STEC strains produce numerous virulence factors, with Shiga toxin (Stx) being the most critical one (3, 4). Typically, clinically relevant STEC strains express one or more Shiga toxins and carry the locus of enterocyte effacement (LEE) and a large plasmid encoding at least the secretory protein EspP and/or the hemolysin Ehx (3, 5, 6). Besides these, LEE-negative STEC strains have caused sporadic cases and outbreaks of HUS (7–10). For a detailed categorization of clinical STEC strains, a collection of 524 STEC strains from patients with HUS was characterized, multilocus sequence typed (MLST), and deposited in the HUS-associated enterohemorrhagic *E. coli* (EHEC) (HUSEC) collection (11). This collection comprises 42 STEC isolates from HUS patients representing all 36 serotypes present in the 524 isolates and all 32 sequence types (STs) identified within these serotypes (www.ehec.org).

The most important pathway of STEC transmission to humans is the ingestion of raw or undercooked foods, such as meat or milk products (12, 13). In 2011, a large outbreak occurred in Germany with the STEC serotype O104:H4, which carried an untypical virulence gene composition on an enteroaggregative *E. coli* backbone (14). This resulted in 53 deaths and more than 800 cases of HUS (15). Sprout seeds were epidemiologically linked to the origin of the outbreak (16–18).

In a study by Werber et al. (19), it was shown that about two-thirds of serogroups from food-associated STEC strains were also present in patient isolates. However, serotypes which have not yet been isolated from patients were also found. STEC strains are genetically very heterogeneous and display enormous genome plasticity (20–23). The virulence profiles of STEC strains isolated from food differ often from those of patient isolates, but a clear estimation of the pathogenicity of food-associated strains was not yet able to be made. Beutin et al. (24) discussed that food-borne STEC strains, which produce only Stx2e, may be considered harmless, because human cases connected with isolates with *stx*$_{2e}$ are only rarely described. However, Bielaszewska et al. (25) described classical Stx2 and activatable Stx2d as the major toxin types found in serious EHEC infections. In a study of Slanec et al. (26), these toxin types were within the major types found in a collection of food-borne STEC isolates. In addition, the EHEC O104:H4 outbreak strain did not fulfill common criteria of a high-risk STEC clone but had a quite high mortality rate. The facts that it did not contain the locus of enterocyte effacement and that it had an enteroaggregative core genome hindered the categorization into classic EHEC subtypes (14, 27, 28). Therefore, differentiation of STEC in harmless and harmful representatives based only on the classification of virulence gene markers is difficult.

Virulence traits of STEC are organized mainly on mobile genetic elements (22, 29), such as plasmids, prophages, and pathogenicity islands (PAIs). Prophages are able to integrate into different chromosomal insertion sites dependent on the availability of their preferred site in the host strain (30, 31). A number of target sites, such as *yehV* (32), *yecE* (33), *wrbA* (31, 34), *z2577* (35, 36), *ssrA* (37), and *sbcB* (38), have been described.

Pathogenicity islands also prefer specific integration sites. The locus of enterocyte effacement (LEE) is a pathogenicity island of ~35 kb and harbors the *eae* gene and a type III secretion machinery whose products are responsible for attaching and effacing lesions on epithelial cells (39, 40). Horizontal transfer of the LEE is possible (41), and comparative analysis of the flanking regions showed chromosomal integration in insertion loci restricted to the tRNA genes *selC*, *pheU*, and *pheV* (42).

A virulence determinant described for LEE-negative STEC is the PAI I$_{CL3}$ (43, 44), which is a hybrid pathogenicity island containing parts of the EDL933 O island 48, O island 122, and other putative virulence factors, such as a hemolysin and adhesin cluster similar to that of *Yersinia pestis* (43). The locus of proteolysis activity (LPA), a genomic island of about 33 kb, was shown to insert near *selC* in *eae*-negative strains containing the Shiga toxin variant *stx*$_{2d}$ (45). Another virulence factor of LEE/*eae*-negative strains is the plasmid-borne *sab* gene, which encodes a protein with features of an autotransporter that supports the adherence to human epithelial (HEp-2) cells and biofilm formation (46). The variety of available virulence genes and the high extent of gene transfer make it difficult to define pathogenic groups, since it became clear that groups with novel gene combinations, as in the O104:H4 outbreak, may rapidly develop (18).

The aim of the study was a further characterization of food-associated STEC isolates from a former study with regard to their potential risk for human infection. Therefore, their phage and LEE integration sites and the presence of three virulence-associated gene loci were examined. Moreover, their phylogenetic distribution and population structure were analyzed by MLST and compared to those of the HUSEC strain collection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Seventy-five food-associated STEC strains were from an earlier study (26). Strains HUSEC003, HUSEC004, HUSEC007, HUSEC008, HUSEC009, HUSEC018, HUSEC019, HUSEC025, HUSEC026, HUSEC028, HUSEC032, and HUSEC034 originate from the HUSEC collection (University of Münster, Germany; www.ehec.org). All strains were cultured in Luria Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7) or on LB agar plates containing 1.5% agar at 37°C. Subtyping of *stx* genes was performed as recently described (47), and some *stx* genes were reclassified from Slanec et al. (26) according to the nomenclature of Scheutz et al. (47).

Multilocus sequence typing. Defined fragments of the seven housekeeping genes (loci) *adk*, *icd*, *fumC*, *recA*, *mdh*, *gyrB*, and *purA* were amplified and sequenced as described previously (11, 20) using oligonucleotides published by Ewers et al. (48). New sequence types (STs) were submitted to the MLST database (http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli). STs were assigned dependent on the allelic profile of each strain and summarized to ST complexes (STCs) defined by at least three STs that differ from their nearest neighbor by no more than one of the seven alleles. MLST data for the HUSEC collection were obtained from www.ehec.org. Based on these data, a minimum spanning tree was generated with SeqSphere software version 0.9.38 beta (Ridom GmbH, Münster, Germany).

DNA amplification and sequencing. All strains were investigated for the insertion of foreign DNA in particular sites by PCR. Therefore, the known LEE and phage integration site genes *selC*, *pheU*, *pheV*, *yehV*, *yecE*, *wrbA*, *ssrA*, and *z2577* (31, 32, 35, 37, 42) were amplified with oligonucleotides and PCR conditions given in Table 1. In addition, the presence of PAI I$_{CL3}$, the locus of proteolysis, and the *sab* gene (44–46) was detected by PCR with the oligonucleotides and PCR conditions given in Table 1. PCR for the detection of *katP* and *etpD* was performed as described previously using the primers w-katF/wkat-B and D1/D13 (49).

PCR was performed in a total volume of 25 μl containing 1.5 U *Taq* DNA polymerase (Genaxxon Bioscience, Germany), 200 μM concentrations of each deoxynucleoside triphosphate (dNTP) (Promega GmbH, Germany), 0.4 μM of each oligonucleotide (Eurofines MWG Operon, Germany), 1-fold PCR buffer S (Genaxxon Bioscience, Germany), and 5 μl 10-fold diluted template DNA (DNA of approximately 10^6 bacterial cells from overnight culture) obtained by heat lysis for 10 min at 95°C.

Antimicrobial susceptibility testing. Antimicrobial susceptibility of strains was tested against 16 antimicrobial substances and antimicrobial combinations by determining the MIC according to the guidelines of the European Committee on Antimicrobial Susceptibility Testing (EUCAST), and breakpoints were applied as described by EUCAST (www.eucast.org). Antibiotics tested are ampicillin (AMP), chloramphenicol (CMP), cefoxitin (COX), cefotiam (CTM), gentamicin (GEN), kanamycin (KAN), mezlocillin (MEZ), mezlocillin-sulbactam (MSU), oxytetracycline (OTE), sulfamethoxazole (SMZ), streptomycin (STR), trimethoprim-sulfamethoxazole (SXT), ceftazidime (CAZ), ciprofloxacin (CIP), nalidixic acid (NAL), and cefotaxime (CTX).

RESULTS

Investigation of LEE integration sites. Since 71/75 food-borne STEC strains are *eae* negative (26), the question of whether the integration sites for the LEE were occupied by foreign DNA or remain empty arose. In this context, the tRNA genes *selC*, *pheU*, and *pheV*, which function as LEE integration sites, were investigated. For the 71 *eae*-negative strains, *selC* was occupied in 54% (38 strains), resulting in negative *selC* PCR results, *pheU* was occupied in 31% (22 strains), and eight (11%) strains resulted in PCR products with larger sizes of approximately 1.5 kb (1 strain) and 1.7 kb (7 strains) instead of the 664-bp size. The highest percentage of occupation was found for *pheV*, with 72% (51 strains) including two strains [WA (WT)5555/08 and LM14960/08] with variable results in repeated analyses. For the four *eae*-positive strains, *selC* was found to be occupied in two cases, *pheU* was occupied in one, and *pheV* was occupied in four of the strains. Therefore, one strain gave a negative result for *selC*, *pheU*, and *pheV* and one strain gave a negative result for *selC* and *pheV*, a finding which keeps open which integration site is used by the LEE. In 13 *eae*-negative strains, all PCRs were negative, meaning that all three sites are occupied by foreign DNA, and 5 strains were positive, meaning that all three sites were empty. All in all, there was a high frequency of occupied integration sites and the strains showed variability in the number and combination of occupied sites.

Analysis of Stx phage integration sites. The five prominent Stx phage integration sites *yecE*, *yehV*, *wrbA*, *z2577*, and *ssrA* were analyzed in all food-associated STEC strains for the integration of DNA fragments. The gene *yecE* was interrupted in 12% (9 strains), *yehV* was interrupted in 3% (2 strains), *wrbA* was interrupted in 15% (11 strains), *z2577* was interrupted in 4% (3 strains), and *ssrA* was interrupted in 28% (21 strains). In summary, 24 strains (32%) had one occupied phage integration site, eight strains (10.6%) had two, two strains (2.6%) had three, and none had either four or all

TABLE 1 Oligonucleotides and PCR conditions used for amplification of LEE and phage integration genes and virulence-associated genes

Target	Oligonucleotide	Nucleotide sequence (5′–3′)	PCR conditions	Reference
selC	K260_for K259_rev	GAGCGAATATTCCGATATCTGGTT CGCCGATTTTTCTTAGCCCA	94°C for 5 min, 30 cycles of 94°C for 90 s, 60°C for 90 s, and 72°C for 120 s, 72°C for 5 min	68
pheU	pheU_for (no. 7) pheU_rev (no. 11)	TTCACCCACGAACTGTTAACC AAATCTCATCAGTCGCCGTTC	95°C for 1 min, 33 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, 72°C 4 min	69
pheV	pheV_for (no. 1) pheV_rev (no. 6)	ACTTCACCGCATGAGCAGTAA GTGCAGCGGCTGGAGTTTGGA	95°C for 1 min, 33 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 60 s, 72°C 4 min	69
yehV	yehV_for yehV_rev	AAGTGGCGTTGCTTTGTGAT AACAGATGTGTGGTGAGTGTCTG	94°C for 5 min, 30 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, 72°C for 5 min	32 (modified)
yecE	yecE_for yecE_rev	CGAAGACGCCTGTAGTGCC CGCAGGGAGAAAACCAACTC	94°C for 1 min, 33 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 90 s, 72°C for 4 min	31
wrbA	wrbA_for wrbA_rev	ATGGCTAAAGTTCTGGTG CTCCTGTTGAAGATTAGC	94°C for 1 min, 30 cycles of 94°C for 60 s, 50°C for 90 s, and 72°C for 30 s, 72°C for 5 min	34 (modified)
ssrA	ssrA_for/B2657 ssrA_rev/B2656	TAGTATTGTGTAACCGCTCATT TGGGGGTAGTGGTAAAAATGAC	94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s, 72°C for 5 min	37
z2577	z2577_for z2577_rev	AACCCCATTGATGCTCAGGCTC TTCCCATTTTACACTTCCTCCG	94°C for 1 min, 30 cycles of 94°C for 60 s, 55°C for 90 s, and 72°C for 60 s, 72°C for 5 min	35
PAI I$_{CL3}$	ms1_for ms1_rev	GCTGATGCGTTACCACACTG GTAATCCTCAACCGCACCAG	94°C for 5 min, 30 cycles of 94°C for 30 s, 53°C for 60 s, and 72°C for 60 s, 72°C for 5 min	44
PAI I$_{CL3}$	ms2_for ms2_rev	GACTGCAGGCGTGGTTAAC TGTATTGTCTGCGCTTCCAG	94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s, 72°C for 5 min	44 (modified) 44
PAI I$_{CL3}$	ms3_for ms3_rev	GCGTGTAGCAGCTCATGCAG GACAACACTGACCGGATAATC	94°C for 5 min, 30 cycles of 94°C for 30 s, 57°C for 60 s, and 72°C for 60 s, 72°C for 5 min	44
PAI I$_{CL3}$	ms4_for ms4_rev	CCTGAAGTGACCGTGAAACAG GTCGGCCGTCACCTTAATAC	94°C for 5 min, 30 cycles of 94°C for 30 s, 57°C for 60 s, and 72°C for 60 s, 72°C for 5 min	44
PAI I$_{CL3}$	ms5_for ms5_rev	CTCCGGACGTACAGGAATATC GTTGGCGTTATCTGACATAC	94°C for 5 min, 30 cycles of 94°C for 30 s, 53°C for 60 s, and 72°C for 60 s, 72°C for 5 min	44
selC-int (locus of proteolysis activity)	selC_F1 int_F1	GTTCGACTCCTGTGATCTTCC ATCGGGGTATACGCCAATAG	94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s, 72°C for 4 min	This work
iha-btuB (locus of proteolysis activity)	iha-B btuB-A	CGTGATGGTGATAACAAAGG CGGAAAAGAGTAAACAGTGG	94°C for 5 min, 30 cycles of 94°C for 30 s, 52°C for 60 s, and 72°C for 90 s, 72°C for 5 min	45 (modified)
sab	LH0147_for LH0147_rev	GGTGGTACAGCAGGTAATG TATCTCACCACCTGCTATCG	94°C for 5 min, 30 cycles of 94°C for 30 s, 53°C for 60 s, and 72°C for 60 s, 72°C for 5 min	46

five analyzed integration sites occupied. All phage integration sites were empty for 41 strains (54.6%). There was no correlation found with the presence of particular *stx* genes in the investigated strains.

Virulence-associated genes. The PAI I$_{CL3}$, which is 27 kb in size, was described by five segments from 324 bp to 880 bp (ms-1 to ms-5) (44). Segments ms-1 and ms-2 were detected together in 35 strains (47%), while ms-3 and ms-5 PCRs were positive in 19 cases each (25%), with one strain difference. Segment ms-4 was found in 30 strains (40%). There were 18 strains (24%) with all segments positive and 34 strains (45%) missing the whole pathogenicity island. Five further different combinations of the present segments were found: six strains with only ms-4, 11 strains with ms-1 and ms-2, one strain with ms-1 to ms-4, one strain missing only ms-3, and four strains with ms-1, ms-2, and ms-4 (see Table S1 in the supplemental material). In summary, high variability was shown for the PAI I$_{CL3}$ segments in the STEC strains analyzed. The locus of proteolysis activity was represented by PCR products of the oligonucleotides selC_F1/int_F1 and iha-B/btuB-A, which detected the left site and the core region of this genomic island, respectively (45). Both regions were present in 17 strains (23%). The left site with parts of the *selC* gene was detectable in 26 strains (35%). Three strains (4%) produced a larger PCR product of about 750 bp, and two strains (LM 14957/08 and TS10/08) revealed a smaller PCR product of about 370 bp instead of 498 bp for the *selC-int* region which was seen as a negative result and not further analyzed. The core region of *iha* and *btuB* gave a PCR product in 30 strains (40%). Eight strains were positive only for the left site, while 12 strains (16%) gave a signal only for the core region. Thirty-four strains were negative for both regions (45%).

The *sab* gene was detectable in three strains (4%) of serotypes O179:H8 (2 strains) and O153:HNT (1 strain).

Characteristics of the most frequent serotype, O113:H21 (8 strains), of the analyzed food-associated strains were the occupation of *selC* (3/8), *pheU* (1/8), and *pheV* (8/8) but none of the phage integration sites *yecE*, *yehV*, *wrbA*, *z2577*, and *ssrA*. The PAI I$_{CL3}$ was complete in all strains, while *sab* was not present in any strain. For the locus of proteolysis activity, 50% of the PCRs were positive for the *selC-int* (4/8) and *iha-btuB* (4/8) regions.

Antibiotic susceptibility testing. Of the 75 food-associated STEC strains, 53 (71%) were susceptible to all antibiotics tested. Of the other 22 strains (29%), eight were resistant to either sulfamethoxazole (7/22) or tetracycline (1/22), four strains were resistant to two antibiotics, two strains were resistant to three antibiotics, and eight strains were resistant to four or more antibiotics (Table 2). Of these strains, TS25/08 and TS29/08 were resistant to seven antibiotics, namely, AMP, CMP, MEZ, OTE, SMZ, STR, and SXT. The most frequent resistance phenotype was against SMZ, found in different combinations for 21 of 22 resistant strains, followed by STR (10/22) and CMP (7/22) as well as SXT (7/22) (Table 2).

Phylogenetic characterization of STEC/HUSEC strains by MLST. In order to characterize the phylogenetic relationship of the food-associated STEC strains and to assess the potential risk for human infection, we compared them by MLST analysis with all 42 strains of the HUSEC collection, containing highly pathogenic STEC strains.

Fifty-nine STs were obtained within the 117 used STEC/HUSEC strains, with 35 STs from the 75 analyzed food strains and 32 STs from the HUSEC collection. Eight STs were identical in the

**TABLE 2** Resistance profiles of food-associated STEC strains

Strain no.	Resistance profile[a]
TS01/07	OTE
18692/1	SMZ
03353/1	SMZ
28504/1	SMZ
13762/1	SMZ
TS03/08	SMZ
LM16092/08	SMZ
TS10/08	SMZ
E918	CMP, SMZ
TS05/08	SMZ, STR
E917	SMZ, STR
CB11597	SMZ, SXT
E921	OTE, SMZ, STR
TS28/08	SMZ, STR, SXT
TS10/07	AMP, MEZ, SMZ, STR
TS07/08	AMP, CMP, MEZ, OTE, SMZ
TS14/08	CMP, OTE, SMZ, STR, SXT
TS15/08	CMP, OTE, SMZ, STR, SXT
RF1a	AMP, MEZ, OTE, SMZ, STR, SXT
TS06/07	CMP, GEN, KAN, SMZ, STR, SXT
TS25/08	AMP, CMP, MEZ, OTE, SMZ, STR, SXT
TS29/08	AMP, CMP, MEZ, OTE, SMZ, STR, SXT

[a] For abbreviations, see Materials and Methods.

two groups. ST10 (9 strains) and ST56 (9 strains) were most frequently found, followed by ST23 (5 strains) and ST297 (5 strains) (Fig. 1). Further, six STs (ST17, ST21, ST201, ST223, ST443, and ST446) were detected in four strains each and three STs (ST11, ST29, and ST442) were detected in three strains each, while 10 STs were found in two strains each and 36 STs were found only once. There were four STCs identified. The most frequent was STC155 (14 strains; ST56 [*n* = 9], ST223 [*n* = 4], ST58 [*n* = 1]), followed by STC10 (12 strains; ST10 [*n* = 9], ST34 [*n* = 1], ST43 [*n* = 1], ST330 [*n* = 1]) (Fig. 1). Additional strains/STs were assigned to the already-described STCs STC29 (11 strains), STC23 (6 strains), and STC469, STC20, and STC11 (5 strains each). A total of 41 strains were not able to be assigned to an already known STC.

For many strains, the serotype correlated well with the ST, meaning that strains with the same serotype have the same sequence type. Serotype O8:H19 was found only with ST201 (4 strains), O178:H19 was found only with ST443 (4 strains), and O22:H8 was found only with ST446 (4 strains). Serotype O113:H21 (8 strains) shared two STs (ST56 and ST223) but was represented exclusively by STC155.

Beyond the STs and STCs, we were able to assign three phylogenetic clusters, containing strains with characteristic properties. Cluster A comprises strains with the well-defined serogroups O26, O111, O103, and O145 as well as the 2011 outbreak strain of serotype O104:H4. Cluster C consists of *E. coli* O157 and O145 strains (cluster C). Members of both clusters are predominantly *eae* positive. STEC food isolates that are *eae* positive and belong to these clusters should be considered potentially harmful. The third cluster (cluster B) in the middle of the tree consists mainly of *eae*-negative food-borne isolates and some *eae*-negative HUSEC strains. This cluster is not uniform, and the strains are quite heterogeneous in their virulence gene composition. Although *eae*-negative STEC strains are considered low risk, we located STEC O113:H21 strains in this cluster which have caused an HUS out-
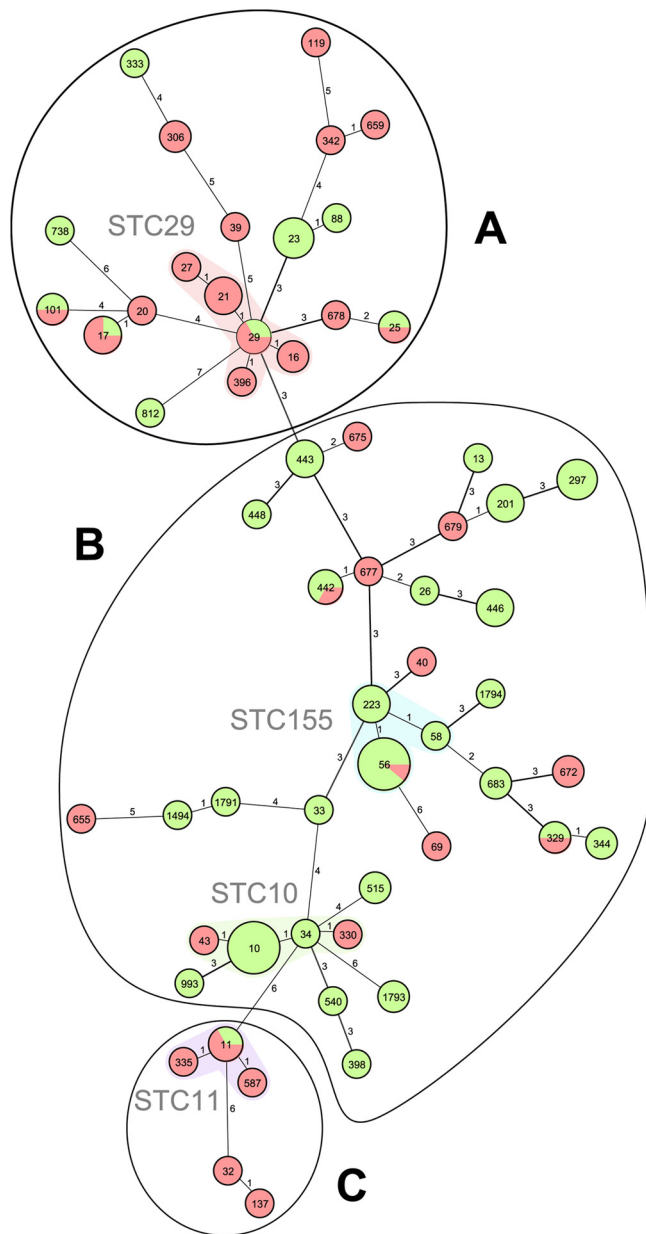
FIG 1 Minimum spanning tree based on MLST data of the analyzed 75 STEC food strains (green) in comparison to the human HUSEC strain collection (red). Each circle represents a sequence type (ST), and the circle size corresponds to the number of strains with the respective ST. Lines connecting the circles show the numbers of different alleles between two STs. Sequence type complexes (STCs) are depicted in light colors. Framed in black are the three phylogenetic clusters, labeled A, B, and C.

break in Australia in 1998 ([8]) and an O91:H21 strain of ST33, the serotype of which has been associated with diarrhea ([50]). For this group, the risk factors are largely unknown, and with respect to their infectious risk, such strains should be investigated with care.

Comparison of food-associated STEC and HUSEC strains. Phylogenetic comparison of the MLST data revealed eight groups of HUSEC and food-associated strains with the same ST ([Table 3]). The largest group based on ST56 contained HUSEC026 (O113:H21) and eight food-associated STEC strains of serotype O113:

H21 and other serotypes, of which two (TS14/08 and TS15/08) lacked all common virulence genes investigated by Slanec et al. ([26]) and in this study. The second-largest group was defined by ST17, including one OR- and two O103 HUSEC strains (HUSEC007, HUSEC008, and HUSEC009) and the food-associated O103:H2 STEC strain LM15814/08. Three groups contained three strains each: the ST11 group, including HUSEC003 and HUSEC004 with serotypes O157:H7 and O157:NM as well as the food-associated O157:H7 strain TS10/08; the ST29 group, including HUSEC018 and HUSEC019 and the food strain LM 14957/08 (strains HUSEC018 and LM14957/08 were both of serotype O26:H11); the ST442 group, including HUSEC034 and the two food isolates 17584/1 and LM3024/08. In this group, a HUSEC strain and a food isolate also shared the serotype O91:H21 ([Table 3]). Furthermore, three groups consisting of a single HUSEC strain and a single food-associated STEC strain, defined by ST25, ST101, and ST329, were detected. There were, in addition, 15 further HUSEC strains, with only one nucleotide difference from food strains in the analyzed MLST genes, which were not further investigated ([Fig. 1]).

Comparing the analyzed integration sites and virulence genes for HUSEC and food strains of the same ST, there is little correlation, but it was noticed that for ST23, four of the five strains had the same unique combination of the 12 virulence characteristics while one strain showed a different result only in one PCR. Similar results were obtained for ST223 and ST443, for which three of four strains had the same combination and one strain had one difference. For the eight ST pairs from food and HUSEC strains, there were the same combinations of present and absent genes/PCR products for HUSEC003 and TS10/08 (ST11), HUSEC018 and LM14957/08 (ST29), and HUSEC026 in comparison with TS21/08 and TS23/08 (ST56) ([Table 3]). Therefore, several particular human strains (HUSEC collection) were found to share their ST with the analyzed food strains, and some STs shared more than one strain from food or human.

In the framework of this study, the *stx* genes of STEC food-associated strains belonging to the eight groups mentioned above have been reclassified according to the new nomenclature published by Scheutz et al. ([47]). It is obvious that in some of the HUSEC/food pair groups, the same *stx* type was present. In some groups, the respective food/HUSEC strains differed by an additional toxin gene, and some groups had a different *stx* composition ([Table 3]). Although virulence gene composition and *stx* types of the HUSEC and food-associated strain pairs differed frequently, strains of STs 11, 17, 29, 101, 56, 25, 329, and 442 should be considered potentially harmful regardless of their origin.

Interestingly, the STEC O26 isolate LM14957/08 of ST29 from a wild boar carries the stx_{2a} gene only and is EHEC hemolysin gene (*e-hlyA*) positive and *espP* negative ([26]), similar to a new emerging O26 clone associated with the development of HUS in Europe ([51]). We therefore performed *katP*- and *etpD*-specific PCR to complete the plasmid profile published in this paper. Whereas the *katP*-specific PCR was negative, the *etpD* gene was present (data not shown). Therefore, LM14957/08 shows the same plasmid gene profile as this O26 EHEC clone.

DISCUSSION

The aim of this study was to perform a further molecular characterization of food-borne STEC strains and to compare their genetic structure with that of strains of the HUSEC collection and to

TABLE 3 PCR typing of virulence gene markers for eight pairs of food-associated strains and strains of the HUSEC collection sharing the same sequence type

ST^a	Strain	Serotype	*eae*	*selC*	*pheU*	*pheV*	*yecE*	*yehV*	*wrbA*	*z2577*	*ssrA*	*ms-1*[b]	*ms-2*[b]	*ms-3*[b]	*ms-4*[b]	*ms-5*[b]	*selC-int*	*iha-btuB*	*sab*	$stx_1{}^c$	$stx_2{}^c$
11	HUSEC003	O157:H7	+	−	+	+	+	−	+	+	+	−	−	−	−	−	−	−	−	−	2a
	HUSEC004	O157:NM	+	−	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	−	2a
	TS10/08	O157:H7	+	−	+	−	+	−	+	+	+	−	−	−	−	−	−	−	−	−	2; lost
17	HUSEC007	O103:H2	+	−	+	−	+	+	+	+	−	−	−	−	−	−	+	−	−	−	2a
	HUSEC008	O103:NM	+	−	+	−	−	+	+	+	−	−	−	−	−	−	+	−	−	−	2a
	HUSEC009	OR:H2	+	−	+	−	−	+	+	+	−	−	−	−	−	−	+	−	−	−	2a
	LM15814/08	O103:H2	+	+	+	−	+	+	+	+	+	−	−	−	+	−	−	−	−	1a	−
29	HUSEC018	O26:H11	+	−	−	−	+	+	−	+	−	−	−	−	−	−	−	−	−	−	2a
	HUSEC019	OR:H11	+	−	−	−	+	−	+	+	−	−	−	−	−	−	−	−	−	1a	−
	LM14957/08	O26:H11	+	−	−	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	2a
101	HUSEC025	O55:HNT	−	−	−[d]	+	−	+	+	+	+	−	−	−	−	−	+	−	−	1a	−
	K30	O55:HNT	−	−	−[d]	+	−	+	+	+	+	−	−	−	−	−	+	−	−	1a	−
56	HUSEC026	O113:H21	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	2d
	TS03/07	O113:H21	−	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	1a	2d
	CB11597	O113:H21	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	2d
	LM14603/08	O21:H21	−	−	+	+	+	+	+	+	+	+	+	−	−	−	−	+	−	−	2b
	LM16092/08	O21:H21	−	−	−	−	+	+	+	+	+	+	+	−	−	−	−	+	−	−	2b
	TS14/08	O153:NM	−	+	+	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	stx_2 NT
	TS15/08	ONT:H4	−	+	+	−	+	+	+	+	+	−	−	−	−	−	−	−	−	−	stx_2 NT
	TS21/08	O113:H21	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	2d
	TS23/08	O113:H21	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	2d
25	HUSEC028	O128:H2	−	−	−	−	−	+	+	−	−	−	−	−	+	−	+	+	−	1c	2b
	LM 27558 Stx2	OR:H43	−	−	−	−	+	+	−	+	−	−	−	−	+	−	+	+	−	−	2b
329	HUSEC032	O136:NM	−	+	+	+	−	+	+	+	+	−	−	−	−	−	−	−	−	1c	2a
	13477/1	O136:H16	−	−	+	−	+	+	+	+	+	−	−	+	+	−	−	−	−	1a	−
442	HUSEC034	O91:H21	−	+	+	−	+	+	−	+	−	+	+	+	+	+	−	+	−	1a	2a, 2d
	17584/1	O91:H21	−	−	+	−	+	+	+	−	−	+	+	+	+	+	+	+	−	1a	2a, 2d
	LM3034/08	O146:H21	−	−	−	−	+	+	+	+	−	+	+	−	+	−	+	+	−	1c	2b

[a] ST, sequence type.
[b] ms-1 to ms-5, oligonucleotides for PAI I_{CL3} segments.
[c] *stx* nomenclature according to Scheutz et al. (47). NT, not typeable.
[d] Unspecific PCR product.

assess the potential infectious risk for food-associated STEC strains.

The PCR analyses of LEE integration sites in *eae*-negative STEC strains showed negative results in 50% of the cases, suggesting that these sites are occupied with foreign DNA. A study by Bertin et al. (52) supports this suggestion. They localized the insertion sites and determined the pathotype of LEE-positive STEC strains. During these studies, they found isolates with altered tRNA loci by insertion of integrase genes, which led them to the suggestion that additional foreign DNA may be integrated here. This was already shown for different PAIs with the respective CP4-*int* integrase genes (23, 53–55).

There are several studies which demonstrated different PAIs in *selC*, *pheU*, and *pheV*. For example, *selC* may also be the integration site for the locus of proteolysis activity (45). In our study, in 40% of the occupied *selC* tRNA genes, the marker genes for the LPA *selC-int* and *iha-btuB* regions were both positive. Schmidt et al. (45) mentioned an exclusive distribution in the STEC subgroup of *eae*-negative strains with the *stx* variant stx_{2d}; this combination with the LPA genes was detected here in eight strains of different serotypes. For each of *pheU* and *pheV*, an integrase gene was also detected (52), and there are other PAIs and DNA fragments noted in these integration sites (41, 55–57). Jores et al. (58) found a new PAI in *pheV* with insertion sequence IS629 and pro-

phage sequences. They hypothesized the transfer of LEE from *pheU* to *pheV* by site-specific recombination (59). During this study, 16 strains were found to have none of the analyzed virulence-associated genes and none of the former analyzed virulence genes (26). The number of occupied integration sites did not correlate with the number of virulence genes in all food-associated STEC strains.

Several strains did not carry interrupted phage integration sites, although they possessed phage genes. Thirteen of these strains were positive for stx_{2e}, two were not typeable, and one harbored only stx_{1c}. Therefore, the presence and usage of other phage integration sites are likely.

Different phage integration sites for Stx phages have been described. Creuzburg et al. (37) found the *ssrA* integration site for CP-1693 prophage in a comparison of different genomes in all cases associated with the presence of foreign DNA. Serra-Moreno et al. (31) also mentioned the different usage of insertion sites by the same phages due to availability. Apart from the most frequently used insertion sites, genes with rare integration are also described. Recktenwald et al. (33) found that the Shiga toxin phage encoded Stx_{2e} in the integration gene *yecE*. This stx_2 variant was the most prevalent in the strains investigated but correlated only for two strains with the occupation of *yecE*. Koch et al. (35) investigated *z2577* as an integration site for different Stx1ox3-

producing EHEC strains. In our study, this gene was occupied in three strains of other *stx* variants.

Girardeau et al. (44) characterized the PAI I$_{CL3}$ and found its exclusive distribution in LEE-negative strains. This was confirmed in this study, with one exception for the food-borne STEC strain LM15814/08, which is *eae* positive and gave a result for segment ms-4 of PAI I$_{CL3}$. Many strains of our study did not carry all segments of the island. This was described previously by Girardeau et al., who investigated strains of different pathotypes, which carried only segments located at the extremities of the PAI. Moreover, they found deleted PAI I$_{CL3}$ gene clusters on mobile genetic elements which were integrated in the *selC*, *pheV*, or *serW* tRNA gene. This is one possible insert in *selC*- or *pheV*-negative strains investigated, of which *pheV* was the most often occupied LEE integration site. Because PAI I$_{CL3}$ is a hybrid genomic island (43), it is imaginable that parts of this island were integrated in different insertion sites analyzed or that oligonucleotides used in this study did not match mutated or deleted parts of the PAI. Comparing single combinations of PAI I$_{CL3}$ segments with the MLST data, no correlation in general was found, but all investigated strains of serotype O113:H21, including HUSEC026, had the complete PAI I$_{CL3}$. Thus, parts of this PAI harboring virulence genes were able to be distributed throughout the genome and distributed to other susceptive strains with empty integration sites.

For the consideration of the risk of human infection, it is important to investigate the susceptibility to antibiotics, and we used typical antibiotics used in human medicine against Gram-negative bacteria. Ewers et al. (60) gave an overview of extended-spectrum-β-lactamase (ESBL)-producing *E. coli* in livestock and companion animals with information about frequency of resistance in different STCs. Here, STC10 was mentioned at position nine with nearly 25% resistant strains. In our study, four of the 10 STC10 strains harbored one or more resistances and two of these strains were among others resistant to AMP and MEZ, but no resistance against cephalosporins was detected (Table 2). The genetic background of these resistances was not analyzed, but as the MIC values for CTX and CAZ are not increased compared to those of other non-ESBL-producing strains, we have no indication of any possible ESBL activity in our strains.

Schroeder et al. (61) conducted a study analyzing the antimicrobial resistance of serogroup O26, O103, O111, O128, and O145 strains from animals and humans with differentiation of STEC and non-STEC strains. In this study, approximately 40% of the STEC strains were resistant to SMZ, tetracycline (TET), or STR. Sulfamethoxazole was also the main resistance found in our study, followed by STR and CMP as well as SXT. In general, 29% of the strains were resistant to one or more antibiotics. Singh et al. (62) used strains from humans and food animals to identify resistance and the distribution and transferability of class 1 integrons in STEC strains. Here, 34% of the strains were resistant to any of the antibiotics. Resistance to STR was most frequently observed, followed by resistance to SMZ, TET, and AMP. Class 1 integrons were detected in 16% of the strains and were transferable in all strains tested. Although the resistances found here were not analyzed for their genetic background and localization, it should be considered that occupied integration sites and foreign DNA may also be the space and origin of antimicrobial resistance. The presence of two strains with resistance against 7 of the antibiotics tested raises the question of their origin. Both strains originated

from minced meat and were probably prone to application of antibiotics during animal husbandry.

Phylogenetic analyses showed that the analyzed food-associated STEC strains carry heterogeneous MLST profiles. Some strains of the human HUSEC collection were found to cluster together with some of the analyzed food isolates.

The most-often-occurring STs in this study were not within the five STs most frequently found by Mellmann et al. (11) during establishment of the HUSEC collection, and the predominant sequence type complex STC29 was here found only in one food strain but was shared by 10 HUSEC strains.

Comparing the most frequently detected STs with the MLST database (http://mlst.ucc.ie/mlst/mlst/dbs/Ecoli), the ST56 reference strain (EcoR30) originates from the *E. coli* reference collection (63) and was isolated from a bison.

Jaureguy et al. (64) analyzed the *E. coli* reference collection (EcoR) (63) and found several STs which have also been found in this study. In their collection of 72 strains, eight strains isolated from dogs (2/8) and humans (6/8) were categorized in STC10 and five strains in STC155 were isolated only from animals. They concluded that clonal complexes represent important phylogenetic units for pathogenesis and comparative genomics.

Wirth et al. (20) performed MLST to analyze evolutionary pathways of pathogenic and nonpathogenic *E. coli* in more than 400 isolates. Comparable to our results, they found STC10, which was the second-most-common STC in our study, most often. They assigned properties based on the study which were diverse, global, pathogroup A with few pathogens and rare recombination events. For STC155, the fourth-most-often found complex, they used properties like global distribution and causing occasional diarrhea. For the strains investigated here, STC10 was found only in food-associated STEC isolates while STC155 included ST56, which was shared by HUSEC026. Another study from Creuzburg et al. (65) used, among others, some of the food-associated strains isolated by Slanec et al. (26) and strains of the HUSEC collection for comparison to analyze the evolutionary relationship and distribution of type III effector genes. Here, STC155 strains, which were negative for all analyzed type III effector genes, and STC10 were found less frequently, but no correlation between the amount and type of effector genes and the risk to cause HUS was shown. This was supported by the three ST pairs between food isolates and the HUSEC collection also mentioned here (HUSEC032 and 13477/1, HUSEC026 and TS03/07, and HUSEC034 and 17584/1).

The clustering of STs into three larger groups gives an opportunity to arrange the strains in a more practical way. In clusters A and C, there are clearly the highest numbers of pathogenic strains used in this study. Most of these strains are *eae* positive and belong to the HUSEC strain collection. Cluster B is so far interesting, since most of the *eae*-negative food-associated strains of this study are included here. Although *eae*, a major marker for virulence, is lacking, it can be shown that in many strains the LEE integration sites are occupied with foreign DNA. Moreover, serotypes O91:H21 and 113:H21 have been shown to cause sporadic infections and outbreaks of diarrhea and HUS (50, 66). In general, STEC food-associated strains carrying the major serogroups O26, O111, O103, O157, and O145 and belonging to the respective STs of clusters A and C may be at risk for human infection. This assumption is underlined by a recent study about clinical EHEC O26 isolates (51). In this study, it was shown that a new clone of EHEC

O26, characterized by the presence of stx_{2a} only, ST29, and a unique plasmid gene profile, has been emerging as an important cause of HUS in Europe. Since O26 strain LM14957/08 belongs to ST29 and carries stx_{2a} and the plasmid gene profile EHEC-$hlyA^+$ $katP$ negative $espP$ negative $etpD^+$, we strongly suggest that it belongs to this new clone. Although food isolates in cluster A are eae negative, the affiliation of the 2011 German outbreak strain O104:H4 with cluster A also supports the suggestion that members of this group are potentially dangerous for humans.

Bettelheim (67) focused on non-O157 STEC strains in his review to raise awareness regarding pathogenicity and environmental importance of these serogroups. In his research, he found serogroup O26 most often, followed by O111, O91, O113, and O103, with each also connected to human disease. For all of these serogroups except O111, pairs of food-associated STEC and HUSEC strains with the same ST were found. In addition, pairs of more-rare serogroups like O136 or O55 were detected. All strains had at least one but most often more than one occupied LEE insertion site with DNA of unknown origin.

In conclusion, STEC food isolates cannot be generally regarded as harmless, since particular serotypes, together with toxin variants and virulence marker compositions, may lead to an infectious or outbreak strain. To date, it is not known which combinations of markers are needed for this. However, as shown by our data and others (8, 50), at least food-borne O113:H21 and O91 should be considered with care.

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