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Evaluation of Major Types of Shiga Toxin 2e-Producing *Escherichia coli* Bacteria Present in Food, Pigs, and the Environment as Potential Pathogens for Humans[▽]

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Shiga toxin 2e (Stx2e)-producing strains from food ($n = 36$), slaughtered pigs ($n = 25$), the environment ($n = 21$), diseased pigs ($n = 19$), and humans ($n = 9$) were investigated for production of Stx2e by enzyme-linked immunosorbent assay, for virulence markers by PCR, and for their serotypes to evaluate their role as potential human pathogens. Stx2e production was low in 64% of all 110 strains. Stx2e production was inducible by mitomycin C but differed considerably between strains. Analysis by nucleotide sequencing and transcription of stx_{2e} genes in high- and low-Stx2e-producing strains showed that toxin production correlated with transcription rates of stx_{2e} genes. DNA sequences specific for the *int*, *Q*, *dam*, and *S* genes of the stx_{2e} bacteriophage P27 were found in 109 strains, indicating cryptic P27-like prophages, although 102 of these were not complete for all genes tested. Genes encoding intimin (*eae*), enterohemorrhagic *Escherichia coli* hemolysin (*ehx*), or other stx_1 or stx_2 variants were not found, whereas genes for heat-stable enterotoxins STI, STII, or EAST1 were present in 54.5% of the strains. Seven major serotypes that were associated with diseased pigs (O138:H14, O139:H1, and O141:H4) or with slaughter pigs, food, and the environment (O8:H4, O8:H9, O100:H30, and O101:H9) accounted for 60% of all Stx2e strains. The human Stx2e isolates did not belong to these major serotypes of Stx2e strains, and high production of Stx2e in human strains was not related to diarrheal disease. The results from this study and other studies do not point to Stx2e as a pathogenicity factor for diarrhea and hemolytic uremic syndrome in humans.

Production of Shiga toxins (Stx), or Vero toxins, is associated with certain *Escherichia coli* strains designated Shiga toxin-producing *E. coli* (STEC) strains or Vero toxin-producing *E. coli* strains. STEC is commonly found in the intestines of ruminant animals such as cattle, sheep, and goats (11). In these domestic animals, STEC normally does not cause disease, and some STEC types were shown to be closely associated with certain animal host species and populations (9, 10, 27, 45). The situation is different in monogastric animals such as pigs. These do not harbor STEC as part of their normal gut flora but may occasionally excrete these bacteria with their feces (16, 54).

In contrast to the case in ruminant animals, STEC plays an important role as pathogens in humans and in pigs. Humans can be infected by the oral route through ingestion of food containing STEC, by contact with an STEC-contaminated environment, or by direct transmission of STEC from infected animals or humans (19). In the human host, STEC may cause diarrhea, and certain STEC strains, designated enterohemorrhagic *E. coli* (EHEC), cause life-threatening hemorrhagic colitis and hemolytic uremic syndrome (HUS) (47). In weaned pigs, STEC plays an important role as agents of edema disease, which is characterized by high contagiousness, neurological disorders, hemorrhagic lesions, and often fatal outcome (1, 19).

Analysis of Shiga toxins produced by different strains of *E. coli* revealed two toxin families, called Stx1 and Stx2, which are genetically and immunologically distinct from each other (35). Toxins of the Stx1 and Stx2 families can be divided further into subtypes differing in the composition of their A or B subunit (40). Studies on human virulent EHEC have revealed that certain toxin types, such as Stx2, Stx2c, and activatable Stx2d, are significantly associated with higher virulence of the bacteria for humans, which may cause severe diseases such as hemorrhagic colitis and HUS (4, 7, 17, 22, 36). In pigs, the toxin type Stx2e was identified as a key factor contributing to the pathogenicity of porcine STEC and therefore was designated the edema disease principle (1). In contrast, Stx2e-producing STEC bacteria are rarely isolated from humans and account for only 0.9 to 1.7% of human STEC isolates (4, 42). A significant association between Stx2e and human diarrhea was not found, and it was suggested that these STEC bacteria are not important as human pathogens (4, 17, 19, 42).

The Shiga toxin variant 2e is produced by STEC isolated from various sources, such as animals, food, the environment, and humans. Stx2e-producing STEC strains belong to different serotypes, and the presence of Stx2e and other virulence attributes was found in a variety of chromosomal backgrounds (12, 33). The occurrence of the stx_{2e} gene in genetically distinct types of *E. coli* could indicate that these are spread by bacte-

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TABLE 1. Origins and properties of STEC strains investigated for stx_{2e} and associated gene sequences

Strain	Serotype	Source[a]	Stx2e production[b]	Virulence marker(s)[c]	GenBank accession no.
CB10282	O8:H9	Milk	Low	—	AM937001
CB10284	O8:H9	Water	Low	—	AM939641
CB10394	O101:H9	HF, D	High	—	AM939642
CB10402	O100:[H30]	Pork	High	STI-p	AM939643
CB8771	O159:H21	PF	High	DA, STI-p, STII	AM940005
CB7671	ONT:H19	HF, As	High	DA	AM940007
CB8770	ONT:H21	PF	High	—	AM940006
CB8810	O8:H9	PF	High	DA	AM940004
2771/97[d]	ONT:NM	HF, D	High	ND	AJ298298

[a] HF, human feces; D, diarrhea; PF, fecal samples from pigs at slaughter; As, asymptomatic.
[b] P_1-g-EIA results for strains grown in presence of mitomycin C.
[c] —, negative for virulence markers investigated here; STI-p, heat-stable enterotoxin STI (animal); DA, diffuse adherence to HEp-2 cells; STII, heat-stable enterotoxin STII; ND, not done.
[d] Reference strain carrying the stx_{2e} bacteriophage P27 (39).

riophages, similar to what was found for other genes of the Stx1 and Stx2 families (21). A lambdoid phage called P27 carrying an stx_{2e} gene was recently isolated from STEC originating from a human patient, but inducible *stx*-carrying phages were not found in other types of Stx2e-producing strains (31, 39). Stx2e-producing strains from humans were shown to differ from porcine pathogenic Stx2e-producing strains in their serotypes and adhesion to epithelial cell lines from humans and pigs (42). Additionally, none of the virulence factors hitherto described for this group of STEC, including production of Stx2e, could be clearly associated with diarrheal disease in humans (15, 42).

However, humans are likely to have contact with Stx2e-producing STEC, as these strains were shown to constitute about 19% of the STEC isolated from meat and milk products in Germany (5). Stx2e-producing strains also frequently occur in pigs (16), in the environment of pig farms (46), and as contaminants of wastewater from abattoirs (26). The low incidence of Stx2e strains in human patients could indicate that most of these strains cannot colonize or may infect but do not cause disease in humans. This is supported by epidemiological findings indicating that Stx2e strains are very rarely isolated from humans, and those patients show mild diarrheal symptoms or are asymptomatic carriers (4, 17, 39, 42). In a previous study we have found that Stx2e-producing strains differ largely in the amount of Stx2e produced (2). In this study, we investigated whether the level of Stx expression is related to serotypes and sources of the Stx2e strains and serves as an indicator for human pathogenicity. We compared Stx2e-producing STEC strains differing in their origin, virulence attributes, and geno- and phenotypes to evaluate the public health impact of this particular group of STEC, which accounts for a major part of STEC isolated from food in Germany.

MATERIALS AND METHODS

Bacteria. A total of 110 Stx2e-producing *E. coli* strains from the collection of the NRL-*E. coli* were investigated. The strains were isolated from human feces ($n = 9$; six patients with uncomplicated diarrhea and three asymptomatic individuals), the environment ($n = 21$; seven strains from pig farms and 14 from river water), food ($n = 36$; 33 strains from meat and meat products and 3 from milk), feces from pigs at slaughter ($n = 25$), and feces or organ samples from pigs with edema disease or diarrhea ($n = 19$) between 1997 and 2005. The origins of some strains from human patients (4, 15), food samples (5), pig farm environments (46), and diseased pigs (50) have been described elsewhere. Detection and characterization of Shiga toxin genes (*stx*) in the strains was performed by PCR following previously published protocols (5). Serotyping of O:H antigens and molecular typing of the flagellar (*fliC*) genes were performed as described previously (5). All strains were investigated for cytotoxicity with the Vero cell toxicity test and for adherence to HEp-2 cells as previously described (4, 42).

The *E. coli* reference strains used as positive controls for PCR detection of virulence markers investigated in this study were used as described previously (3, 20, 50). The human fecal *E. coli* strain 2771/97 (ONT:NM), which carries the Stx2e-encoding bacteriophage P27, was used as positive control for detection of P27-associated genes (31, 39). The properties of representative Stx2e-producing strains that were investigated for stx_{2e} and associated gene sequences are summarized in Table 1.

Production of Shiga toxins. All strains were tested for cytotoxic activity in the Vero cell test, which was performed as described previously (2). The strains were investigated quantitatively for production of Stx1 and Stx2 by the P_1-glycoprotein receptor enzyme immunoassay (P_1-g-EIA) (2). This assay is based on binding of the Stx B subunit to the P_1-glycoprotein receptor. Inducible production of Stx was measured by comparing P_1-g-EIA results obtained with bacteria grown in tryptic soy broth (TSB) to those obtained with cultures growing in TSB supplemented with 50 ng/ml mitomycin C. EIA results were recorded photometrically as described previously (2). Test results were recorded as negative (0) if the extinction was ≤ 0.1 above the negative control, as weakly positive (1+) if the extinction was >0.1 to 0.5 above, as moderately positive (2+) if the extinction was >0.5 to 1.0 above, and as strongly positive (3+ or 4+) if the extinction was >1.0 to 2.0 above or extinction >2.0 above. Cultures of representative strains belonging to toxicity groups 1+ to 4+ were examined by end point titration in the P_1-g-EIA. Twofold dilutions of broth cultures still showing a positive result in the P_1-g-EIA were as follows: 1+, 1:0 to 1:8; 2+, 1:16 to 1:64; 3+, 1:128 to 1:512; and 4+, $\geq$1:1,024.

Preparation of total DNA, RNA, and cDNA from bacteria. Total DNA of bacteria was prepared from 1-ml overnight cultures (approximately 1×10^9 bacteria) with the RT Spin Bacteria DNA minikit (Invitek, Berlin, Germany). DNA preparations were stored at 4°C for use. For preparation of RNA, bacteria were grown to late-exponential phase in TSB without and with mitomycin C as described above. Total RNA was isolated from 5-ml bacterial cultures with the RNeasy minikit (Qiagen, Hilden, Germany). RNA preparations were repeatedly digested with RNase-free DNase I (Roche Applied Systems, Mannheim, Germany) for 30 min at 37°C as described by the manufacturer. Next, DNase I was inactivated by heat treatment for 10 min at 75°C, and the samples were purified on RNeasy columns following the instructions of the provider (Qiagen). The absence of DNA from RNA samples was controlled by real-time PCR (35 cycles) amplification of the *icdA* and the stx_{2e} genes. The RNA preparations were stored at −20°C for use. Preparation of cDNA from the RNA samples was performed with the High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA) following the instructions supplied by the provider.

Detection of virulence genes in *E. coli* by PCR. PCRs for detection of genes of the Stx1 and Stx2 families and for detection of the *eae* gene, encoding intimin, were performed as described previously (5). In addition, a number of virulence genes known to be associated with porcine pathogenic *E. coli* strains were investigated. PCR for specific detection of genes encoding Stx2e (stx_{2e}), alpha-hemolysin (α-*hlyA*), EHEC hemolysin (*ehxA*), heat-labile enterotoxin LT-I, heat-stable enterotoxin STI (STa), cytolethal distending toxins (*cdt*), cytotoxic necro-

TABLE 2. Detection of virulence genes by PCR

Target gene (phenotype)	Accession no.	Primer	Nucleotide sequence (5′→3′)	PCR conditions	No. of PCR cycles	PCR product (bp)	Reference
stx_{2e} (Stx2e, B subunit)	M21534	SLT-IIBv1	ATGAAGAAGATGTTT ATAGCG	94.0°C, 30 s; 62.0°C, 60 s; 72.0°C, 30 s	30	261	2
		SLT-IIBv2	GTTAAACTTCACCTG GGCAAAG				
astA (EAST1)	L11241	EAST11a	CCATCAACACAGTAT ATCCGA	95°C, 30 s; 55°C, 120 s; 72°C, 120	30	111	52
		EAST11b	GGTCGCGAGTGACG GCTTTGT				
STII (STb)	M35586	STb-f	CGCATTTCTTCTTGC ATCTATG	94°C, 40 s; 55°C, 40 s; 72°C, 40 s	30	189	This work
		STb-r	TGCTGCAACCATTAT TTGGG				
STI-h (human)	M34916	STIh-f	TCCCTCAGGATGCT AAAC	94°C, 40 s; 47°C, 60 s; 72°C, 40 s	30	244	6
		ST1h-b	GCAACAGGTACATA CGTT				
STI-p (animal)	M25607	STIp-f	TTCTGTATTATCTTT CCCC	94°C, 40 s; 51.3°C, 40 s; 72°C, 40 s	30	258	6
		ST1p-b	TTATGATTTTCTCAG CACC				
faeG (F4 [K88])	AJ616236	K88-f	GGTGATTTCAATGGT TCGGTC	94°C, 30 s; 61.8°C 60 s; 72°C 45 s	30	766	This work
		K88-r	ATTGCTACGTTCAGC GGAGCG				
fan (F5 [K99], fimbrial subunit)	M35282	K99-f	GACTACCAATGCTTC TGCGA	95.0°C, 30 s; 60.0°C 45 s; 72.0°C 45 s	30	459	This work
		K99-b	GGTGGATATAAAGCT GGCGT				
fasA (F6 [987p], fimbrial subunit)	M35257	987p-f	CTGCCAGTCTATGCC AAGTG	94°C, 30 s; 61.8°C, 60 s; 72°C, 45 s	30	459	This work
		987p-b	ACGGTGTACCTGCTG AACGAATAG				
fedA (F18 [F107], fimbrial subunit)	M61713	F107-f	CTTTCACATTGCGTG TGGAG	94°C, 30 s; 61.8°C, 60 s; 72°C, 45 s	30	513	This work
		F107-r	ACCACCTTTCAGTTG AGCAG				
Fim41a (F41, fimbrial subunit)	X14354	F41-f	GCATCAGCGGCAGT ATCT	95.0°C, 30 s; 60.0°C 45 s; 72.0°C 45 s	30	380	14
		F41-b	AGGTGATAATACTGA GCTAGGGAC				

tizing factors (*cnf*), cell cycle inhibition factor (*cif*), and intimin (*eae*) was performed as described previously (3, 6, 50). PCR protocols for detection of genes encoding fimbrial adhesins F4 (K88), F5 (K99), F6 (987p), F18 (F107), and F41 and heat-stable enterotoxins (STIh, STIp, STII and EAST1) are listed in Table 2. PCR primers were developed with the help of Accelrys Gene v2.5 software (Accelrys Inc., Cambridge, United Kingdom).

Detection of gene sequences associated with stx_{2e}-carrying bacteriophage P27 and insertion of P27 into the *E. coli* chromosome. The presence of stx_{2e}-carrying bacteriophage P27-associated gene sequences was investigated by PCR. PCR primers specific for the corresponding gene sequences of P27 which did not amplify related genes of other (*stx*-carrying) bacteriophages were selected by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). Strain C600 (*E. coli* K-12) (2) was used as a negative control and strain 2771/97 (31) as a positive control. The open reading frame labels (L01, L21, L24-25, and L26-27) are from the published P27 bacteriophage sequence (39). Primers P27-1 (5′ GTTATTT TTCTGTATCGGCTGC 3′) and P27-2 (5′ TGTTGAGTCGAAAAGTCT ATCG 3′) were used for amplification of a 772-bp region flanked by the phage DNA methylase gene (L24) and the $stxA_{2e}$ subunit gene (L25). Primers P27-3 (5′ GAGAATACTGGACGAACAGATG 3′) and P27-4 (5′ CACTGTCAAACCC TGTGT 3′) were used to amplify the 799-bp downstream region between the $stxB_{2e}$ subunit gene (L26) and the *S* (holin) gene (L28) of P27. The amplification conditions for both PCRs were 30 cycles of 94°C for 40 s, 57.7°C for 40 s, and 72°C for 60 s.

The P27 antitermination *Q* gene (L21)-specific PCR (30 cycles of 94°C for 40 s, 50.5°C for 40 s, and 72°C for 60 s), yielding a 204-bp amplicon, was performed with primers P27-5 (5′ GTTTACTACTCACCGATAGCAG 3′) and P27-6 (5′ CCTTGTCTTTTGGCGATATTTC 3′). The insertion of P27 in the chromosomal *E. coli yecE* gene was investigated by PCR with primer P27-7 (5′ TGCG GGTCAAAAATTCAGTCAC 3′), located in the *yecE* gene, and primer P27-8 (5′ GGCATCGAAAGGCATACCAGTC 3′), located in the P27 integrase (L01) gene. The PCR was performed for 30 cycles (94°C for 40 s, 56.5°C for 40 s, and 72°C for 60 s), resulting in a 450-bp fragment with strains carrying the phage P27

inserted into the *yecE* gene. The presence of an intact *yecE* gene in P27 integrase-negative strains was investigated by PCR with primers P27-9 (5′ AATGGTCG CATCCTAAATGG 3′) and P27-10 (5′ GTACAGCATGTACCGGAAC 3′), resulting in a 549-bp fragment spanning the insertion site of P27 into the *yecE* gene (39). The PCR was performed for 30 cycles (94°C for 40 s. 56.6°C for 40 s, and 72°C for 60 sec).

Transcriptional analysis of $stxA_{2e}$ genes by qRT-PCR. Quantitative real-time reverse transcription-PCR (qRT-PCR) was performed with the Applied Biosystems 7500 real-time PCR system (Applied Biosystems) with cDNA samples from bacteria. Transcription rates of the $stxA_{2e}$ gene in bacteria were compared to those of the *icdA* housekeeping gene (51). Primers and TaqMan MGB probes were developed with ABI Prism Primer software (Applied Biosystems) and produced by Applied Biosystems (Applera Deutschland, Darmstadt, Germany). Primers icdA-F and icdA-R were used as described previously (51) together with the 6-carboxyfluorescein-labeled *icdA* MGB probe (5′ ACCCTGCAAAACG GCAA 3′). Primers Stx2-f (5′ CAGGCAGATACAGAGAGAATTTCG 3′) and Stx2-b (5′ CCGGCGTCATCGTATACACA 3′) were used together with the VIC-labeled Stx2-specific TaqMan MGB probe (5′ ACTGTCTGAAACTGCTC 3′) for amplification of the $stxA_{2e}$ gene. Real-time PCR amplifications were performed for 35 cycles in 50-μl reaction volumes using TaqMan universal PCR master mix (Applied Biosystems). Relative quantification assays were performed with dilutions of cDNA in an *icdA* and stx_{2e} multiplex assay. qRT-PCR assays were analyzed with the 7500 system SDS software version 1.4 (Applied Biosystems).

Determination of the sequence of the region upstream of the stx_{2e} gene in low- and high-toxin-producing strains. The complete stx_{2e} genes and adjacent DNA segments of two low (CB10282 and CB10284)- and two high (CB10394 and CB10402)-Stx2e-producing *E. coli* strains were analyzed for their nucleotide sequences (Table 1). A 2,385-bp DNA stretch encompassing the homologous region between the putative DNA methylase gene (L24) and the putative holin gene (L28) of stx_{2e} bacteriophage P27 (accession no. AJ298298) was sequenced in Stx2e-producing strains CB10282 and CB10284 using primers and PCR products derived from the sequence under accession no. AJ298298. Strains CB10394 and CB10402 were found negative for sequences homologous to AJ298298 in the region upstream of the $stxA_{2e}$ gene as tested by PCR using primers P27-1 and P27-2 (see above). To obtain the nucleotide sequence of the DNA region upstream of the $stxA_{2e}$ gene in strains CB10394 and CB10402, total DNA was isolated and digested with restriction enzymes which cut only once each in different positions downstream of the $stxA_{2e}$ gene. Restriction-digested DNA fragments were ligated with T4 ligase and used as templates in a PCR using primer Stx2e out1 (5′ GTAACAGGCACAGTATCCAC 3′) (positions 571 to 552 in the sequence under accession no. AM939642) in combination with primer Stx2e out2 (5′ GACAACTATTTCCATGACAACG 3′) (positions 906 to 927 in the AM939642 sequence) or with stx2e out3 (5′ CAGCCATTGTTACAAA GTGC 3′) (positions 1666 to 1685 in the AM939642 sequence). A PCR product derived from amplification with these primers is generated only if the DNA sequences adjacent to the 3′ ends of the primers are joined to form a circular DNA molecule. PCR products were obtained with ligation mixtures of NciI- and PstI-digested total DNA. The PCR products were purified and analyzed for their nucleotide sequences. The nucleotide sequence data were used to generate primer Stx2e 4f (5′ AGGATAATAAGACTCTCTCGCC 3′), which was used in combination with primer P27-2 (see above) to generate a 643-bp amplicon of the region upstream of the $stxA_{2e}$ gene in strains CB10394 and CB10402. The same primers were also used for amplification and nucleotide sequencing of this region present in the Stx2e-producing strains CB8770, CB8771, CB8810, and CB7671. Oligonucleotides for sequencing of the stx_{2e} gene and the region downstream of $stxB_{2e}$ in strains CB10394, CB10402, CB10394, and CB10402 were derived from the published sequence of the stx_{2e} bacteriophage P27 from strain 2771/97 (accession no. AJ298298) (39).

PCR products were purified and used for sequencing by applying the dye terminator chemistry (PE Applied Biosystems, Darmstadt, Germany) and separated on an automated DNA sequencer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems, Foster City, CA). The sequences were analyzed using the Lasergene software (DNASTAR, Madison, WI) and Accelrys Gene v2.5 software.

Statistical analysis. The H test (Kruskal-Wallis) followed by the U test (Mann-Whitney) was performed. The Holm correction method (α-correction) assesses significance assuming that each test is not independent from the other. We selected the Holm correction method to identify significant differences between the strains according to their source. We also applied the Bonferroni correction by adjusting the *P* value. Bonferroni corrections gave similar results.

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences determined in this study are listed in Table 1.

TABLE 3. Relative amounts of Stx produced by stx_{2e} strains as measured with the P_1-g-EIA

Stx production[a]	No. (%) of strains grown in:	
	TSB	TSB + mitomycin C
Undetectable (0)	34 (30.9)	1 (0.9)
Weak (1+)	37 (33.6)	25 (22.7)
Moderate (2+)	20 (18.2)	9 (8.2)
High (3+)	13 (11.8)	14 (12.7)
High (4+)	6 (5.5)	61 (55.5)

[a] 0 to 4+ correspond to the P_1-g-EIA extinction rates as listed in Materials and Methods. Stx2e production was significantly higher ($P < 0.01$ to $P < 0.001$) in strains from all sources when grown in the presence of mitomycin C.

RESULTS

Presence and expression of *stx* genes in stx_{2e}-positive STEC. All 110 strains showed cytotoxic activity in the Vero cell assay. Investigation for genes encoding Shiga toxins (Stx) was performed as described previously (5). The 110 strains were all positive in a PCR specific for the stx_{2e} gene (Table 2), and other *stx* genes of the Stx1 or Stx2 family were not detected. Production of Stx was measured quantitatively in the P_1-g-EIA, and the bacteria were investigated for induction of Stx production by use of mitomycin C as described in Materials and Methods. The results are summarized in Table 3. When grown without induction, about one-third of the strains showed no detectable Stx and another third showed only weak (1+) production of toxin in the P_1-g-EIA. The remaining strains showed moderate (2+) to high (3+ to 4+) production of Stx2e. Growing bacteria in TSB plus mitomycin C resulted in increased toxin production in all but one strain. Stx2e production was enhanced more than 1,000-fold in some strains, and 1,000-fold differences in toxin production were detected between the strains (Table 4 and unpublished data). Strains were divided in low producers (0 and 1+) and high producers (3+ and 4+) according to their Stx2e production upon mitomycin induction. High toxin producers were frequently found among strains belonging to serotypes O101:H9 (100%), O141:H4 (100%), and O100:[H30] (94.4%). Low toxin producers were more frequent among serotype O8:H4 (87.5%), O138:H14 (80%), and O8:H9 (40%) isolates (data not shown). Interestingly, Stx2e production was significantly higher in strains isolated from humans than in those isolated from diseased pigs ($P < 0.001$ with and without mitomycin C induction).

Nucleotide sequence analysis of the Stx2e-encoding region in low- and high-Stx2e-producing STEC strains. We were interested in whether the major differences found between Stx2e strains for Stx production could be related to alterations in the nucleic acid sequences of stx_{2e} and adjacent genes. In order to explore this, we analyzed representative low- and high-Stx2e-producing strains (Table 1) for the nucleotide sequences of stx_{2e} and adjacent genes. The 2,385-bp region encompassing the stx_{2e} gene (see Materials and Methods) was identical in low-Stx2e-producing strains CB10282 (accession no. AM937001) and CB10284 (AM939641) and differed in only four nucleotides (99.8% similarity) from the corresponding region of stx_{2e} from phage P27 (AJ298298) (Fig. 1A).

The 2,274 bp region encompassing the stx_{2e} gene was identical in both high-Stx2e-producing strains CB10394 (accession

TABLE 4. Production of Stx2e and relative quantification of $stxA_{2e}$ gene transcription in high- and low-Stx2e-producing strains

Strain	TSB		TSB + mitomycin C	
	P_1-g-EIA[a]	Relative quantification by RT-PCR (ratio of $stxA_{2e}$ to *icdA* gene expression)[b]	P_1-g-EIA	Relative quantification by RT-PCR (ratio of $stxA_{2e}$ to *icdA* gene expression)
CB10282	0 (negative)	1.0 ± 0.0	1+ (1:0)	1.0 ± 0.2
CB10284	0 (negative)	0.16 ± 0.1	1+ (1:0)	1.0 ± 0.2
CB10394	1+ (1:4)	2.01 ± 0.4	4+ (1:2,048)	1,179.0 ± 286
CB10402	1+ (1:2)	1,19 ± 0.0	4+ (1:1,024)	9.84 ± 0.8
2771/97[c]	2+ (1:32)	5.71 ± 2.9	4+ (1:4,096)	151.86 ± 17.7

[a] The highest dilution of culture medium showing a positive result in the P_1-g-EIA is indicated in parentheses.
[b] Means and standard deviations from two separate experiments with measurements performed in duplicate.
[c] Reference strain carrying the stx_{2e} bacteriophage P27.

no. AM939642) and CB10402 (AM939643). High (CB10394 and CB10402)- and low (CB10282 and CB10284)-Stx2e-producing strains were identical in their $stxA_{2e}$ gene sequences and differed from the P27 sequence (strain 2771/97) by one nucleotide at position 1523, leading to change in the amino acid composition (lysine to threonine) (Fig. 1A and B). The high- and low-Stx2e-producing strains were not different from phage P27 in the amino acid sequence of their Stx2e B subunit.

On the other hand, the high-Stx2e-producing strains CB10394 and CB10402 differed in the region upstream of the $stxA_{2e}$ gene from the corresponding sequences of 2771/97 (phage P27), with CB10282 and CB10284 showing only 88.1% homology in the region starting with the tRNA-Ile to the beginning of the $stxA_{2e}$ gene. Nineteen alterations were observed in the region encoding tRNA-Ile (76 nucleotides) and six changes in the region encoding tRNA-Arg (77 nucleotides) (Fig. 2). No homology to any DNA sequences deposited in GenBank was found for the 192-bp DNA stretch upstream of the tRNA-Ile region present in strains CB10394 and CB10402 (Fig. 1).

Besides CB10394 and CB10402, 13 other strains from our study belonging to different serotypes were found to be altered in the region upstream of the $stxA_{2e}$ gene compared to phage P27. All these strains were high (4+) Stx2e producers when grown in TSB with mitomycin C. In order to investigate the possible genetic changes in these strains more specifically, we determined the nucleotide sequences in this region of four of these strains, CB8770, CB8810, CB7671, and CB8771 (Table 1), that were unrelated in their origins and serotypes. The sequences of the regions upstream of the $stxA_{2e}$ genes of strains CB10394, CB10402, CB8770, CB8810, and CB7671 were found to be identical. Strain CB8771 showed the same alterations in the tRNA-Ile region but differed from all other strains in the region downstream of tRNA-Ile into the $stxA_{2e}$ gene (Fig. 2).

Transcription of $stxA_{2e}$ genes in low- and high-Stx2e-producing strains. The similarity of the stx_{2e} coding sequences present in high and low Stx2e producers (see above) indicates that the differences in Stx production are not due to mutations in the Stx2e-coding sequences. In order to investigate whether

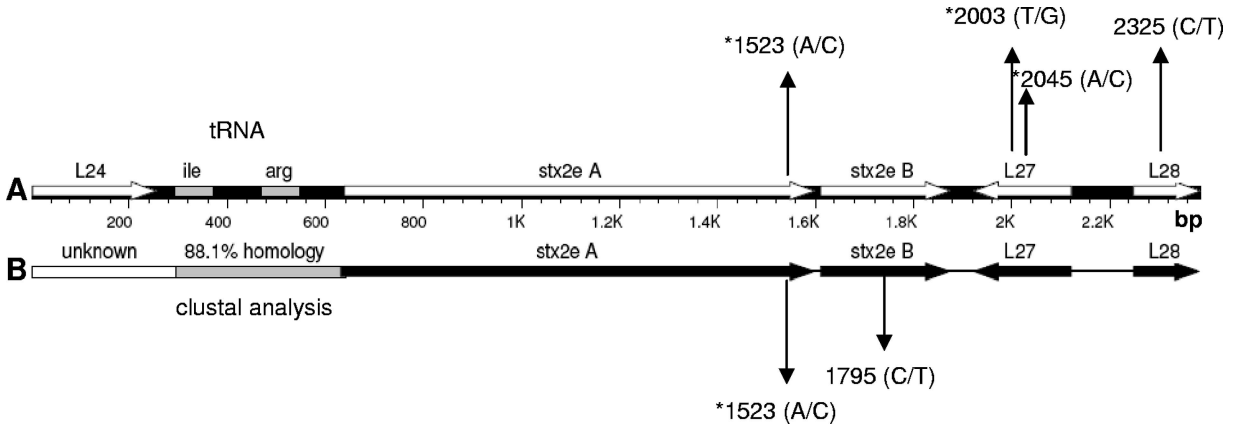

FIG. 1. A DNA segment corresponding to positions 16866 to 19250 of the bacteriophage P27 genome (accession no. AJ298298) was investigated in strains CB10282 (AM937001) and CB10284 (AM939641) (A) as well as in strains CB10394 (AM939642) and CB10402 (AM939643) (B). (A) The low-Stx2e-producing strains CB10282 and CB10284 differed in only four nucleotides, at positions 1523, 2003, 2045, and 2325, from the AJ299298 sequence. Nucleotide changes leading to alterations in the amino acid sequence are indicated by asterisks. (B) The high-Stx2e-producing strains CB10304 and CB10402 differed in two nucleotides (positions 1523 and 1795) in the 1,749-bp stretch between $stxA_{2e}$ and L28. These strains, as well as the high Stx2e producers CB8771 (AM940005), CB7671 (AM940007), CB8770 (AM940006), and CB8810 (AM940004), shared only 88.1% genetic homology with AJ298298 in the 344-bp tRNA region located upstream of $stxA_{2e}$ (for details, see the Clustal analysis in Fig. 2). The "unknown" 241-bp region upstream left of the tRNA region (identical in strains CB10304, CB10402, CB8771, CB7671, CB8770, and CB8810) does not show similarity to any sequence deposited in GenBank.

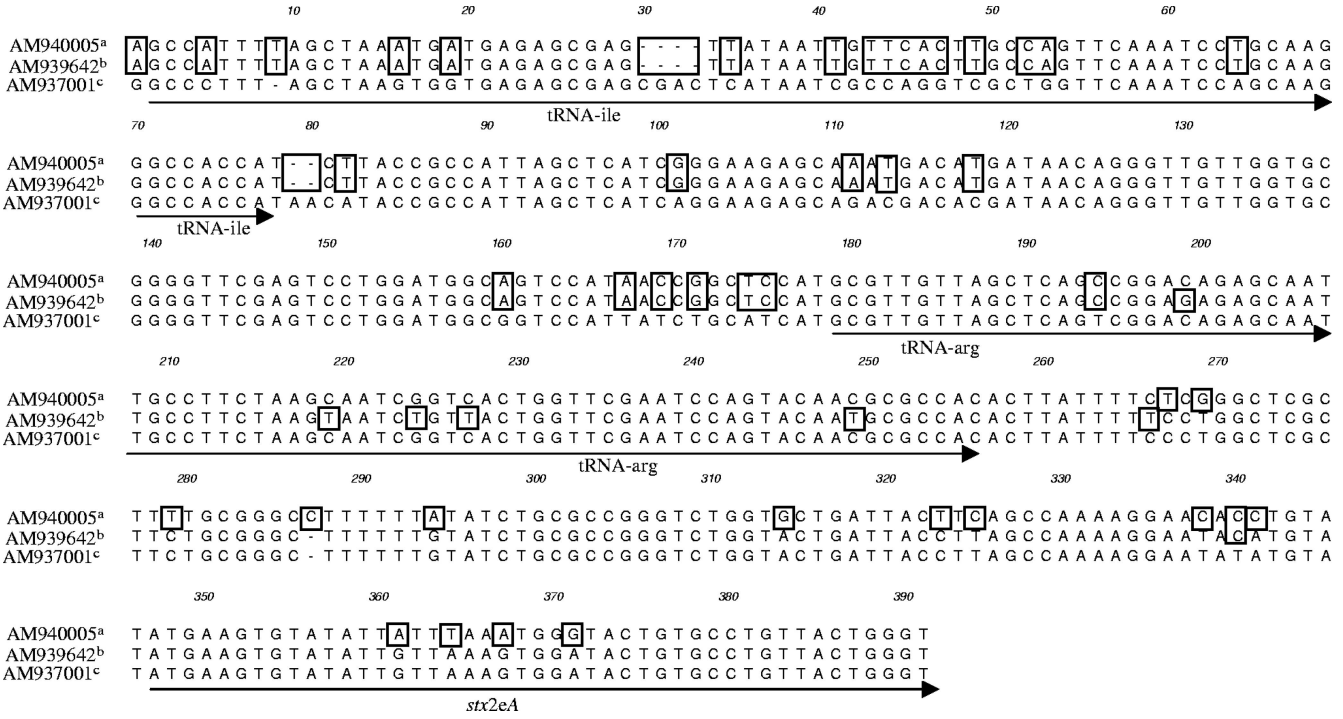

FIG. 2. (a and b) Clustal analysis of the tRNA region present in high-Stx2e-producing strains CB7671 (accession no. AM940007) (a) and CB10394 (AM939642) (b) and identical sequences present in CB10402 (AM939643), CB8771 (AM940005), CB8770 (AM940006), and CB8810 (AM940004). (c) Sequence present in low-Stx2e-producing strains CB10282 (AM937001) and CB10284 (AM939641) and in the high Stx2e producer 2771/97, which harbors the stx_{2e}-carrying phage P27 (AJ298298). The positions of tRNA genes and the start of the $stxA_{2e}$ gene are indicated by bold arrows. Nucleotide changes and gaps are indicated by frames at the corresponding positions.

the differences resulted from transcriptional regulation, we compared the mRNA transcription level of the $stxA_{2e}$ gene with that of the *icdA* housekeeping gene as a standard by RT-PCR in a relative quantification assay as described in Materials and Methods. Two low (CB10282 and CB10284)- and two high (CB10394 and CB10402)-Stx2e-producing strains were compared. Strain 2771/97, which has previously been described to be inducible for Stx2e production by mitomycin C (31), was used as positive control. The results are presented in Table 4. When grown in TSB without mitomycin C, only strains 2771/97, CB10394, and CB10402 showed detectable Stx2e production with the P_1-g-EIA. Growth in TSB supplemented with mitomycin C enhanced Stx2e production clearly in these strains; in contrast, only small amounts of Stx2e (1+ in the P_1-g-EIA) became detectable with low toxin producers CB10282 and CB10284. The differences in Stx2e production in these strains were found to relate to the transcription rates of the $stxA_{2e}$ gene in relative-quantification RT-PCR assays. Upon mitomycin C induction, the transcription rates of the $stxA_{2e}$ gene were increased between 10- and 1,000-fold over that of the *icdA* gene in the high-Stx2e-producing strains (CB10394, CB10402, and 2771/97), whereas the low Stx2e producers CB10282 and CB10284 did not show an increase in $stxA_{2e}$ transcription.

Relationship of Stx2e production to presence of bacteriophage P27-specific gene sequences. The transducible bacteriophage P27 was previously isolated from STEC 2771/97 and found to carry an Stx2e-encoding gene (31). The complete sequence of bacteriophage P27 was published, and corresponding sequences located upstream and downstream of the stx_{2e} gene were found to be present in other Stx2e-producing strains (39). We were interested in whether the presence and expression of the $stxA_{2e}$ gene are correlated with the presence of bacteriophage P27 sequences in the 110 *E. coli* strains investigated in this work. The occurrence of phage P27-related sequences was tested in PCRs amplifying four different gene loci of the phage genome (Table 5). Only one (CB8810 [Table 1]) of the 110 strains was negative for all P27 phage-specific genes (except the stx_{2e} gene). The 772-bp region between the phage DNA-methylase gene (L24) and the $stxA_{2e}$ gene (L25) was found in 95 (86.4%) of the strains. All 15 strains that were negative for this specific region of the P27 genome gave a PCR product of 643 bp with primers Stx2e 4f and P27-2. The same primers were used to characterize the altered $stxA_{2e}$ upstream regions of strains CB10394 and CB10402 by nucleotide sequence analysis. The specificity of the Stx2e 4f and P27-2 PCR products in another four Stx2e-producing strains (CB8770, CB8771, CB8810, and CB7671) was tested by nucleotide sequencing (Fig. 2).

A region homologous to phage P27 downstream from $stxB_{2e}$ (L26) to the *S* (holin) gene (L27) was present in 44 (40.0%) of the strains. Forty strains (36.6%) gave a PCR product spanning from the P27 integrase gene (L01) to the chromosomal *yecE* gene. Twenty strains (18.2%) carried the P27 *Q* gene homologue (L21). On the other hand, only seven of the strains (6.4%) were positive for all P27-specific sequences investigated here. Most of the strains carried one to three of the gene loci tested in different combinations (Table 5). The results indicate

TABLE 5. Presence of stx_{2e} bacteriophage-specific genes in stx_{2e}-positive STEC strains

No. of P27 gene loci	PCR detection of bacteriophage P27 genes outside $stxAB_{2e}$[a]				No. of positive strains
	Integrase (*yecE-int* [L01]) (0–283)[b]	Putative P27 *Q* gene (L21) (13854–14056)	DNA methylase-$stxA_{2e}$ (L24) (16828–17599)	Holin ($stxB_{2e}$-S [L28]) (18607–19405)	
0	0	0	0	0	1
1	+	0	0	0	6
1	0	0	+	0	46
2	0	0	+	+	23
2	+	0	0	+	8
3	+	0	+	+	6
3	+	+	+	0	13
4	+	+	+	+	7
No. of positive strains	40	20	95	44	

[a] 0, PCR negative; +, PCR positive.
[b] PCR product position in bacteriophage P27 sequence (accession no. AJ298298).

that a P27-like phage was probably present in all of the strains at an earlier stage but that changes resulting in loss or modification of P27-specific genes have occurred in at least 93.6% of the 110 strains investigated.

We were interested in whether the amount of Stx2e production is correlated with the integrity of the P27 genome in the strains. Of the 61 strains producing large amounts (4+) of Stx2e in TSB plus mitomycin C, one (CB8810) was negative for all, 25 were positive for one, 20 for two, 10 for three, and 5 for all four P27 gene loci, as listed in Table 5. On the other hand, all 15 strains that were altered in the upstream region of the stx_{2e} gene (see above) were significantly higher (4+) Stx2e producers ($P < 0.001$) than the 95 strains that carried the region which was homologous to the P27 sequence.

Virulence profiles and serotypes associated with Stx2e-producing strains. Besides Shiga toxin genes, we investigated the presence of the intimin gene (*eae*) and the plasmid-carried EHEC hemolysin (*ehxA*) gene, as both of these genes are closely associated with human EHEC strains. None of the 110 Stx2e-producing strains was positive for *eae* or for *ehxA*. Similarly, none of the strains belonged to *E. coli* serotypes that are frequently associated with typical or atypical EHEC strains that are pathogenic for humans. By serotyping, the 110 STEC strains could be divided into nine major serotypes representing 72 (65.5%) of the strains (Table 6). Thirty-one (28.2%) of the strains were not typeable (ONT) with O antisera covering O1 to O181. The remaining seven strains belonged to rare *E. coli* serotypes, and one was O-rough.

We investigated virulence markers that are frequently found in human and animal pathogenic STEC and enterotoxigenic *E. coli* strains, such as adhesins, cytotoxins, and enterotoxins. Except for F18 fimbriae (21.8% positive), all strains were negative

TABLE 6. Serotypes, origins, and virulence markers of Stx2e-producing strains from this study

Serotype	Total no. of strains	No. of strains from:					No. of strains with the following virulence marker[a]				
		Slaughter pigs	Food[b]	Environment[c]	Diseased pigs[d]	Humans[e]	α-*hlyA*	F18	STI-p	STII	EAST1
O2:[H32]	4	0	4	0	0	0	1	0	0	0	0
O141:H4	10	1	1	5	3	0	10	8	1	1	0
O139:H1	10	0	0	0	10	0	10	9	0	0	1
O138:H14	5	0	0	0	5	0	4	5	3	2	0
O101:H9	5	3	0	0	0	2	0	0	0	0	3
O100:H30	18	6	5	7	0	0	0	0	16	0	2
O8:H9	10	3	6	1	0	0	0	0	4	0	2
O8:H4	8	1	6	1	0	0	0	0	7	0	1
O8:H19	2	0	0	2	0	0	0	0	0	0	1
ONT[f]	31	10	12	4	0	5	0	1	4	1	12
Single[g]	7	1	2	1	1	2	1	1	2	2	2
Total	110	25	36	21	19	9	26	24	37	5	24

[a] Virulence markers were investigated by PCR as described in Materials and Methods. Virulence markers which tested negative for all strains are listed in Results.
[b] Strains from meat ($n = 33$) and milk ($n = 3$).
[c] Strains from pig farms ($n = 7$) and river water ($n = 14$).
[d] Strains isolated from feces or organs of pigs with edema disease.
[e] Strains from feces of human asymptomatic carriers ($n = 3$) and patients with diarrhea ($n = 6$).
[f] O-untypeable strains.
[g] Single isolates of the following serotypes, with the origin and virulence marker(s) in parentheses: O36:19 (meat, α-*hlyA*), O59:H21 (meat, STI-p, STII), O60:H4 (human diarrhea, EAST1), O137:H1 (diseased pig), O121:H19 (pig farm environment, F18, EAST1), O159:H21 (slaughter pig, STI-p, STII), and Or:H19 (asymptomatic human).

for genes encoding adhesins K88, K99, 987p, and F41, which play a role as colonization factors for cattle and pigs. Diffuse adherence (DA) was observed with 31 strains (28.2%) in assays performed on cultured HEp-2 cells. The DA type was not associated with other phenotypical traits and adhesins investigated here or with the origin of the strains. By performing the HEp-2 cell assay, complete detachment of HEp-2 cells from coverslips within a 3-h incubation period with bacteria was found with 11 (10.0%) of the strains. We have looked for the genes encoding cytotoxins, such as α-*hlyA*, *cnf*, *cdt*, and *cif*, which could be responsible for this effect, but only alpha-hemolysin was found to be present in 7 (63.6%) of these 11 strains.

Among the heat-stable enterotoxins STI-p, STI-h, STII, and EAST1, only STI-p (33.6%), EAST1 (21.8%), and STII (5.4%) were detected in some Stx2e strains. No strain was positive for heat-labile enterotoxin LT-I. The results are summarized in Table 6.

Twenty-six (23.6%) of the STEC strains belonged to serotypes that are commonly associated with porcine pathogenic *E. coli* types (O137:H1, O138:H14, O139:H1, and O141:H4). Most of these strains (73.1%) were isolated from diseased pigs, and virulence attributes such as alpha-hemolysin (92.3%) and F18 fimbriae (84.6%) were frequently associated with these strains. Seven (26.9%) of strains belonging to porcine pathogenic types (two O138, two O139, and three O141) provoked rapid detachment of HEp-2 cells in adherence assays (see above). In contrast, alpha-hemolysin and F18 fimbriae were present each in only two (2.3%) and rapid detachment of HEp-2 cells was observed with only four (4.8%) of the 84 Stx2e strains from other sources.

Strains belonging to serotypes O8:H4, O8:H9, and O100:[H30] ($n = 36$) were frequently isolated from pigs at slaughter, from food, and from surface water and soil. All these were negative for the virulence markers tested except for STI-p (75.0%) and EAST1 (13.9%). Serotypes O8:H9 and O100:H30 were also described as frequent in swine feces in a nationwide study performed in the United States (16).

The nine Stx2e-producing strains originating from humans were heterogeneous for their serotypes, and only three of these (two O101:H9 and one O60:H4) were typeable for their O antigen. Five of the human Stx2e strains showed DA and one showed rapid killing of HEp-2, cells as described above. Other virulence markers were not present, except for EAST1 in two (22.2%) of the strains.

DISCUSSION

Shiga toxin 2e-encoding genes are present in *E. coli* strains from various sources and with different genetic backgrounds, indicating that the stx_{2e} genes are spread horizontally in *E. coli*. A lysogenic stx_{2e} bacteriophage designated P27 was recently identified and shown to transduce the stx_{2e} gene to *stx*-negative *E. coli* strains (31). Although P27 shows a typical lambdoid structure, it is only remotely related to other lambdoid *stx*-carrying phages (39). The presence of bacteriophage P27-specific genes in almost all of the strains investigated in this study indicates that the stx_{2e} determinant is spread in *E. coli* by P27-like phages. It was previously suggested that P27-like stx_{2e} phages were acquired early in the evolution of Stx2e strains and have undergone multiple recombination events since then (39). This is in agreement with the finding that most (93.6%) of the 110 Stx2e strains from this study lacked one or more of the P27-specific genes, perhaps by successive deletions of phage sequences. It is therefore likely that the P27-like phages are present as cryptic prophages or phage remnants in most of the strains. This suggestion is also supported by the finding that stx_{2e} genes are generally not transducible from their host strains, which is in contrast to the case for other types of *stx* genes (29, 31).

Besides the genomic differences, the host range within the *E. coli* strains of the stx_{2e} phage type differs from that of strains of the other *stx* phage types. The stx_{2e} gene is associated with *E. coli* serotypes that have not been described as bacterial hosts for other types of Stx so far (4, 16, 17, 39, 42). Additionally, the stx_{2e} gene was not reported to be present in typical and atypical EHEC strains causing bloody diarrhea and HUS, except for one case reported in 1994 (11, 36, 43).

In our study the stx_{2e} gene was not found in combination with other *stx* genes in the bacteria. Similar findings were reported from a study on STEC prevalence in pigs in the United States; the stx_{2e} determinant was rarely found in association with other *stx* genes (16). In contrast to that, other variants of the stx_2 family occur frequently in combination with stx_1 and stx_2 genes in STEC from patients and ruminant animals (11). The reason for these differences between Stx2e-producing and other STEC strains is not known.

The stx_{2e}-positive strains investigated here differed largely in the amount of Stx produced by the bacteria. Without induction, two-thirds of the strains were identified as poor producers of Stx (0 to 1+), but 99% of the strains were inducible for Stx2e production by mitomycin C. Mitomycin C was chosen as a potent inductor for phage-encoded gene expression in vitro. The inducibility of Stx production is likely to play a role in vivo for pathogenesis, since Stx production was shown to be triggered by neutrophils and norepinephrine in the gastrointestinal tract (13, 49). The inducible phenotype could not be related to phage production (data not shown) or to the integrity of the phage P27 genome in these strains. The presence of the phage-carried *Q*-gene was reported to be required for effective transcription and expression of stx_2 genes on *stx*-carrying phages of O157:H7 strains (25). In our study, the absence of the P27 *Q* gene homologue was not associated with low Stx2e production ($P = 0.292$), since it was absent in 82.6% of the high-producing (3+ and 4+) Stx2e strains. Our data confirm previous findings indicating that the P27 *Q* gene is frequently disconnected genetically from the stx_{2e} gene (39). It cannot be excluded that the P27 *Q* gene is replaced by another phage *Q* homologue by recombination. It is also possible that induction of Stx2e production in the strains is generally triggered by phage-independent functions such as RecA, as previously described for EHEC O157 strains (18).

The amount of Stx2e produced by the strains was measured with the P1-g-EIA, a binding assay that measures the quantity and function of the various allelic Stx B proteins. However, it is not likely that the differences found in the amount of Stx2e produced by the strains are attributable to mutations in the stx_{2e} genes, since translation of the nucleotide sequence of the $stxAB_{2e}$ genes in high- and low-Stx2e-producing strains resulted in identical amino acid sequences. On the other hand,

production of Stx2e was associated with the transcription efficiency of the stx_{2e} gene in these strains, which was found to be drastically enhanced by mitomycin C. Similar findings were reported previously for stx_{2d} and stx_{2e} variants (53). Only five (4.5%) of the 110 Stx2e strains showed high production of Stx2e with noninduced and induced cultures, indicating that constitutive high expression of stx_{2e} genes is not common in natural isolates.

The upstream regions flanking the 5′ ends of the *stx* genes were reported to be conserved and continuous with phage sequences in a number of Shiga toxin-encoding bacteriophages (32, 39, 44). Genes for rare tRNAs are located in the neighborhood of the stx_2 genes, and it was suggested that these could play a role in effective expression of Stx2 (23, 38). Correspondingly, the region located upstream from the $stxA_{2e}$ gene was found to be conserved in 95 (86.4%) of the Stx2e strains investigated here, pointing to a common origin of the stx_{2e} genes in most of the strains. However, large differences in Stx2e production were observed despite identical tRNA regions (Table 4 and Fig. 1 and 2), indicating that other functions interfere with the expression of stx_{2e} genes. The 15 stx_{2e} strains that were genetically different from P27 in this region were all high Stx2e producers, indicating that the conserved tRNA region of the P27 genome is not essential for production of Stx2e in these strains. Although most of these 15 strains were unrelated in their serotypes, virulence attributes, and origins (data not shown), they were highly similar in the DNA region upstream of $stxA_{2e}$. This could indicate that these were spread by a recombinant type of P27 phage to genetically different *E. coli* host strains and supports the suggestion of an early uptake of stx_{2e} phages in the evolution of this group of STEC strains (39).

Evaluation of Stx2e strains as potential human diarrheal pathogens. There are only few data available on the role of Shiga toxins in human diarrhea. In vitro production of Stx has been shown to be positively correlated with the severity of diarrheal symptoms in patients infected with EHEC O157 (30, 34), and Stx2 was found to cause damage and fluid secretion in intestinal epithelial cells (41). Therefore, it cannot be excluded that Stx2e might have an effect on fluid secretion in the human intestine.

There are no data available on the toxicity of Stx2e for the human organism. Three of the nine human Stx2e strains produced large amounts (3+ to 4+) of Stx2e when grown without mitomycin C and eight strains (all 4+) did so when grown with mitomycin C, indicating that most of the human strains were good Stx2e producers. Stx2e differs from other Shiga toxins by having an altered receptor specificity (Gb4) (24), and effective binding of Stx2e to porcine red blood cells was found to be essential for development of edema disease in pigs (28). Stx2e was also found to bind as efficiently as Sx1 and Sx2 to human erythrocytes (8), but in contrast to Stx2, Stx2e is not associated with bloody diarrhea and HUS in humans (4, 17, 36). In the pig intestine, Stx2e does not cause fluid accumulation, which indicates that the toxin itself does not cause diarrhea in the animals (48). It was suggested that the lack of enterotoxicity of Stx2e in pigs is due to the absence of toxin receptors in the villus absorptive enterocytes (48). Accordingly, diarrhea is not a typical sign in pigs with edema disease, and it was reported to precede edema disease only in infections with strains producing enterotoxins besides Stx2e (1). The finding that high-Stx2e-producing strains were isolated from humans with no or only mild symptoms of diarrhea indicates that Sx2e has no or little potential to elicit diarrhea in humans, possibly because of a lack of receptors Gb4 and Gb3, which could serve as targets for Stx2e (24) on human enterocytes.

Are Stx2e strains pathogenic for humans? Six of the human excretors suffered from uncomplicated diarrhea, and three individuals showed no symptoms of enteric disease (data not shown). The amounts of Stx2e produced in strains from humans with diarrhea and from asymptomatic cases were similar (data not shown). These findings correspond to other studies reporting that Stx2e-producing strains are rarely isolated from humans, are not significantly associated with diarrhea, and do not cause severe disease (4, 15, 17, 37, 39, 42). The low prevalence of these strains in humans is in clear contrast to their high prevalence in meat and its products in Germany (5). This indicates that most of the Stx2e strains present in food are not good colonizers of the human intestine. Stx2e strains from humans were found to differ from the corresponding porcine pathogenic strains by their adherence to human and swine epithelial cells, but the adhesins of human Stx2e strains were not identified (42). DA to HEp-2 cells, which was used as an indicator for colonization of human epithelial cells, was observed with five human Stx2e strains in this study. The DA type was found in 28.2% of all Stx2e strains investigated here but could not be associated with a particular serotype or with other virulence markers (42). Toxicity resulting in rapid detachment of epithelial cells was reported to be associated with porcine pathogenic types of Stx2e strains (42) and was found to be associated with O138, O139, O141, and O101 strains in our study. On the other hand, only one of the nine human Stx2e strains (O60:H4) provoked rapid detachment of HEp-2 cell monolayers.

Certain more rarely occurring serogroups of Stx2e strains, such as O60 and O101, were reported more frequently in association with human infections (15, 17, 37, 39). It is possible that some of the Stx2e-producing strains have the ability to cause diarrhea in humans, but the underlying mechanism remains unknown. Enterotoxins, which could be a cause of diarrhea in humans, were not investigated in previous studies dealing with human STEC infections (15, 37, 39). It is conceivable that enterotoxins may play a role as a cause for human diarrhea. In contrast to all other types of STEC, more than 50% of Stx2e-producing strains from this study were positive for heat-stable enterotoxins such as STI-p, STII, and EAST1. The majority of the Stx2e strains from food carried STI-p and/or EAST1 genes, and it is thus likely that human consumers frequently come in contact with ST- or EAST1-positive Stx2e strains. In our study, EAST1 was present in two of six Stx2e strains (ONT:H4 and O60:H4) from human patients with diarrhea (4, 17, 42). We also cannot exclude that genetic variants of enterotoxins and adhesion factors that were not detected with the PCR primers used in the study are present in some of the strains.

In conclusion, the results from this study complement others indicating that Stx2e is not a pathogenicity factor for diarrhea and HUS in humans. This finding is important for assessment of the public health impact of STEC from food, because Stx2e-producing *E. coli* accounted for 19% of all STEC isolated from food in a previous study (5).

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