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Shiga Toxin 2e-Producing *Escherichia coli* Isolates from Humans and Pigs Differ in Their Virulence Profiles and Interactions with Intestinal Epithelial Cells

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Thirteen *Escherichia coli* strains harboring stx_{2e} were isolated from 11,056 human stools. This frequency corresponded to the presence of the stx_{2e} allele in 1.7% of all Shiga toxin-producing *E. coli* (STEC) strains. The strains harboring stx_{2e} were associated with mild diarrhea ($n = 9$) or asymptomatic infections ($n = 4$). Because STEC isolates possessing stx_{2e} are porcine pathogens, we compared the human STEC isolates with stx_{2e}-harboring *E. coli* isolated from piglets with edema disease and postweaning diarrhea. All pig isolates possessed the gene encoding the F18 adhesin, and the majority possessed adhesin involved in diffuse adherence; these adhesins were absent from all the human STEC isolates. In contrast, the high-pathogenicity island encoding an iron uptake system was found only in human isolates. Host-specific patterns of interaction with intestinal epithelial cells were observed. All human isolates adhered to human intestinal epithelial cell lines T84 and HCT-8 but not to pig intestinal epithelial cell line IPEC-J2. In contrast, the pig isolates completely lysed human epithelial cells but not IPEC-J2 cells, to which most of them adhered. Our data demonstrate that *E. coli* isolates producing Shiga toxin 2e have imported specific virulence and fitness determinants which allow them to adapt to the specific hosts in which they cause various forms of disease.

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) isolates, which cause diarrhea and hemolytic-uremic syndrome (HUS) in humans (19, 26, 50), generally cause minimal or no injury in their animal host reservoirs (26). The only naturally occurring diseases in animals caused by STEC are swollen head syndrome in chickens (44) and edema disease in piglets (20). Edema disease is characterized by vascular necrosis, edema, and neurological signs and can be fatal (20). Although the exact mechanisms that lead to edema disease are unknown, Stx2e and adherence-mediating virulence factors such as the F18 adhesin, F4 fimbriae, and adhesin involved in diffuse adherence (AIDA) seem to be common among strains isolated from diseased pigs (21, 35). In one study, the presence of Stx2e in the erythrocyte fraction was strongly associated with clinical disease (30). stx_{2e} is the most frequent stx_2 variant found in fecal samples from pigs (14), and it was the second most common stx_2 variant in environmental STEC isolates (54). In the latter study, the stx_{2e} variant was found not only in STEC strains isolated from pig samples but also in isolates from a dairy cattle herd, suggesting that such strains spread from pigs to cattle (54).

Stx2e-producing *E. coli* strains have also occasionally been isolated from humans (5, 15, 40, 52). The majority of the patients had uncomplicated diarrhea (5, 15, 40), and some had HUS (52). However, the frequency with which Stx2e-producing STEC strains occur in humans, their virulence factors, their mechanisms of interaction with the human host, their reservoir(s), and their mode(s) of transmission are poorly understood.

Here, we compared the putative virulence genes in Stx2e-producing *E. coli* strains isolated from humans and diseased pigs in order to assess the extent to which they are related. We also analyzed the interactions of the two groups of organisms with homologous and heterologous intestinal epithelial cells in vitro in a search for characteristics that might be related to adaptation in the host.

MATERIALS AND METHODS

Bacterial strains. After screening 11,056 stools (9,206 from patients with diarrhea or HUS and 1,850 from asymptomatic individuals), we isolated 13 *E. coli* strains containing the stx_{2e} gene. These isolates were obtained from patients with uncomplicated diarrhea ($n = 9$) or from asymptomatic carriers ($n = 4$) and were recovered at the Institute of Hygiene and Microbiology, University of Würzburg, Würzburg, Germany, and the Institute of Hygiene, University Hospital Münster, Münster, Germany, during routine diagnostic examinations and epidemiological investigations between January 1997 and December 2003. The procedures used for STEC isolation from stools have been described previously (15). Briefly, enriched primary stool cultures were screened using PCRs for *stx* and *eae* genes, and STEC strains were isolated from PCR-positive stools using colony blot hybridization with digoxigenin-labeled *stx* probes (15). The 13 human isolates showed no geographical or temporal linkage. A subset of these strains was investigated for stx_{2e} transcription in a previous study (57). Twelve porcine STEC strains harboring stx_{2e} were isolated from German piglets with edema disease or postweaning diarrhea (35), while one strain (strain E57) was isolated from a pig with diarrhea in Canada (27).

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***stx* subtyping.** The isolated strains were tested for stx_1 and stx_2 using the primer pairs KS7-KS8 ($stxB_1$ and $stxB_{1c}$) and LP43-LP44 ($stxA_2$ and $stxA_2$ variants) (Table 1). stx_1 and stx_{1c} were distinguished by HhaI restriction of the KS7-KS8 PCR products (16, 56). The strategy used to distinguish stx_2 from its variants has been described previously (15). Briefly, STEC strains positive in the PCR with primers LP43 and LP44 were subjected to PCR with primers GK3 and GK4 (Table 1), and the amplification products were digested with HaeIII (New England Biolabs, Frankfurt, Germany) to differentiate between stx_2 and stx_{2c} (15). Isolates in which amplification products could not be elicited with primers GK3 and GK4 were tested further for the presence of the stx_{2d} (41) and stx_{2e} (55) genes using primers VT2-cm and VT2-f and primers FK1 and FK2, respectively (Table 1). Strains positive in the PCR with primers FK1 and FK2, which target the $stxB_{2e}$ subunit gene (15), were confirmed to contain the $stxA_{2e}$ subunit gene using the PCR with primers FK9 and FK10 (13) (Table 1). The presence of stx_{2e} in PCR-positive isolates was confirmed by Southern blot hybridization with digoxigenin-labeled $stxA_{2e}$ and $stxB_{2e}$ probes derived from stx_{2e}-harboring human isolate VUB-EH60 (40) by PCRs with primer pairs FK9-FK10 and FK1-FK2 (Table 1), respectively. Moreover, the identity of stx_{2e} genes was verified by nucleotide sequence analysis performed as described previously (57).

PCR. PCRs were performed with a Biometra TGradient 96 cycler (Biometra GmbH, Göttingen, Germany) (16, 48). The PCR primers, target sequences, conditions, and positive controls are shown in Table 1. *E. coli* C600 was used as a negative control. The specificity of PCR products was confirmed by analyzing the sequences of representative amplicons (6, 48).

Southern blot hybridization. Southern blot hybridization of plasmid DNA with digoxigenin-labeled enterohemorrhagic *E. coli* (EHEC) *hlyA*, *katP*, *espP*, and *etpD* probes was performed as described previously (58).

Phenotypic methods. Isolates were serotyped using antisera against *E. coli* O antigens 1 to 181 and H antigens 1 to 56 (42). Stx production was tested using a commercial latex agglutination assay (verotoxin-producing *E. coli* reverse passive latex agglutination; Denka Seiken Co., Ltd., Tokyo, Japan). Fermentation of sorbitol was detected on sorbitol MacConkey (SMAC) agar plates after overnight incubation. The enterohemolytic phenotype was investigated on enterohemolysin agar containing 5% defibrinated and washed sheep erythrocytes and 10 mM $CaCl_2$ (45). Resistance to tellurite was determined from the ability of isolates to grow on cefixime-tellurite (CT)-SMAC agar (Oxoid, Hampshire, United Kingdom) (7). Urease activity was examined in urea degradation broth (Heipha) after 24 h of incubation at 37°C (9, 17).

Cell cultures. The T84 cell line (human colonic carcinoma epithelial cells; ATCC CCL-248) and the HCT-8 cell line (human ileocecal adenocarcinoma cells; ATCC CCL-244) were used. The culture medium for T84 cells contained a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 medium (Cambrex Bioscience, Verviers, Belgium) supplemented with 10% (vol/vol) fetal calf serum (FCS) (Cambrex). HCT-8 cells were grown in RPMI 1640 (Cambrex) supplemented with 10% FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate (Cambrex). The IPEC-J2 cell line (4) from jejunal epithelial cells of a neonatal piglet was maintained in a 1:1 mixture of Dulbecco's modified Eagle medium and Ham's F-12 medium (Cambrex) supplemented with 5% FCS. All cell cultures were grown at 37°C in 5% CO_2 until they reached confluence, and then they were subcultured using a 0.1% trypsin–EDTA solution (Cambrex).

Interaction of stx_{2e}-harboring *E. coli* with intestinal epithelial cells. For the adherence assay, cells were grown on coverslips in six-well plates (Corning Inc., Corning, N.Y.) which were seeded with 1×10^6 T84 or HCT-8 cells/well or 2.5×10^5 IPEC-J2 cells/well; the plates were incubated at 37°C with 5% CO_2 until the cultures were semiconfluent. One hundred fifty microliters of a bacterial overnight culture in Luria-Bertani broth (8×10^7 to 1×10^8 CFU) was added to the cells and allowed to attach for 5 h. The cells were then thoroughly washed with phosphate-buffered saline (Cambrex), fixed with 70% ethanol, and stained with 10% Giemsa stain (Merck). The adherence assay was performed in parallel in the absence and presence of 0.5% (wt/vol) D-mannose (Roth, Karlsruhe, Germany) in the growth medium. For quantitative analysis, the numbers of bacteria attached to one cell were determined, and the results were scored as follows: ++++, >100 bacteria attached; +++, 50 to 100 bacteria attached; ++, 10 to 50 bacteria attached; +, 1 to 10 bacteria attached; −, no bacteria attached. Enteropathogenic *E. coli* strain 2348/69 (O127:H6) (33) and *E. coli* K-12 strain C600 were used as positive and negative controls, respectively. To ensure that the bacteria interacted specifically with the intestinal epithelial cells, the assays with all strains were also performed in wells without cells. To test the effects of culture supernatants on the intestinal epithelial cells, strains were grown with aeration (180 rpm) in Luria-Bertani broth overnight, the bacterial cells were removed by centrifugation (8,000 rpm, 15 min), and the supernatants were filter sterilized (pore size, 0.22 μm; Schleicher & Schuell GmbH, Dassel, Germany). The presence of Stx2e was verified by the latex agglutination assay as described above.

RESULTS

Frequency of stx_{2e}-containing *E. coli* in human stools. A total of 747 STEC strains were isolated from 11,056 stools from patients with HUS or diarrhea or asymptomatic individuals. The 13 stx_{2e}-harboring STEC strains isolated during the period studied thus accounted for 1.7% of all STEC isolates and were in 0.12% of all stool samples investigated. All STEC strains harboring stx_{2e} were isolated from patients with mild diarrhea ($n = 9$) or from asymptomatic carriers ($n = 4$). None was associated with HUS.

Diagnostic characteristics of stx_{2e}-harboring strains. Table 2 compares the serotypes of human STEC isolates containing stx_{2e} with those of stx_{2e}-positive STEC isolates from pigs. The human isolates belonged to none of the serotypes associated with edema disease in piglets, and more than one-half were nontypeable with antisera against currently known *E. coli* O antigens, suggesting that they might represent new serotypes. All but one human strain and all but three porcine strains produced Stx2e, as demonstrated by a commercial latex agglutination assay. All isolates fermented sorbitol on SMAC agar within 24 h, and none grew on CT-SMAC agar. This is consistent with the absence in all of the strains of *terF* (Table 2), which is used as a marker for the *ter* cluster that encodes tellurite resistance (7, 51). Similarly, in accordance with the absence of the EHEC *hlyA* gene in all 26 strains (Table 2), none displayed an enterohemolytic phenotype on enterohemolysin agar. None of the strains investigated possessed the *ureC* gene (Table 2), a marker for the *ure* gene cluster (18, 32), and accordingly none of them produced urease.

Distribution of virulence genes. Genes encoding various adhesins, such as intimin, the iron-regulated gene A homologue adhesin (Iha) (49), EHEC factor for adherence (Efa1) (22, 34), STEC autoagglutinating adhesin (Saa) (37), and Sfp fimbriae (17), which are frequently found in STEC strains harboring stx_2 and the variants stx_{2c} and stx_{2d} (15, 16, 17, 22), were not found in any of the 26 *E. coli* strains harboring the stx_{2e} allele (Table 2). All of the porcine isolates, but none of the human isolates, possessed *fedA*, which encodes the major subunit of F18 fimbrial adhesin (35). Most of the porcine isolates (11 of 13) contained the *orfB* gene, which encodes AIDA (2) (Table 1). The *orfA* gene encoding a 45-kDa protein, which is required to modify AIDA so that it adheres to target cells (3), was present in all porcine isolates (Table 2). Ten of the 13 porcine isolates but none of the human isolates contained *espI* (Table 2), which is located on a pathogenicity island termed the locus of proteolysis activity which is inserted into *selC* of locus of enterocyte effacement-negative stx_{2d}-harboring STEC strains (46). *espI* encodes a novel serine protease (EspI) which cleaves swine pepsin A and human apolipoprotein A-I (46). In contrast, the *espP* gene encoding plasmid-encoded serine protease (EspP) in *E. coli* O157:H7, as well as the other plasmid-borne genes of STEC strains, such as EHEC *hlyA*, *katP*, and *etpD* (48, 58), were absent from all 26 strains investigated (Table 2). Similarly, various alleles encoding cytolethal distending toxin (*cdt-I*, *cdt-II*, *cdt-III*, and *cdt-V*), some of which were previously identified in a subset of *eae*-negative STEC strains from pa-

TABLE 1. PCR primers and conditions used in this study

Primer	Sequence (5′-3′)	Target(s)	PCR conditions[a]			Size of PCR product (bp)	Reference	Positive control[b]
			Denaturation	Annealing	Extension			
KS7	CCCGGATCCATGAAAAAAACATTATTAATAGC	$stxB_1$	94°C, 30 s	52°C, 60 s	72°C, 40 s	285	15	EDL933
KS8	CCCGAATTCAGCTATTCTGAGTCAACG	$stxB_{1c}$						
LP43	ATCCTATTCCCGGGAGTTTACG	$stxA_2$ and variants	94°C, 30 s	57°C, 60 s	72°C, 60 s	584	15	EDL933
LP44	GCGTCATCGTATACACAGGAGC							
GK3	ATGAAGAAGATGTTTATG	$stxB_2$, $stxB_{2c}$	94°C, 30 s	52°C, 60 s	72°C, 40 s	260	15	EDL933
GK4	TCAGTCATTATTAAACTG							
VT2-cm	AAGAAGATATTTGTAGCGG	$stxB_{2d}$	94°C, 30 s	55°C, 60 s	72°C, 60 s	256	41	EH250
VT2-f	TAAACTGCACTTCAGCAAAT							
FK1	CCCGGATCCAAGAAGATGTTTATAG	$stxB_{2e}$	94°C, 30 s	55°C, 60 s	72°C, 40 s	280	15	VUB-EH60
FK2	CCCGAATTCTCAGTTAAACTTCACC							
FK9	CCCGGATCCATGAAGTGTATATTGTTA	$stxA_{2e}$	94°C, 30 s	52°C, 60 s	72°C, 60 s	260	13	VUB-EH60
FK10	CCCGAATTCAGCACAATCCGCCGCCAT							
SK1	CCCGAATTCGGCACAAGCATAAGC	*eae*	94°C, 30 s	52°C, 60 s	72°C, 60 s	863	15	EDL933
SK2	CCCGGATCCGTCTCGCCAGTATTCG	Conserved						
Iha-I	CAGTTCAGTTTCGCATTCACC	*iha*	94°C, 30 s	56°C, 60 s	72°C, 90 s	1,305	46	4797/97
Iha-II	GTATGGCTCTGATGCGATG							
SAADF	CGTGATGAACAGGCTATTGC	*saa*	94°C, 30 s	52°C, 60 s	72°C, 40 s	119	16	3937/97[c]
SAADR	ATGGACATGCCTGTGGCAAC							
E643f	TATCAGGCCAATCAAAACAG	*efa-1*[d]	94°C, 30 s	50°C, 60 s	72°C, 60 s	974	22	493/89
E1598r	AGACACTGGTAAATTTCGC							
E5242f	TAAGCGAGCCCTGATAAGCA	*efa-2*[d]	94°C, 30 s	55°C, 60 s	72°C, 60 s	630	22	493/89
E5854r	CGTGTTGCTTGCCTTTGC							
E7044f	TGTCTAACTGGATTGTATGGC	*efa-3*[d]	94°C, 30 s	56°C, 60 s	72°C, 60 s	685	22	493/89
E7710r	ATGTTGTTCCCGGCCCAGT							
sfpA-U	AGCCAAGGCCAAGGGATTATTA	*sfpA*	94°C, 30 s	59°C, 60 s	72°C, 60 s	440	17	493/89
sfpA-L	TTAGCAACAGCAGTGAAGTCTC							
HlyA1	GGTGCAGCAGAAAAAGTTGTAG	EHEC *hlyA*	94°C, 30 s	57°C, 60 s	72°C, 90 s	1,550	45	EDL933
HlyA4	TCTCGCCTGATAGTGTTTGGTA							
esp-A	AAACAGCAGGCACTTGAACG	*espP*	94°C, 30 s	56°C, 60 s	72°C, 150 s	1,830	56	EDL933
esp-B	GGAGTCGTCAGTCAGTAGAT							
D1	CGTCAGGAGGATGTTCAG	*etpD*	94°C, 30 s	56°C, 60 s	72°C, 70 s	1,062	56	EDL933
D13R	CGACTGCACCTGTTCCTGATTA							
wkat-B	CTTCCTGTTCTGATTCTTCTGG	*katP*	94°C, 30 s	56°C, 60 s	72°C, 150 s	2,125	56	EDL933
wkat-F	AACTTATTTCTCGCATCATCC							
Cdt I-f	TGGTGAGAATCGGAACTG	*cdt-IA*	94°C, 30 s	51°C, 60 s	72°C, 60 s	418	6	6468/62
Cdt I-r	CATTCCATCAGGTTTGTC							
Cdt II-f	AATCCCTATCCCTGAACC	*cdt-IIA*	94°C, 30 s	52°C, 60 s	72°C, 60 s	542	6	9142/88
Cdt II-r	GTTCTATTGGCTGTGGTG							
Cdt III-f	AAACAGGACGGTAATAATGACTAATA	*cdt-III*	94°C, 30 s	54°C, 60 s	72°C, 180 s	2,230	6	1404
Cdt III-r	GTGATCTCCTTCCATGAAAATATAGT	Complete						
c338f	AGCATTAAATAAAAGCACGA	*cdt-VA*[e]	94°C, 30 s	52°C, 60 s	72°C, 60 s	1,329	23	493/89
c2135r	TACTTGCTGTGGTCTGCTAT							
c1309f	AGCACCCGCAGTATCTTTGA	*cdt-VB*[e]	94°C, 30 s	52°C, 60 s	72°C, 60 s	1,363	23	493/89
c2166r	AGCCTCTTTTATCGTCTGGA							
P105	GTCAACGAACATTAGATTAT	*cdt-VC*[e]	94°C, 30 s	49°C, 60 s	72°C, 60 s	748	23	493/89
c2767r	ATGGTCATGCTTTGTTATAT							
RTsubAF	CGAATGTTTTTCTTGCTCCAG	*subA*	94°C, 30 s	53°C, 60 s	72°C, 60 s	220	38	3706/02[f]
RTsubAR	ACACTGCTGACAGGATGATAAG							
espI-I	ATGGACAGAGTGGAGACAG	*espI*	94°C, 30 s	52°C, 60 s	72°C, 60 s	560	46	4797/97
espI-II	GCCACCTTTATTCTCACCA							
Irp2 FP	AAGGATTCGCTGTTACCGGAC	*irp2*[g]	94°C, 30 s	60°C, 60 s	72°C, 60 s	280	25	5720/96
Irp2 RP	TCGTCGGGCAGCGTTTCTTCT							
FyuA f	TGATTAACCCCGCGACGGGAA	*fyuA*[g]	94°C, 30 s	63°C, 60 s	72°C, 90 s	880	25	5720/96
FyuA r	CGCAGTAGGCACGATGTTGTA							
UN19	CTGGGTGACATTATTGCTTGG	*orfA*[h]	94°C, 60 s	64°C, 60 s	72°C, 90 s	370	35	2787
UN20	TTTGCTTGTGCGGTAGACTG							
UN21	TGAAAACATTAAGGGCTCG	*orfB*[i]	94°C, 60 s	64°C, 60 s	72°C, 90 s	450	35	2787
UN22	CCGGAAACATTGACCATACC							
UN23	CAGTTTATCAATCAGCTCGGG	*orfB*[j]	94°C, 60 s	64°C, 60 s	72°C, 90 s	543	35	2787
UN24	CCACCGTTCCGTTATCCTC							
fedA1	GTGAAAAGACTAGTGTTTATTTC	*fedA*	94°C, 60 s	56°C, 60 s	72°C, 60 s	230	35	2787
fedA2	CTTGTAAGTAACCGCGTAAGC							
TerF1	TTACAATCCGGACAAAACA	*terF*	94°C, 30 s	55°C, 60 s	72°C, 60 s	244	51	EDL933
TerF2	CAATGACAACGGTGATCG							
UreC-f	TCTAACGCCACAACCTGTAC	*ureC*	94°C, 60 s	60°C, 60 s	72°C, 60 s	398	32	EDL933
UreC-r	GAGGAAGGCAGAATATTGGG							

[a] All PCRs included 30 cycles, followed by a final extension of 5 min at 72°C.
[b] The PCR positive control strains were the strains described in the references unless indicated otherwise.
[c] *saa*$^+$ *E. coli* O91:NM (16).
[d] Three different regions of *efa1* were targeted to detect the whole gene (9,996 bp) (22).
[e] The presence of three open reading frames encoding cytolethal distending toxin V was investigated.
[f] *subA*$^+$ *E. coli* O113:H21 from our collection (H. Karch, unpublished).
[g] Markers for the HPI. The presence of additional HPI genes, their links, and the insertion site of HPI were determined previously (25).
[h] *orfA* encodes a 45-kDa protein which is required to modify AIDA-I to adhere to target cells (3).
[i] The primer amplifies a fragment from the coding region for AIDA-I (35).
[j] The primer amplifies a fragment from the coding region for AIDA^C (35).

TABLE 2. Distribution of putative virulence genes and other genes investigated among stx_{2e}-harboring *E. coli* strains isolated from humans and pigs

Gene	Predicted product or marker[a]	Human isolates ($n = 13$)[b]		Porcine isolates ($n = 13$)[c]	
		No. positive	No. negative	No. positive	No. negative
stx_{2e}	Stx2e	13	0	13	0
eae	Intimin	0	13	0	13
iha	Iha	0	13	0	13
efa1	Efa1	0	13	0	13
saa	Saa	0	13	0	13
sfpA	SfpA	0	13	0	13
fedA	Major subunit of F18	0	13	13	0
orfA	AIDA-MP	0	13	13	0
orfB	AIDA	0	13	11	2
espI	Serine protease EspI	0	13	10	3
espP	Serine protease EspP	0	13	0	13
EHEC *hlyA*	EHEC hemolysin	0	13	0	13
etpD	Type II secretion system	0	13	0	13
katP	Catalase peroxidase	0	13	0	13
cdt[d]	CDT	0	13	0	13
subA	Subtilase cytotoxin A subunit	0	13	0	13
irp2	HPI	5	8	0	13
fyu A	HPI	5	8	0	13
terF	Tellurite resistance	0	13	0	13
ureC	Urease	0	13	0	13

[a] Iha, iron-regulated gene A homologue adhesin; Efa1, EHEC factor for adherence; Saa, Shiga toxin-producing *E. coli* autoagglutinating adhesin; SfpA, major pillin subunit of sorbitol-fermenting STEC O157 plasmid-encoded fimbriae (Sfp); AIDA-MP, AIDA modifying protein (3); F18, fimbrial adhesin; CDT, cytolethal distending toxin.

[b] The serotypes were O8:H10 (one strain), O8:H19 (one strain), O8:H- (one strain), O8:HNT (two strains), ONT:H10 (one strain), ONT:H19 (two strains), and ONT:H- (five strains).

[c] The serotypes were O138:K81 (one strain), O139:K12 (one strain), O139:K82 (eight strains), O141:K45 (one strain), O141:K85 (one strain), and O149:K91 (one strain).

[d] The presence of *cdt-I*, *cdt-II*, *cdt-III*, and *cdt-V* alleles (6) was investigated.

tients (6), were absent from all human and porcine stx_{2e}-harboring *E. coli* isolates (Table 2). Also, none of the strains investigated possessed the *subA* gene encoding the A subunit of the subtilase cytotoxin (38).

HPI is present in human STEC isolates that harbor stx_{2e}. Five of the 13 human *E. coli* isolates that possessed stx_{2e}, but none of the corresponding porcine isolates, contained *irp2* and *fyuA* (Table 2), which are components of an iron uptake-mediating gene cluster located on the high-pathogenicity island (HPI) (25). This prompted us to investigate whether a complete HPI is present in these five human isolates. Moreover, we compared HPIs of stx_{2e}-harboring *E. coli* strains with previously characterized HPIs in STEC isolates belonging to serogroups O26 and O128 and *Yersinia pestis* (25). To do this, all five stx_{2e}-harboring *E. coli* strains were subjected to 14 additional PCRs which target the other HPI genes or link consecutive genes (25). The results of the HPI analysis of these strains and a comparison of the HPIs of stx_{2e}-harboring STEC isolates with the HPIs of other STEC strains and *Y. pestis* are summarized in Table 3. Each of the five stx_{2e}-containing STEC isolates yielded amplicons that were of the same size as those detectable in the positive control STEC and *Y. pestis* strains in each of the PCRs targeting single HPI genes or links of the genes that constitute the siderophore yersiniabactin biosynthetic cluster (*ybtS*, *ybtQ*, *ybtA*, *irp2*, *irp1*, *ybtU*, *ybtT*, and *ybtE*) and the *fyuA* gene encoding the yersiniabactin receptor (Table 3) (PCRs IV to X and XII to VIII). Moreoever, similar to HPI of *Y. pestis*, but unlike HPIs of the STEC strains belonging to serogroups O26 and O128, the HPI in each of the five stx_{2e}-harboring *E. coli* strains contained the insertion element IS*100* (PCR XI) (Table 3). The sizes of the amplicons elicited from the integrase gene (*int*) (PCR III) (Table 3) in four of the five stx_{2e}-containing STEC strains were identical to the size of the amplicon elicited from STEC O26 strain 5720/96 (Table 3), which was previously shown to possess a truncated *int* gene (25). In contrast, one remaining strain possessing stx_{2e} (24059/97) yielded an *int* amplicon that was the same size as the amplicons of STEC O128 strain 3172/87 and *Y. pestis* strain (Table 3), both of which contain an intact *int* gene (25).

The integration site of HPI in stx_{2e}-harboring STEC strains was investigated using PCRs (25) linking the *int* gene of HPI with three different tRNA loci (*asnT*, *asnU*, and *asnV*). These sites are used by HPI to integrate into the chromosomes of pathogenic yersiniae (10). Amplicons that were 900 and 1,100 bp long, similar to those in STEC O26 strain 5720/96, were obtained from four strains in two different PCRs connecting *asnT* with the *int* gene (PCRs I and II) (Table 3). In these two PCRs, the remaining stx_{2e}-positive strain yielded amplicons that were 1,200 and 1,500 bp long and were similar to those in *Y. pestis* and STEC strain 3172/97 (Table 3). *asnU-int* and *asnV-int* PCRs were negative for all strains investigated (data not shown). These findings demonstrate that in STEC strains harboring stx_{2e} HPI is located near *asnT*, similar to the location in STEC O26 and O128 and *Y. pestis* (25). These PCR analyses also confirmed that four of the five strains harboring stx_{2e} (3357/98, 665/00, E01/233, and E02/25), like STEC O26 strain 5720/96, possess an HPI with a truncated *int* gene. In contrast, the remaining stx_{2e}-positive strain, 24059/97, like STEC O128 strain 3172/97 and *Y. pestis*, contains an HPI with an intact *int* gene (Table 3). Together, these data demonstrate that each of

the five STEC human isolates harboring stx_{2e} possesses a complete HPI whose structure is closer to that of *Y. pestis* than to that of STEC strains belonging to serogroups O26 and O128.

Interaction of stx_{2e}-containing STEC with cultured intestinal epithelial cells. The known STEC adhesins are absent from *E. coli* strains containing stx_{2e} (Table 2). Therefore, we investigated whether stx_{2e}-containing STEC can adhere to intestinal epithelial cells in vitro. As shown in Table 4, all human strains adhered with various intensities to human cell lines T84 and HCT-8. The presence of D-mannose in the culture medium did not inhibit the adherence of most of these strains; the only exception was strain 3096/00 (Table 4), which adhered more strongly to T84 cells in the absence than in the presence of 0.5% D-mannose. This suggests that an as-yet-unidentified adhesin(s), different from type 1 pili, plays a role in the adherence of human STEC strains harboring stx_{2e} to human intestinal epithelial cells. However, none of the human stx_{2e}-containing STEC strains adhered to pig intestinal epithelial cell line IPEC-J2 (Table 4).

In contrast to human strains, which lysed none of the intestinal epithelial cell lines investigated, most pig isolates completely lysed human intestinal epithelial cells during a 5-h incubation (Table 4). However, with an equally long incubation period, the pig isolates did not lyse IPEC-J2 cells (Table 4), but 12 of the 13 strains adhered (Table 4). Representative patterns of the interaction of human and pig STEC strains with intestinal epithelial cells from homologous and heterologous hosts are shown in Fig. 1. No lysis of human intestinal epithelial cells was observed with sterile-filtered culture supernatants of the 26 strains, 22 of which contained Stx2e as determined by the latex agglutination assay.

DISCUSSION

STEC ecology is complex and only partially understood. This is due to the marked heterogeneity of STEC strains. Epidemiological studies and molecular profiling indicate that most STEC infections in humans are food-borne and that the source of the pathogen is a nonhuman reservoir (26). Recent studies indicate that besides ruminants, swine also harbor STEC capable of causing human illness (11, 14, 43). A study conducted in the United States showed that 13% and 6% of STEC strains isolated from swine feces during a farm survey possessed the stx_1 and stx_2 genes, respectively (14). These genes are typically found in human STEC strains; however, the majority (80%) of these strains harbored the stx_{2e} variant (14) known to cause edema disease in weaned pigs (20). Although strains causing pig edema disease have been extensively characterized (1, 21, 35), to our knowledge this is the first detailed analysis of phenotypic and molecular characteristics of human stx_{2e}-containing STEC isolates. Both serotyping and molecular profiling demonstrated that Stx2e-producing STEC strains that cause human diseases are different from the strains that cause edema disease in pigs. Moreover, the two groups vary in their interactions with intestinal epithelial cells.

Most laboratories do not routinely screen for Stx2e-producing STEC in the way that they screen for *E. coli* O157:H7. The former strains would be overlooked on sorbitol MacConkey agar because all isolates investigated ferment sorbitol. They also do not grow on CT-SMAC agar, a medium routinely used to isolate *E. coli*

TABLE 3. PCR analysis of HPIs in stx_{2e}-harboring *E. coli* strains of human origin and comparison with HPIs of other STEC strains and *Y. pestis*

Strain or species[a]	Serogroup	Results of PCRs targeting HPI genes or their links[b]																	
		PCR I, *asnT-int(1)*	PCR II, *asnT-int(2)*	PCR III, *int*	PCR IV, *ybtS*	PCR V, *ybtQ*	PCR VI, *ybtA*	PCR VII, *irp2*	PCR VIII, *irp1*	PCR IX, *ybtE-fyuA*	PCR X, *fyuA*	PCR XI, IS*100*	PCR XII, *int-ybtS*	PCR XIII, *ybtS-ybtQ*	PCR IV, *ybtQ-ybtA*	PCR XV, *ybtA-irp2*	PCR XVI, *irp2-irp1*	PCR XVII, *irp1-ybtT*	PCR XVIII, *ybtT-fyuA*
5720/96[c]	O26	900	1,100	900	160	800	230	280	240	360	780	–	800	2,800	2,800	1,300	300	1,700[d]	2,500
3172/97[c]	O128	1,200	1,400	1,200	160	800	230	280	240	360	780	–	800	2,800	2,800	1,300	300	1,700[d]	2,500
Y. pestis[c]	NA[g]	1,255	1,500	1,203	160	797	233	286	237	359	780	100	830	2,797	2,805	1,340	300	1,762	2,518
24059/97	ONT	+[e]	+[e]	+[e]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3357/98	ONT	+[f]	+[f]	+[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
665/00	O8	+[f]	+[f]	+[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E01/233	ONT	+[f]	+[f]	+[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E02/25	ONT	+[f]	+[f]	+[f]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. coli C600	NA[g]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a] *E. coli* strains 5720/96 and 3172/97 and *Y. pestis* strain KIM6 were analyzed previously to determine their HPI structures (25) and were used as positive controls in this study; *E. coli* C600 was a negative control. The other five strains are human STEC isolates containing stx_{2e}.

[b] The numbering of the PCRs and the sequences targeted are the same as described by Karch et al. (25). *int* is the integrase gene; *ybtS*, *ybtQ*, *ybtA*, *irp2*, *irp1*, *ybtE*, and *ybtT* are components of the siderophore yersiniabactin biosynthetic gene cluster; *fyuA* encodes yersiniabactin receptor; and IS*100* is an insertion element. PCR results: +, amplicon of the same size as that elicited from the positive control strains was obtained, unless indicated otherwise; –, no PCR product was obtained.

[c] The values for PCRs I to XVIII indicate the sizes (in bp) of amplicons obtained in the PCRs with the positive control strains. –, no PCR product was obtained. The sizes of the *Y. pestis* PCR products are the sizes in the previously published HPI sequence (25).

[d] The 1,700-bp amplicon indicates that the *ybtU* gene located between *irp1* and *ybtT* is present and intact.

[e] +, amplicons that were the same size as those from *Y. pestis* and *E. coli* strain 3172/97 were obtained.

[f] +, amplicons that were the same size as those from *E. coli* strain 5720/96 were obtained.

[g] NA, not available.

TABLE 4. Interaction of stx_{2e}-harboring STEC strains from humans and pigs with intestinal epithelial cells from homologous and heterologous hosts

Strain	Serotype	Source	Adherence to cell line[a]:		
			T84	HCT-8	IPEC-J2
2771/97	ONT:H-	Human	+++	++	−
3054/97	O8:HNT	Human	++	+++	−
3583/97	ONT:H-	Human	+	+++	−
24059/97	ONT:H10	Human	++	+++	−
24066/97	ONT:H-	Human	+	+	−
26725/97	ONT:H-	Human	+	+++	−
3229/98	O8:H-	Human	+	++	−
3357/98	ONT:H19	Human	+	++	−
3615/99	O8:H10	Human	++	+++	−
665/00	O8:H19	Human	+++	+	−
3096/00	O8:HNT	Human	++	++	−
E01/233	ONT:H-	Human	+	++	−
E02/25	ONT:H19	Human	++	+++	−
S103G	O141:K45	Pig	CL[b]	CL	+
S105G	O139:K12	Pig	80%[c]	CL	+
S115G	O139:K82	Pig	CL	CL	++
S116G	O139:K82	Pig	CL	CL	++
S123G	O139:K82	Pig	CL	CL	++
S125G	O139:K82	Pig	CL	CL	++
S126G	O139:K82	Pig	CL	CL	−
S128G	O139:K82	Pig	90%[c]	CL	++++
S130G	O149:K91	Pig	90%[c]	CL	+
S131G	O139:K82	Pig	CL	CL	+
S132G	O139:K82	Pig	CL	CL	+++
S138G	O139:K82	Pig	CL	CL	+++
E57	O138	Pig	CL	CL	++
2348/69	O127:H6	Human EPEC (positive control)[d]	++	++++	−
C600	Not available	Laboratory strain (negative control)	−	+	−

[a] After 5 h of incubation of bacteria with cells in the presence of 0.5% D-mannose, the adherence was quantified based on the number of bacteria attached to one cell, as follows: ++++, >100 bacteria; +++, 50 to 100 bacteria; ++, 10 to 50 bacteria; +, 1 to 10 bacteria; −, no bacteria attached.
[b] CL, complete lysis of cells after 5 h of incubation with bacteria.
[c] Percentage of cells which underwent lysis after 5 h of incubation with bacteria.
[d] EPEC, enteropathogenic *E. coli*.

O157:H7 (26, 50), and this is due to their tellurite sensitivity, as demonstrated in this study. Furthermore, EHEC *hlyA*, the structural gene encoding EHEC hemolysin (45), was not present in any of the stx_{2e}-harboring strains investigated. As a result, none of the strains showed the characteristic enterohemolytic phenotype. Although EHEC hemolysin production is a useful marker for the detection of STEC (9, 48, 53), it cannot be used to detect strains producing Stx2e. In addition, all Stx2e-producing STEC investigated lacked *ureC*, which we used as a marker for the *ure* operon (18, 24). It has recently been reported (32) that the presence of *ureC* distinguishes STEC strains belonging to the major serogroups associated with human diseases (O157, O26, O103, O111, and O145) from diarrheagenic *E. coli* belonging to other pathogroups (32). On the basis of this finding, *ureC* has been recommended as a target in the screening for such STEC strains (32). Our data, on the other hand, demonstrate that STEC strains harboring stx_{2e} would be missed by this screening procedure because of the absence of *ureC*. Thus, because of the poor repertoire of diagnostically useful phenotypic markers in stx_{2e}-positive STEC, the detection of the stx_{2e} gene with PCR, as used in this study, followed by colony blot hybridization, should be superior to the culture methods for screening primary stool cultures. The PCR approach enabled us to show that the frequency with which strains possessing the stx_{2e} allele occur in human stools is very low (0.12%) and that such strains can be present in stools of asymptomatic subjects. This raises a question about the etiological role of these strains in human diseases. In this study, we were unable to identify stx_{2e}-positive STEC strains in association with bloody diarrhea or HUS, although such strains have been isolated from an HUS patient by other workers (52). The absence of data on the anti-O157 lipopolysaccharide antibody response in the HUS patient of Thomas et al. (52), however, does not allow exclusion of a coinfection with *E. coli* O157:H7, which might have caused the HUS.

It is well established that STEC strains produce factors other than Stx that are potentially injurious to the human host (6, 8, 22, 23, 28, 34, 37, 38, 49). Intimin, the best-characterized STEC adhesin (33, 59), mediates attaching and effacing lesions in vitro and in animal models (33), but there are several other putative adherence factors, including Iha (49), Saa (37), and Efa1 (34), which also mediate adherence in vitro. However, all of these factors are absent from the stx_{2e}-containing strains investigated here. Notably, we observed different patterns when the strains interacted with intestinal epithelial cells. Whereas stx_{2e}-containing STEC strains from humans adhered to human epithelial cells, they did not adhere to pig intestinal epithelial cells. Although the molecular basis of this phenomenon is not known, differences in receptor-binding capacities

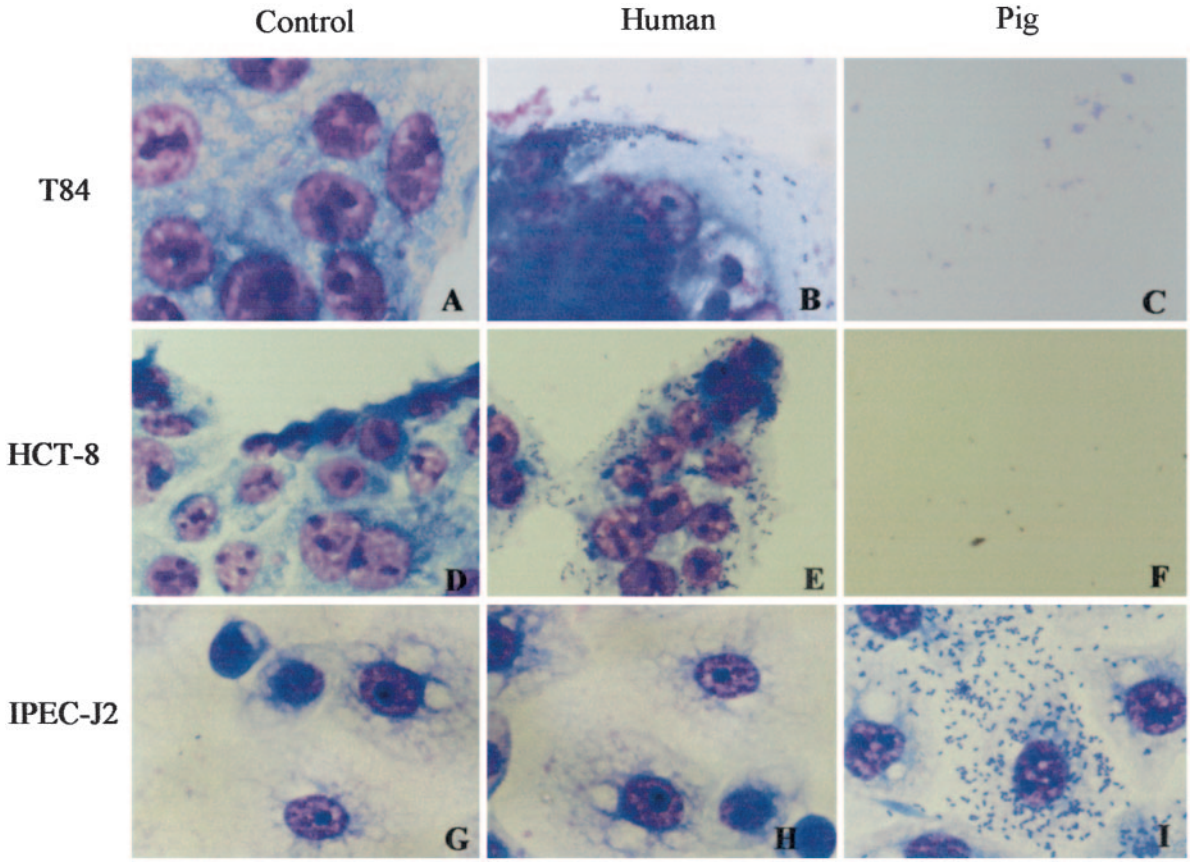

FIG. 1. Interaction of human and pig stx_{2e}-containing STEC isolates with intestinal epithelial cells from homologous and heterologous hosts. (A, D, and G) Control (untreated) cells of cell lines T84 (human), HCT-8 (human), and IPEC-J2 (pig), respectively. (B) Adherence of human strain 24059/97 to T84 cells. (E) Adherence of human strain 3583/97 to HCT-8 cells. (H) Lack of adherence of human strain E02/25 to IPEC-J2 cells. (C and F) Lysis of T84 and HCT-8 cells by pig strains S103G and S115G, respectively. (I) Adherence of pig strain S128G to IPEC-J2 cells. No adherence was observed with any of the strains tested in wells without intestinal epithelial cells, indicating that the adherence is cell dependent.

of intestinal epithelial cells from different hosts are likely to be involved. Moreover, porcine strains were able to lyse human, but not porcine, intestinal epithelial cells. The cell lysis is not attributable to Stx2e because of its rapid occurrence (after 5 h) and because T84 cells do not express Gb3 and Gb4, the receptors for Stx2e (29, 53, 55). Moreover, sterile culture supernatants containing Stx2e displayed no visible lysis of the intestinal epithelial cells used in this study. The factors determining the interaction of Stx2e-producing STEC strains with intestinal epithelial cells from the homologous and heterologous species are not known, but it is likely that the differences in the interaction are due to differences in the molecular mechanisms involved. We have initiated transposon mutagenesis experiments to determine which genes are involved in the ability of porcine strains to cause the cell lysis.

It is noteworthy that the plasmid-encoded cytolysin EHEC hemolysin and cytolethal distending toxin, a potent toxin produced by a subset of STEC strains associated with human disease (6, 23, 39) and by Stx2f-producing STEC strains found in pigeons (31), are absent from STEC strains producing Stx2e. Furthermore, in addition to having no EHEC *hlyA*, all Stx2e-producing strains also lack other plasmid-borne genes, such as *katP*, *espP*, and *etpD*, which are usually present in STEC strains harboring stx_1 and stx_2 (9, 48, 58) and their variants (stx_{1c}, stx_{2c}, or stx_{2d}) (16, 48). Our finding that known putative virulence determinants of STEC strains pathogenic for humans are absent from Stx2e-producing human isolates extends a previous observation by our group (13). Although we found a close relatedness between one human and four porcine *E. coli* O101 strains by DNA fingerprinting, the virulence factors typically found in porcine STEC (i.e., heat-stable and heat-labile enterotoxins and F107 fimbriae) were absent from the human isolate (13). Moreover, this isolate also lacked virulence factors (*eae* and EHEC hemolysin) typical of STEC pathogenic for humans (13). This indicated that the pathogenicity of the human Stx2e-producing *E. coli* O101 strain might involve different mechanisms. Taken together, these data demonstrate that the mechanisms of pathogenicity of Stx2e-producing STEC strains associated with human diseases warrant further investigation.

Although swine are a potential reservoir of STEC strains that cause human illness (11, 14, 43), in an analysis of 11,056 stool samples from humans we were unable to detect the Stx2e-producing strains belonging to serogroups O138, O139, and O141 which are associated with edema disease in piglets (1, 35). Furthermore, a detailed characterization of the Stx2e-producing strains isolated from humans showed that they lack virulence factors, such as AIDA and F18 adhesins, that are frequently found in Stx2e-producing strains associated with pig

edema disease (1, 21, 35). Therefore, it is unlikely that the Stx2e-producing STEC strains that cause pig edema disease are human pathogens. Moreover, only some of the serotypes identified among the Stx2e-producing STEC strains from humans in this study and in a study by Beutin et al. (O43:H30, O60:H4, O91:H21, Ont:H10, and Ont:H19) (5) have been found among the stx_{2e}-harboring STEC strains isolated from healthy pigs (14). These data indicate that there may be additional, as-yet-unknown reservoirs of STEC strains harboring stx_{2e} and that the extent to which these porcine strains play a role in the epidemiology of human infections needs further investigation. The majority of Stx2e-producing STEC strains isolated from humans in this study failed to agglutinate with O antisera currently available for serotyping of *E. coli*. Therefore, the development of diagnostic sera against such strains, which might represent novel O serogroups, would improve laboratory diagnosis of them and thus increase our understanding of their epidemiology.

The presence of the HPI of pathogenic yersiniae in a subset of the human stx_{2e}-containing STEC strains is noteworthy. This island confers virulence in highly pathogenic *Yersinia* species. HPI is also widely distributed among other *Enterobacteriaceae* (36), especially extraintestinal pathogenic *E. coli* strains that cause bacteremia and urosepsis in humans (47) and septicemia in poultry (12), and it contributes to the virulence of such strains (47). Recently, HPI was also found in certain serotypes of STEC pathogenic to humans, and it has been hypothesized that HPI can contribute to the fitness of such strains in diverse environments under iron limitation conditions (25). HPI contains a P4-like integrase (*int*) gene at the 5′ end and the *fyuA* gene encoding the receptor for yersiniabactin and pesticin at the 3′ end of the HPI core. A cluster of genes encoding the siderophore yersiniabactin is located between *int* and *fyuA* (25). Moreover, HPI in *Y. pestis* contains the insertion element IS*100* upstream of *fyuA* (25). Our examination of the structure of HPI, identified in the five human stx_{2e}-containing STEC strains, showed that each of these strains contained a complete HPI structurally similar to HPI in *Y. pestis*. However, three of the five HPI-positive STEC strains in this study were isolated from patients with diarrhea, and two were isolated from asymptomatic carriers, making it impossible to speculate on the putative contribution of HPI to the pathogenicity of such strains.

In conclusion, Stx2e-producing *E. coli* strains, although having stx_{2e} in common, appear to have independently imported and exchanged virulence determinants. This has led to differences in the pathogenicity profiles and forms of disease, suggesting that there has been specific host adaptation.

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