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Clonal structure of Shiga toxin (Stx)-producing and β-D-glucuronidase-positive *Escherichia coli* O157:H7 strains isolated from outbreaks and sporadic cases in Hokkaido, Japan

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A total of 22 clonal phenotypic variants of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) O157:H7 was isolated from six different locations in Hokkaido, Japan. These isolates were negative for sorbitol fermentation but positive for β-D-glucuronidase (GUD$^+$). They carried *eaeA*, EHEC-*hlyA*, *pas* and *etpD* genes like typical *E. coli* O157:H7 and, in addition, *stx*1 and *stx*2 genes. However, they were shown to lack *katP* and *espP* genes that are present in typical STEC O157:H7. All these atypical GUD$^+$ STEC O157:H7 isolates had very similar antimicrobial susceptibilities. Pulsed-field gel electrophoresis analysis with *Xba*I, *Sfi*I, *Swa*I, *Spe*I and *Not*I indicated that they were identical or closely related to one another. From their phenotypic and genotypic features, these GUD$^+$ STEC O157:H7 isolates may represent a distinct clone among STEC O157.

Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) or enterohaemorrhagic *E. coli* (EHEC) O157:H7 is now recognised as a serious food-borne pathogen, causing severe bloody diarrhoea with abdominal cramps and, occasionally, haemolytic-uraemic syndrome (HUS) [1]. STEC has an ability to produce one or more Stxs (Stx1, Stx2, or variants) responsible for serious illness in man [2, 3]. It is thought that HUS results from the systemic action of Stx on vascular endothelial cells [4] but other factors which have an influence on the virulence of toxigenic *E. coli* O157 also seem to be required. The locus of enterocyte effacement (LEE) in this bacterium encodes *eae* and *tir* genes, which are committed to the production of attaching and effacing lesions. The LEE also contains genes for a type III secretion system [5, 6] and for other secretory proteins (*esp* loci) [5] and *pas* which is responsible for the secretion of Esp proteins [7]. The large plasmid of STEC O157 also carries several potential virulence determinants characteristic of STEC [8–13]. In addition to EHEC haemolysin which acts as a pore-forming cytolysin of eukaryotic cells [8, 9], a bifunctional catalase peroxidase (KatP) [10] and a human coagulation factor V-cleaving serine protease (EspP) [11] have been identified [12]. The *etp* gene cluster that presumably encodes a type II secretion pathway system has also been found [13].

In Japan, after relatively large outbreaks of STEC O157:H7 in Okayama and Hiroshima prefectures, in 1996, Sakai city experienced a huge outbreak of STEC O157:H7 when >5000 individuals were infected [14–16]. Since then, >1000 patients have been infected annually with STEC of different serotypes including O157:H7 [15]. In Hokkaido prefecture, before 1996 there were only a few cases of STEC infection, but 1 year later the number of patients infected with STEC O157:H7 increased to 100.

Unlike other *E. coli* isolates, STEC O157 isolates are negative for sorbitol fermentation (SOR$^-$) within 24 h and do not exhibit β-D-glucuronidase activity (GUD$^-$) [17, 18]. This enables their efficient differential selection from stool samples on sorbitol-containing MacConkey agar (SMAC) [19], i.e., the colonies of *E. coli* which are negative for these biochemical activities are most probably STEC O157 [20, 21]. However, in recent years phenotypic variants of non-motile STEC O157, SOR$^+$ and GUD$^+$, have been isolated in Germany [22] and the Czech Republic [23], and a motile SOR$^-$ and GUD$^+$ atypical STEC O157:H7 strain has been isolated

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in the USA [24]. In particular, Karch *et al.* [25] reported novel SOR^+ and GUD^+ O157:H^- strains that caused an outbreak of HUS in Germany and showed their DNA patterns by pulsed-field gel electrophoresis (PFGE) to be unique and distinct from those of typical O157:H7 strains. In Japan also, particularly in Hokkaido, SOR^- and GUD^+ phenotypic variants of STEC O157:H7 have been isolated since 1996. These GUD^+ STEC O157:H7 isolates have been examined for their virulence determinants as well as their genetic features by PFGE. Evidence that the GUD^+ STEC O157:H7 isolates represent a distinct clone within STEC serogroup O157 is presented in this report.

Materials and methods

Bacterial isolates

Twenty-two isolates of GUD^+ STEC O157:H7 were obtained for phenotypic and genotypic studies. Two isolates of typical GUD^- STEC O157:H7 from subject numbers 23 and 24 with symptoms of bloody diarrhoea and abdominal cramps were used as controls. The epidemiological data for these isolates are summarised in Table 1. A further 49 typical STEC O157 isolates (Table 2) from persons with or without clinical symptoms (watery diarrhoea, bloody diarrhoea, abdominal cramps and other signs) and from cattle were subjected to PFGE for dendrogram clustering analysis.

Isolation of enteropathogenic bacteria from stool specimens

Stool specimens were cultured for bacterial enteric pathogens such as *Salmonella*, *Shigella*, *Campylobacter*, *Bacillus*, *Vibrio*, *Aeromonas*, *Plesiomonas* and *Yersinia* spp. by standard procedures [26]. For isolation of *E. coli* O157, the stool samples were streaked directly on SMAC (Difco) or SMAC supplemented with cefixime (Dynal, Oslo, Norway) 0.05 mg/L and potassium tellurite (Dynal) 2.5 mg/L (CT-SMAC), or on both media. On other occasions, stool samples were cultured in 10 ml of GN broth (Merck, Darmstadt, Germany) at 37°C for 6 h, followed by mixing 1 ml of the culture with magnetic beads coated with rabbit anti-O157 antibodies (Dynal). The magnetic beads were then washed and resuspended in 100 μl of a buffer according to the supplier's instructions. One half of the resuspended beads was streaked on to a SMAC plate and the rest on to a CT-SMAC plate. After incubation for 16–20 h at 37°C, the resulting SOR^- colonies, and a few SOR^+ colonies, were checked for the presence of the *stx* gene by PCR. Colonies that gave a positive reaction by PCR were examined further. They were identified as *E. coli* from their biochemical properties and were serotyped with rabbit anti-O157 and anti-H7 sera (Denkaseiken, Tokyo, Japan).

Phenotypic characterisation of E. coli isolates

The bacterial isolates were stored on heart infusion agar slants and freshly cultured on SMAC for each test. The biochemical properties of the isolates were examined with the API 20E system (bioMérieux, Marcy l'Etoile, France). The GUD activity of the isolates was examined on three media containing 4-methylumbelliferyl-β-D-glucuronide (MUG): (i) EC medium with MUG (Difco Laboratories), (ii) CLIG agar (Kyokuto seiyaku, Tokyo, Japan) and (iii) Fluorocult^{TM} *E. coli* O157:H7-Agar (Merck). An overnight culture at 37°C which fluoresced under UV light was judged to be GUD positive. Production of Stx was tested by the reverse passive latex agglutination (RPLA) test according to manufacturer's instructions (Denkaseiken). The haemolytic activity of the isolates was detected after culture on blood agar plates containing defibrinated and washed sheep red blood cells 5% and 10 mM $CaCl_2$ [9, 27, 28].

Antimicrobial susceptibility test

The susceptibility of the isolates to antimicrobial agents was examined by the 'E test' (AB Biodisk, Solna, Sweden).

Standard DNA methods

E. coli DNA was isolated from 1 ml of an overnight culture with a Wizard Genomic DNA Purification kit (Promega, WI, USA) according to the manufacturer's instructions. The amount of DNA was calculated from its absorption at 260 nm. For regular Southern blot hybridisation, DNA was digested with *Bam*HI (Toyobo, Tokyo, Japan) and electrophoresed through agarose 0.8% gels in half-strength Tris-borate-EDTA (TBE) buffer, pH 8.3 [29]. The separated DNA fragments were transferred to Magna nylon membranes (Micron Separation, MA, USA) by standard methods [29]. When checking for clonality of the GUD^+ STEC O157:H7 isolates, DNA samples in agarose plugs were digested with the restriction endonuclease *Xba*I, separated by PFGE and transferred to nylon membranes in the same way [29]. Non-radioactive direct DNA labelling and Southern blot hybridisation were performed with a Gene Images AlkPhos Direct labelling and detection kit (Amersham Pharmacia, Uppsala, Sweden) following the manufacturer's procedures. The probes used in this study were prepared by PCR amplification of stx_1 (primers LP30/LP31 [30]), stx_2 (primers LP43/LP44 [30]), *espP* (primers esp-A/esp-B [12]) and *katP* (primers wkat-B/wkat-F [10]).

PCR

PCR for detecting the specific genes listed in Table 3 was performed with the GeneAmp PCR System 9600 (Perkin-Elmer Applied Biosystems) in a volume of 20 μl containing 1 μl of purified bacterial DNA

Table 1. Epidemiological data, plasmid and chromosomal genes encoding accessory virulence factors of atypical isolates of *E. coli* O157:H7 and control typical *E. coli* O157:H7 isolates from individuals in Hokkaido, Japan

Subject no.	Isolate no.	Age (years)	Sex	Specimen collection date	City of isolation	Clinical symptom	Serotype	Phenotype			Chromosomal genes*				Plasmid genes†			
								SF	GUD	Haemolysis	*stx*$_1$	*stx*$_2$	*eae*	*pas*	EHEC-*hly*	*etpD*	*KatP*	*espP*
1	EC96012	61	M	Aug. 1996	Chitose	LF	O157:H7	–‡	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
2	EC96020	58	M	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
3	EC96021	53	F	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
4	EC96022	59	M	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
5	EC96023	50	M	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
6	EC96024	47	F	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
7	EC96025	80	F	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
8	EC96026	63	F	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
9	EC96027	84	F	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
10	EC96029	64	M	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
11	EC96028	27	M	Aug. 1996	Chitose	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
12	EC96032	2	F	Aug. 1996	Akkeshi	D	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
13	EC96038	26	F	Aug. 1996	Akkeshi	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
14	EC96073	49	F	Sept. 1996	Engaru	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
15	EC96110	6	F	Oct. 1996	Kushiro	D	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
16	EC96111	6	F	Oct. 1996	Kushiro	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
17	EC96112	6	F	Oct. 1996	Kushiro	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
18	EC96113	4	M	Oct. 1996	Kushiro	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
19	EC97144	22	F	July 1997	Asahikawa	D	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
20	EC97119	2	F	Aug. 1997	Ebetsu	D	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
21	EC97120	8	M	Aug. 1997	Ebetsu	No	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
22	EC98206	74	F	Oct. 1998	Kushiro	D	O157:H7	–	+	EHEC-Hly	+	+	γ1	+	+	+	–/–	–/–
23	EC96031	23	F	Aug. 1996	Sapporo	BD	O157:H7	–	–	EHEC-Hly	+	+	γ1	+	+	+	+/25.4, 9.0	+/8.4
24	EC96017	8	M	Aug. 1996	Obihiro	BD	O157:H7	–	–	EHEC-Hly	–	+	γ1	+	+	+	+/25.4, 9.0	+/8.4

SF, fermentation of sorbitol; GUD, β-glucuronidase activity; F, female; M, male; LF, loose faeces; No, no symptom; D, diarrhoea; BD, bloody diarrhoea.
*These genes were examined by PCR.
†Detection of the gene by PCR/size (in kb) of the genome DNA fragments hybridising to the respective probe; +, positive result by PCR; –, no signal obtained.
‡Results obtained after overnight culture of bacteria on SMAC agar plates.

Table 2. *E. coli* O157 isolates in Hokkaido examined by PFGE*

Isolate no.	Age (years)	Sex	Specimen collection date	City of isolation	Clinical symptom	Serotype	Stx type
EC96004	2	M	Aug. 1996	Sapporo	BD	O157:H7	1†
EC96037	9	M	Aug. 1996	Chitose	D	O157:H7	1&2
EC96056	7	F	Aug. 1996	Obihiro	D	O157:H7	1&2
EC96057	2	F	Aug. 1996	Muroran	D	O157:H7	2
EC96061	8	M	Aug. 1996	Shikaoi	BD	O157:H7	2
EC96086	1	M	Sept. 1996	Obihiro	D	O157:H7	2
EC96107	4	M	Oct. 1996	Obihiro	D	O157:H7	2
EC97005	67	F	Jan. 1997	Sapporo	D	O157:H7	1&2
EC97026	38	F	June 1997	Kitahiroshima	D	O157:H7	1&2
EC97032	48	F	June 1997	Sapporo	NS	O157:H7	2
EC97090	4	F	Aug. 1997	Obihiro	BD	O157:H7	2
EC97092	18	M	Aug. 1997	Sapporo	D	O157:H7	1&2
EC97093	18	M	Aug. 1997	Engaru	D	O157:H7	2
EC97101	1	F	Aug. 1997	Sapporo	BD	O157:H7	1&2
EC97103	16	F	Aug. 1997	Kuchan	BD	O157:H7	2
EC98123	56	F	June 1998	Obihiro	NS	O157:H-	1&2
EC98147	9	M	Aug. 1998	Sapporo	BD	O157:H7	1&2
EC98152	21	F	Aug. 1998	Sapporo	D	O157:H7	1&2
EC98159	5	M	Aug. 1998	Makubetsu	BD	O157:H7	1&2
EC98162	21	F	Aug. 1998	Otofuke	D	O157:H7	1&2
EC98167	2	M	Aug. 1998	Iwamizawa	BD	O157:H-	2
EC98168	65	F	Aug. 1998	Horonobe	BD	O157:H7	1&2
EC98166	4	F	Sept. 1998	Tohbetsu	HUS	O157:H7	2
EC98187	58	F	Sept. 1998	Kuriyama	BD	O157:H7	1&2
EC98193	2	M	Sept. 1998	Noboribetsu	D	O157:H7	1&2
EC98200	12	M	Sept. 1998	Iwamizawa	BD	O157:H7	1&2
EC98221	14	F	Oct. 1998	Asahikawa	D	O157:H7	1&2
EC98226	73	M	Oct. 1998	Yakumo	BD	O157:H7	1&2
EC99039	2	M	May 1999	Akabira	BD	O157:H7	1&2
SREC9901	1	M	June 1999	Urakawa	D	O157:H7	2
EC99109	9	M	July 1999	Tomakomai	BD	O157:H7	1&2
EC99120	2	M	July 1999	Date	D	O157:H7	1&2
SREC9904	10	F	July 1999	Sapporo	D	O157:H-	1&2
EC99129	10	F	Aug. 1999	Chitose	BD	O157:H7	1&2
SREC9910	14	F	Aug. 1999	Asahikawa	D	O157:H7	1&2
SREC9918	37	M	Sept. 1999	Sapporo	NS	O157:H7	2
SREC9920	4	F	Oct. 1999	Obihiro	BD	O157:H7	1&2
B342	NK	C	Dec. 1999	Shikaoi	No	O157:H7	2
EC00003	3	F	Feb. 2000	Sapporo	BD	O157:H7	1&2
SREC0003	10	F	May 2000	Otofuke	BD	O157:H-	1&2
EC00044	10	F	June 2000	Bihoro	D	O157:H7	2
EC00064	14	M	Aug. 2000	Asahikawa	BD	O157:H7	1&2
EC00069	2	M	Aug. 2000	Makkari	BD	O157:H7	1&2
EC00101	1	M	Aug. 2000	Asahikawa	BD	O157:H7	1&2
SREC0021	73	F	Aug. 2000	Asahikawa	BD	O157:H7	1&2
B497	NK	C	Aug. 2000	Shikaoi	No	O157:H7	2
EC00098	31	M	Sept. 2000	Eniwa	D	O157:H7	1&2
B539	NK	C	Dec. 2000	Shintoku	No	O157:H7	2
B515	NK	C	Dec. 2000	Kushiro	No	O157:H7	2

Abbreviations used in this table are the same as those in Table 1 except C (cattle) and NK (not known).
*All O157 isolates listed here did not ferment sorbitol within 24 h on SMAC and had the GUD$^-$ phenotype.
†Isolate was stx_1 and stx_2 positive by PCR but production of Stx2 was not observed by RPLA test.

(*c*. 200 ng), 200 mM deoxynucleotide triphosphates (dATP, dCTP, dGTP and dTTP), 30 pmol of each primer, 2 μl of a 10-fold concentrated buffer mixture and 2.5 U of Taq polymerase (Takara). The PCR conditions, primer designations and sequences are given in Table 3.

DNA fingerprint analysis by PFGE

PFGE analysis was conducted according to the methods described previously [14] but with a slight modification. Agarose plugs containing bacterial cells were incubated in 0.5 ml of lysis buffer containing lysozyme 5 mg/ml, 0.5 M EDTA (pH 8.0) at 37°C for 3 h. After incubation, the lysis buffer was removed and 0.5 ml of proteinase buffer – 0.5 M EDTA, pH 8.0, sodium sarkosyl 1% containing proteinase K (Roche Diagnostics, Mannheim, Germany) 1 mg/ml – was added to the plugs, followed by incubation at 50°C for 18–24 h. Proteinase activity was stopped by adding 1 ml of 4 mM PefablocSC (Roche) in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The genomic DNA in the agarose plugs was digested with *Xba*I (Takara) following the conditions indicated by the manufacturer. The resulting DNA fragments were electrophoresed through agarose 1% with the CHEF-DRIII apparatus (BioRad Laboratories, Richmond, CA, USA) at 6 V/cm and 14°C for 22 h with switch times ranging from 4 to 50 s. λ Concatamers (BioRad) were used as DNA size markers. After staining with ethidium

Table 3. PCR primers used and PCR conditions

Primer designation	Nucleotide sequence (5′–3′)	Target	PCR conditions*			PCR product size (bp)	Reference
			Denature	Annealing	Extension†		
LP30	CAG TTA ATG TGG TGG CGA AGG	*stx*$_1$	94°C, 90 s	64°C, 90 s	72°C, 90 s	348	30
LP31	CAC CAG ACA ATG TAA CCG CTG						
LP43	ATC CTA TTC CCG GGA GTT TAC G	*stx*$_2$	94°C, 90 s	64°C, 90 s	72°C, 90 s	584	30
LP44	GCG TCA TCG TAT ACA CAG GAG C						
PT-2	GCG AAA ACT GTG GAA TTG GG	*uidA*	94°C, 90 s	64°C, 90 s	72°C, 90 s	252	30
PT-3	TGA TGC TCC ATA ACT TCC TG						
SK1	CCC GAA TTC GGC ACA AGC ATA AGC	*eae* (SK1/SK2)	94°C, 30 s	62°C, 60 s	72°C, 90 s	881	32
SK2	CCC GGA TCC GTC TCG CCA GTA TTC G						
LP2	CCC GAA TTC TTA TTT TAC ACA AGT GGC	*eae* α (SK1/LP2)	94°C, 30 s	62°C, 60 s	72°C, 90 s	2807	32
LP3	CCC GAA TTC TTA TTC TAC ACA AAC CGC	*eae* γ (SK1/LP3)	94°C, 30 s	62°C, 60 s	72°C, 90 s	2792	32
LP4	CCC GTG ATA CCA GTA CCA ATT ACG GTC	*eae* β (SK1/LP4)	94°C, 30 s	62°C, 60 s	72°C, 90 s	2287	32
LP5	AGC TCA CTC GTA GAT GAC GGC AAG CG	*eae* ε (SK1/LP5)	94°C, 30 s	62°C, 60 s	72°C, 90 s	2608	32
wkat-B	CTT CCT GTT CTG ATT CTT CTG G	*katP*	94°C, 30 s	56°C, 60 s	72°C, 150 s	2125	10
wkat-F	AAC TTA TTT CTC GCA TCA TCC						
hlyA1	GGT GCA GCA GAA AAA GTT GTA G	*hlyA*	94°C, 30 s	57°C, 60 s	72°C, 90 s	1551	8
hlyA4	TCT CGC CTG ATA GTG TTT GGT A						
D1	CGT CAG GAG GAT GTT CAG	*etpD*	94°C, 30 s	52°C, 60 s	72°C, 70 s	1062	13
D13R	CGA CTG CAC CTG TTC CTG ATT A						
esp-A	AAA CAG CAG GCA CTT GAA CG	*espP*	94°C, 30 s	56°C, 60 s	72°C, 150 s	1830	12
esp-B	GGA GTC GTC AGT CAG TAG AT						
ANK49	GCA GGA TCC ATG TTA TCC TCA TAT AAA ATA AAC	*pas*	94°C, 30 s	56°C, 60 s	72°C, 150 s	1239	7
ANK50	CCA GGT ACC TTA ATA CGA CAG TGG AAT ATG						

*For 30 cycles.
†After 30 cycles, final extension step was done for 5 min.

bromide, the agarose gel was photographed and the band image was digitised for computer analysis. The GelCompar software package (Applied Maths, Kortrijk, Belgium) was used for cluster analysis. For further analysis of the clonal structure of these atypical GUD$^+$ STEC O157:H7 isolates, genomic DNA was digested with *Not*I, *Sfi*I, *Spe*I and *Swa*I restriction endonucleases (Takara). For the PFGE analyses of digests with these enzymes, switch times and electrophoresis times were modified: for *Not*I and *Spe*I digests, the switch time was 4–8 s for 9 h followed by 8–50 s for 13 h; and for *Sfi*I and *Swa*I digests, the switch time was 2–20 s for 22 h.

Results

Isolation of GUD$^+$ E. coli O157:H7 strains and their epidemiological background in Hokkaido

A total of 22 isolates of GUD$^+$ *E. coli* O157:H7 was obtained from two outbreaks and four sporadic cases that occurred between Aug. 1996 and Oct. 1998 as listed in Table 1. The isolates were obtained from the residents of six cities in Hokkaido (Fig. 1). The first outbreak occurred in the psychiatric ward of a hospital in Chitose city, producing 10 isolates from 163 inpatients (subject nos 1–10) and one isolate from 108 staff members (subject no. 11). The second outbreak occurred in a kindergarten in Kushiro city, producing four isolates from 41 children (subject nos 15–18).

The first sporadic case in a kindergarten in Akkeshi city produced two isolates: one from an infant (subject no. 12) and the other from the teacher (subject no. 13). In the second sporadic case in Asahikawa city, one isolate was obtained from a 22-year-old woman with loose stools (subject no. 19). In the third sporadic case in Ebetsu city, two isolates were obtained from siblings; one had loose stools (subject no. 20) and the other had no symptoms (subject no. 21). In the fourth sporadic case in Kushiro city, one isolate was obtained from a 74-year-old man with loose stools (subject no. 22). Lastly, one isolate was obtained from a healthy woman in Engaru (subject no. 14). In total, six persons passed loose stools or had diarrhoea but none of them had bloody stools or abdominal cramps. They received antibiotic therapy for a few days and they were cured shortly afterwards. Sixteen persons were completely asymptomatic. No other enteric pathogens were identified in the 22 stool specimens.

Biochemical properties of the GUD$^+$ E. coli O157:H7 isolates

All 22 of these *E. coli* O157:H7 isolates produced GUD after incubation for 16–24 h. However, none of them had rhamnose fermentation activity as shown by API 20E. No other differences in biochemical activities were seen between the GUD$^+$ and GUD$^-$ isolates of STEC O157.

Detection of accessory virulence genes

PCR reactions were performed to detect virulence genes in the atypical GUD$^+$ *E. coli* O157:H7 isolates. As shown in Table 1, in all the isolates, fragments of EHEC-*hlyA*, *etpD*, *stx$_1$*, *stx$_2$* and *pas* genes were amplified by each specific primer to the same predicted sizes as for the typical GUD$^-$ STEC O157:H7 isolates. However, *katP* and *espP* sequences that are usually

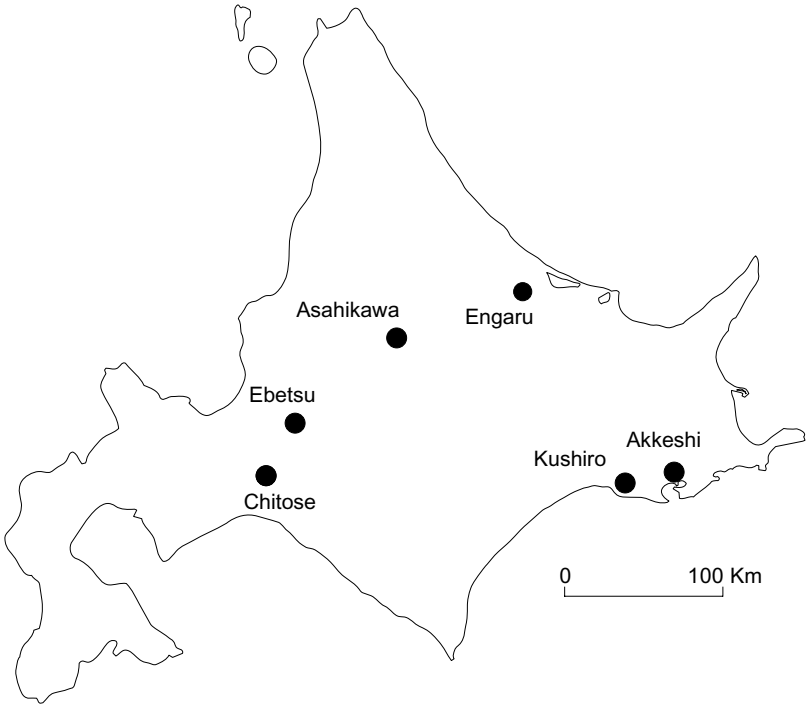

Fig. 1. Locations of the cities where the GUD$^+$ STEC O157:H7 isolates were obtained.

detected in typical GUD– STEC O157:H7 isolates were not detectable in the GUD+ STEC O157:H7 isolates obtained in Hokkaido. To confirm this, blots of *Bam*HI-restricted DNA of these isolates were hybridised with either *katP* or *espP* DNA fragments. As shown in Table 1, none of the isolates produced signals for *katP* or *espP*, whereas the GUD– control isolates EC96017 and EC96031 DNA gave positive signals at 25.4 plus 9.0 kb (*katP*) and 8.4 kb (*espP*) positions on the *Bam*HI blot.

For identification of the *eaeA* gene, PCR amplification was conducted initially with primers SK1 and SK2 [31]. Later, additional primers (SK1/LP2, SK1/LP3, SK1/LP4 and SK1/LP5) were used to determine the subclass of intimin which is encoded by this gene [31]. Of these primer sets, only SK1/LP3 produced 2972-bp DNA fragments, which were cleaved with *Pvu*II to give the specific restriction fragment length polymorphism (RFLP) patterns reported by Oswald *et al*. [31]. This indicated that the GUD+ STEC O157:H7 isolates possessed a γ1 type intimin gene similar to that of the GUD– STEC O157:H7 isolates (Table 1).

Phenotypic characteristics in GUD+ STEC O157:H7 isolates

Phenotypic determinants were characterised and are shown in Table 1. Stx production was confirmed by the RPLA test. All the GUD+ STEC O157:H7 isolates produced Stx1 and Stx2. These isolates also produced enterohaemolysin on blood agar but were unable to ferment sorbitol on SMAC agar plates within 24 h.

Antimicrobial susceptibility testing

All the atypical GUD+ STEC O157:H7 isolates showed very similar antimicrobial susceptibility patterns. Differences in MIC values to each antimicrobial agent were no more than two-fold among the isolates and they were judged susceptible to the antimicrobial agent used (Table 4).

PFGE analysis

The *Xba*I digestion patterns of the 11 GUD+ STEC O157:H7 isolates from the outbreak in the psychiatric ward of a hospital in Chitose are shown in Fig. 2. They were identical to one another as regards the number and size of the *Xba*I DNA fragments, indicating a clonal origin. The *Xba*I digestion patterns of the four GUD+ STEC O157:H7 isolates from the outbreak in the kindergarten in Kushiro were identical as shown in Fig. 3 (lanes 15, 16 and 18) or almost identical (lane 17) to one another and were essentially the same as those of the isolates from Chitose city (lane 1). The *Xba*I digestion patterns of the isolates from the sporadic cases in Akkeshi city (lanes 12 and 13), Engaru city (lane 14), Asahikawa city (lane 19), Ebetsu city (lanes 20 and 21) and Kushiro city (lane 22) were very similar to those of the isolates from Chitose city (lane 1). The differences among these *Xba*I digestion patterns were less than two DNA fragments [32]. Thus, the identical or closely similar *Xba*I digestion patterns

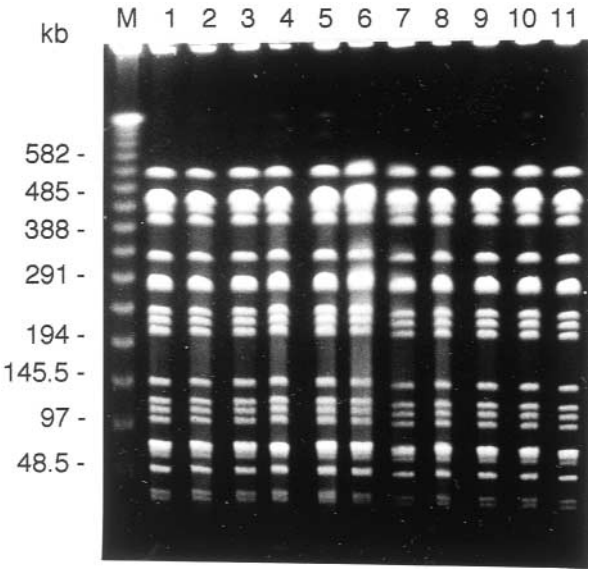

Fig. 2. *Xba*I-digested genomic DNA from GUD+ STEC O157:H7 isolates from in-patients (lanes **1**–**10**) and a staff member (**11**) on the psychiatric ward of a hospital in Chitose city. Lane **M**, mol. wt marker of λ DNA concatamers.

Table 4. Comparison of antimicrobial susceptibility patterns of GUD+ and GUD– STEC O157:H7 isolates

	MIC (μg/ml) for isolates		
Antimicrobial agent	GUD+*	EC96031 (GUD–)	EC96017 (GUD–)
Ampicillin	2–4 (S)	2 (S)	2 (S)
Cefotaxime	0.032–0.064 (S)	0.064 (S)	0.047 (S)
Gentamicin	0.5–1 (S)	0.25 (S)	0.25 (S)
Ciprofloxacin	0.008–0.016 (S)	0.016 (S)	0.016 (S)
Norfloxacin	0.064 (S)	0.125 (S)	0.125 (S)
Tetracycline	0.5–1 (S)	2 (S)	1 (S)
Sulphamethoxazole-trimethoprim	0.032 (S)	0.032 (S)	0.032 (S)
Trimethoprim	0.125 (S)	0.125 (S)	0.125 (S)
Fosfomycin	0.5–1 (S)	1 (S)	0.5 (S)
Chloramphenicol	2–4 (S)	4 (S)	6 (S)

S, susceptible.
*MIC of 22 GUD+ isolates.

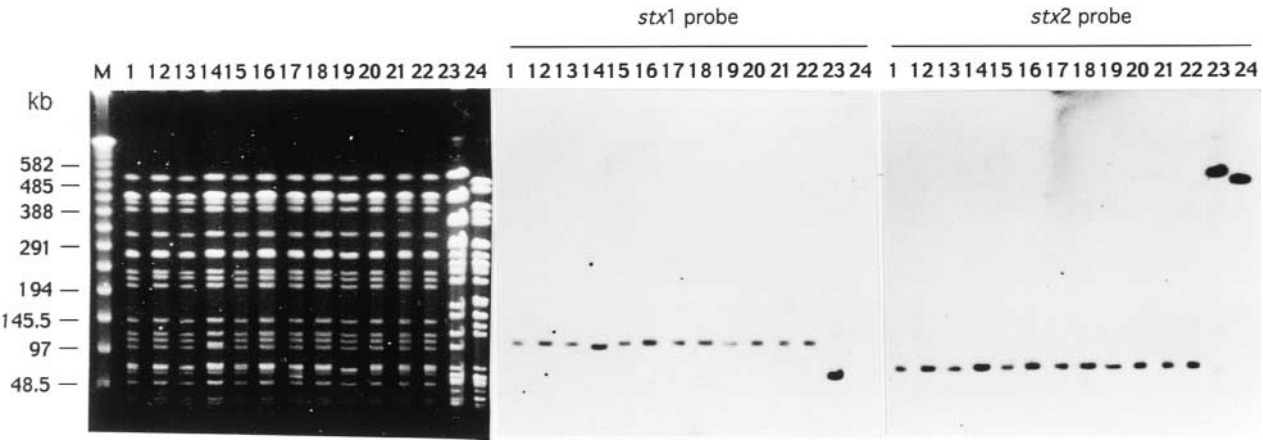

Fig. 3. PFGE patterns of *Xba*I-digested genomic DNA from GUD$^+$ STEC O157:H7 isolates from Chitose city (lane **1**), Akkeshi city (**12**, infant; **13**, teacher), Engaru city (**14**), Kushiro city (**15**–**18**), Asahikawa city (**19**), Ebetsu city (**20**, **21**) and Kushiro city (**22**). Control GUD$^-$ STEC O157:H7 isolate digests are in lanes **23** (EC96031) and **24** (EC96017). Lane **M**, mol. wt marker of λ DNA concatamers. The separated *Xba*I restriction DNA fragments were blotted on to nylon membrane and probed with PCR-amplified stx_1 and stx_2 products. Labelling of the probe DNA, hybridisation with the blotted DNA and visualisation of the signals were performed according to the manufacturer's instructions (Gene Images AlkPhos Direct labelling and detection kit: Amersham Pharmacia, Uppsala, Sweden).

of the 22 isolates of GUD$^+$ STEC O157:H7 indicated that they belong to a single clone. For further confirmation of this clonality, PFGE analysis was repeated on these GUD$^+$ STEC O157:H7 isolates after digestion with the restriction endonucleases *Not*I, *Swa*I, *Sfi*I and *Spe*I. As shown in Fig. 4, the difference in each of the PFGE patterns among the isolates was four bands or less. The blot of PFGE-separated chromosomal DNAs of these isolates was also probed with the PCR products of stx_1 and stx_2. The *Xba*I restriction fragments of 110 kb and 80 kb were shown to contain DNA sequences homologous to stx_1 and stx_2, respectively (Fig. 3). This provided further evidence of the high degree of clonality of the isolates.

The *Xba*I-restricted PFGE patterns of the 51 GUD$^-$ STEC O157 isolates from individuals with symptoms of watery diarrhoea (19 isolates), bloody diarrhoea (24), HUS (1) or with no symptoms (3) and from cattle (4) were compared with these of the GUD$^+$ STEC O157:H7 isolates. A dendrogram of the PFGE patterns was constructed with GelCompar software and is depicted in Fig. 5. The GUD$^+$ STEC O157:H7 isolates formed a cluster distinct from the other typical STEC O157. They showed similarities of $\geqslant$91.9% within the GUD$^+$ STEC O157:H7 cluster, but only 59.7% similarity with the closest other cluster. These results showed that GUD$^+$ STEC O157 isolates made a unique clone among the STEC O157 complex.

Discussion

This report provides evidence that 22 GUD$^+$ STEC O157:H7 isolates from Hokkaido, Japan obtained during 1996–1998 make a distinct clonal subgroup within the O157 complex. PFGE analyses with several restriction endonucleases revealed that the isolates were closely related to one another. They showed similar antimicrobial susceptibility patterns and retained a large plasmid that carried *etpD* and EHEC-*hlyA* genes but, unlike typical GUD$^-$ STEC O157, had lost *espP* and *katP* genes. They also exhibited the same phenotypes of virulence determinants such as production of Stx1, Stx2 and enterohaemolysin. To our knowledge, this is the first report of the isolation of well-characterised GUD$^+$ STEC O157:H7 strains in Japan.

These atypical STEC O157:H7 isolates were shown to possess some interesting characteristics in addition to GUD$^+$ activity. STEC strains produce several virulence factors responsible for the clinical symptoms distinctive to the infected person. Some of the factors are coded in the chromosomal DNA and the others in plasmid DNA [5-13]. There is a locus in the chromosomal DNA containing a cluster of genes for the expression of specific pathogenicity, called attaching-and-effacing (A/E) histopathology, which was first found in enteropathogenic *E. coli* (EPEC) strain E2348/69 and named LEE [33]. To obtain more information on the pathogenicity of the STEC isolates, the type of *eaeA* gene and the presence of the *pas* gene in STEC O157:H7 were examined. All the GUD$^+$ STEC O157:H7 isolates had the *pas* gene and were shown to possess the *eaeA* gene coding for γ1 type intimin, as do the typical GUD$^-$ STEC O157 strains. Thus, chromosome-encoded virulence factors could not discriminate the GUD$^+$ STEC O157:H7 isolates from the GUD$^-$ STEC O157 strains.

In general, plasmid DNA encodes various virulence factors in enteropathogenic bacteria such as EPEC, enterotoxigenic *E. coli* and enteroinvasive *E. coli* as well as STEC. The STEC have been characterised in terms of a large plasmid of 93.6–104 kb [34], designated pO157 [35]. pO157 is reported to have heterogeneity with respect to gene composition and

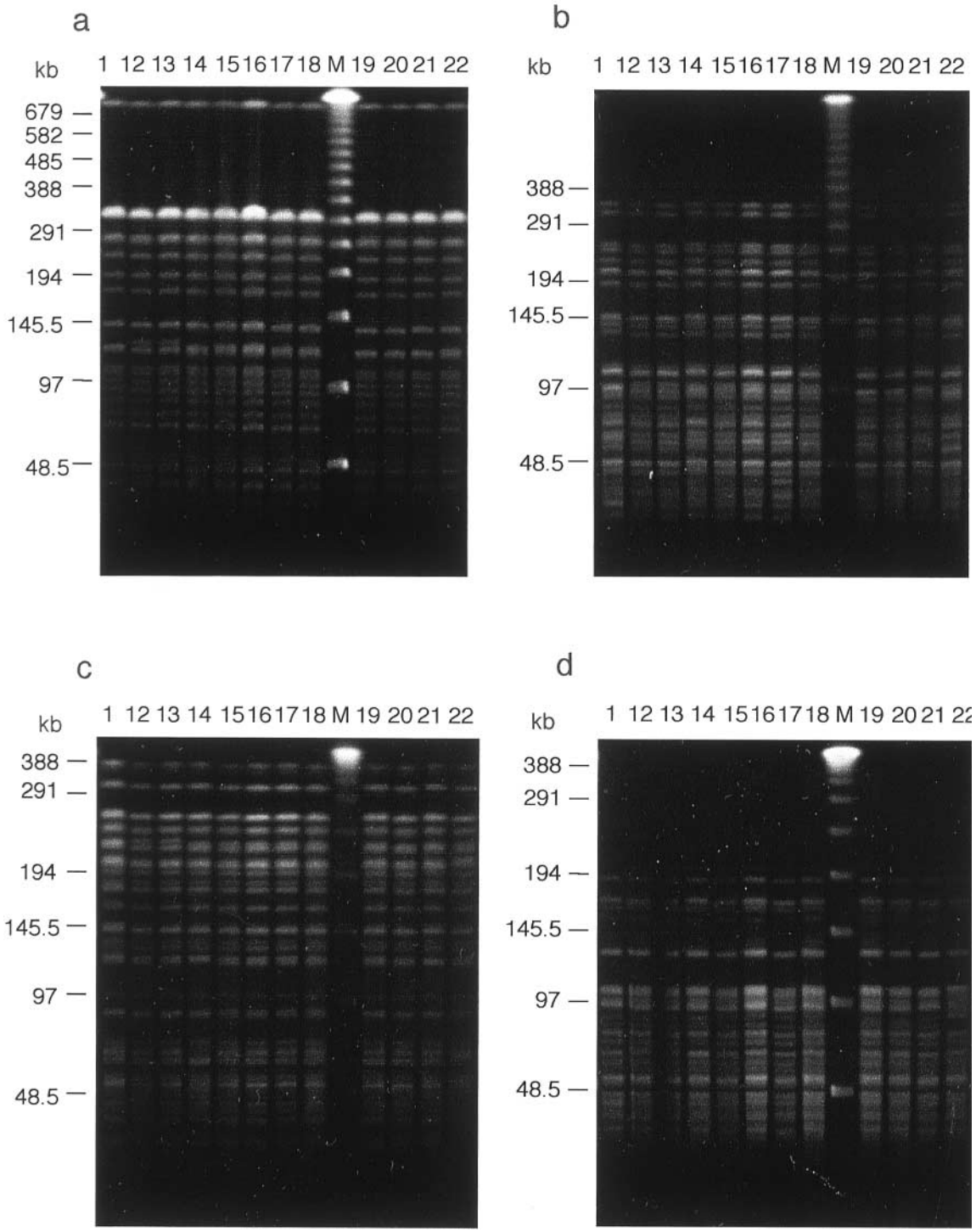

Fig. 4. PFGE patterns of the DNA of GUD+ STEC O157:H7 isolates from Hokkaido digested with *Not*I (**a**), *Spe*I (**b**), *Sfi*I (**c**), and *Swa*I (**d**). Lane **M**, mol. wt marker of λ DNA concatamers. Lane numbers correspond to the subject numbers shown in Table 1.

arrangement among different serotypes of STEC [12]. In Germany and the Czech Republic, some GUD+ STEC O157:H− strains have been isolated from patients suffering from HUS and diarrhoea. Fingerprinting analysis indicated that these isolates belonged to a distinct clone in the STEC O157 complex [23, 25]. However, Bielaszewska *et al*. [36] described two types of large plasmid present in SOR+ and GUD+ STEC O157 strains in Europe: one type possessed a combination of EHEC-*hlyA* and *etpD* but not *katP* nor *espP* genes, whereas the other type contained none of these four genes. The virulence gene profile of the large plasmid in the GUD+ STEC O157:H7 isolates from Hokkaido corresponded to the former type of plasmid described above. The present study failed to find heterogeneity in the pO157 of GUD+ STEC O157, suggesting a high degree of clonality in the GUD+ STEC O157:H7 isolates. There were further properties that distinguished between the European GUD+ STEC O157 isolates and those from Hokkaido: the isolates from Hokkaido were motile, did not ferment sobitol within 24 h and produced both Stx1 and Stx2, whereas

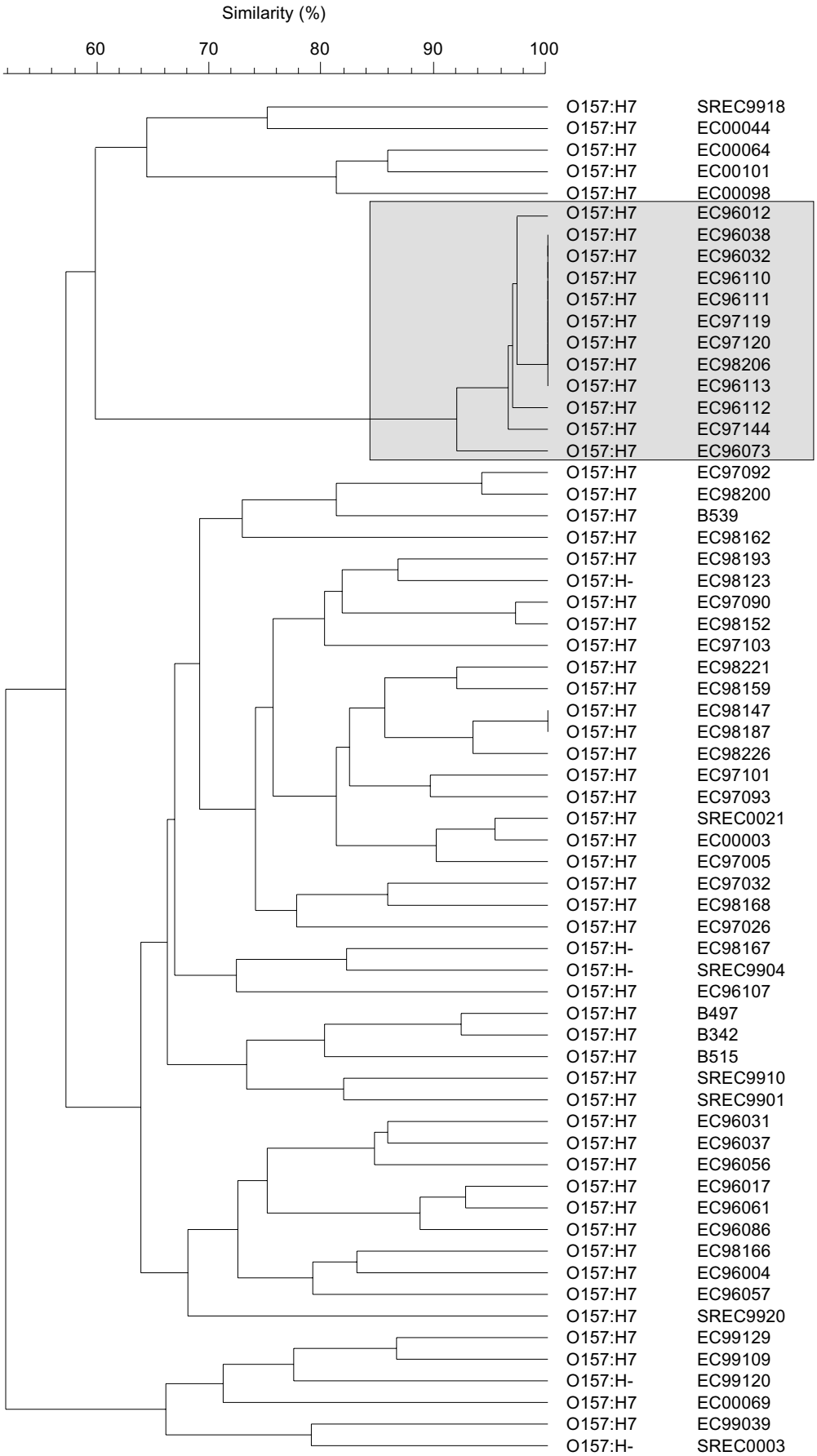

Fig. 5. Dendrogram derived from similarities by PFGE of *Xba*I-digested DNA for Hokkaido GUD$^+$ and GUD$^-$ STEC O157 isolates with GelCompar software package. The characteristics of the isolates are given in Tables 1 and 2. The GUD$^+$ STEC O157:H7 isolates are clustered in the shaded box.

the European isolates produced only Stx2, were non-motile and fermented sorbitol within 24 h.

SOR$^+$ and GUD$^+$ STEC O157:H$^-$ strains in Europe have been isolated from patients with bloody diarrhoea or HUS, whereas the GUD$^+$ STEC O157:H7 isolates in Hokkaido were from individuals without HUS. Moreover, of 22 residents shedding GUD$^+$ STEC O157, 16 people (72.7%) were symptomless. The manifestation of clinical symptoms seems to be dependent on the patient's age [37]. Of the persons infected with STEC O157, 82% of those aged $<$10 years (475 of 576) and 68% of those aged $>$70 years (47 of 69) showed one or more symptoms. In contrast to this, examination of the 22 persons infected with the GUD$^+$ STEC O157:H7 isolates revealed that only 43% of those aged $<$10 years (3 of 7) and 33% of those aged $>$70 years (1 of 3) manifested clinical symptoms (Table 1). These epidemiological data suggest that the GUD$^+$ STEC O157:H7 isolates cause mild symptoms, if any, in young and old patients who are thought to be the most susceptible.

At present, increasing numbers of healthy people are carriers of STEC strains and are probably the source of many sporadic infections. In contrast to the European SOR$^+$ and GUD$^+$ STEC O157:H$^-$ strains, the GUD$^+$ STEC O157:H7 isolates from Hokkaido produced enterohaemolysin and grew on CT-SMAC like the typical STEC O157 strains. Therefore, these GUD$^+$ STEC O157:H7 strains can be isolated from stool samples as easily as the typical STEC O157 strains [36, 38].

It is well known from PFGE analysis that STEC O157 strains have many genotypes [14]. We have also observed genome diversity in typical GUD$^-$ STEC O157 strains in Hokkaido. By contrast, the SOR$^+$ and GUD$^+$ STEC O157:H$^-$ isolates from Germany and the Czech Republic displayed very similar PFGE patterns [25, 36]. In the same way, PFGE analysis showed that the GUD$^+$ STEC O157:H7 isolates from Hokkaido were more closely related to one another than to the typical GUD$^-$ STEC O157 (Fig. 4). There was no known link between any of these outbreaks and the sporadic cases. The clonal nature of the GUD$^+$ STEC O157:H7 isolates seems to indicate that either the strains have evolved independently to one very similar clone or they originated from the same clone. Recently, it was reported that the atypical SOR$^+$ and GUD$^+$ STEC O157:H$^-$ strain isolated in the Czech Republic was also isolated from cattle [36]. Similarly, a wild deer captured in Hokkaido, Japan, was found to carry almost the same GUD$^+$ STEC O157:H7 strain as those reported here (unpublished data). It is conceivable that deer are another natural reservoir and the source of the atypical GUD$^+$ STEC isolates in Hokkaido.

Feng *et al*. [39] have proposed an evolutionary model for the emergence of the *E. coli* O157:H7 complex of strains based on mutation in *uidA*, Stx production, SOR and GUD phenotypes, and their multilocus enzyme electrophoretic profile. In it, the EPEC-like strain of O55:H7 is a common ancestor of the O157:H7 complex, although it has the *uidA* -10 mutation (A to T) and SOR$^+$ and GUD$^+$ phenotypes. The model predicts that the atypical SOR$^+$ and GUD$^+$ STEC O157:H$^-$ isolates in Europe and the GUD$^+$ STEC O157:H7 isolate in USA were derived from the EPEC-like O55:H7 strain because these two atypical strains had an identical mutation at *uidA* $+92$ allele (T to G) [39]. PCR performed on the atypical STEC O157:H7 Hokkaido isolates for a possible mutation in the same *uidA* $+92$ allele with a primer set covering this site [30] indicated the presence of the same mutation at the *uidA* $+92$ site (data not shown). This feature, together with the traits of SOR$^-$ and GUD$^+$ phenotypes and Stx1 and Stx2 production, suggests that the GUD$^+$ STEC O157:H7 isolates obtained in Hokkaido were derived from *E. coli* O55:H7. Therefore, they locate more closely to the atypical GUD$^+$ O157:H7 US strain than to the GUD$^+$ O157:H$^-$ European strains in their evolution.

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