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Verotoxinogenic *Citrobacter freundii* associated with severe gastroenteritis and cases of haemolytic uraemic syndrome in a nursery school: green butter as the infection source

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SUMMARY

A summer outbreak of severe gastroenteritis followed by haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura in a nursery school and kindergarten is described. Sandwiches prepared with green butter made with contaminated parsley were the likely vehicle of infection. The parsley originated from an organic garden in which manure of pig origin was used instead of artificial fertilizers. Clonally identical verotoxinogenic *Citrobacter freundii* were found as causative agents of HUS and gastroenteritis and were also detected on the parsley.

INTRODUCTION

Verotoxinogenic *Escherichia coli* (VTEC) strains of different serovars have been recognized as causative agents of severe gastrointestinal infections with life-threatening post infectious syndromes such as haemolytic uraemic syndrome (HUS), thrombotic thrombocytopenic purpura (TTP) or haemorrhagic colitis (HC) [1]. The broad range of serovars necessitates a diagnostic approach different to the conventional serological or biochemical tests used in routine medical microbiological investigations. The molecular identification of the various verotoxins (Shiga-like toxins or VTs) was introduced as a diagnostic tool for clinical and epidemiological purposes [2–7].

As with other enteric pathogens the major reservoir of VTEC seems to be animal husbandry since outbreaks and single cases of the respective infectious diseases have been aligned epidemiologically to contaminated milk and meat products [8–15]; however, other routes of infection have been additionally identified [16–18]. The risks of VTEC infections acquired via the food chain increase when hygienic standards decline [17].

In this paper an outbreak of HUS and severe gastroenteritis in a nursery school and adjacent kindergarten is described. Microbiological and epidemiological investigations demonstrated verotoxinogenic *C. freundii* as the aetiologic agent and sandwiches as the infection source. The sandwiches were prepared with green butter which was made from contaminated parsley originating in an organic garden.

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MATERIALS AND METHODS

Origin of strains

A severe outbreak of HUS and gastroenteritis in a nursery school of a small town in northwest Germany resulted in extensive microbiological investigations by our laboratory. A total of 152 persons were investigated. The following diagnostic approach was applied which was found appropriate for epidemiological purposes [19]: 1. faecal samples from patients and healthy members of the nursery school (1–3 years old, group I) and adjacent kindergarten (4–5 years old, group II and 6 years old, group III), from staff members, and from family members of the respective children were tested for the presence of verotoxin I (VT1) and verotoxin II (VT2) using the faecal-toxin-ELISA technique (see below): in the case of toxin-positive stool samples the patients were reinvestigated between two and five times on the following days; 2. toxin-positive samples were streaked onto selective bile-chrysoidine agar (Institut für Immunpräparate und Nährmedien GmbH, Berlin) and 10–50 colonies per sample as far as available (total of 535 colonies) were further investigated for the presence of verotoxinogenic determinants using gene probes specific for *sltI* and *sltII* (see below); verotoxinogenic colonies isolated during follow-up investigations were not recorded; 3. positive bacterial strains were subsequently characterized by their serofermentative properties, by plasmid pattern and by restriction length polymorphism (RFLP) analysis of their chromosomal DNA. Verotoxinogenicity was confirmed using the respective gene probes and primers for polymerase chain reaction (PCR), as well as antitoxin-antibodies in ELISA and Western blots.

Serofermentative characterization of E. coli *and* citrobacter *strains*

The fermentative subdifferentiation of the bacterial isolates was carried out according to the methods of Farmer and colleagues [20] and Sakazaki [21]. Serological typing was performed according to the method of Sedlak [22] by commercial sera purchased from the Institute of Sera and Vaccines (USOL) Prague.

DNA techniques, plasmids and Southern hybridization

Plasmid DNA isolation, DNA cleavage with restriction enzymes, agarose gel electrophoresis and elution of DNA fragments from agarose gels were performed according to the method of Maniatis and colleagues [23]. The methods of determination of plasmid numbers and size of plasmid DNA (plasmid profiles), together with the plasmid grouping indicative of their phenotypic traits (plasmid pattern) have been described before [24]. Southern hybridization and DNA probing specific for *sltI* and *sltII* were carried out as described earlier [25].

Pulsed field electrophoresis

The pulsed field electrophoresis and the generation of genomic DNA used for pulsed field electrophoresis was carried out essentially as described earlier [25]. Electrophoresis was carried out on CHEF DrII equipment (Bio-Rad) at 10 °C in 1 % agarose gels for 28 h with a constant voltage of 200 V. Pulse times were 5 sec

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at the beginning and 40 sec at the end with a ramping. As size markers yeast chromosome (Saccharomyces cerevisiae YPH 149 and lambda ladder (Bio-Rad)) were used.

PCR

The PCR was applied as described by Schmidt and colleagues [5] and Karch and co-workers [26] using the primers designed for *sltI* A, *sltII* A, *sltI* B, *sltII* B. Amplification was performed using the AmpliTaq-DNA polymerase, Stoffel fragment and the GeneAmp PCR Reagent Kit (Perkin Elmer Cetus) according to the manufacturer's protocol.

Faecal toxins-ELISA

The search for faecal toxin was carried out by means of a sandwich ELISA. Microtitre plates (Greiner) were percolated with 100 μl/well of 0·1 % nitrocellulose solution in methanol. For coating the first antitoxin-antibody the respective sheep antibody was used diluted 1:3000 in 0·05 M carbonate buffer pH 9·6 overnight at 4 °C (coating buffer). After incubation overnight at 4 °C the non-adsorbed antibodies were washed off three times with phosphate buffered saline containing 0·05 % Tween 20 (PBS-T). Non-specific absorption was blocked by incubation for 1 h at 37 °C with coating buffer containing 1 % bovine serum albumin. Faecal samples were suspended in PBS-T (1 g in 2·5 ml) and 200 μl of the suspensions were incubated for 2 h at 37 °C. Following three washings with PBS-T incubation with the second antitoxin-antibody (rabbit) was carried out for 1 h at 37 °C. After washing three times with PBS-T, peroxidase labelled goat anti-rabbit IgG (Sigma) diluted 1:4000 in PBS-T-BSA was added and incubated for 1 h at 37 °C. Plates were then washed six times in PBS-T and 1·2 phenylenediamine (Fluka) substrate was added. Colour was developed at room temperature for approximately 30 min and the optical density (OD) at 490 nm was measured with a Dynatech Reader MR 7000. As positive controls crude toxins VT1 and VT2 derived from pVP48 and pIE988 [25], respectively, were taken. As a negative control sonicated *E. coli* K12 J53 was applied.

Western blot

Western blots were carried out after SDS-PAGE (Mini-PROTEAN, Bio-Rad) using a semi dry blotter (BioMetra, Fast Blot Type B32) at 150 mAp for 45 min. After blocking with PBS-T and 1 % BSA, the respective rabbit antibodies (1:50) were added and incubated for 2 h at room temperature with shaking. After washing, peroxidase labelled goat anti-rabbit IgG (Sigma) diluted 1:300 in PBS-T-BSA was added, and incubated and washed as above. Finally, colour was developed at room temperature with 4-chloronaphthol substrate.

Purification of toxins and generation of antitoxin-antisera

The purification of VT1 and VT2 was carried out as described by Dickie and colleagues [27] using sonicated preparations derived from E. coli K12 C600 carrying the plasmids pVP48 (VT1) and pIE988 (VT2) [25], respectively. Rabbit and sheep antibodies raised against VT1 and VT2 were generated according to the method of Dickie and colleagues [27].

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RESULTS

Microbiological investigations

In total, 152 persons were tested for the presence of verotoxins in faecal samples using the 'faecal toxin' ELISA method. The results are presented in Table 1. Thirty-six persons had positive faecal samples, 16 of them in repeated investigations. The highest positivity rate (85%) was found in the 1–3 year old children of the nursery school (group I). Within this group the clinical symptoms of HUS were also registered. Toxin positive faecal samples (65%) were found also among the 4–5 year old children of the kindergarten (group II), among the 6 year old children (15%), the staff members (60%), and among family members (8%). However, clinical symptoms were not registered among the personnel and the family members (Table 1).

Surprisingly, mainly *C. freundii* (59/72 isolates) and only a low number of *E. coli* strains (13/72) were detected from the positive faecal samples using dot blot hybridization techniques for their selection (Table 2). All *C. freundii* isolates gave positive signals only with the gene probe for *sltII* (Table 3) which was subsequently confirmed by PCR (Fig. 1). In contrast, *E. coli* isolates were found to be positive for *sltI*, *sltI/sltII* or *sltII* (Table 3). Verotoxinogenic *C. freundii* and *E. coli* strains were submitted for serofermentative characterization, plasmid profile and RFLP analysis.

The *C. freundii* isolates were found to be indistinguishable by serofermentative testing, however, not belonging to any of the 42 serovars described by Sedlak [22]. Also, identical RFLPs (Fig. 2) and plasmid patterns (Table 3) supported the clonal nature of the *C. freundii* isolates. In contrast, the *E. coli* isolates had heterogeneous plasmid profiles (Table 3), RFLP patterns (Fig. 2), and other membrane proteins (data not shown), all providing evidence in favour of genetic diversity of the isolates. Moreover, O157:H7/H$^-$ or O26:H11 *E. coli* serovars could not be detected among them but serovars such as O139, O141 and O147 were isolated (Table 3). The genetic heterogeneity of the verotoxinogenic *E. coli* isolates together with their scarcity suggest a marginal involvement in the outbreak. Interestingly, a very high instability of the *sltII* determinant was observed. Beginning with a 10^6/ml inoculum of *sltII* positive *C. freundii*, about 40% of bacterial cells within 6 h ($\sim 10^9$/ml) lost the toxin determinant. However, non-verotoxinogenic *C. freundii* colonies were never detected from faeces but were isolated from parsley (see below).

The finding of verotoxinogenic *C. freundii* strains in the faecal samples of patients suffering with HUS and gastroenteritis prompted us to investigate *C. freundii* strains from the culture collection of our laboratory with respect to the presence of *sltI* and *sltII* determinants. A total of 256 strains were examined which originated mainly from wound infections, urinary tract infections and in some cases from gastroenteritis. No verotoxin positive strains were found among the 256 strains by means of gene probes, PCR, and ELISA.

Epidemiological consideration

The outbreak of HUS and severe gastroenteritis was recorded at the beginning of June. Three children, two boys, 2 years old and a girl, 3 years old, fell ill with

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Table 1. *Faecal toxin positive samples in patients, healthy members of the institution and their respective families*

Group	Number of patients positive/total (%)	Symptoms		
		HUS	Gastroenteritis	Healthy
1–3 years	11/13 (85)	8*	2	1
4–5 years	10/15 (65)	0	5	5
6 years	3/20 (15)	0	1	2
Personnel	4/7 (60)	0	0	4
Family members	8/97 (8)	0	0	8

* One patient (S.S.) died 1 day after the first investigations, however, positive faecal sample was not available (see Table 2).

Table 2. *Frequency of isolation of* C. freundii *among children attending a nursery school*

Patient/age/symptoms	Stool samples positive/total	Number* of strains tested positive/total	Toxinogenic isolates identified (number)
K.J./3/HUS	1/4	5/35	*Citrobacter freundii* (5)
S.S./2/HUS	1/1	1/10	*Citrobacter freundii* (1)
E.R./3/HUS	5/5	5/27	*Citrobacter freundii* (3), *E. coli* (2)
E.I./8 Healthy sister of E.R.	1/7	4/10	*Citrobacter freundii* (2), *E. coli* (2)
E.V./27 Healthy father of E.R.	0/7	—	—
K.O./3/HUS	1/3	12/18	*Citrobacter freundii* (8), *E. coli* (4)
T.M./2/HUS	2/5	8/35	*Citrobacter freundii* (8)
T.P./4 Healthy sister of T.M.	0/5	—	—
K.M./3/HUS	2/5	5/28	*Citrobacter freundii* (5)
D.M./3/healthy†	1/1	3/50	*Citrobacter freundii* (2), *E. coli* (1)
/enteritis	1/1	2/50	*Citrobacter freundii* (1), *E. coli* (1)
/HUS	3/6	4/50	*Citrobacter freundii* (4)
F.M.C/3/gastroenteritis	5/8	—	—
Sch.K./3/HUS	1/8	7/55	*Citrobacter freundii* (7)
R.P./2/enteritis	2/4	3/70	*Citrobacter freundii* (2), *E. coli* (1)
L.F./5/diarrhoea	1/4	0/50	—
R.M./4/diarrhoea	2/3	3/35	*Citrobacter freundii* (3)
H.S./6/healthy	1/3	5/22	*Citrobacter freundii* (3), *E. coli* (2)
Y.B./28 Healthy staff member	2/2	5/40	*Citrobacter freundii* (5)

* The numbers of isolates refer only to the first positive stool samples.
† Numbers given for three stool samples (see text).

clear signs of acute renal failure, thrombocytopenia and microangiopathic haemolytic anaemia which urgently led to hospitalization and dialysis. Within the next 5 days a further six children, all 3 years old, had to be hospitalized due to the same disease.

Among the nine HUS patients one boy died shortly after the onset of illness due to renal failure and no further inspection of faecal specimens was possible. All other children recovered after several weeks treatment and dialysis. From the remaining eight patients toxin positive faecal samples containing mainly

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Table 3. *Genetic characteristics of verotoxinogenic* C. freundii* *and sporadic VTEC isolated from the patients*

Patients	Number of isolates	Plasmid pattern†	DNA probes		PCR				ELISA‡	
			sltI	*sltII*	*sltI* A	*sltII* A	*sltI* B	*sltII* B	VT1	VT2
C. freundii§										
K.O.	8	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·05	0·98
T.M.	8	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·07	1·10
K.J.	5	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·05	1·05
E.R.	3	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·03	0·05
K.M.	5	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·06	0·80
D.M.	5	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·01	0·95
Sh.K.	7	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·09	1·30
R.P.	2	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·09	1·30
E.I.	2	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·02	1·05
H.S.	3	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·05	0·95
Y.B.	5	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·03	1·20
Parsley	2	150 (IncH2), 3·2, 2·8	−	+	−	+	−	+	0·07	1·25
E. coli‖ (serovar)										
K.O. (Ont)	2	60, 2·5	+	−	+	−	+	−	0·95	0·05
(O139)	1	112, 50, 3·0, 1·5	−	+	−	+	−	+	0·03	1·20
(O114)	1	55, 3·8, 2·0, 2·2	+	+	+	+	+	+	1·25	1·00
E.R. (Ont)	1	100, 2·9, 2·2	+	−	+	−	+	−	0·98	0·05
(O147)	1	48	+	+	+	+	+	+	1·05	1·10
E.I. (Ont)	2	32, 1·6	+	+	+	+	+	+	1·20	0·90
D.M. (Ont)	2	32, 1·6	+	+	+	+	+	+	1·20	0·85
(O139)	1	60, 3·5, 3·0	−	+	−	+	−	+	0·05	1·25
(Ont)	1	60, 2·5	+	+	+	+	+	+	1·05	1·30
R.P. (Ont)	1	3·4, 18	+	+	+	+	+	+	1·00	1·20
H.S. (O139)	1	80, 60, 3·0	−	+	−	+	−	+	0·01	1·10
(O139)	1	15, 2·6	−	+	−	+	−	+	0·03	1·05

* A very high instability of the *sltII* determinants among the citrobacter strains was registered (see also [8, 21].

† Number and size in Md, plasmid incompatibility groups in brackets.

‡ ELISA factors are given positive control was set 1·0, negative control 0·05.

§ *C. freundii* could not be serotyped with the commercial 42 sera according to Sedlak [22].

‖ Various *E. coli* nontypable 0-serotypes, but no O157:H7/H$^-$, O26:H11, O55 or O111 serovars were detected.

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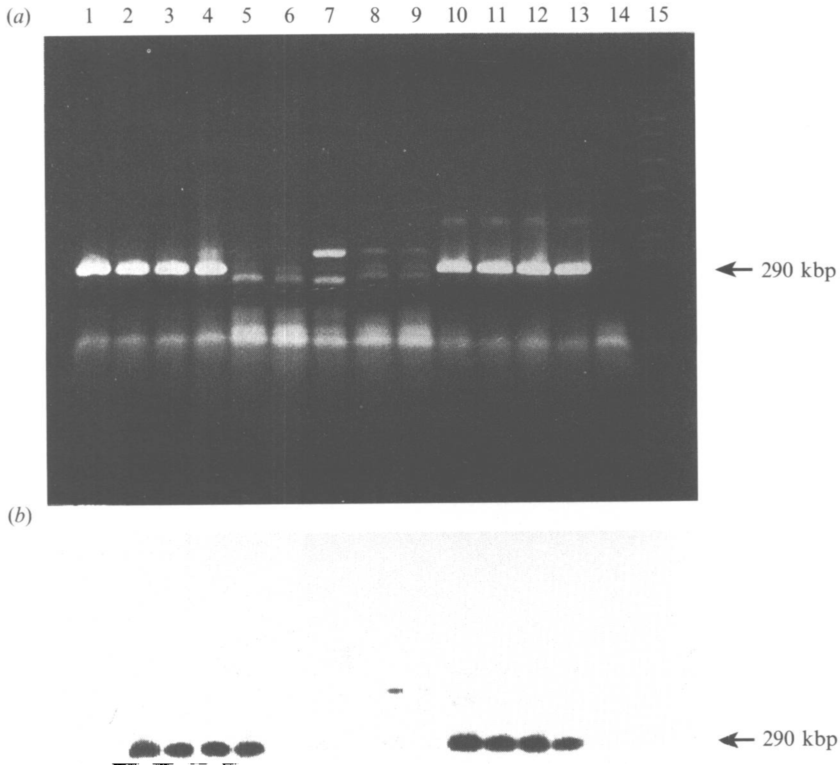

Fig. 1. PCR amplification (A) and hybridization (B) of *sltII* genes in selected *C. freundii* and *E. coli* isolates: 1. *C. freundii* from K.O., HUS; 2, *C. freundii* from T.M., HUS; 3, *C. freundii* from K.J., HUS; 4, *C. freundii* from parsley; 5, *E. coli* from K.O. (only *sltI* positive); 6, *E. coli* from E.R. (only *sltI* positive); 7, *E. coli* from R.P., Enteritis; 8, *E. coli* from O.M., HUS; 9, *E. coli* from E.R., HUS; 10, *C. freundii* from E.R., HUS; 11, *C. freundii* from Y.B., staff member; 12, *C. freundii* from H.S., healthy member of kindergarten; 13, positive control (pIE988); 14, negative control (*E. coli* C600); 15, marker.

verotoxinogenic *C. freundii* (and in some cases only *E. coli*) were detected repeatedly (Table 2). It is of interest to note the observations concerning the patient D.M. (see Table 2) a 3-year-old girl who gave toxin positive stool samples and verotoxinogenic *C. freundii* but without any clinical signs. However, 1 day after the first inspection symptoms of gastroenteritis and 2 days later symptoms of HUS developed.

According to the epidemiological questionnaire and the fact that all three groups including the staff members were affected (Tables 1, 2), sandwiches were implicated as the most probable infection source. These sandwiches prepared with green butter were provided to all of the members of nursery school and kindergarten including the personnel. The green butter was prepared with parsley derived from a private organic garden and slurry of animal origin was used as fertilizer. However, the green butter was not any longer available for inspection. Attempts to isolate verotoxinogenic *C. freundii* from parsley of the organic garden were therefore made. Leaves were harvested and found to be highly contaminated with many bacterial species including *E. coli*, *Proteus mirabilis*, *Pseudomonas* spp.

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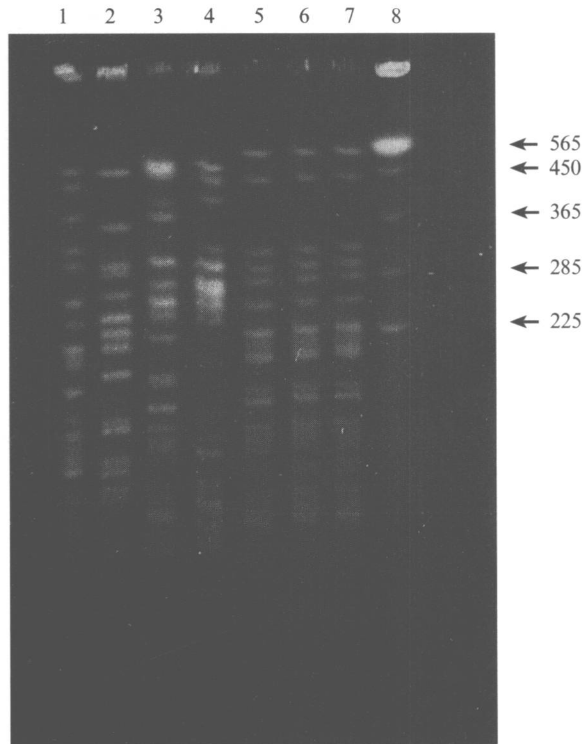

Fig. 2. RFLP of selected *E. coli* and *C. freundii* isolates using Xba digestion of genomic DNA after pulsed field electrophoresis: 1, *E. coli* from E.R., HUS; 2, *E. coli* from K.O., HUS; 3, *E. coli* from D.M., HUS; 4, *E. coli* from R.P., enteritis; 5, *C. freundii* from K.O., HUS; 6, *C. freundii* from T.M., HUS; 7, *C. freundii* from parsley; 8, marker.

and *C. freundii*. Two out of 13 *C. freundii* isolates were subsequently detected as verotoxinogenic by the respective DNA probes and PCR. Both were found to be genetically indistinguishable from the isolates from patients (Table 3, Fig. 2). This suggests strongly that the green butter prepared by means of contaminated parsley was the infection source of the verotoxinogenic *C. freundii* strains. However, verotoxinogenic *E. coli* (4/35) were detected from parsley, too, all four colonies belonging to *E. coli* O139.

DISCUSSION

An outbreak of severe gastroenteritis followed by HUS and TTP in a nursery school and kindergarten is described. Verotoxinogenic *C. freundii* was found as the aetiologic agent. Verotoxinogenic *E. coli* were also detected, but they appeared as contaminants additionally found in the stool samples. This interpretation is supported by their clonal heterogeneity and their irregular appearance in the patients' stool samples. Sandwiches prepared with green butter were the likely vehicle of infection. The green butter was provided through the kitchen of the nursery school and made from parsley. The parsley originated from an organic garden in which manure of pig origin was used instead of artificial fertilizers. Parsley was found contaminated with the same verotoxinogenic *C. freundii* clone as was isolated from patients and from some healthy excreters.

Sandwiches were considered to be the infection source because of statements of

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the staff members that only sandwiches were provided to both the children in the nursery school (group I) and kindergarten (groups II and III) as well as to staff members. The finding of faecal verotoxin positive stool samples containing verotoxinogenic *C. freundii* in all these groups supports this hypothesis.

The onset of the illness 4 days after consumption of sandwiches and the severity of infections (9 HUS, 1 died) together with the frequently observed faecal toxin positivity suggests a high rate of contamination. Since the staff member convinced us that vigorous washing of the parsley was carried out before preparing the green butter, the verotoxinogenic *C. freundii* must have multiplied before consumption. Since the outbreak took place in early summertime the environmental temperature might have been sufficient for such a propagation.

Contaminated food, particularly, milk and meat products have been demonstrated frequently as a source of verotoxinogenic bacteria [8–15] but the data presented here underline the fact that plant products may also become contaminated, in particular when associated with organic plant cultivation or when hygienic conditions become inadequate [17].

Verotoxinogenicity seems not to be common among *C. freundii* as a search among 256 strains from the stock culture collection revealed no positives. However, these *C. freundii* isolates originated mainly from hospital infections (septicaemia, urinary tract and wound infections) and only 48 of them from cases of gastroenteritis. The high instability of the verotoxinogenicity among *C. freundii* reported in this paper and also by Schmidt and colleagues [5] might explain these negative results.

C. freundii is frequently reported as an environmental bacterium and as a nosocomial agent with a broad range of virulence factors including verotoxins [5, 28, 29]. In this respect, *C. freundii* resembles *E. coli* and it would be interesting to demonstrate *C. freundii* strains as good recipients for horizontal gene transfer. Since a λ phage DNA-probe hybridized with genomic DNA from the clinical verotoxinogenic *C. freundii* isolates but did not with the respective toxin-negative segregants (unpublished observations) it is possible that *sltII* carrying lambdoid phages infected *C. freundii* cells, perhaps, during their growth in manure. Due to instability of λ phages in *C. freundii* the frequent loss of *sltII* determinants might be explained. It is important to remember that enteric strains other than *E. coli* may be clinically and epidemiologically associated with verotoxinogenicity.

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