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Shiga-Like Toxin II-Related Cytotoxins in *Citrobacter freundii* Strains from Humans and Beef Samples

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By hybridizing colonies grown from 928 individual stool samples of patients suffering from diarrhea with oligonucleotide probes 772 and 849 complementary to Shiga-like toxin I (SLT-I) and SLT-II gene sequences, respectively, we identified two strains that hybridized with probe 849, which biochemical identification revealed as *Citrobacter freundii*. An additional five *slt-II* probe-positive isolates were screened from 81 beef samples. Polymerase chain reaction analysis and restriction of amplified products provided evidence for *slt-II*-related genes in all seven strains. From *C. freundii* LM 76, the genes encoding the A and B subunits were cloned in pUC 18 vectors and sequenced. The gene encoding the A subunit differed from that of *Escherichia coli slt-IIvhc* in 4 bases, resulting in two amino acid residue differences. In 11, 13, and 11 nucleotides, differentiation of *slt-IIA*, *slt-IIcA*, and *vtx2haA*, respectively, was found. These differences affected the predicted amino acid sequence as follows: there were six amino acid differences with SLT-IIA, five with SLT-IIcA, and four with VTx2haA. The nucleotide sequence of the gene encoding the B subunit is identical to *slt-IIvhcB* and differed from *slt-IIcB* and *vtx2haB* by only a single nucleotide base, but this resulted in a predicted amino acid sequence identical to that reported for these toxins. We therefore termed the toxin genes *C. freundii slt-IIcA* and *slt-IIcB*. Culture filtrates inoculated with material from the colonies from primary cultures were cytotoxic to Vero cells. Neutralization assays with antisera to *E. coli* SLT-I, SLT-II, and SLT-IIvhc revealed that antibodies against SLT-IIvhc reduced the *C. freundii* cytotoxic activity specifically and to the same degree as with the *E. coli* SLT-IIvhc control strain. In five of the seven strains tested, subcultivation on both a liquid or solid medium resulted in loss of cytotoxic activity. With polymerase chain reaction, we demonstrated that loss of cytotoxic activity ran parallel with the loss of *slt* genes. These data demonstrate the intergeneric occurrence of SLT-II-related toxins, which may well be a new marker of enteropathogenicity in *C. freundii*. Our findings that the toxin genes belong to the *slt-II* family plus their evident instability in the majority of strains should help pave the way to a better understanding of their role in diarrhea or food poisoning.

Citrobacter freundii strains are widely distributed in nature and are found in soil, water, foodstuffs, and a wide variety of animals, including household pets, birds, and cattle (6, 32, 33). A member of the family *Enterobacteriaceae*, this organism may cause urinary tract infections, wound infections, and (sometimes) pneumonia in humans, especially in immunocompromised hosts (16, 24, 32, 33). *C. freundii* has also been isolated to various degrees from the feces of healthy people (6, 10, 32).

In addition to being normal components of the intestinal microfloras, *C. freundii* strains have constantly been linked with sporadic cases and outbreaks of diarrhea (1, 6, 10, 12). Heat-stable and heat-labile enterotoxins have been identified as possible candidates for enteropathogenicity (10, 11, 12, 39). However, in a previous clinico-epidemiological study, although *C. freundii* organisms were present significantly more often in the stools from children with diarrhea than in the stools from controls, *C. freundii* patient isolates produced heat-stable enterotoxins in only 3 of 46 cases, and none produced heat-labile enterotoxins (10). In addition to enterotoxins, much earlier studies of *Citrobacter* species reported on cytotoxic effects on Vero and HeLa cells with a lethal effect on mice (27). Such effects have been demonstrated by molecular methods in *Shigella dysenteriae* type I to be due to Shiga toxin (34, 36) and in *Escherichia coli* are caused by Shiga-like toxins (SLTs; verocytotoxins) (4, 28, 41). Whereas Shiga toxin and SLT-I have nearly identical nucleotide sequences (34, 35), in *E. coli* strains, *slt* sequences that have considerable gene sequence heterogeneity to Shiga toxin I and SLT-I have been identified (8, 14, 17, 18, 26, 31, 40). These toxins are termed SLT-II (VT2) or SLT-II variants, examples of which are SLT-IIc, made by *E. coli* O157:H$^-$ strain E 32511 (31); SLT-IIvhc, present in *E. coli* O157:H7 strain 7279 (26); VTx2ha and VTx2hb, produced by *E. coli* O91:H21 isolate B2F1 (17); and SLT-IIva, produced by the O128:B12 strain H.I.8 (8). Additional SLT-II variants in porcine *E. coli* isolates have been recorded (14, 40).

Here, we demonstrate the presence of *slt-II*-related genes in *C. freundii*.

MATERIALS AND METHODS

Bacterial strains. SLT-I- and SLT-II-producing reference strains *E. coli* C600(H19J) and *E. coli* C600(933W) were provided by A. D. O'Brien, Bethesda, Md. The *E. coli* O157:H7 isolate 7279 producing SLT-IIvhc was described recently (26). SLT-IIc reference strain E 32511 was provided by M. A. Karmali, Toronto, Canada. The isolates from M. A. Karmali were previously shown to harbor only SLT-IIc (15), the *slt-II* genes detected by Schmitt et al. (31) having been lost. *E. coli* O157:H7 strain 7279 also harbors

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TABLE 1. Biochemical reactions of SLT-producing *C. freundii* strains

Biochemical reaction	*C. freundii* strain[a]						
	LM 30	LM 54	LM 64	LM 70	LM 76	214/89	015/91
Hydrogen sulfide (triple sugar iron)	+	+	+	+	+	+	+
Indole production	−	−	−	−	−	−	−
Citrate	+	+	+	+	+	+	+
Urea hydrolysis	+	+	+	(+)	(+)	+	+
Phenylalanine deaminase	−	−	−	−	−	−	−
Lysine decarboxylase	−	−	−	−	−	−	−
Ornithine decarboxylase	−	−	−	+	+	−	−
Arginine dihydrolase	+	+	+	+	+	+	+
Growth in KCN	+	+	+	+	+	+	+
D-Glucose, acid	+	+	+	+	+	+	+
D-Glucose, gas	+	+	+	+	+	+	+
Lactose fermentation	+	+	+	+	+	+	+
Sucrose fermentation	+	+	+	−	−	+	+
D-Mannitol fermentation	+	+	+	+	+	+	+
Salicin fermentation	−	−	−	−	−	−	−
Adonitol fermentation	−	−	−	−	−	−	−
D-Sorbitol fermentation	+	+	+	+	+	+	+
Mucate fermentation	+	+	+	+	+	+	+
myo-Inositol fermentation	+	+	+	−	−	−	−
L-Arabinose fermentation	+	+	+	+	+	+	+
Voges-Proskauer	−	−	−	−	−	−	−
Methyl red	+	+	+	+	+	+	+
Dulcitol fermentation	−	−	−	+	+	−	−
L-Rhamnose fermentation	+	+	+	+	+	+	+
D-Xylose fermentation	+	+	+	+	+	+	+
Trehalose fermentation	+	+	+	+	+	+	+
Cellobiose fermentation	+	+	+	+	+	+	+
Esculin hydrolysis	−	−	−	−	−	−	−
Acetate utilization	+	+	+	+	+	+	+
Nitrate→nitrite	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+

[a] + and −, positive and negative for biochemical reactions; (+), delayed positive reaction.

only one *slt* gene (3). Strain A9167/1 of *E. coli* serotype O157:H7 used for sequence analysis experiments as a control was previously shown to hybridize with *slt-I-* but not with *slt-II*-specific probes (19). The SLT-producing *C. freundii* strains LM 30, LM 54, LM 64, LM 70, and LM 76 were isolated from hamburger meat, whereas strains 214/89 and 015/91 are from patients with diarrhea. In addition, five *slt* probe-negative strains from beef and two strains from patients' stool samples were used as controls.

Stool samples and bovine meat. A total of 688 stool samples from hospitalized patients with diarrhea were collected from October 1988 to October 1991 and have been described in some detail (13). An additional 240 samples were analyzed from November 1991 to August 1992. Beef samples (hamburger meat) were obtained from grocery stores in Northern (Hamburg) and Southern (Würzburg) Germany. The samples were initially homogenized and resuspended in phosphate-buffered saline (PBS) and subsequently were vortexed and plated either undiluted or diluted (1:10, 1:100, 1:1,000) onto MacConkey agar. When possible, a maximum of 64 colonies were analyzed for *slt* genes by hybridization of single colonies. To achieve this, material from lactose-fermenting colonies was transferred with a toothpick onto nitrocellulose filters laid on top of Trypticase soy agar plates in duplicate. One of the filters was hybridized with oligonucleotide 772 (*slt-IA* specific) and the other with oligonucleotide 849 (*slt-IIA* specific) as described previously (19). In order to avoid the loss of toxin genes by subcultivation, material from the original colonies was suspended in 100 μl of PBS and glycerol was subsequently added to make a final concentration of 50%. These cultures were stored at −80°C. For subcultivation experiments, 10 μl of these glycerol stocks was used to inoculate 5 ml of trypticase soy broth (TSB) that was incubated overnight. If single colonies grown from the original material were to be analyzed, 100 μl of a 1:10^6 dilution from the glycerol stock was spread on agar plates.

Biochemical tests. The biochemical properties of the *slt* probe-positive strains were determined by the method described by Farmer et al. (7). The reactions tested are shown in Table 1.

Cytotoxicity assay and neutralization assay. Culture filtrates were tested for cytotoxic activity on Vero cells and HeLa cells as previously described (21). The titer of the cytotoxic activity was specified as the reciprocal of the highest dilution of the culture filtrates causing 50% cell death (CD_{50}) per well after being incubated for 2 days at 37°C. The CD_{50} was estimated by microscopic examination of the Vero cells and was confirmed macroscopically by staining residual Vero cells with crystal violet (9). After incubation of the broth cultures for 24 h with agitation at 37°C, the bacteria were harvested by centrifugation (8,000 × *g* for 10 min at 4°C) and the supernatant was filter-sterilized. After dilution with cell culture, medium aliquots (100 μl each) were transferred to microtiter plates and examined for cytotoxic effects. To monitor the stability of SLT production, culture filtrates were prepared by inoculating TSB with material either from single *slt* probe-positive colonies from the primary MacConkey agar plate or from colonies that were

TABLE 2. Nucleotide sequences of primer pairs and PCR strategies used for amplification of *C. freundii slt* genes

Primer	Nucleotide sequence	PCR conditions (°C, s)			Expected size of fragment (bp)	Expected nature of amplification product
		Denaturing	Annealing	Extension		
GK1	5′-CCGGATCCATGAAGTGTATATTATTTAAATGG-3′	94, 30	52, 60	72, 120	1,260	Complete operon of *slt-II* or *slt-II*-related toxin genes
GK4	5′-CCCGAATTCTCAGTCATTATTAAACTGCAC-3′					
MK1	5′-TTTACGATAGACTTCTCGAC-3′	94, 30	43, 60	72, 60	230	All *slt-IA*- or *slt-IIA*-related sequences
MK2	5′-CACATATAAATTATTTCGCTC-3′					
GK1	5′-CCCGGATCCATGAAGTGTATATTATTTAAATGG-3′	94, 30	52, 60	72, 120	960	*slt-IIA* or *slt-IIA*-related toxin genes
GK2	5′-CCCGAATTCTTATTTACCCGTTGTATATAAAAA-3′					
GK3	5′-CCCGGATCCATGAAGAAGATGTTTATGGCG-3′	94, 30	43, 60	72, 60	290	*slt-IIB* or *slt-IIB*-related toxin genes
GK4	5′-CCCGAATTCTCAGTCATTATTAAACTGCAC-3′					

subcultured on Trypticase soy agar. To follow the stability of SLT production after growth in broth culture, 10 µl of the glycerol stocks was employed for inoculating 5-ml portions of TSB; these samples were then incubated overnight. To ascertain *slt* gene stability from this culture, 10 µl was employed to inoculate 5-ml portions of fresh TSB.

Cytotoxins were identified by neutralization tests as described (19) with minor modifications. Briefly, 100 µl of culture filtrate diluted twofold (range, 1:20 to 1:640) was mixed with 100 µl of antiserum that was diluted (1:1,000 dilutions for anti-SLT-I and SLT-II; 1:2,000 for anti-SLT-IIvhc). Formalin-treated purified SLT-I and SLT-II (22) and crude SLT-IIvhc were used to obtain antibodies. Rabbits were treated with multiple intradermal injections of culture supernatants from *E. coli* O157:H7 strain 7279 that were concentrated by ammonium sulfate precipitation (50%, vol/vol), detoxified, and emulsified with equal volumes of Freund's incomplete adjuvant. Preimmune rabbit serum mixed with serially diluted supernatants of the respective strains was used as a control in the neutralization assays. All culture supernatants were adapted, in that a 1:80 dilution resulted in 50% detachment of cell monolayers.

PCR and restriction analysis. The oligonucleotides used as primers, purified by high-performance liquid chromatography, were purchased from MWG-Biotech, Ebersberg, Germany. Primer pair MK1/MK2 was described previously (20); primers GK1/GK2 and GK3/GK4 were described previously by Gunzer et al. (13). For amplifying the complete *slt-II* or *slt-II*-related toxin operons, the primer combination GK1/GK4 was employed. For amplification of the *slt-IB* gene, the primer pair KS7 (5′-CCC GGA TCC ATG AAA AAA ACA TTA TTA ATA GC-3′) and KS8 (5′-CCC GAA TCC AGC TAT TCT GAG TCA ACG-3′) was used. The sequences of all the primers used to amplify *C. freundii slt* sequences, the precise polymerase chain reaction (PCR) conditions prevalent here, the expected size fragments, and the expected nature of the amplification products are depicted in Table 2.

Amplifications were performed in a total volume of 50 µl containing each deoxynucleoside triphosphate at 200 µM, 50 pmol of each primer, 5-µl 10-fold concentrated polymerase synthesis buffer, and 2.5 U of *Taq* (DNA polymerase) (Amersham Laboratories, Buckinghamshire, United Kingdom). The samples were overlaid with 50 µl of mineral oil to prevent condensation. Thermal cycling was carried out in a thermostatically controlled water bath. After 30 cycles, the amplification products were subjected to submarine gel electrophoresis on 1% agarose gels and visualized by staining with ethidium bromide.

To distinguish between *slt-II* and *slt-II*-related sequences, restriction analysis of the PCR amplification products obtained after PCR with primers GK3/GK4 was carried out according to Tyler et al. (37) with the minor modifications described previously (13). Digestion with *Hae*III (Boehringer GmbH, Mannheim, Germany) was performed as recommended by the supplier.

Southern blot hybridization. For Southern blot analysis, total bacterial DNA was prepared as described by Sambrook et al. (29), digested with *Bam*HI, and subjected to electrophoresis through a 0.7% agarose gel. After agarose gel electrophoresis, the DNA was denatured by soaking the gel for 1 h in 4 volumes of a solution of 1.5 M NaCl and 0.5 M NaOH at room temperature. Subsequent to neutralization by the gel being washed in 4 volumes of a solution of 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl at room temperature for 1 h, the DNA was transferred to a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) by capillary blotting as described by Sambrook et al. (29).

Finally, the filter was rinsed in twofold sodium chloride–sodium citrate buffer (150 mM NaCl, 15 mM Na citrate [pH 7.0]) and heated at 80°C for 2 h. Hybridization was conducted with digoxigenin-labeled DNA probes as described in the Boehringer manual (Boehringer GmbH, Mannheim, Germany). A 960-bp DNA fragment of the *slt-IIA* gene resulting from amplification with the primer pair GK1/GK2 was labeled with digoxigenin and used as a hybridization probe. Probe labeling was performed by the PCR program for the SLT-IIA subunit gene, but instead of dTTP only, a mixture of 130 µM dTTP and 70 µM digoxigenin-11–dUTP was added. Hybridization and detection of bound probes were performed with the digoxigenin labeling and detection kit (Boehringer GmbH) according to the manufacturer's instructions.

Cloning PCR products. The PCR products from distinct amplifications with primer pairs GK1/GK2 and GK3/GK4 were separated on a 0.8% Tris-acetate-EDTA agarose gel. Gel slices with the appropriate DNA fragments were ex-

cised, the DNA was eluted and purified with the Gene Clean kit (Dianova, Hamburg, Germany). Purified DNA was digested with *Eco*RI and *Bam*HI and again purified as described above. These purified fragments were then ligated in plasmid vector pUC 18 (38, 42), restricted with the same enzymes by using T4 DNA ligase (New England Biolabs, Inc.). The ligation mixture contained fragment and vector DNA in a 5:1 ratio. Ligations were carried out at 10°C overnight in a total volume of 20 μl. Aliquots of the distinct ligation mixtures were used to transform *E. coli* DH5α (GIBCO BRL) with the $CaCl_2$ method of Mandel and Higa (25). Selection of transformants carrying recombinant plasmids was on Trypticase soy agar containing ampicillin (100 μg/ml), isopropyl-β-D-thiogalactopyranoside (Sigma Chemical Co.), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma Chemical Co.). Before DNA was added, competent cells were separated in different tubes, the cells in each tube being transformed with a different ligation mixture to obtain independent transformants. From each of the distinct transformation plates, colonies were picked, from which plasmid DNA was prepared for PCR and nucleotide sequence analysis by using the Qiagen Plasmid-Midi kit (Diagen, Düsseldorf, Germany).

Nucleotide sequence analysis. DNA was sequenced by the dideoxy chain-termination method (30) with [α-^{35}S]dATP (Dupont, Dreieich, Germany), the T7 sequencing kit (Pharmacia LKB, Heidelberg, Germany), standard and reverse primer for M13 and pUC 18 (Pharmacia LKB and Boehringer GmbH, respectively). For the A subunit gene, internal 15-mer primers KS5 (5′-TGC TGT GGA TAT ACG-3′) and KS6 (5′-CAA TTC AGT ATA ACG-3′) were designed and purchased from MWG-Biotech. Sequencing was adopted for pUC 18 vectors as furnished by Chen and Seeburg (5). Sequencing products were separated by electrophoresis on 5 to 6% denaturing polyacrylamide gels and detected by autoradiography. Both strands and at least two independent PCR clones were sequenced from each of the *C. freundii slt* genes to prevent any PCR identification error.

Nucleotide sequence accession number. The nucleotide sequences of *C. freundii slt-IIcA* and *slt-IIcB* have been submitted to the EMBL and have been assigned the accession numbers X 67514 and X 67515, respectively.

RESULTS

Identification of *slt-II* sequences in *C. freundii* strains from patients' stools and beef by colony hybridization. Lactose-positive colonies on MacConkey agar from stool samples of 928 patients with diarrhea were analyzed by colony hybridization with oligonucleotides 772 and 849, which are complementary to *slt-IA* and *slt-IIA* sequences, respectively. With two stool samples, colonies hybridized with oligonucleotide 849, whereby three probe-positive colonies tested biochemically proved to be *C. freundii*. To check these rather unusual findings, all of the remaining colonies (13 colonies in one patient, 28 colonies in another) were tested by Kligler agar, revealing H_2S production. No common enteric pathogens were detected in the stool samples of these patients, none of the hybridizing colonies being *E. coli*. A second source for *C. freundii* strains hybridizing with the *slt-II*-derived DNA probe was discovered in bovine meat samples from different grocery stores. A total of 82 beef samples were tested by the DNA probes, 5 samples being identified as containing *C. freundii* that hybridized with the *slt-II*- but not with the *slt-I*-derived probe.

Biochemical characterization of *slt* probe-positive *C. freundii* strains. The biochemical characteristics of the seven *slt* probe-positive *C. freundii* strains are listed in Table 1. In all respects, the biochemical reactions confirmed those reported by Farmer et al. (7) for *C. freundii*. Of note are the differences between *C. freundii* strains in their various capacities for fermenting sucrose, inositol, and dulcitol. The biochemical profiles of the meat isolates LM 30, LM 54, and LM 64 were identical to those of the patient isolates 214/89 and 015/91 (Table 1).

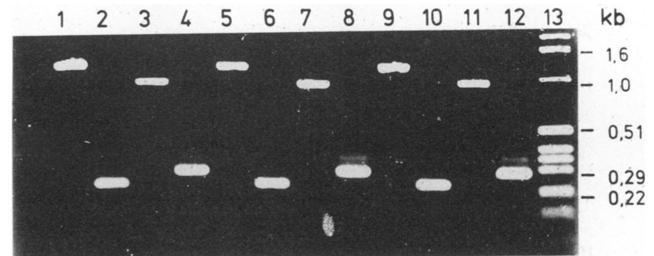

FIG. 1. PCR with *slt-II*-derived primers created with *E. coli* or *C. freundii* DNA as a template. The amplification products in lanes 1 to 4 were obtained with *E. coli* C600(933W), the products in lanes 5 to 8 with *C. freundii* LM 30, and the products in lanes 9 to 12 with *C. freundii* LM 76. PCR amplification products with primers GK1/GK4 used to amplify the complete *slt-II* operon are shown in lanes 1, 5, and 9, and those with universal primers MK1/MK2 are shown in lanes 2, 6, and 10. Primers GK1/GK2 were used to amplify the *slt-IIA* genes shown in lanes 3, 7, and 11, and primers GK3/GK4 were used to amplify the *slt-IIB* genes shown in lanes 4, 8, and 12. In lane 13, the molecular size standard, a 1-kb DNA ladder (GIBCO BRL) is shown.

PCR amplification, restriction analysis, and hybridization of *slt* genes in *C. freundii*. To confirm the presence of *slt* genes, PCR was performed with the four primer pairs shown in Table 2. They were designed to amplify both *slt-II* as well as *slt-II*-related sequences. As shown in Fig. 1 with the SLT-II-producing reference strain C600(933W), these primers did amplify the sizes of fragments expected (Table 2); i.e., about 1,260 bp was amplified when primer pair GK1/GK4 was used (Fig. 1, lane 1), 230 bp with primer pair

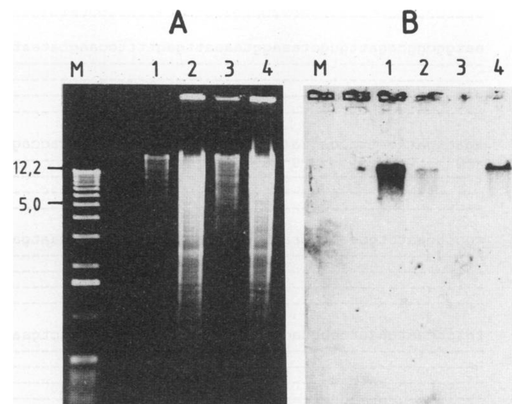

FIG. 2. Agarose gel electrophoresis (A) and Southern blot analysis (B) of *Bam*HI-restricted total cellular DNA from *E. coli* C600(933W) (lane 1), *C. freundii* LM 30 (lane 2), *E. coli* C600(H19J) (lane 3), and *C. freundii* LM 76 (lane 4) probed with the digoxigenin-labeled *slt-IIA* gene probe. M is the molecular size marker (1-kb DNA ladder; GIBCO BRL). Numbers to the left are size markers in kilobase pairs.

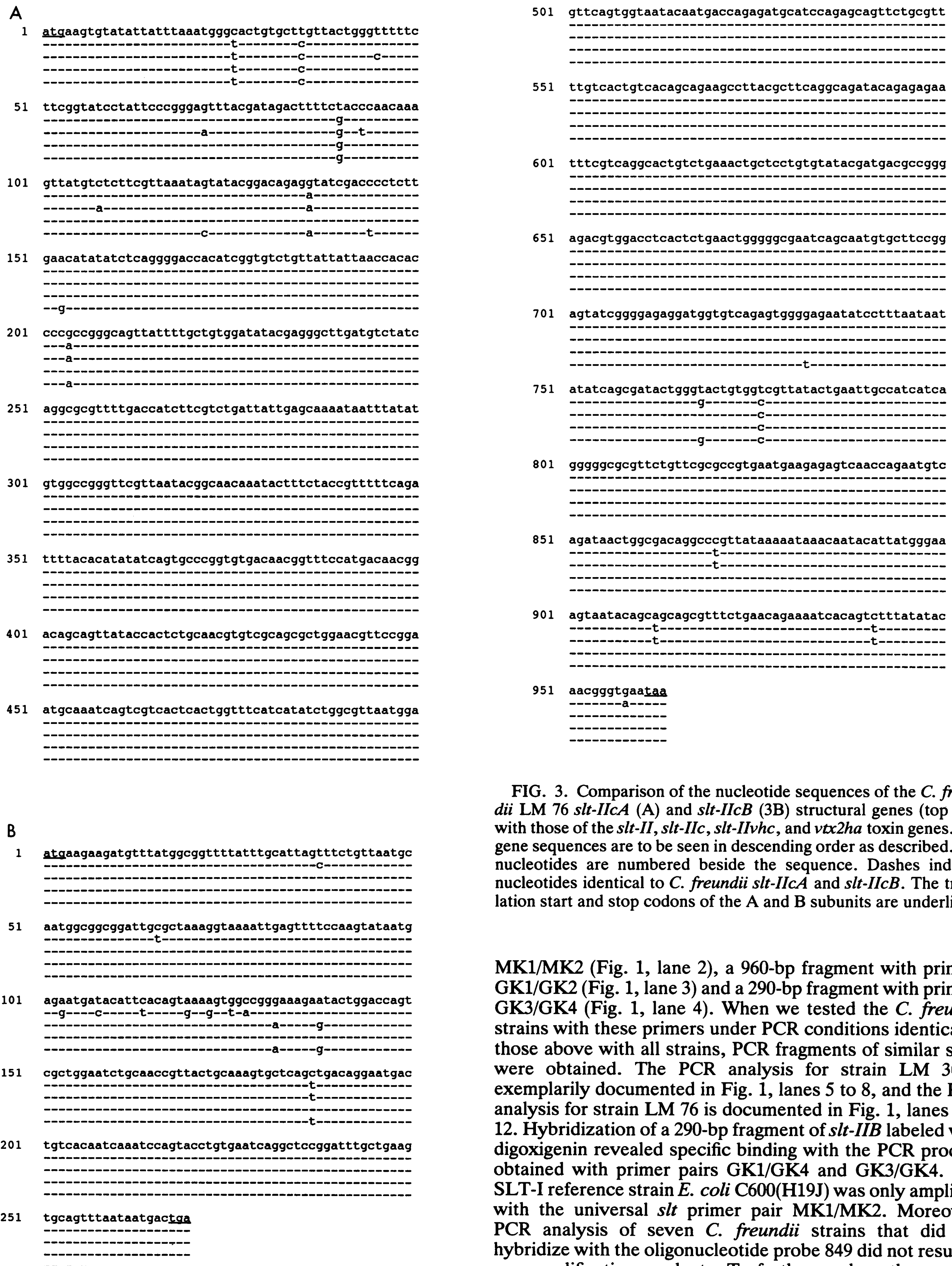

FIG. 3. Comparison of the nucleotide sequences of the *C. freundii* LM 76 *slt-IIcA* (A) and *slt-IIcB* (3B) structural genes (top line) with those of the *slt-II*, *slt-IIc*, *slt-IIvhc*, and *vtx2ha* toxin genes. The gene sequences are to be seen in descending order as described. The nucleotides are numbered beside the sequence. Dashes indicate nucleotides identical to *C. freundii slt-IIcA* and *slt-IIcB*. The translation start and stop codons of the A and B subunits are underlined.

MK1/MK2 (Fig. 1, lane 2), a 960-bp fragment with primers GK1/GK2 (Fig. 1, lane 3) and a 290-bp fragment with primers GK3/GK4 (Fig. 1, lane 4). When we tested the *C. freundii* strains with these primers under PCR conditions identical to those above with all strains, PCR fragments of similar sizes were obtained. The PCR analysis for strain LM 30 is exemplarily documented in Fig. 1, lanes 5 to 8, and the PCR analysis for strain LM 76 is documented in Fig. 1, lanes 9 to 12. Hybridization of a 290-bp fragment of *slt-IIB* labeled with digoxigenin revealed specific binding with the PCR product obtained with primer pairs GK1/GK4 and GK3/GK4. The SLT-I reference strain *E. coli* C600(H19J) was only amplified with the universal *slt* primer pair MK1/MK2. Moreover, PCR analysis of seven *C. freundii* strains that did not hybridize with the oligonucleotide probe 849 did not result in any amplification products. To further analyze the amplification products, restriction analysis with *Hae*III was performed to distinguish whether they were derived from *slt-I* or *slt-II*-related genes. In all *C. freundii* strains, analysis with

TABLE 3. Comparison of the nucleotide and predicted amino acid sequences of the A and B subunit genes of *C. freundii* SLT-IIc with those of SLT-II[a] and SLT-II subtypes[b]

Designation of SLT	% Homology[c]			
	Nucleotide sequence encoding:		Predicted amino acid sequence	
	A subunit genes	B subunit genes	A subunit	B subunit
C. freundii SLT-IIc	100	100	100	100
SLT-II	98.7	96.1	98.1	96.5
SLT-IIc	98.7	98.8	98.4	100
SLT-IIvhc	99.58	100	99.4	100
VTx2ha	98.85	98.8	98.7	100

[a] See reference 31.
[b] See references 17, 26, and 31.
[c] Values represent percent homology with the indicated sequences from the A and B subunit genes of *C. freundii* SLT-IIc.

restriction endonuclease *Hae*III of the PCR fragment obtained with primer pair GK3/GK4 yielded 137- and 157-bp fragments, whereas the amplification product of the *E. coli* SLT-II reference strain remained undigested (data not shown). These experiments revealed that the *C. freundii* strains harbored *slt-II*-related genes. Southern blot analysis of *Bam*HI-digested whole-cell DNA from *C. freundii* strains LM 30 (Fig. 2B, lane 2) and LM 76 (Fig. 2B, lane 4) with an *slt-IIA*-derived probe showed a single fragment band with a size of about 12 kb, demonstrating the existence of a single chromosomal copy of the *C. freundii slt* gene.

Cloning and sequence analysis of *slt* genes from *C. freundii* LM 76. The PCR products obtained after amplification of cells from *C. freundii* LM 76 with primers GK1/GK2 and GK3/GK4 were cloned in vector pUC 18. After the subunit genes were cloned, double-stranded sequencing could be performed. Two independent clones were analyzed for each of the A and B subunit genes. Complementary strands of the B subunit gene were sequenced by using pUC 18 standard and reverse primers. In addition, for the A subunit genes two internal primers, KS5 and KS6, were employed. Both PCR clones from each subunit gene showed the same DNA sequence. Figure 3 compares the *C. freundii slt* nucleotide sequences with those published for *slt-II* (18), *slt-IIc* (31), *slt-IIvhc* (26), and *vtx2ha* (17) genes. Several base differences were found in the 5′ region (first 200 bp) and in the 3′ region (last 200 bp) but not in the middle portion of the A subunit gene. The *C. freundii* SLT-IIA subunit gene was found to be most similar to that of SLT-IIvhc from *E. coli* O157:H7 strain 7279 (Fig. 3A). Four base differences were found within the structural gene for the A subunit when compared with *slt-IIvhc*, 11 differences with *slt-II*, 13 with *slt-IIc*, and 11 with *vtx2ha*. They affected the predicted amino acid sequences as follows: there were two amino acid differences compared with SLT-IIvhc, six with SLT-II, five with SLT-IIc, and four with VTx2ha. The *C. freundii slt-IIB* nucleotide sequence is identical to that reported for *slt-IIvhc* (26), revealing one single base change to *slt-IIcB* and *vtx2haB* (Fig. 3B). The B subunits of these toxins are, however, identical at the amino acid level. The homology of the *C. freundii slt-IIA* and *-B* genes to the published DNA sequences as well as to the predicted amino acid sequences of the SLTs (17, 18, 26, 31) is summarized in Table 3.

To obtain controls, we sequenced the SLT-IB subunit gene from *E. coli* O157:H7 strain A9167/1 and the SLT-IIvhcA as well as SLT-IIvhcB subunit genes according to the same strategy described above. The nucleotide sequences obtained for *slt-IB* were identical to those reported by Calderwood et al. (4) for *slt-IB* from bacteriophage H19B, the nucleotide sequences determined for *slt-IIvhcA* and *slt-IIvhcB* being in accordance with those reported by Meyer et al. (26).

TABLE 4. Cytotoxicity to Vero and HeLa cells of culture supernatants from *E. coli* and *C. freundii* strains

Organism	CD_{50}/ml for[a]:	
	Vero cells	HeLa cells
E. coli		
C600(933W)	20,480	10,240
C600(H19J)	10,240	10,240
7279	2,560	160
E32511	20,480	640
C. freundii		
LM 30	1,280	80
LM 54	640	40
LM 64	640	40
LM 70	640	40
LM 76	1,280	40
214/89	640	40
015/91	640	40

[a] Assays were performed three times; data from a representative experiment are shown.

Cytotoxicity of the *C. freundii* strains. All seven *C. freundii* strains hybridizing with the *slt-II* probes were tested for cytotoxicity to Vero and HeLa cells, this cytotoxic activity being compared with that of seven *C. freundii* strains that did not hybridize with DNA probes as well as with the SLT-I and SLT-II reference strains *E. coli* C600(H19J) and C600(933W), the SLT-IIvhc-producing strain 7279, and the SLT-IIc-producing strain E32511. None of the DNA probe-negative *C. freundii* control strains yielded any cytotoxic activity (CD_{50} < 20), whereas the probe-positive *C. freundii* strains revealed moderate cytotoxic activity, the CD_{50} being 640 in five strains and 1,280 in two strains. The *E. coli* strains cultured under the same conditions yielded higher levels of cytotoxic activity (Table 4). Toxins from *E. coli* C600(H19J) and *E. coli* C600(933W) gave comparable CD_{50}s per milliliter on both cell types. By contrast, toxins from *E. coli* E32511 and 7279 were more than 10-fold less cytotoxic for HeLa cells than for Vero cells. Similarly, the *C. freundii* toxins were less toxic to HeLa cells than to Vero cells.

Neutralization of *C. freundii* culture filtrates with anti-SLT-I, anti-SLT-II, and anti-SLT-IIvhc was performed, revealing that antibody against SLT-I did not affect the cytotoxic activity of all strains, whereas neutralization with anti-SLT-II did slightly reduce the titers. When antibodies against SLT-IIvhc were used, the titers were reduced dramatically to the same extent as with the SLT-IIvhc-producing *E. coli* 7279. Figure 4 shows the neutralization capacity of the antibodies employed when they were incubated with serially diluted culture filtrates of the SLT-I-producing strain C600(H19J) (Fig. 4A), the SLT-II-producing strain C600 (933W) (Fig. 4B), the SLT-IIvhc-producing strain 7279 (Fig. 4C), and *C. freundii* LM 76 (Fig. 4D). It can readily be seen that anti-SLT-I neutralizes the cytotoxic activity of the SLT-I-producing strain (Fig. 4A) but has no neutralizing effect on the SLT-II or the SLT-II-related toxins (Fig. 4B, C, and D). The culture filtrate containing SLT-II of *E. coli* C600 (933W) is neutralized by anti-SLT-II, but neutralization also

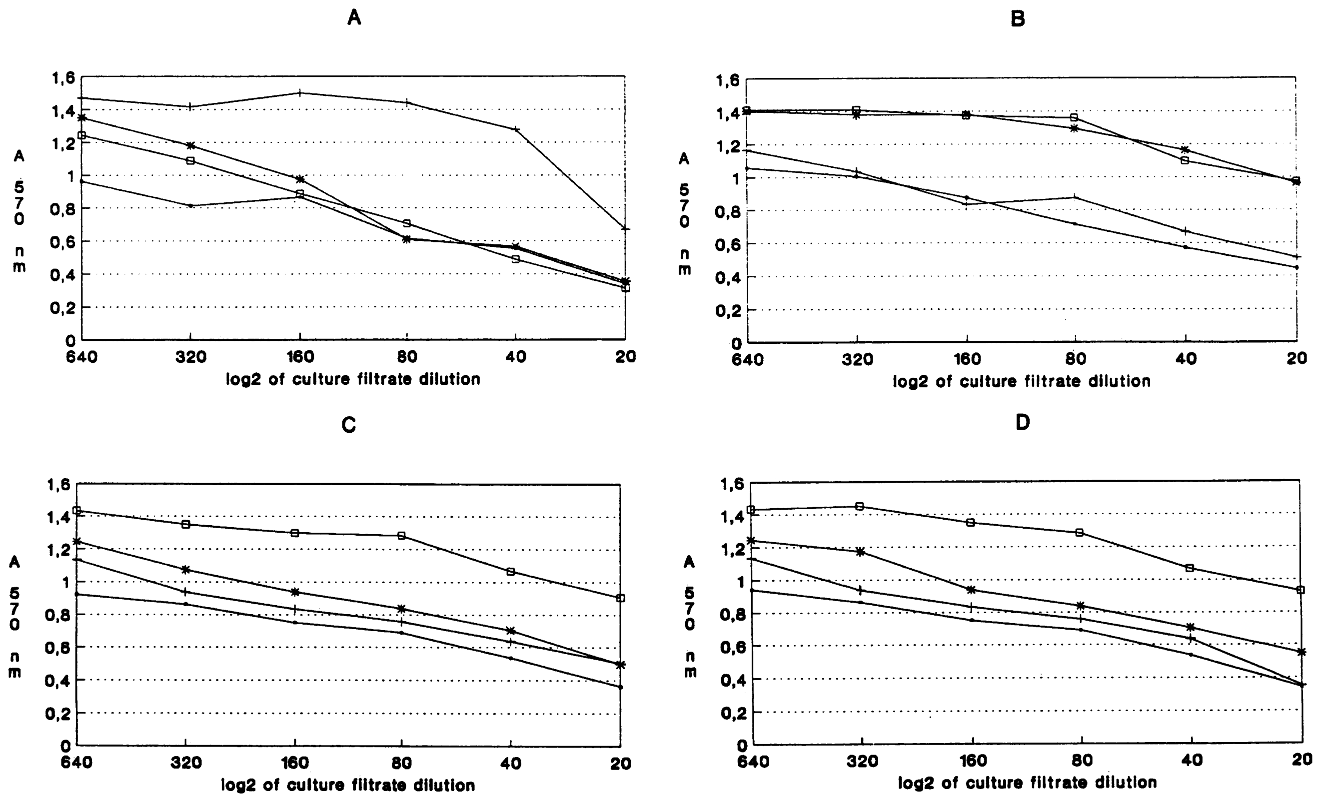

FIG. 4. Neutralization of toxins derived from *E. coli* C600(H19J) (A), *E. coli* C600(933W) (B), *E. coli* 7279 (C), and *C. freundii* LM 76 (D). Neutralization was performed with culture supernatants from each strain diluted 1:20, 1:40, 1:80, 1:160, 1:320, and 1:640 with antibodies against purified SLT-I and SLT-II or crude SLT-IIvhc as well as preimmune serum. The profiles were obtained by plotting A_{570}s of the eluted dye versus $\log_2$ of the culture filtrate dilutions. The CD_{50} value was obtained by extrapolating from one-half of the control dye absorbance value (Vero cells without antibodies) to the scale of the toxin dilutions with preimmune serum. The CD_{50} values in all experiments have been estimated by extrapolation and were in the range of 80 to 90. Each value represents one of at least three experiments. ●, preimmune; +, anti-*slt-I*; *, anti-*slt-II*; □, anti-*slt-IIvhc*.

occurs with anti-SLT-IIvhc (Fig. 4B). In contrast, SLT-IIvhc is more effectively neutralized by antibodies against SLT-IIvhc than by anti-SLT-II (Fig. 4C). The same holds true for the culture filtrate of *C. freundii* LM 76 (Fig. 4D).

Loss of *C. freundii* SLT in five of seven strains as demonstrated by cytotoxicity assay and PCR. We noted that subculturing in one strain (not under consideration in this study) bore the risk of cytotoxic activity being lost. Therefore, in all strains described here, the following experiments were carried out to monitor the stability of *slt* genes in *C. freundii*. (i) Colony material from a single colony grown on MacConkey agar was used to inoculate TSB, and the supernatant was tested for cytotoxicity. By this method, all strains exhibited cytotoxic activity. (ii) Culture material (10 μl) from the glycerol stock was deployed to inoculate 5 ml of broth. Again, cytotoxic activity in the culture supernatants was observed. (iii) Ten microliters from this overnight culture was employed for inoculating 5 ml of fresh medium. These experiments revealed no cytotoxic activity in five of the seven strains, two strains (LM 30 and LM 76) maintaining cytotoxic activity. (iv) Material from colonies passaged once on solid agar was used to inoculate TSB. Again, in five strains no cytotoxic activity was observed, whereas in the two strains mentioned above activity was again detected.

Monitoring *slt* gene loss by PCR. Since we could not detect cytotoxic activity in broth subcultures from five strains (see third experiment above), we subjected 10^6 cells from these strains to PCR analysis with the primers shown in Table 2. An exemplary result with primer pair GK3/GK4 and cells of strains LM 54 and 214/89 is shown in Fig. 5. No amplification product was present in either strain tested after subcultivation (Fig. 5, lanes 1 and 2). Cultures grown from the glycerol stock (see second experiment above) revealed a fragment of

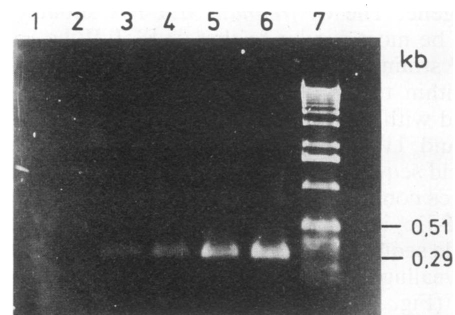

FIG. 5. Influence of subculturing *C. freundii* on *slt* gene stability. Amplification signals from cells of strains LM 56 (lane 1) and 214/89 (lane 2) are not detectable after a second subcultivation. Weak signals are present after a single subcultivation of strain LM 56 (lane 3) and strain 214/89 (lane 4). When cells from the glycerol stocks of strains LM 56 (lane 5) and 214/89 (lane 6) were tested, strong signals occurred.

the expected size (Fig. 5, lanes 3 and 4) which was weaker than fragments obtained with the same number of cells from the glycerol stock (Fig. 5, lanes 5 and 6). When these experiments were carried out with strains LM 30 and LM 76, which were shown above to maintain their cytotoxic stability with respect to subculturing, bands of about 290 bp could be detected on agarose gels after subcultivation. The PCR detection limit was shown to be 10 cells.

DISCUSSION

In this study, evidence has been furnished that *C. freundii* strains on primary cultures harbor *slt* genes as demonstrated by colony hybridization, PCR analysis, and nucleotide sequencing. Evidence from cytotoxicity tests and neutralization studies has likewise been provided that *C. freundii* strains produce functional toxins. The demonstration that *slt* genes closely related to the *E. coli slt-II* family are present in *C. freundii* has diagnostic implications, posing the questions as to whether SLT-producing *C. freundii* can be considered obligate enteropathogens and whether gene transfer between *E. coli* and *C. freundii* is directly responsible for their presence.

Loss of the toxin genes in five of seven strains after subcultivation resulted in loss of cytotoxic activity. A similar phenomenon in about one-third of clinical SLT-producing *E. coli* strains has recently been described (21). Neither in *E. coli* nor in the *C. freundii* strains described here did the phenomena lead to loss of the genes, a fact not hitherto known and requiring further experimentation. Another difficult aspect to explain here is why strains LM 30 and LM 76 retain their cytotoxic activities and their *slt* genes, whereas the others, including isolates that are phenotypically indistinguishable, lose their cytotoxic nature after subculturing. In the latter unstable strains, the PCR showed weaker signals after the first subcultivation that disappeared after the second one. The question is posed as to how these phenomena came about. One explanation for the reduction in cytotoxic activity and copy number of the *slt* genes might be that an *slt* gene-harboring cell does not pass on the *slt* genes to all of its progeny. Bacteriophages causing pseudolysogeny (2) could be responsible for this phenomenon. However, the apparently complete loss of *slt* genes after subcultivation means that there must be fewer than 10 copies per 10^6 cells; otherwise we would have detected them by PCR. With *E. coli* we have recently demonstrated that spontaneous induction of toxin-converting phages leads to the loss of those bacterial cells carrying *slt-II* genes (21). Analysis of the DNA regions flanking the *slt* genes is expected to give more information as to whether the *slt* genes are phage encoded or whether insertion sequences could be responsible for gene transfer.

By neutralization studies, we were able to show that the cytotoxic activity from all seven *C. freundii* strains was neutralized better by antibodies against SLT-IIvhc produced by *E. coli* O157:H7 strain 7279 than by anti-SLT-II. In accordance with these findings, restriction analysis discriminated the *C. freundii* B subunit genes from the SLT-II genes. Moreover, to definitely show the relationship between *C. freundii* SLT and *E. coli* SLTs, we cloned and sequenced the genes from strain LM 76. Since replication errors brought about by the misincorporation of bases are a common potential source of concern when PCR products are cloned and sequenced as was done here, we sequenced two independent PCR clones. For controls, we sequenced the A and B subunit genes from *E. coli* O157:H7 strain 7279 and the B subunit genes from the *E. coli* O157:H7 strain A 9167/1 according to the same strategy performed with *C. freundii* LM 76. With strain 7279, we confirmed our previously published sequence obtained by directly sequencing PCR products by the asymmetric priming method (26). Moreover, the hitherto unknown sequence of the B subunit of strain A 9167/1 was found to be identical to that described for *slt-IB* present on phage H19B (4). The sequence of the latter was obtained by traditional cloning methods (4). We consequently believe that our sequence analysis is highly valid. In order not to further confuse the present "Shiga-like toxin-verocytotoxin" nomenclature, we designated the toxin genes *C. freundii slt-IIcA* and *slt-IIcB*. The basis for this terminology is the identity of the predicted amino acid sequence with SLT-IIcB, although the A subunit sequence differs from that of this toxin. The prefix *C. freundii* should merely state the organism from which the toxin is produced.

Although *C. freundii* strains have been linked with diarrheal diseases (1, 6, 10, 12, 32, 33), the virulence mechanisms by which these organisms cause diarrhea have been poorly investigated. Production of enterotoxins has been demonstrated (10–12, 39), but it was only in a minority of the strains from diarrheal patients that enterotoxins could be shown (10). SLT production is well known as a marker of virulence in *E. coli* (23) and may function as such here. There is evidence in the literature from Nestorescu's earlier work that *Citrobacter* species are able to express cytotoxicity on Vero cells (12) that is heat labile and toxic for mice. Unfortunately, there is no indication as to whether such toxins were expressed stably nor were precise culture conditions stated. We have tried several growth conditions, including incubation of original cultures in a microaerophil or anaerobic atmosphere and invariably failed to prevent loss of *slt* genes in five of seven strains. Interestingly enough, as corroboration of our findings, Farmer (6) mentions a personal communication from J. Wells that cytopathic effects seen in *C. freundii* are not stably expressed. The DNA probes and PCR conditions described here, which identify and amplify the *slt* genes from *C. freundii*, as well as recognition of the instability in the majority of strains should facilitate future epidemiological studies on the role SLT-producing *C. freundii* strains play in diarrheal diseases. This is essential to know because our study was not systematic in the sense of selectively detecting these organisms. The human isolates were only recognized because pure cultures of *C. freundii* were present on the MacConkey agar plates, necessarily leading to all three of the colonies tested for biochemical reactions being identified as such. However, it is equally possible that we overlooked low numbers of *slt-II* probe-positive *C. freundii* cells among SLT-II-producing *E. coli* strains. Therefore, the criterion for prevalence of SLT-producing *C. freundii* strains in diarrheal stools should not be the findings for 2 of 928 samples. We assume that this figure could be considerably higher if we selectively isolated *C. freundii* and performed hybridization studies with these colonies. Unlike the findings for human samples, in beef samples *C. freundii* strains were frequently found, and it is difficult to understand why they were not recognized by other investigators analyzing colonies with *slt-II*-specific DNA probes. An aspect that should be borne in mind here is that these findings may only be due to local phenomena. Another explanation is that *C. freundii* strains hybridize well on replica blots and they still hybridize when material from the original plate is transferred to nitrocellulose before hybridization. However, as shown here and already mentioned above, five of the seven strains did not contain

slt-specific DNA after subcultivation. If hybridization signals disappear, such an observation should not be misinterpreted because of the nonreproducibility of hybridization experiments.

For diagnostic purposes and further research, it is therefore recommended that cytotoxicity assays, DNA hybridization, and PCR be invariably performed with original cultures, these being conserved; subculturing in broth or on solid agar should be avoided under all circumstances if the original material is no longer available because it is not conserved. Finally, the presence of SLTs in *C. freundii* should be taken into due account if stool samples are directly screened by molecular methods or serological means for *E. coli* SLTs. Without identifying the organisms harboring the genes, it is at present very difficult to establish whether the genes or toxins found in the fecal samples originate from *E. coli* or *C. freundii*.

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