Production of *Escherichia coli* STa-Like Heat-Stable Enterotoxin by *Citrobacter freundii* Isolated from Humans

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Citrobacter species are often present in the stools of children and are generally considered a normal component of the intestinal microflora. Previous reports suggested that they might act as enteric pathogens. Aiming at defining the role of *Citrobacter* species in inducing diarrhea, we looked for their presence in the stools of 328 children with diarrhea and in 108 controls. *Citrobacter* strains were isolated from 46 patients (14%) and 7 controls (6.5%) ($P < 0.05$). All isolates were tested for heat-stable (ST) and heat-labile (LT) enterotoxin. No LT-producing organisms were found. Three *C. freundii* strains, all isolated from children with diarrhea, elaborated an enterotoxin detected by the suckling mouse assay. A crude extract was prepared by acetone precipitation and a sequential ultrafiltration technique. The enterotoxin was heat stable, and its estimated molecular weight was between 2,000 and 10,000. *Citrobacter* enterotoxin was soluble in methanol and stable at acid and neutral pHs but not above pH 8, and its activity was destroyed by treatment with 2-mercaptoethanol. *Citrobacter* enterotoxin was inactive in the 18-h rabbit ileal loop test. All these characteristics closely resemble STa produced by *Escherichia coli*. The time course of *Citrobacter* enterotoxin-induced intestinal secretion in suckling mice was similar to that of *E. coli* STa. The enterotoxin produced by *C. freundii* cross-reacted with monoclonal antibodies raised against *E. coli* STa. These results suggest that *C. freundii* is capable of inducing diarrhea through the production of an *E. coli*-like STa, and its presence in the stools of patients with diarrhea should be considered as that of a possible etiologic agent.

Citrobacter are gram-negative enterobacteria classified as a genus in the family *Enterobacteriaceae* (29). *Citrobacter* species are often found in the stools of children, where they are generally regarded as normal components of the intestinal microflora. *Citrobacter* species have been reported previously (3, 4) in association with enteritis in human, but thus far little effort has been made to clarify the nature of this association.

It has been well established that *Escherichia coli* and some non-*E. coli* enterobacteria can cause diarrhea by producing either heat-stable (ST) or heat-labile (LT) enterotoxins (2). Therefore, the isolation of some strains of LT-producing *Citrobacter* species (38) suggested that it might act as an enteric pathogen. However, no clinical significance is generally given to the finding of *Citrobacter* species in the stools of patients with diarrhea.

Aiming at defining the role of *Citrobacter* in producing diarrhea, we looked for its presence in the stools of 328 children with diarrhea and 108 controls. The strains isolated were screened for the presence of both ST and LT. Three strains of *Citrobacter* produced an ST enterotoxigenic activity. One such isolate was further investigated to obtain more information on the nature of the enterotoxigenicity. Investigations were carried out to compare and contrast *Citrobacter* enterotoxin with the well-known *E. coli* STa (6) (see below). The purpose of this study was twofold: (i) to report the possible pathogenicity of *Citrobacter* species in the pediatric age group and (ii) to outline the major characteristics of the enterotoxigenic activity produced by this microorganism.

MATERIALS AND METHODS

Patients. Patients in this study consisted of 328 children (mean age, 15 months; range, 1 to 36 months) admitted to the University Childrens Hospital, University of Naples, both as inpatients and outpatients during the period May 1983 to September 1984 and presenting with diarrhea. Diarrhea was defined as a greater frequency of stools, diminished consistency of stools, or both in comparison with the previous usual state of the patients. At admission there was no detectable cause of the diarrhea, and the patients were enrolled in the study irrespective of its duration. Subjects known to have received antibiotics were excluded from the study. Each patient underwent a routine diagnostic evaluation for children with either acute or chronic diarrhea (9). Another 108 children, matched for age and without gastrointestinal symptoms in the previous 6 weeks, were enrolled as the control group. All the children came from the Naples area.

Isolation and identification of enteric pathogens. Fresh stool specimens were examined for the genus *Enterobacteria* by plating directly on MacConkey agar and, after Selenite broth enrichment, on xylose-lysine-deoxycholate (XLD), salmonella-shigella, and MacConkey agars. One to four different colony types from each plate were subcultured on triple sugar iron agar, and biochemical identification was performed with a multitest system (API 20E, Ayerst Italiana). These strains were stored in nutrient agar in the dark and tested for the production of ST and LT within 30 days. A total of 8 to 12 colonies of *Enterobacteria* were usually tested for each specimen. Approximately 80% of the colonies were *E. coli*. The presence of *Campylobacter* species (Butzler medium [8]), *Yersinia enterocolitica* (11), *Aeromo-*

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TABLE 1. *Citrobacter* isolation and enterotoxin production of isolates from children with diarrhea and from healthy controls

Organism or enterotoxin	No./total (%)	
	Patients	Controls
<i>Citrobacter</i> species ^a	46/328 (14)	7/108 (6.5)
ST	3/46 (6.5)	0/7
LT	0/46	0/7

^a The difference between the frequency of isolation of *Citrobacter* strains from patients and controls was statistically significant ($P < 0.05$).

nas hydrophila (7), and rotavirus (Rotazyme; Abbott Laboratories, North Chicago, Ill.) was also investigated. Ova and parasites were sought by microscopic examination of fresh stools and after enrichment (13). All media were purchased from Oxoid Italiana, Milan, Italy.

Detection of enterotoxin production. The suckling mouse assay (SMA) was used as described previously (14). *Citrobacter* strains were grown in Casamino Acids (Difco Laboratories, Detroit, Mich.) yeast extract medium at 37°C for 18 h in a shaker incubator, as reported previously (14). The culture was then centrifuged and tested. Each strain was tested in three mice, and positive strains were retested 3 times. A gut weight to body weight (GW/BW) ratio greater than 0.085 was considered positive. The SMA was also used to monitor the enterotoxin effect in the physicochemical and biological characterization experiments. Strains producing enterotoxin were stored in the dark in nutrient agar and retested every month for 3 successive months. The Biken test was performed as described previously (20) to detect toxins immunochemically related to *E. coli* LT. A known LT-producing *E. coli* strain (NA ISS 123, serotype O159:H4) served as a positive control. The kit for the Biken test was purchased from Virion (Virion Italia S.P.A., Milan, Italy).

Preparation of the UM-2 retentate from the culture supernatant. *Citrobacter* no. 2 (API code 1404532) was incubated at 37°C in a rotary shaker for 18 h in a minimal medium supplemented with asparagine, as described by Staples et al. (31). The culture was continuously bubbled with oxygen at a rate of 300 ml/min. The medium was then centrifuged at $35,000 \times g$ and processed as described by Klipstein et al. (24). Briefly, the supernatant was filtered through a 0.45- μ m-pore-size membrane (Waters Associates, Milford, Mass.), and enterotoxin was precipitated by adding 8 volumes of acetone. The acetone precipitate, dissolved in distilled water and centrifuged at $11,300 \times g$, was sequentially filtered through ultrafiltration members (PM-10 and UM-2; Amicon Corp., Lexington, Mass.), and each fraction was lyophilized and stored at -20°C.

Biological activity. The minimal effective dose (MED), i.e., the minimum amount of toxin inducing a positive response in the SMA, was established by testing serial twofold dilutions of the culture supernatant and the different ultrafiltration fractions. Protein was measured by the method described by Lowry et al. (24). The time course of ST activity was determined by sacrificing the mice at 60-min intervals up to 7 h after injection of 1 MED of the culture supernatant. The culture supernatant from ST-producing *E. coli* 214-C1 grown under the same conditions was used as a positive control. The rabbit ileal loop test for LT was performed as described by Evans et al. (12) by sacrificing the animals 18 h after the injection of 70 MEDs of toxin.

Physicochemical characterization. The heat stability of the UM-2 retentate, which was dissolved in phosphate-buffered saline, was tested in the SMA after exposure at 60°C for 1 h

or 100°C for 10 min. The culture supernatants of *Citrobacter* sp. strains 1 and 2 were also tested. pH stability was tested by titrating the toxin with NaOH or HCl. After 1 h at 37°C, the preparation was adjusted to pH 7 and immediately injected into the mice. Methanol solubility was determined as described by Mullan et al. (26), and the methanol-soluble and insoluble fractions were tested in suckling mice. The effect of chemical reduction on enterotoxin activity was tested after the addition of 2-mercaptoethanol, as previously described by Staples et al. (31).

Immunological studies. Serial dilutions of *Citrobacter* supernatant were tested by an enzyme-linked immunosorbent assay (ELISA) for the detection of *E. coli* STa, as described by Thompson et al. (36). We used monoclonal antibody 20C1B8, which was prepared against STa purified from *E. coli* 18D (5). The antibodies were obtained from H. Brandwein (Genetic Diagnostics, Great Neck, N.Y.). High-pressure liquid chromatography-purified STa from *E. coli* 18D was used to generate the standard curve in the competitive assay (36). All the results are means $\pm$ standard errors of at least three separate experiments.

RESULTS

Epidemiology and clinical picture. Strains of *Citrobacter* were isolated from the stools of 46 of 328 (14%) children with diarrhea and from 7 (6.5%) children belonging to the control group (Table 1). The difference was statistically significant ($P < 0.05$). In 9 of 46 children positive for *Citrobacter* species, an enteric pathogen was detected. These included rotavirus (4), *Salmonella* species (3), *Campylobacter jejuni* (1), and ST-producing *E. coli* (1). No seasonal distribution was observed in the isolation rate of *Citrobacter* strains. The patients from whom enterotoxin-producing *Citrobacter* strains were isolated were admitted to the hospital in different periods of the year and lived in different neighborhoods.

Three strains of *Citrobacter freundii*, all isolated from children with diarrhea, showed a positive response in the SMA, with mean GW/BW ratios of 0.098, 0.119, and 0.093. The tests were repeated monthly for 3 months, with repeated positive responses for two strains, whereas the third strain became negative after 2 months. None of the strains produced LT.

In two of the children (4 and 22 months of age) symptomatology was mild, consisting of watery diarrhea lasting 4 to 7 days without fever or dehydration and subsiding with no therapy. The third SMA-positive *C. freundii* strain was isolated from an 8-month-old girl. At admission the child was dehydrated and required intravenous correction of metabolic acidosis. The diarrhea lasted 6 days and abated with supportive therapy only. None of the patients showed apparent abdominal pain. The fecal smear for leukocytes was negative in all patients.

No other enteric pathogens were detected in two patients, while in one patient (the 4-month-old child), an ST-producing *E. coli* strain was found associated with *C. freundii*. No follow-up stool cultures were available.

Enterotoxin activity in the ultrafiltration fractions. Enterotoxin activity was present both in the retentate and in the filtrate of the PM-10 membrane, whereas no activity was found in the UM-2 filtrate. The preparation of the UM-2 retentate led to an approximately twofold purification of STa activity (data not shown). Because most of the enterotoxin activity was recovered in the UM-2 retentate fraction, this was used to study the biological and the physicochemical characterization of *C. freundii* ST.

Biological characteristics of *C. freundii* ST. The time course of the enterotoxigenic activity in the suckling mouse assay showed a positive response as early as 60 min after the injection of the culture supernatant (Fig. 1). Peak activity was reached 3 to 4 h after injection. After 3 to 4 h the GW/BW ratio slowly returned toward the base line. In all the time course was very similar to that obtained with an ST-producing *E. coli* strain (Fig. 1). The time course of the *C. freundii* UM-2 retentate showed a similar pattern.

The 18-h rabbit ileal loop test for *E. coli* LT failed to demonstrate intestinal fluid accumulation either after the injection of *C. freundii* UM-2 retentate (70 MEDs) from *C. freundii* 2, or of culture supernatant from all three ST-producing strains.

Major physicochemical characteristics of *C. freundii* ST. (i) **Heat stability.** The biological activity of the culture supernatants from SMA-positive *Citrobacter* strains or of the UM-2 retentate prepared from *C. freundii* 2 was not affected by heating at either 60°C for 60 min or 100°C for 10 min, as assessed by SMA (Table 2).

(ii) **pH stability.** Maximal ST activity was seen in the pH range of 5 to 7, with decreasing activity down to pH 2 and up to pH 8; above pH 8 the toxin was completely inactivated (Fig. 2).

(iii) **Methanol solubility.** Biological activity was found only in the methanol-soluble fraction. No enterotoxigenic activity was found in the methanol-insoluble fraction.

(iv) **Effect of chemical reduction.** Treatment with the sulfhydryl reagent 2-mercaptoethanol led to a complete loss of biological activity. Animals injected with 2-mercaptoethanol alone showed no adverse effects. The GW/BW ratio of the untreated control was 0.104. The GW/BW ratio after reduction was 0.058. The GW/BW ratio for 2-mercaptoethanol treatment alone was 0.045.

Immunological studies. *C. freundii* toxin cross-reacted with monoclonal antibody against *E. coli* STa, as demonstrated in a competitive ELISA. Serial dilutions of culture supernatant of *C. freundii* resulted in a competition displacement curve parallel to that observed for pure *E. coli* STa (Fig. 3), suggesting an immunological similarity of the two toxins. The amount of *C. freundii* toxin in the culture

TABLE 2. Heat stability of *Citrobacter* enterotoxin

Treatment	Biological activity ^a		
	Culture supernatant		UM-2 retentate (strain 2)
	Strain 1	Strain 2	
60°C, 60 min	0.095	0.113	0.102
100°C, 10 min	0.094	0.102	0.100
Untreated	0.098	0.119	0.108

^a GW/BW ratio by the SMA. Three mice were used for each test. One MED of culture supernatant or retentate of UM-2 ultrafiltration membrane was injected into each mouse. Values are means of three experiments.

supernatant, estimated by using the standard curve obtained as described above, was 0.521 ± 0.098 μ g/ml.

The physicochemical, biological, and immunological characteristics in *Citrobacter* ST and comparison with *E. coli* STa are summarized in Table 3.

DISCUSSION

ST production is regarded as a marker of enteropathogenicity (28). In addition to *E. coli*, other bacteria have been shown to be capable of producing an ST type of activity. *Klebsiella pneumoniae* (22), *Aeromonas hydrophila* (7), *Yersinia enterocolitica* (27), *Enterobacter cloacae* (23), *Proteus* spp. (2), and, more recently, *Vibrio cholerae* non-O1 (19) have been shown to produce STa or ST-related enterotoxins.

E. coli STs are currently divided into two major classes (6, 26). The first, STa, is active in the SMA, methanol soluble, and negative in the rabbit ileal loop assay; it acts through an increase of cyclic GMP; and it is pathogenic for humans. STa has been studied extensively, and its amino acid sequence as well as its mechanism of action are largely known (15, 16, 37). The second, STb, is insoluble in methanol, negative in the SMA, and positive in the porcine ileal loop assay; has no effect on cyclic nucleotides; and is without proven pathogenicity in humans (6, 21).

The toxin produced by *C. freundii* fulfills all of the criteria originally proposed by Burgess et al. (6) to describe *E. coli* STa: it is heat stable, soluble in methanol, active in the suckling mouse assay, and inactive in the 18-h rabbit ileal loop assay for *E. coli* LT.

Other chemical and biological characteristics of the ST produced by *C. freundii* also closely resemble those of *E. coli* STa (Table 3). The estimated molecular weights, i.e.,

FIG. 1. Time course of enterotoxigenic activity of *C. freundii* enterotoxin. The culture supernatant of *C. freundii* (0.1 ml) was prepared as described in the text, and 1 MED was injected into each mouse. The animals were sacrificed at various times, and the GW/BW ratio was determined. The culture supernatant from a known STa-producing *E. coli* strain (214-C1; 0.1 ml), grown under the conditions described above, was injected in parallel animals as a positive control. A GW/BW ratio greater than 0.085 was considered positive. Values are means of three separate experiments.

FIG. 2. Effect of pH on enterotoxigenic activity of *C. freundii* enterotoxin. Each animal received 1 MED of the UM-2 retentate fraction that was incubated for 1 h at the desired pH and neutralized immediately prior to injection in mice. A GW/BW ratio greater than 0.085 was considered positive (dashed line). Values are means $\pm$ standard errors of three different experiments.

FIG. 3. Comparison of pure *E. coli* STa and the *C. freundii* ST-containing Supernatant by monoclonal antibody ELISA. Symbols: ●, standard competition curve with pure *E. coli* STa (lower scale on x axis); Δ, serial dilutions of *C. freundii* culture supernatant (upper scale on x axis).

between 2,000 and 10,000; the pattern of the effect of pH; the loss of activity after reduction with 2-mercaptoethanol; and the time course of enterotoxigenic activity of *C. freundii* enterotoxin are very similar to those reported for *E. coli* STa (1, 31, 35).

C. freundii toxin is immunologically similar to *E. coli* STa. We found that monoclonal antibodies raised against an 18-amino-acid *E. coli* STa (5) recognize a component in the culture supernatant of *C. freundii* in a competitive ELISA (36). By using this ELISA, the quantity of toxin estimated in culture filtrate (approximately 0.5 µg/ml) is well within the range reported previously (36) with STa-producing *E. coli* strains (0.02 to 1.8 µg/ml). However, the precise quantity of *C. freundii* toxin is unknown since the monoclonal antibody assay actually measures the presence of a single epitope on the toxin. It is clear from the data shown in Fig. 3 that *C. freundii* toxin demonstrates identical binding kinetics to those of *E. coli* STa for this monoclonal antibody. Thus, the cross-reactivity with the *E. coli* STa epitope appears to be complete. Since this assay is specific for 18- and 19-amino-acid *E. coli* STa's and does not detect STb (36), these data

TABLE 3. Physicochemical, biological, and immunological characteristics of *Citrobacter* ST enterotoxin and similarities *E. coli* STa

Characteristic	<i>Citrobacter</i> ST ^a	<i>E. coli</i> STa ^b
Mol wt	2,000–10,000	2,000–5,000
Heat stability	Yes	Yes
Inactivation pH	pH >8	pH >8
Inactivated by 2-mercaptoethanol	Yes	Yes
Methanol soluble	Yes	Yes
Peak of activity in SMA (h)	3–4	2–4
Activity in 18-h rabbit ileal loop assay	No	No
Reactivity with anti-STa antibodies	Yes	Yes

^a As judged by enterotoxigenic activity in the UM-2 retentate fraction.

^b References 1, 15, 30, and 34.

strongly suggest that *C. freundii* produces an STa which is closely related to the *E. coli* STa's.

Interestingly, in our clinicoepidemiological studies, *Citrobacter* species were found with significantly greater frequency ($P < 0.05$) in the patients with diarrhea than in controls, although not all such isolates from subjects with diarrhea had enterotoxins. Thus, we do not have conclusive evidence of a cause-effect relationship between the ST-producing *C. freundii* and the observed diarrhea. However, in addition to the findings of ST-producing *Citrobacter* strains in children with diarrhea and not in controls, there are two additional lines of evidence that are consistent with such a relationship. First, in all the cases the symptomatology was consistent with enterotoxigenic pathogenesis, as characterized by profuse water diarrhea and the lack of abdominal pain, fecal blood, or leukocytes. Second, no other cause of diarrhea was detected, and furthermore, all the patients recovered without specific therapy. Interestingly, even though the number of observations is limited, the prevalence of enterotoxigenic *C. freundii* in the Naples area seems to be similar to that of enterotoxigenic *E. coli* (9).

The simultaneous presence of an ST-producing *E. coli* strain and another enterotoxigenic bacterial strain in one of the patients needs further comment. The simultaneous isolation of two different enterotoxigenic bacteria from the same patient has been previously reported (32). It is possible that the plasmid encoding for ST could be transferred in vivo between different bacteria by conjugation or transduction processes.

We did not find LT-producing *C. freundii* strains. Such strains have been previously detected by the CHO and the Y1 cell assay systems (17, 33), while we used the Biken test. Even though the Biken test is considered as sensitive as the tissue culture assays (18), no reports are thus far available on its validity in detecting enterotoxigenic bacteria other than *E. coli*. Therefore, we cannot establish whether the lack of LT-producing *C. freundii* reflects the absence of such strains in Italy. However, the ST-positive *C. freundii* strains did not produce LT, as indicated by the results of the 18-h rabbit ileal loop test.

The ELISA that we used for our immunological studies was rapid and easy to perform and can be a useful method for the rapid detection of ST-producing bacteria. We suggest, as proposed previously (30), that the conventional sequence of laboratory steps be reversed such that the enterotoxigenicity is determined prior to the specific identification of fecal coliforms. Such an approach might offer the advantage of considerably reducing the time for a proper diagnosis, thus allowing the clinician to make more logical and full use of adequate therapeutic strategies. Our findings suggest that *C. freundii* should be added to the list of enterobacteria that are capable of acting as enteropathogens through the production of enterotoxins, and its presence in the stools of patients with diarrhea should be regarded as that of a possible etiologic agent.

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