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Purification and Characterization of an Enterotoxin from *Bacteroides fragilis*

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An enterotoxin produced by *Bacteroides fragilis* was purified to homogeneity and characterized as to its biological activity and basic molecular properties. Toxin preparations were prepared by growing *B. fragilis* VPI 13784 in brain heart infusion broth to early stationary phase, immediately precipitating the culture supernatant fluid with 70% ammonium sulfate, and stabilizing the precipitate with the protease inhibitor TPCK (tolylsulfonyl phenylalanyl chloromethyl ketone). The toxin was sequentially purified by anion-exchange chromatography on Q-Sepharose, hydrophobic interaction chromatography on phenyl-agarose, and high-resolution ion-exchange chromatography on Mono Q. The toxin appeared homogeneous as judged by polyacrylamide gel electrophoresis. The estimated molecular weight of the highly purified toxin as determined by gel filtration chromatography on Superose-12 and sodium dodecyl sulfate-polyacrylamide gel electrophoresis is 19,000. It has an isoelectric point of approximately 4.5 and is stable at pHs 5 to 10. The purified toxin is stable at −20 and 4°C and upon freeze-drying, but it is unstable at temperatures above 55°C. It is sensitive to proteinase K and *Streptomyces* protease but is resistant to trypsin and chymotrypsin. The activity of the purified toxin is neutralized by antiserum to a toxigenic strain of *B. fragilis* but not by antiserum to nontoxigenic strains. N-terminal amino acid analysis reveal an unambiguous sequence of Ala-Val-Pro-Ser-Glu-Pro-Lys-Thr-Val-Tyr-Val-Ile-Xxx-Leu-Arg-Glu-Asn-Gly-Ser-Thr. The highly purified toxin induced a strong fluid accumulation response in the lamb ileal-loop assay as well as a cytotoxic response (cell rounding) on HT-29 colon carcinoma cells. Thus, the purified toxin can cause both enterotoxic and cytotoxic activities.

Bacteroides fragilis is the anaerobic bacterial species most commonly isolated from human clinical specimens, causing abscesses, soft-tissue infections, and bacteremias, especially following gastrointestinal surgery (24). It is also a normal inhabitant of the human colonic flora, constituting about 1% of the normal flora (9), and this is the source from which it infects tissue. The virulence mechanisms of this species have been studied by many investigators, but the pathogenicity is not easy to explain. The most obvious pathogenic mechanism is a thin capsular-polysaccharide layer that appears to protect the cells from phagocytosis (4), but until recently, there have been no reports of toxins or other overt mechanisms of cellular destruction or invasiveness. In 1984, the first report of a *B. fragilis* toxin was made by a veterinary research team headed by Lyle L. Myers. This report concerned not an invasive toxin but rather an enterotoxic activity which was reported to cause diarrhea in lambs (14). This activity was assayed by using ligated lamb ileal loops, since rabbit ileal loops consistently failed to give a positive fluid accumulation response (20). Since 1984, these researchers have reported in several publications that *B. fragilis* causes diarrhea in calves (3, 20), piglets (5, 15), and foals (17). In 1989, enterotoxigenic isolates were shown to kill 84% of infant rabbits when the organisms were given orally (19). Subsequent pathology studies showed that the organisms caused characteristic crypt hyperplasia and exfoliation throughout the colon (18).

In 1987, the same group reported that some cases of diarrhea in humans appeared to be caused by *B. fragilis* (21). In one study, enterotoxigenic strains of *B. fragilis* were isolated from 8 of 44 individuals with diarrhea of unknown cause. These individuals had watery diarrhea of 1 to 4 weeks duration, and no other enteric pathogens were isolated from the stools of seven of these eight people. Enterotoxigenic strains have now been isolated from 15% (19 of 123) of the patients with diarrhea and from only 7% of matched controls without diarrhea (21). Furthermore, the toxigenic organisms appear to be carried in the general population. Of over 200 isolates from a municipal sewage plant, 22 (9%) produced the enterotoxin in culture, as determined by the lamb ileal-loop assay (23). Recently, it was shown that culture filtrates from most of the enterotoxigenic strains caused a transient cytotoxic reaction on the colon carcinoma cell line HT-29. The cytotoxic activity causes cell rounding within 2 to 4 h, and the cells revert to a normal appearance after further incubation (25).

In this report, we describe the methods for purification of a toxin from culture filtrate of a highly enterotoxigenic strain of *B. fragilis* and characterize the toxin as to its size and its physical and molecular properties. We also demonstrate that the enterotoxic activity in lamb ileal loops and the cytotoxic activity in HT-29 cells can be caused by this one protein toxin.

MATERIALS AND METHODS

Chemicals and reagents. Trizma base, glycine, sodium acetate, sodium hydroxide, sodium chloride, ammonium sulfate, sodium dodecyl sulfate (SDS), acrylamide-bisacrylamide (premixed for a 40% stock solution), and *N,N,N′,N′*- and tetramethyl-ethylenediamine were obtained from Sigma Chemical Co. (St. Louis, Mo.). Tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK) and *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK) were obtained from Boehringer GmbH (Mannheim, Germany). Ammonium persulfate was

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obtained from Bio-Rad Laboratories (Richmond, Calif.). Unless otherwise specified, the Tris HCl buffer used throughout this work was 0.05 M, pH 7.5.

Culture media and bacterial strains. All broth media and yeast extract were obtained from Difco (Detroit, Mich.) and were prereduced and anaerobically sterilized according to standard methods described in the *Anaerobe Laboratory Manual* (8).

Thirteen enterotoxigenic *B. fragilis* strains (13784, 13760, 13785, 13919, 13920, 13953, 13969, 13974, 14315, 14316, 14317, 14318, and 14319) were obtained from our culture collection (Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg) with the permission of Lyle L. Myers, who originally deposited the strains for identification. The identity of all strains as species of *B. fragilis* was confirmed by reanalyzing the strains using standard analyses involving gas chromatography of volatile fatty acids (8) and cellular fatty acids (22) and polyacrylamide gel electrophoresis of soluble proteins (13). Three other enterotoxigenic strains, 43858, 43859, and 43860, which had been deposited by L. L. Myers, were obtained from the American Type Culture Collection. In addition, we obtained and screened 40 other clinical and intestinal strains of *B. fragilis* and 20 strains each of the other most common colonic *Bacteroides* species (*B. vulgatus*, *B. thetaiotaomicron*, and *B. distasonis*) from our collection. Pure stock cultures of all strains were maintained at room temperature in chopped meat broth.

Protein determinations. Protein concentrations were determined by using the Pierce Coomassie Protein Assay kit (Pierce, Rockford, Ill.) according to the instructions of the manufacturer. Bovine gamma globulin was used to generate the standard curves for determining the protein concentrations of samples.

Cytotoxicity assay. HT-29 cells were grown and maintained in McCoy's 5A medium supplemented with 10% fetal calf serum according to standard tissue culture procedures. For the cytotoxicity assay, we harvested maintenance cultures grown at 37°C under 5% CO_2 in 100-mm-diameter plates using nonenzymatic cell dissociation solution (Sigma), washed the cells from the plate surface, diluted them in McCoy's medium, and aliquoted 180 μl of the diluted suspensions into each well of 96-well microtiter plates. The cells were allowed to settle, attach, and grow for 2 to 3 days prior to use. Samples whose titers were to be determined were diluted twofold in sterile aerobic or prereduced Tris HCl buffer, and 20 μl of each serial dilution was added to the tissue culture wells with thorough mixing. The plates were incubated for 3 to 4 h at 37°C under 5% CO_2, after which time they were examined for the cytotoxic effect (i.e., clusters of cells exhibiting rounding). Cytotoxicity titers were expressed as the reciprocal of the highest dilution of toxin that caused more than 50% cell rounding. A cytotoxic unit (CU) is the lowest amount of toxin that elicits a positive response, i.e., more than 50% cell rounding. The viability of the toxin-treated cells was determined by using the tetrazolium blue method (6).

We screened 14 other mammalian cell lines for sensitivity to the purified toxin of *B. fragilis* VPI 13784 and toxic culture supernatants of VPI 13784 and VPI 2633. All cell lines were obtained from the American Type Culture Collection and prepared in 96-well plates by using the media for each cell line suggested by the supplier. The cell lines included CCD-33CO (human colon fibroblast), MDCK (canine kidney), PA-1 (human ovary carcinoma), CHO-K1 (Chinese hamster ovary), EAT (mouse ascites tumor), HS-27 (human foreskin), Vero (green monkey kidney), NCI-H508 (human cecal adenocarcinoma), BHK (baby hamster kidney), Caco-2 (human colon carcinoma), OTF9-63 (mouse teratocarcinoma), LS174T (human colon adenoma), NIH 3T3 (Swiss mouse fibroblast), and T-84 (human colon carcinoma). Each cell line was tested for sensitivity to (i) the purified toxin (2,500 CU, 0.5 μg/ml) and (ii) culture supernatant fluids of all of the enterotoxigenic strains, using the cytotoxicity assay format described above for HT-29 cells.

Ileal-loop assay. Preparations were tested for their abilities to induce a fluid accumulation response in ligated lamb and rabbit ileal loops by using the procedures previously described (11, 14, 21). Into lamb loops, we injected 1 ml of early-stationary-phase brain heart infusion (BHI) cultures (approximately 10^9 CFU) of the five most toxigenic strains of *B. fragilis* (VPI strains 2633, 13784, 13953, 14317, and 14318) and 1 ml of filter-sterilized preparations obtained at each stage of purification of the toxin from culture supernatant. In rabbits, we tested only the purified toxin (5 μg). For the lamb ileal-loop assay, the reactions were determined at 16 h, and in the rabbit assay, reactions were determined at 12 h. The enterotoxic response was expressed as a volume-to-length ratio (milliliters per centimeter), with a ratio greater than 1.0 indicating a strong positive response. A negative response was defined as loops with no fluid accumulation and a volume-to-length ratio of 0.2 or less.

Production of toxin in broth cultures. We grew each enterotoxigenic strain to maximum turbidity in prereduced BHI broth and assayed the culture supernatant fluid for cytotoxic activity. Prereduced BHI broths (10 ml) were inoculated under CO_2 from chopped meat stock cultures of each strain, and the tubes were anaerobically incubated at 37°C. The turbidity of each culture was monitored in a Spectronic 20 (Milton Roy, Rochester, N.Y.) by A_{600}. When the turbidity of each culture was greater than an absorbance of 1.2, the cells were removed by microcentrifugation (15,000 × *g*, 5 min, 20°C) and the supernatant fluids were assayed for cytotoxicity on HT-29 cells.

The various broth media tested for production of toxin included chopped meat, BHI, peptone-yeast extract, and trypticase-yeast extract, all with and without supplementation with 0.5% glucose. A chopped meat stock culture of each of the strains was diluted 1:10 in anaerobic dilution fluid, and 25 ml of prereduced broth was inoculated under CO_2 with 1 drop of the dilution. During incubation at 37°C, we removed 1 ml of each culture at mid-log phase (A_{600} = 0.6 to 0.8), late log phase (A_{600} = 1.0 to 1.2), early stationary phase (1 to 2 h after maximum turbidity; A_{600} = 1.3 to 1.5), and late stationary phase (8 to 10 h after maximum turbidity).

Filtrates of 25 ml of BHI cultures of VPI 13784 and VPI 2633, inoculated as described above, were harvested at early stationary phase, stored under a variety of conditions, and assayed as follows: 4°C, with assays for cytotoxicity daily for 5 days; −20°C, with assays for cytotoxicity every other week for 3 months; and freeze-dried at 4°C, with assays for cytotoxicity weekly for 1 month. Culture filtrates supplemented with the protease inhibitor(s) TPCK, TLCK, or both (0.1 mg/ml) were also stored at 4°C and assayed daily for 5 days.

In order to check for intracellular toxin, cell pellets obtained from harvesting late-log phase and early stationary phase 1-liter cultures of VPI 13784 and VPI 2633 were disrupted and assayed for toxin. The cell pellets were washed three times with 20 volumes of Tris HCl buffer, suspended in 20 ml of Tris HCl buffer, and disrupted by passage through a French pressure cell at 16,000 lb/in^2.

Cellular debris was removed by centrifugation, and the lysate supernatant was stored at −20°C until assayed for cytotoxic activity.

Phage typing of enterotoxigenic strains. The toxigenic *B. fragilis* strains were typed against a set of 10 specific phages (Bf-1, -13, -15, -20, -25, -29, -31, -32, -51, and -52) by using the soft-agar-overlay technique as previously described (2). The strains were grown in BHI for 14 to 16 h, and 0.1 ml of each culture was mixed with 3 ml of 0.7% BHI agar poured onto prereduced 2% BHI agar plates. The seeded overlays were spotted with 10 μl of high-titer lysates of each phage (10^8 to 10^9 PFU/ml), incubated anaerobically for 24 h at 37°C, and scored for the degree of phage susceptibility of the bacterial strain by observing for clear or turbid plaque formation. Lawns of each bacterial strain in which each phage was propagated were also tested to control for efficiency of plaque formation.

Purification of toxin. (i) Production of culture supernatant fluid. *B. fragilis* VPI 13784 was grown in 1 liter of prereduced BHI broth. One-tenth milliliter of a chopped meat stock culture was anaerobically transferred to 10 ml of BHI broth and incubated for 8 h at 37°C. This fresh BHI culture was then diluted 1:10 in fresh BHI broth, and 0.1 ml of the dilution was inoculated, under CO_2, into 1 liter of BHI broth. The flask was incubated without shaking at 37°C for 16 h to early stationary phase (A_{600} = 1.0 to 1.2). The cells were removed by centrifugation at 12,000 × *g* for 60 min at 4°C, and the supernatant fluid was immediately precipitated with ammonium sulfate.

(ii) Ammonium sulfate precipitation. The culture supernatant was brought to 70% ammonium sulfate saturation by the slow addition of powdered ammonium sulfate. After 2 h of mixing at room temperature, the precipitate was collected by centrifugation at 10,000 × *g* for 30 min at 4°C and dissolved in 25 ml of Tris HCl buffer containing 1 mg of TPCK per ml. The preparation was dialyzed against 5 liters of Tris HCl overnight at 4°C.

(iii) Preparative ion-exchange chromatography on Q-Sepharose. The dialysate was applied to a freshly packed 20-ml Q-Sepharose column (1.5 by 12 cm) (Sigma) equilibrated with Tris HCl buffer on an automated fast protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden). The column was washed (2 ml/min) with buffer until the absorbance of the column eluent returned to zero. A 100-min, 0.0 to 0.5 M NaCl gradient then was applied to the loaded column at a flow rate of 2 ml/min, and the eluent was monitored at 280 nm. Fractions (10 ml each) were collected and assayed for cytotoxicity.

(iv) Hydrophobic interaction chromatography on phenyl-agarose. The six most cytotoxic fractions from the Q-Sepharose separation were pooled, adjusted to 1.5 M NaCl, and applied to a 50 ml phenyl-agarose column (2.5 by 8 cm) equilibrated with Tris HCl–1.5 M NaCl. The column was sequentially washed with 100 ml each of Tris HCl supplemented with 1.5, 1.0, and 0.5 M NaCl. The toxin was eluted with 25% ethanol into 10-ml fractions, which were collected and assayed for cytotoxicity. The three most cytotoxic fractions were pooled and dialyzed against 200 volumes of Tris HCl buffer overnight at 4°C to remove the ethanol.

(v) High-resolution ion-exchange chromatography on Mono Q. Ten milliliters of the dialyzed toxic phenyl-agarose pool was applied to a 1-ml Mono Q column (Pharmacia) equilibrated with Tris HCl on an automated FPLC. The column was washed (1 ml/min) with buffer until the absorbance of the eluent returned to zero. A 20-min, 0.0 to 0.5 M NaCl gradient was applied to the loaded column at a flow rate of 1 ml/min, and the eluent was monitored at 280 nm. Fractions (1 ml each) were collected and assayed for cytotoxic activity. When more-concentrated preparations of purified toxin were required for characterization studies, we concentrated the Mono Q fractions by using a Centricon-10 concentration device (Millipore, Bedford, Mass.), with centrifugation at 5,000 × *g* at 4°C.

Analytical PAGE. Determination of protein homogeneity was performed by nondenaturing polyacrylamide gel electrophoresis (PAGE) using the discontinuous system of Laemmli (11). Electrophoresis was performed at 10°C in 0.025 M Tris–0.192 M glycine buffer, pH 8.3, at 25 mA per gel. After electrophoresis, the gels were fixed and stained by using the Bio-Rad Silver Staining Kit according to the instructions provided by the manufacturer. To locate the cytotoxic activity, the gels were sliced horizontally into 0.5-cm segments, and each gel slice was homogenized with 1 ml of Tris HCl buffer and allowed to incubate at 4°C for 3 to 4 h. The acrylamide was remove by microcentrifugation, and the supernatant fluids were assayed for cytotoxic activity on HT-29 cells.

Estimations of molecular weights. Estimations of the molecular weight of the toxin by gel filtration chromatography were made by using an HR 10/30 Superose-12 column (Pharmacia LKB) at room temperature on an automated FPLC with detection at 280 nm. One-half milliliter of the dialyzed ammonium sulfate precipitate and approximately 2.5 μg of purified toxin in 0.5 ml of Tris-HCl buffer were each run in the system using Tris HCl–0.15 M NaCl at a flow rate of 0.5 ml/min. Fractions (1 ml each) were collected and assayed for cytotoxic activity. The following low-molecular-weight standards were used at a concentration of 1 mg/ml; aprotinin (M_r, 6,500), cytochrome *c* (M_r, 12,400), carbonic anhydrase (M_r, 29,000), bovine albumin (M_r, 67,000), and alcohol dehydrogenase (M_r, 150,000).

Estimation of the molecular weight of the purified toxin by denaturing PAGE was performed by the method of Laemmli (10). Approximately 2.5 μg of purified toxin containing a final concentration of 2.5% SDS and 5% 2-mercaptoethanol was heated to 100°C for 2 min before the bromphenol blue-glycerol was added. Conditions for electrophoresis were the same as described above for nondenaturing PAGE. Bio-Rad Prestained SDS-PAGE Molecular Weight Standards (M_rs, 3,000, 6,000, 14,000, 17,500, 26,000, and 42,000) were used as standards. After electrophoresis, the gels were fixed and stained by using the Bio-Rad Silver Staining Kit according to the instructions provided by the manufacturer (Bio-Rad).

Isoelectric focusing. Flat-bed isoelectric focusing was performed using an LKB Multiphor system according to specifications of the manufacturer (LKB, Bromma, Sweden). Purified toxin was dialyzed against 0.01 M Tris HCl, pH 7.5, and run on a 100-ml flat bed of LKB Ultrodex gel containing a 2.5% mixture of ampholine designed to develop a pH range of 3.5 to 7. Approximately 3 μg of purified toxin in 3 ml of gel was applied to the center of the gel bed, and the gel was focused for 18 h at a constant power of 8 W at 8 to 10°C. After focusing, the gel bed was divided into 30 fractions, and the pH of each fraction was determined with a microelectrode. Each gel fraction was washed with 2 ml of Tris HCl buffer, and each gel eluent was assayed for cytotoxic activity.

Effect of storage at different temperatures. Aliquots of purified toxin (0.2 μg/ml) were stored in Tris HCl buffer at 20, 4, and −20°C. Samples also were freezed-dried and stored at 4°C. Samples were assayed for residual cytotoxic

activity daily for 1 week and then once per week for 4 weeks. Samples were also incubated at 55 and 65°C for 1 h and assayed for residual cytotoxic activity.

Effect of pH. Approximately 10.0 μg of purified toxin per ml was dialyzed overnight against 0.01 M Tris HCl, pH 7.5, and was diluted 1:10 in the following buffer systems: 0.2 M glycine HCl, pH 2.0; 0.1 M acetate, pH 4.0; 0.05 M Tris HCl, pH 7.5; 0.2 M Tris-NaOH, pH 10.0; and 0.2 M glycine-NaOH, pH 11.0. The samples were incubated for 1 h at 37°C, diluted 1:10 in 0.2 M Tris HCl, pH 7.5, and assayed for residual cytotoxic activity. The pH of each sample was determined to ensure that each sample was brought back to pH 6.5 to 7.5 before being assayed for cytotoxic activity.

Effects of enzymes. Purified toxin was incubated with each of the following enzymes: bacterial α-amylase (type II-A, *Bacillus* spp.), bacterial lipase (type VIII, *Pseudomonas* spp.), trypsin (type XIII TPCK treated, bovine pancreas), chymotrypsin (type VII TLCK treated, bovine pancreas), proteinase K (type XI-A, *Tritirachium album*), purified bacterial protease (type XIV, *Streptomyces griseus*), and Dispase (purified neutral protease from *Bacillus polymyxa*). All enzymes were obtained from Sigma with the exception of Dispase, which was obtained from Boehringer Mannheim (Indianapolis, Ind.). All proteases were tested at final concentrations of 1 mg/ml in 0.05 M Tris HCl, pH 7.5. The bacterial amylase and lipase were tested at 10 mg/ml in 0.05 M Tris HCl, pH 6.8. Toxin (1.0 μg/ml) was diluted with an equal volume of each enzyme solution, incubated at 37°C for 1 h, and assayed for residual cytotoxic activity. Enzyme solutions that did not contain toxin had no effect on the HT-29 cells.

N-terminal amino acid analysis of the purified toxin. The first 20 amino acids at the N terminus of the purified toxin were determined at the Virginia Polytechnic Institute and State University Protein Sequencing Facility with a model 477A Protein Sequenator (Applied Biosystems, Foster City, Calif.) with on-line identification of phenylthiohydantoins according to the manufacturer's specifications. Samples from two independent purifications were used for the analyses.

Neutralization of purified toxin by polyvalent rabbit antiserum. Antiserum to the toxigenic strain VPI 2633 and the nontoxigenic strain VPI 2533 was available in our antiserum repository and was used in the preliminary screening of the enterotoxigenic strains. The antiserum, made for use in a previous study on *B. fragilis*, was prepared by using a crude-lysate vaccine according to one of our previous procedures (7). Briefly, overnight BHI cultures of each strain were disrupted by sonication and filtered-sterilized (0.45-μm-pore-size filter). The cell-free-lysate vaccine was mixed with an equal volume of Freund's incomplete adjuvant, and 1 ml was injected subcutaneously weekly for 10 weeks into adult New Zealand rabbits. Sera were collected and analyzed by crossed-immunoelectrophoresis, using previously described methods (1), to determine the presence of precipitating antibodies.

Neutralization studies were performed by diluting each antiserum and neutral rabbit serum 1:10 in buffer and mixing equal volumes of each dilution with (i) 1,000 to 1,500 CU of crude or purified toxin (0.25 μg) from VPI 13784 and (ii) undiluted crude toxic culture filtrates of eight other toxigenic strains which produced cytotoxic titers of 40 or greater. Each mixture was incubated at 37°C for 30 min and assayed for residual cytotoxic activity. Control mixtures consisted of (i) diluted serum mixed 1:1 with buffer instead of toxin and (ii) toxin mixed 1:1 with buffer instead of serum.

TABLE 1. Production of enterotoxin by *B. fragilis* VPI 13784 in culture supernatant fluids

Prereduced broth medium	Cytotoxic titer[a] at phase[b]:			
	Mid log	Late log	Early stationary	Late stationary
Chopped meat	40	160	640	320
BHI	40	160	640	160
Peptone yeast extract	<20	40	80	40
Trypticase yeast extract	<20	20	80	40

[a] Expressed as the reciprocal of the dilution causing more than 50% rounding.

[b] Growth phase was defined on the basis of A_{600}. Mid-log phase, A_{600} of 0.5 to 0.8; late-log phase, A_{600} of 0.8 to 1.2; early stationary phase, 1 to 2 h after maximum turbidity, A_{600} of 1.2 to 1.5; late stationary phase, 8 to 10 h after maximum turbidity.

RESULTS

Analysis of *Bacteroides* species for production of toxin. The cytotoxic titers of culture supernatant fluids of the 16 enterotoxigenic *B. fragilis* strains ranged from 40 to 640, with VPI 13784 supernatant fluid consistently having the highest titer. Of the other 40 *B. fragilis* strains screened, strains VPI 2633, VPI J14-7, and VPI 2556 had titers of 320, 80, and 40, respectively. Phage typing of all 19 toxigenic *B. fragilis* strains revealed that all of the strains were sensitive to at least five of the phages and that enterotoxigenicity and cytotoxic activity were not restricted to any particular phage sensitivity pattern, nor was cytotoxic activity associated with sensitivity to any one particular phage. However, because of the specificity of the *B. fragilis* bacteriophages (2), we may consider the sensitivity to the phages a fourth set of criteria confirming that these strains are indeed *B. fragilis*. On the basis of patterns of resistance of the 16 strains from the Virginia Polytechnic Institute and State University culture collection to penicillin, tetracycline, erythromycin, chloramphenicol, and clindamycin (12), determined as part of the standard identification procedures, we saw no correlation between toxigenicity and antibiotic resistance. All of the strains were resistant to penicillin. In addition, eight were resistant to tetracycline and one was resistant to erythromycin. No cytotoxic activity was observed in the culture supernatant fluids of any of the strains of *B. vulgatus*, *B. thetaiotaomicron*, and *B. distasonis*.

The production of toxin by VPI 13784 in the different culture broths is described in Table 1. Maximum production of toxin was obtained in chopped meat and BHI broths at early stationary phase. This pattern was similar to that obtained with the other highly toxigenic strain, VPI 2633. In all broths supplemented with glucose, both strains produced less than 10% of the toxin produced in unsupplemented broths. No significant amounts of toxin were found in the lysed cell pellets of either strain.

The cytotoxic activities of the culture supernatant fluids of both VPI 13784 and VPI 2633 were stable when the fluids were stored at −20°C or freeze-dried and stored at 4°C. However, supernatant fluids stored at 4°C in liquid form lost cytotoxicity by about 50% each day over 4 to 5 days. Culture supernatant fluids supplemented with the protease inhibitor TPCK or TLCK immediately after the cells were removed retained virtually all of their cytotoxic activity when stored at 4°C over the 5 days. When the toxigenic strains were grown in BHI broth in the presence of TPCK or TLCK, the cytotoxicity of the resulting supernatant fluids was only about half of that obtained in cultures grown without the

inhibitors, but the cytotoxic activity was stable at 4°C over the 5-day period.

Purification of toxin from *B. fragilis* VPI 13784. Because of the ability of VPI 13784 to produce high titers of toxin in broth culture, we chose it for subsequent studies on the purification of the toxin. When we grew the organism in 1 liter of BHI, we routinely obtained titers of 320 which were stabilized by the addition of TPCK prior to ammonium sulfate precipitation. More than 90% of the toxin precipitated at 70% saturation, and virtually none was lost upon dialysis.

Since the cytotoxic activity of culture supernatant fluids stabilized with protease inhibitor was not sensitive to exposure to air, we took no precautions during purification to maintain anaerobic conditions. The initial steps during purification involved anion-exchange and hydrophobic interaction chromatography. Upon semipreparative chromatography on Q-Sepharose, most of the toxic activity eluted at 0.3 to 0.4 M NaCl. The pooled fractions had a clear, light-brown appearance and a cytotoxic titer of 2,560 to 5,120 (Fig. 1a). After pooling the Q-Sepharose fractions and adjusting the salt concentration to 1.5 M, we applied the pool to a phenyl-agarose column. We washed the phenyl-agarose with a step gradient of decreasing ionic strength and eluted the toxin with 25% ethanol (Fig. 1b). At this stage, the material was colorless and had a cytotoxic titer of 5,120 to 10,240.

The final step in the purification consisted of ion-exchange chromatography on an analytical Mono Q column. The toxic activity eluted at 0.20 to 0.24 M NaCl as a single peak resolved to baseline (Fig. 1c). The preparation had a titer of 20,000 to 40,000 and a protein concentration of 5 to 10 μg/ml. A representative summary of the purification data is shown in Table 2. Analysis of the preparation after Mono Q fractionation revealed a single protein band by native PAGE (Fig. 2). The cytotoxic activity was localized at the same region as the single toxin band on stained gels.

Characterization of purified toxin. The molecular weight of the purified toxin as determined by gel filtration chromatography on an HR 10/30 Superose-12 column was in the range of 18,000 to 22,000. This was also the range in which the cytotoxicity eluted when the crude toxin preparation was fractionated. By denaturing SDS-PAGE, the purified toxin gave a single band with an estimated molecular weight of 19,000.

The isoelectric point was in the range of 4.4 to 4.6. Virtually all of the cytotoxic activity was recovered from the gel bed, indicating that precipitation at this pH did not occur, or if it did, it did not affect the ability of the toxin to be solubilized and active.

In the absence of protease inhibitors, the purified toxin was stable in solution in 0.05 Tris HCl, pH 7.5, at 20 and 4°C over a 4-week period. Over 90% of the cytotoxicity of the purified toxin was lost when it was incubated at 55°C for 1 h, and over 99% of the activity was lost when it was incubated at 65°C for 1 h. The purified toxin was stable within the pH range of 5 to 10. Over 90% of the cytotoxicity was lost when the toxin was incubated at pHs 4.0 and 11.0 for 1 h, and over 99% of the activity was lost at pHs 3.0 and 12.0 after 1 h.

Purified toxin retained virtually all of its activity when incubated with trypsin, chymotrypsin, and Dispase for 1 h at 37°C. However, over 99% of the cytotoxicity was lost when it was incubated with proteinase K and the bacterial protease from *S. griseus*. We observed no effects on cytotoxic activity with either the bacterial amylase or lipase.

Antiserum prepared against enterotoxigenic *B. fragilis* VPI 2633 completely neutralized the cytotoxicity of both the crude toxin preparations and the purified toxin of VPI 13784. In addition, the antiserum neutralized the cytotoxic activity of crude culture supernatant fluids of all eight other enterotoxigenic strains tested. Antiserum prepared against the nonenterotoxigenic strains, as well as neutral rabbit serum, had no effect on the cytotoxic activity.

These characteristics of the purified toxin are summarized in Table 3.

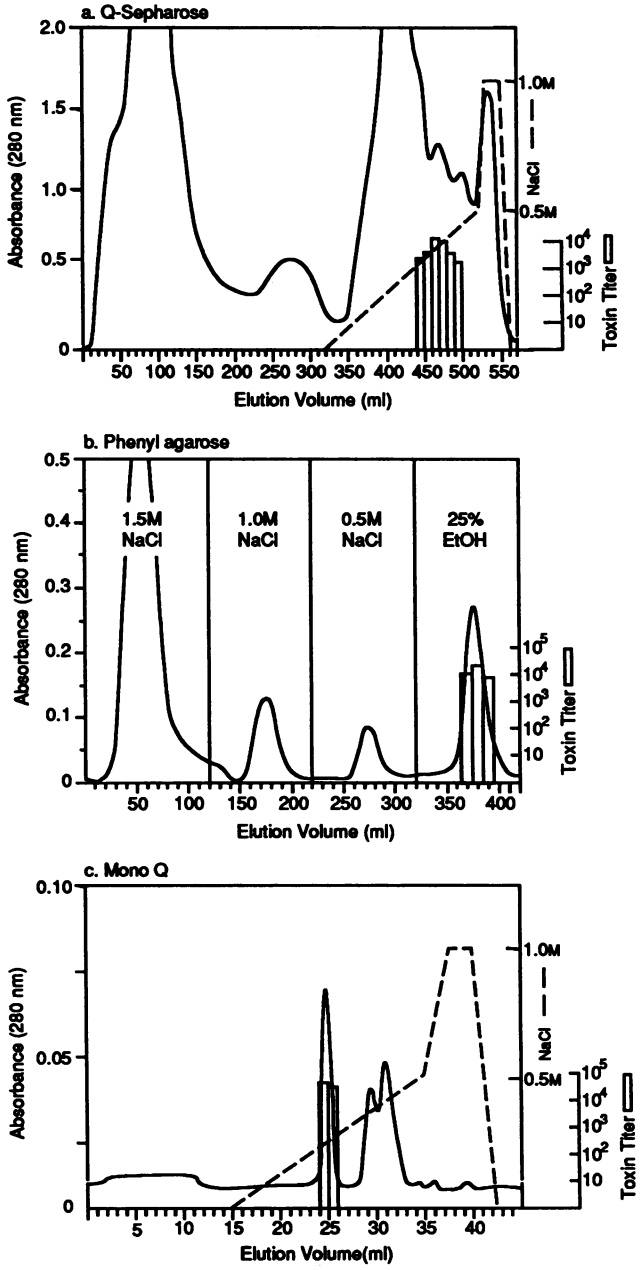

FIG. 1. Chromatographic profiles showing the purification of the *B. fragilis* enterotoxin from culture supernatant fluid of VPI 13784. (a) Anion-exchange chromatography on Q-Sepharose of dialyzed ammonium sulfate precipitate of culture supernatant fluid; (b) hydrophobic interaction chromatography on phenyl-agarose of pooled cytotoxic fractions from Q-Sepharose; (c) high-resolution Mono Q chromatography of pooled cytotoxic fractions from phenyl-agarose. EtOH, ethanol.

TABLE 2. Purification of enterotoxin of *B. fragilis* VPI 13784 from culture supernatant fluid

Method of purification	Total protein (μg)	Total CU[a]	Sp act (CU/μg)	Fold purification[b]	% Recovery[c]
Culture supernatant fluid[d]	160,000	320,000	2		
NH_4SO_4 precipitate (dialyzed)	22,750	294,000	13	6.5	92
Q-Sepharose	2,500	255,000	102	51	79
Phenyl-agarose	180	196,000	1,088	544	61
Mono Q FPLC	32	154,000	4,815	2,408	48

[a] Calculated as cytotoxic titer × total volume of sample.
[b] Based on the CU per microgram of protein in the BHI culture supernatant fluid.
[c] Based on the total CU in the BHI culture supernatant fluid.
[d] Total protein was determined on BHI culture supernatant fluid dialyzed overnight against Tris HCl at 4°C.

N-terminal amino acid sequence of purified toxin. The first 20 amino acid residues at the amino terminus were determined by using two independently purified toxin preparations. The unambiguous sequence Ala-Val-Pro-Ser-Glu-Pro-Lys-Thr-Val-Tyr-Val-Ile-Xxx-Leu-Arg-Glu-Asn-Gly-Ser-Thr was identical for each sample. The amino acid at position 13 gave no detectable signal during the respective sequence cycle, although the other cycles yielded strong "clean" signals, indicating that there was sufficient material for analysis. The N-terminal sequence was analyzed by using the National Biomedical Research Foundation Protein Database to determine any similarities with known sequences, and there were no significant matches.

Cytotoxic activity of the purified toxin. The cytotoxic response of HT-29 cells to the purified toxin developed between 15 min and 4 h after exposure to the toxin, depending on the toxin concentration. After 12 to 18 h, the cells treated with low doses of purified toxin reverted back to their normal clustered growth state and grew to confluence.

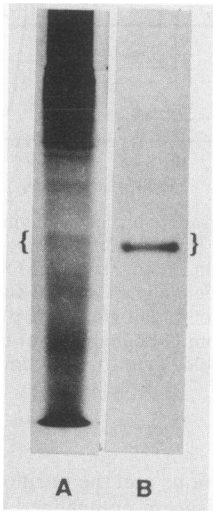

FIG. 2. Native PAGE of dialyzed ammonium sulfate precipitate (A) and purified enterotoxin (B) from culture supernatant fluid of *B. fragilis* VPI 13784. Approximately 100 μg of ammonium sulfate precipitate and 2.5 μg of purified toxin were analyzed on a 12.5% resolving gel with a 4% stacking gel. Bands were detected by silver staining. Brackets indicate the region from which cytotoxic activity was eluted from the gel.

This reversion also was observed regularly with cells treated with the crude culture supernatant fluids. At concentrations of purified toxin greater then 0.5 μg/ml, the cells remain rounded. However, at both high and low concentrations, the cells remained viable.

When toxin-treated cells were allowed to revert to normal and were incubated a second time with crude or purified toxin, they responded as normal cells, i.e., rounded within 0.25 to 2 h and reverted after 12 to 18 h at the lower doses of toxin. Again, all the cells remained viable. This process could be repeated until the cells reached confluence. When cells were incubated with toxin and the cell culture medium was assayed for residual cytotoxic activity, a rapid decrease in activity was observed. By 18 h, no residual activity was detected in the culture wells even at the higher doses. In a control experiment, when crude or purified toxin was incubated in McCoy's medium without cells, more than 99.0% of the cytotoxic activity was lost over 18 h.

No significant cytotoxic effects were observed with the other 14 mammalian cell lines when the purified toxin or the culture filtrates were used. At the highest doses of purified toxin (0.5 μg/ml), the four colonic cell lines (CCD-33CO, T84, LS174T, and Caco-2) appeared to exhibit mild swelling, but no significant rounding was observed. To determine if the HT-29 cells secreted into their culture any factor which might facilitate or promote a cytotoxic response, we removed the normal culture medium from each of the other cell lines and replaced it with conditioned medium from confluent HT-29 cultures prior to exposing the cell lines to toxin. As before, the cell lines did not exhibit any distinct cytotoxic response.

Enterotoxigenicity of the purified toxin and bacterial strains in ligated loops. Preparations at each stage of the toxin purification, including the purified toxin, caused strong nonhemorrhagic fluid accumulation responses in the ligated lamb ileal loops. However, the unconcentrated culture filtrate cause only a very weak response. All fresh cultures of the five most cytotoxic strains also caused strong fluid accumulation responses. The results for the lamb loop assay are summarized in Table 4. In the rabbit ligated-ileal-loop assay, we observed only a very weak fluid accumulation response with the purified toxin (volume-to-loop ratio = 0.43).

TABLE 3. Summary of properties of purified enterotoxin of *B. fragilis*

Property	Observation
Mol wt	
Gel filtration	18,000 to 22,000
SDS-PAGE	19,000
Isoelectric point	4.4–4.6
Temperature for storage	
65°C	>99% reduction
55°C	>90% reduction
20°C	No effect
4°C	No effect
4°C (freeze-dried)	No effect
−20°C	No effect
pH stability	Between pH 5 and 10
Enzyme susceptibility	
Trypsin, chymotrypsin, Dispase	No effect
Proteinase K, *Streptomyces* protease	>99% reduction
Bacterial amylase, bacterial lipase	No effect

TABLE 4. Fluid accumulation response to *B. fragilis* cultures and enterotoxin in ligated lamb ileal loops

Sample	CU[a]	Loop response[b]	Protein[c] (μg)
Bacterial cultures[d]			
VPI 2633	320	1.14	
VPI 13953	320	1.31	
VPI 14316	160	1.18	
VPI 14315	80	0.76	
VPI 13784	640	1.37	
Toxin preparations[e]			
Culture supernatant	320	0.50	160
Ammonium sulfate	10,000	1.04	728
DEAE Sepharose	20,000	1.23	250
Phenyl-agarose	20,000	1.15	42
Mono Q	20,000	1.11	5

[a] Number of CU in the sample at the time of injection.
[b] Loop volume/loop length.
[c] Total amount of protein injected into loop.
[d] One milliliter of BHI culture containing approximately 10^9 CFU.
[e] Preparations from a 1-liter culture of VPI 13784.

DISCUSSION

The role of *B. fragilis* as a major clinical pathogen has been realized for many years. Recently, however, the results from several studies suggested that this organism may also play a role in diarrheal disease. This observation is based on findings showing that certain isolates of *B. fragilis* cause an enterotoxic response in animals and that the activity is due to the production of a toxic factor. Up to now, studies on this factor have been done with crude or only partially purified material, thus hindering the characterization of the factor. It also was unclear if the enterotoxic factor that caused the positive loop response could also cause the cytotoxic effect on the colonic carcinoma cell line HT-29. In this report, we described a purification procedure that yielded highly purified toxin which is homogeneous by several criteria. The toxin purified by this procedure was cytotoxic for HT-29 cells and elicited a positive fluid response in the lamb ligated-loop assay. Thus, both effects can be caused by this same purified toxin. Since we did not screen all the column fractions in the lamb loop assay, we cannot rule out the possibility that there may be another enterotoxic factor in the culture supernatants. However, the toxin we purified was the only other cytotoxic factor observed during the purifications in which all fractions at all stages of purification were screened for cytotoxic activity.

The toxin is a relatively small protein with a molecular weight of 19,000 with no subunits as determined by SDS-PAGE. This is similar to the molecular weight of 19,500 estimated by Myers et al. (21). Our results for the effects of temperature and pH on the highly purified toxin also correlate well with those reported by Myers et al. for crude culture filtrates and show that the toxin is stable at cold temperatures but is inactivated at elevated temperatures of more than 55°C. Judging by its pI, the toxin is an acidic protein. Interestingly, the highly purified toxin is resistant to trypsin and chymotrypsin. However, the toxin appears to be degraded in culture supernatant fluids unless it is protected by the protease inhibitors TLCK or TPCK, which inactivate trypsin and chymotrypsin, respectively. These results, along with the finding that late-stationary-phase cultures contain only very low levels of toxin, suggest that the toxin is degraded by proteases in the crude material. *Bacteroides* species produce various levels of proteases, including some that are highly specific.

The results from our studies to determine the optimal in vitro conditions for production of the toxin showed that glucose was inhibitory. This may indicate that some type of catabolite repression is occurring when the bacteria grow fermentatively. On the other hand, the glucose may be inducing the production of different proteases which are inactivating the toxin before it can accumulate in the supernatant. Before any conclusions on the true effects of glucose can be made, the physiology and biochemical reactions involved must be studied in much greater detail.

Our results with cultures and culture filtrates in the lamb ileal-loop assay (Table 4) are consistent with those of the L. L. Myers group (21). Whereas pure cultures of enterotoxigenic *B. fragilis* elicit a strong fluid accumulation response, presumably because of the continued growth and toxin production within the loop, the crude culture filtrates usually do not. The toxin contents of unconcentrated crude culture filtrates are too low to elicit a good loop response. In addition, the purified toxin did not induce fluid accumulation in the rabbit loops, which is consistent with the fact that rabbit loops have not proven useful for studying the cytotoxic activity of culture filtrates of enterotoxigenic *B. fragilis*.

The only tissue cell line reported to be sensitive to the toxin is the HT-29 cell line. Of the 14 cell lines we examined, none exhibited the dramatic cytotoxic response to the culture filtrates or purified toxin of the HT-29 cells. The high degree of specificity toward the HT-29 cells suggests a highly specific binding mechanism, possibly a very specific receptor. The availability of highly purified toxin should now allow studies on receptors and mechanism of action to be initiated.

Despite the fact that the antiserum against strain VPI 2633 was made for a previous study by using a crude-lysate vaccine which was not optimized for toxin content, the antiserum was capable of neutralizing the cytotoxic activities of all culture filtrates and toxin preparations tested. Thus, it appears that the different enterotoxigenic strains are producing at least one antigenically related toxin, although enterotoxigenic and nonenterotoxigenic *B. fragilis* strains have been shown to be an antigenically diverse group (16). We are currently producing antisera against partially and highly purified toxin. Once these antisera are available, we hope that they will be useful in immunoassays for confirming the presence of toxin in clinical specimens.

Of the 40 other isolates of *B. fragilis* from the Virginia Polytechnic Institute and State University anaerobe laboratory collection that we examined, 3 were positive for toxin production. Although the number of pure culture strains tested was relatively small, this toxigenicity rate is similar to what others have reported for *B. fragilis* isolates (23). We are now developing DNA probes based on our N-terminal sequencing results, and these probes should be useful for (i) screening *B. fragilis* isolates and other *Bacteroides* species for the toxin gene and (ii) locating and sequencing toxin gene fragments.

In conclusion, our results confirm the results of studies by other investigators and demonstrate that a relatively high percentage of strains of *B. fragilis* and not the other related *Bacteroides* spp. produce a toxin that exhibits both enterotoxic and cytotoxic activities. These observations raise new concerns about the role of *B. fragilis* as an enteropathogen. Diarrhea kills more than 10 million people a year, yet the cause of the diarrhea remains unknown in many cases. How

many of these cases may be associated with the *B. fragilis* enterotoxin remains unknown. These observations also raise additional possibilities concerning the invasiveness and tissue-damaging ability of *B. fragilis*. The organism produces a capsule that probably increases its virulence, but this does not satisfactorily explain the accompanying tissue damage. The production of a toxin with cytotoxic activity could be one of the unknown factors which enables the organism to cause the cellular destruction associated with soft-tissue infections. Now that purified material is available, we can begin to develop the tools to study the *B. fragilis* enterotoxin in stools and other clinical specimens and to determine if the toxin plays a role in the etiology of diarrheal disease, anaerobic infections, or both.

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