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Cloning and Characterization of the *Bacteroides fragilis* Metalloprotease Toxin Gene

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Strains of *Bacteroides fragilis* that produce a ca. 20-kDa heat-labile protein toxin (termed *B. fragilis* toxin [BFT]) have been associated with diarrheal disease of animals and humans. BFT alters the morphology of intestinal epithelial cells both in vitro and in vivo and stimulates secretion in ligated intestinal segments of rats, rabbits, and lambs. Previous genetic and biochemical data indicated that BFT was a metalloprotease which hydrolyzed G (monomeric) actin, gelatin, and azocoll in vitro. In this paper, the cloning and sequencing of the entire *B. fragilis* toxin gene (*bft*) from enterotoxigenic *B. fragilis* (ETBF) 86-5443-2-2 is reported. The *bft* gene from this ETBF strain consists of one open reading frame of 1,191 nucleotides encoding a predicted 397-residue holotoxin with a calculated molecular weight of 44,493. Comparison of the predicted BFT protein sequence with the N-terminal amino acid sequence of purified BFT indicates that BFT is most probably synthesized by ETBF strains as a preproprotein. These data predict that BFT is processed to yield a biologically active toxin of 186 residues with a molecular mass of 20.7 kDa which is secreted into the culture supernatant. Analysis of the holotoxin sequence predicts a 20-residue amphipathic region at the carboxy terminus of BFT. Thus, in addition to the metalloprotease activity of BFT, the prediction of an amphipathic domain suggests that oligomerization of BFT may permit membrane insertion of the toxin with creation of a transmembrane pore. Comparison of the sequences available for the *bft* genes from ETBF 86-5443-2-2 and VPI 13784 revealed two regions of reduced homology. Hybridization of oligonucleotide probes specific for each *bft* to toxigenic *B. fragilis* strains revealed that 51 and 49% of toxigenic strains contained the 86-5433-2-2 and VPI 13784 *bft* genes, respectively. No toxigenic strain hybridized with both probes. We propose that these two subtypes of *bft* be termed *bft-1* (VPI 13784) and *bft-2* (86-5433-2-2).

In 1984, strains of *Bacteroides fragilis* associated with diarrheal disease in lambs were found to stimulate a secretory response in lamb ligated intestinal segments (19). Additional field and experimental work revealed that these diarrheagenic strains of *B. fragilis* (termed enterotoxigenic *B. fragilis* [ETBF]) could also cause intestinal disease, predominantly colonic, in calves, piglets, foals, and rabbits (reviewed in reference 34). Investigation of the role of ETBF strains in human diarrheal disease revealed an association of ETBF with diarrheal disease in children between the ages of 1 and 5 years (27, 28, 32). However, studies of the role of ETBF in adult diarrheal disease are not yet available. The secretory response to ETBF was attributed to the expression by these strains of a ca. 20-kDa protein which, to date, has exhibited biologic activity only on epithelial cell lines in vitro (1, 21, 23, 34, 41). Purification and determination of the N-terminal amino acid sequence of this protein (termed the *B. fragilis* toxin [BFT]) from strain VPI 13784 (originally isolated from a lamb) (37) enabled the use of a single specific primer-PCR to generate a portion of the *bft* (*B. fragilis* toxin) gene (17). Sequence analysis of this PCR product revealed a zinc-binding consensus motif (HEXXHXXGXXH) characteristic of metalloproteases termed metzincins. In addition, purified BFT hydrolyzed G (monomeric) actin, gelatin, and azocoll in vitro; these results are consistent with its predicted enzymatic activity (17).

In our laboratory, cell-free culture supernatants of another toxigenic strain of *B. fragilis* (strain 86-5443-2-2) originally isolated from a piglet with diarrheal disease routinely contain higher levels of biologic activity when assayed on HT29/C1 cells than do cell-free culture supernatants of strain VPI 13784 (18, 43). Potential explanations for this difference in activity include accessory virulence factors, varying regulation of BFT production or secretion, *bft* gene copy number, and/or variation in the structure of the protein toxin. Thus, to begin to analyze the biologic differences identified between these two strains, the purpose of the current work was to clone and sequence the complete *bft* gene from *B. fragilis.*

MATERIALS AND METHODS

Bacterial strains, media, and vectors. ETBF 86-5443-2-2 (piglet isolate) was used for cloning the toxin gene (*bft*). ETBF VPI 13784 (lamb isolate) and the nontoxigenic *B. fragilis* 077225-2 (human isolate) were used in some experiments. The additional human *B. fragilis* strains used in these studies had been previously characterized as toxigenic or nontoxigenic by the lamb ligated ileal loop (LLIL) assay (19, 28) and/or the HT29/C1 cell assay (18, 41). *B. fragilis* was propagated anaerobically on BHC medium (3.7 g of brain heart infusion BHI base [Difco Laboratories, Detroit, Mich.] per liter, 0.5 g of yeast extract [Difco Laboratories] per liter, 0.1 mg of vitamin K per liter, 0.5 mg of hemin per liter, 50 mg of L-cysteine per liter [all from Sigma, St. Louis, Mo.]), and *Escherichia coli* strains were grown on Luria agar (L agar) or in L broth. Selection for antibiotic resistance was performed on L agar plates supplemented with ampicillin (200 μg/ml). Bacterial strains were stored at −70°C in L broth supplemented with 30% glycerol or 10% dimethyl sulfoxide. *E. coli* DH5-α (GIBCO/BRL Inc., Gaithersburg, Md.) was used as the recipient for all transformations. The cosmid vector pHC79 was used to construct the *B. fragilis* genomic library, and DNA

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fragments were cloned into the vector pBluescriptII SK+ (pBSII; Stratagene Inc., La Jolla, Calif.).

Purification of BFT. BFT was purified from culture supernatant of ETBF 86-5443-2-2 as previously described (37). Purified BFT was stored at −70°C in 0.05 M Tris–0.2 M NaCl buffer (pH 7.5).

Determination of the N-terminal amino acid sequence of BFT. In preparation for N-terminal amino acid sequencing, purified BFT was precipitated in ice-cold 100% trichloroacetic acid; after removal of the trichloroacetic acid supernatant, BFT was washed with acetone at −20°C overnight and the pellet was dried. The N-terminal amino acid sequence of BFT from ETBF 86-5443-2-2 was determined by automated Edman chemistry with an Applied Biosystems Instruments protein sequencer (model 477) at the Beckman Center of Stanford University (Stanford, Calif.).

PCR. Degenerate primers designed from the N-terminal amino acid sequence of BFT (forward primer, 5′-GARCCNAARACNGTNTAYGTNATH-3′; 6,144-fold degeneracy) and the zinc-binding consensus motif (HEXXH) characteristic of metalloproteases (reverse primer, 5′-ATCTTCWGCWCCAAGWATGTG WCCWARTTC-3′; 64-fold degeneracy) were used to generate a fragment of the *bft* gene by PCR. PCRs with *Taq* polymerase (2.5 U) were performed with 50 μl containing chromosomal DNA template (100 ng), primers (100 ng), deoxynucleoside triphosphates (200 μM), and $MgCl_2$ (2 mM). The amplification cycle was denaturation at 94°C for 2 min, annealing at 51°C for 2 min, and extension at 72°C for 1 min. The amplification cycle was repeated 29 times and followed by final extension at 72°C for 5 min. Amplified fragments were cloned with the TA cloning vector as described by the manufacturer (Invitrogen, San Diego, Calif.).

PCR was also used to obtain the DNA fragment encoding the protoxin sequence of *bft* from strain VPI 13784. Exact primers derived from the sequence of the 86-5443-2-2 *bft* were used in this reaction (forward primer, 5′-TGTGGAA TCACATCGTGCATCA-3′ corresponding to bp 1666 to 1687 in Fig. 2; reverse primer, 5′-AGTTCGCCGCATCCTGCATCTG-3′ corresponding to the inverse complement of bp 2555 to 2576 in Fig. 2). PCRs were performed with *Taq* polymerase (1.25 U) in 50 μl containing chromosomal DNA (50 ng), primers (80 pmol), deoxynucleoside triphosphates (200 μM), and $MgCl_2$ (1.5 mM). The amplification cycle was denaturation at 94°C for 1 min, annealing at 66°C for 2 min, and extension at 72°C for 1 min. The amplification cycle was repeated 29 times and was followed by a final extension at 72°C for 5 min. The amplified 900-bp product was directly sequenced as described below.

General cloning techniques. Plasmid DNA was extracted by the alkali lysis method (31) or with Qiagen columns (Stratagene Inc., La Jolla, Calif.). Purification of DNA fragments and extraction from gel slices were performed with Geneclean (Bio 101, La Jolla, Calif.). Plasmid DNA was introduced into *E. coli* DH5-α by transformation of competent cells obtained from GIBCO/BRL, as specified by the manufacturer. Restriction enzymes and T4 DNA ligase (GIBCO/BRL) were used as specified by the manufacturer.

Southern hybridization analysis was performed with nitrocellulose filters under high-stringency conditions. Fragment probes were labeled with [α-^{32}P]dATP by random priming (random-prime DNA-labeling kit; Boehringer GmbH, Mannheim, Germany), hybridized in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS)–1 mM EDTA–1× Denhardt's solution at 37°C, and washed with 5× SSC–0.1% SDS. Synthetic oligonucleotide probes were end labeled with [γ-^{32}P]dATP by using T4 polynucleotide kinase (31).

Colony blots were prepared by the technique described by Wright et al. (42) with slight modifications. Briefly, *E. coli* colonies grown overnight on L agar supplemented with ampicillin (200 μg/ml) were overlaid with Whatman 541 filters for 30 min to transfer the colonies to the filters. The filters were processed by microwaving in alkali lysis solution (0.5 M NaOH, 1.5 M NaCl) followed by neutralization in 2 M ammonium acetate. The filters were hybridized in 6× SSC–5× Denhardt's solution–1 mM EDTA (pH 8) with the oligonucleotide probe for 1 h at 56°C and washed twice in 1× SSC–1% SDS at 56°C for 10 min.

For *B. fragilis* colony blots, organisms were grown on BHC prior to transfer to nitrocellulose filters. The filters were processed by being soaked in 0.5 M NaOH for 10 min and then subjected to sequential washes in 1 M Tris (pH 7.4) (three times for 1 min each) and then 1 M Tris (pH 7.4)–1.5 M NaC1 for 10 min. The filters were hybridized with either a fragment probe encoding the entire *bft* gene or specific oligonucleotide probes (see Results) and washed as described above.

Construction and screening of the cosmid library. Chromosomal DNA from *B. fragilis* 86-5443-2-2 was partially digested with *Sau*3AI under conditions where the majority of fragments were 30 to 45 kb in size and ligated into cosmid vector pHC79 digested with *Bam*HI. Ligated DNA was packaged into λ phage by using the Giga Pack II packing extract (Stratagene) and transduced into *E. coli* DH5-α. The cosmid library was screened with an oligonucleotide probe derived from internal *bft* sequences determined from the PCR product sequence (5′-CAGA TGCAGGATGCGGCGAACTC-3′).

Sequence analysis. Plasmid DNA and PCR-amplified products were sequenced by the fluorescent dideoxy terminator method of cycle sequencing (13) on a PE/ABd 373a automated DNA sequencer at the DNA Analysis Facility of Johns Hopkins University. Sequences were compiled with Sequencher software (Gene Codes Corp., Ann Arbor, Mich.). DNA and amino acid sequences were analyzed with programs developed by the Genetic Computer Group at the University of Wisconsin (2).

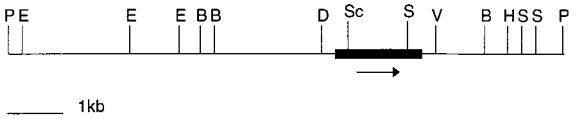

FIG. 1. Restriction map showing the position of *bft* gene (shaded box) and relevant restriction sites. The arrow indicates the direction of transcription. P, *Pst*I; E, *Eco*RI; B, *Bgl*II; S, *Sma*I; V, *Eco*RV; Sc, *Sac*II; D, *Dra*III.

RESULTS

Identification of the *bft* gene by PCR. N-terminal amino acid sequencing of BFT purified from ETBF 86-5443-2-2 revealed a sequence of Ala-Val-Pro-Ser-Glu-Pro-Xxx-Thr-Val-Tyr-Val-(Tyr)-Ile-Cys-Leu-Arg. This sequence is identical to the N-terminal amino acid sequence of BFT purified from VPI 13784 except in position 7 (Xxx [unidentified] and Lys in ETBF 86-5443-2-2 and VPI 13784, respectively). In addition, Cys was identified at position 13 of BFT from ETBF 86-5443-2-2 but was unidentified (Xxx) in the N-terminal amino acid sequence previously published for VPI 13784 (37). Initial attempts to identify the *bft* gene from a genomic library by using either a degenerate oligonucleotide probe or an inverse PCR strategy based on the N-terminal sequence of BFT were unsuccessful. A strategy was then designed to amplify part of the *bft* gene by using direct PCR and two degenerate oligonucleotide primers, one based on the N-terminal sequence and the other based on a zinc-binding consensus sequence initially reported to be present in BFT (10). A PCR in which ETBF 86-5443-2-2 DNA was used as the template yielded multiple products when analyzed by ethidium bromide-stained gel electrophoresis (data not shown). In contrast, no bands were identified when DNA from nontoxigenic *B. fragilis* 077225-2 was used as the template. The entire amplified PCR mixture was ligated into the TA cloning vector and transformed into *E. coli* DH5-α. Plasmid analysis of the resulting colonies yielded five distinct classes of clones with inserts varying in size from 400 to 900 bp, which were then sequenced. The DNA sequence of a 437-bp insert shared extensive homology with a partial *bft* gene sequence that had previously been published for VPI 13784 (17).

Cloning of the *bft* gene. A genomic *B. fragilis* cosmid library in *E. coli* DH5-α was screened with an oligonucleotide probe designed from the partial *bft* gene sequence obtained from the 437-bp PCR product. Of 2,000 colonies screened, 6 cosmid clones reacted with the probe. Southern blot analysis of restriction digests of the cosmid clones revealed that five of the clones contained a 7.8-kb *Hin*dIII fragment that reacted with the probe, and a homologous *Hin*dIII fragment of the identical size was seen in Southern blots of *B. fragilis* 86-5443-2-2 chromosomal DNA digested with this enzyme. One of the cosmids containing the 7.8-kb *Hin*dIII fragment was further mapped, and two subclones, B3 and B5, containing a 4.8-kb *Bgl*II fragment in opposite orientations, were constructed with pBSII (Fig. 1). The nucleotide sequence of the fragment revealed one large ORF of 1215 bp with two in-frame ATG start codons within 24 bp (Fig. 2). No obvious ribosomal binding site that might indicate which start codon is actually used was identified upstream of either potential start codon. When the predicted protein sequence was examined, the use of the second in-frame ATG as the initiation codon yielded a protein with a perfect consensus signal peptide whereas the use of the first ATG did not yield a product with an apparent signal peptide. Since the toxin is clearly secreted from the cytoplasm of ETBF strains (18, 21, 37, 41), we chose the second ATG as the most likely start codon for the *bft* gene. The use of this codon predicts a protein product of 397 residues (1,191 bp) with a predicted

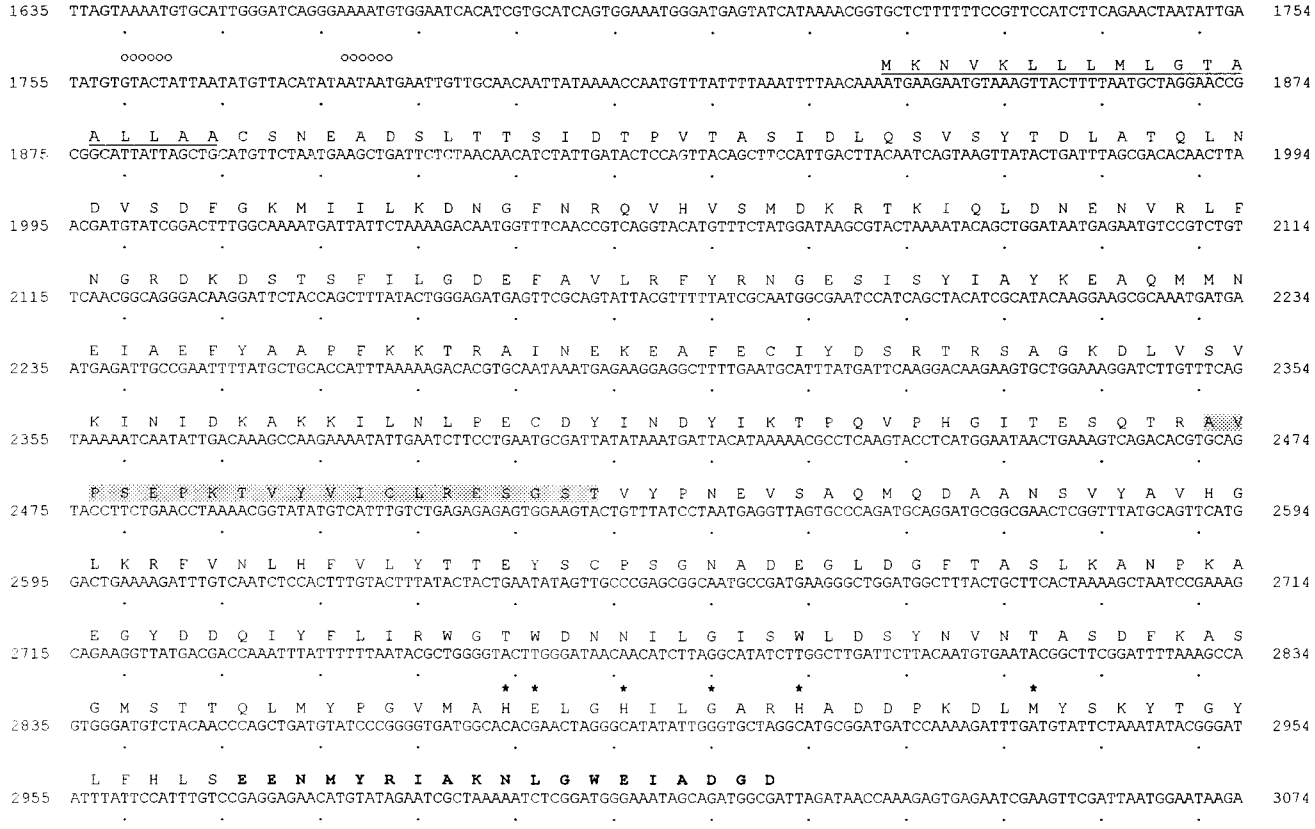

FIG. 2. Nucleotide sequence and predicted protein product of the *bft* gene from *B. fragilis* 86-5443-2-2. A potential signal peptide sequence is underlined, and the amino-terminal sequence of the purified protein is enclosed in the shaded box. The carboxy-terminal 20 residues which are predicted to form an amphipathic region are shown in boldface type. Residues forming the zinc-binding signature motif are marked with *. Potential −35 and −10 promoter sequences are indicated by open circles.

molecular weight of 44,493. Consistent with the sequence of previously reported *B. fragilis* genes, the G+C content of *bft* was 39%.

Comparison of the amino-terminal sequence determined from the purified BFT protein with the protein sequence predicted from the nucleotide sequence revealed a perfect match between the two analyses. However, the first codon of the amino-terminal sequence of the purified BFT protein is residue 212 of the 397-residue protein predicted from *bft*. Thus, BFT is apparently produced by ETBF strains as a preproprotein. The final processed product would be a 186-residue protein with a predicted molecular weight of 20,741 and pI of 4.75. Interestingly, the carboxy-terminal 20 amino acids of the processed BFT protein form a predicted amphipathic structure (Fig. 3).

Identification of *bft* gene diversity. The predicted amino acid sequences of the 86-5443-2-2 *bft* gene were aligned with the predicted amino acid sequences derived from the PCR-generated protoxin region (this study) and the reported partial VPI 13784 *bft* nucleotide sequence (17) (Fig. 4). Overall, the partial sequence from VPI 13784 and the corresponding region from 86-5443-2-2 share 92% identity and 95.4% similarity in protein sequence and 94.6% identity in nucleotide sequence. However, the distribution of the predicted amino acid differences is not random, with only 2 conserved amino acid differences in the 211-amino-acid preprotoxin region and 29 amino acid differences predicted in the mature toxin protein sequence. The relative paucity of amino acid substitutions in the preprotoxin region suggests that this region is comparatively intolerant of even conservative amino acid changes. Comparison of the preprotoxin region (i.e., residues 1 through 211) with previously reported sequences revealed no compelling homologies.

The reduced homology between the predicted 86-5433-2-2 and VPI 13784 BFT sequences is clustered in two regions of the mature protein (region 1, residues 355 to 362; region 2, residues 368 to 384 [Fig. 4]). To further assess this diversity, colony blot hybridizations were performed with a fragment *bft* probe and specific 22-bp oligonucleotide probes to region 1 of each *bft* (Fig. 5). These data revealed that the fragment probe identified both toxigenic strains (but not a nontoxigenic *B. fragilis* strain) whereas each specific oligonucleotide probe hybridized only with its parent strain.

To determine if these probes were sensitive and specific for identification of ETBF strains, 139 human *B. fragilis* strains isolated from intestinal or extraintestinal sites and previously characterized as toxigenic or nontoxigenic by either the LLIL assay (19, 28) and/or the HT29/C1 cell assay (18, 41) were examined (Table 1). These data revealed that the *bft* fragment probe was 93 and 100% sensitive and specific, respectively, in identifying ETBF strains when compared to the LLIL assay and 95% and 92% sensitive and specific, respectively, when compared to the HT29/C1 cell assay. Furthermore, of the toxigenic strains identified by all three techniques (LLIL, the HT29/C1 cell assay, and the *bft* fragment probe), 51.4% hybridized to the 86-5443-2-2 *bft* oligonucleotide probe and 48.6% hybridized to the VPI 13784 *bft* oligonucleotide probe. No strain hybridized to both oligonucleotide probes.

The biologic activity detectable in cell-free culture superna-

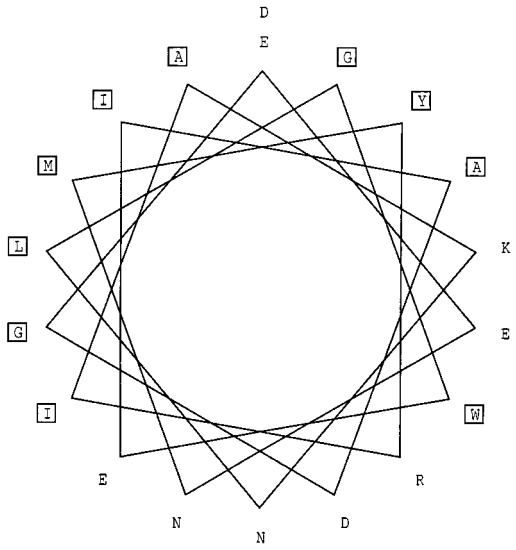

FIG. 3. Potential amphipathic region of BFT. The C-terminal end of the BFT was arranged in the form of a wheel by using the program HELICALWHEEL. Hydrophobic residues are boxed. The glutamic acid (E) residue at the top of the wheel is residue 379 of the predicted amino acid sequence of BFT.

tants of ETBF strains is known to vary (18, 21, 39, 41). To assess if expression of a particular *bft* subtype correlated with the biologic activity detectable in cell-free culture supernatants, *B. fragilis* strains expressing either the VPI 13784 or 86-5443-2-2 *bft* were assessed for toxigenicity by the HT29/C1 cell assay (Table 2). These data revealed that the *bft* subtype did not correlate with the level of detectable HT29/C1 biologic activity in culture supernatants of this selected group of strains.

Determination of *bft* gene copy number. To determine whether the difference in biologic activity detected between ETBF 86-5443-2-2 and VPI 13784 was due to a difference in

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86-5443-2-2  MKNVKLLLMLGTAALLAACSNEADSLTTSIDTPVTASIDLQSVSYTDLAT 50
VPI 13784    MKNVKLLLMLGTAALLAACSNEADSLTTSIDAPVTASIDLQSVSYTDLAT
             QLNDVSDFGKMIILKDNGFNRQVHVSMDKRTKIQLDNENVRLFNGRDKDS 100
             QLNDVSDFGKMIILKDNGFNRQVHVSMDKRTKIQLDNENVRLFNGRDKDS
             TSFILGDEFAVLRFYRNGESISYIAYKEAQMMNEIAEFYAAPFKKTRAIN 150
             TSFILGDEFAVLRFYRNGESISYIAYKEAQMMNEIAEFYAAPFKKTRAIN
             EKEAFECIYDSRTRSAGKDLVSVKINIDKAKKILNLPECDYINDYIKTPQ 200
             EKEAFECIYDSRTRSAGKDIVSVKINIDKAKKILNLPECDYINDYIKTPQ
             VPHGITESQTRAVPSEPKTVYVICLRESGSTVYPNEVSAQMQDAANSVYA 250
             VPHGITESQTRAVPSEPKTVYVICLRENGSTIYPNEVSAQMQDAANSVYA
             VHGLKRFVNLHFVLYTTEYSCPSANADEGLDAFTASLKANPKAEGYADQI 300
             VHGLKRYVNFHFVLYTTEYSCPSGDAKEGLEGFTASLKSNPKAEGYDDQI
             YFLIRWGTWDNNILGISWLDSYNVNTASDFKASGMSTTQLMYPGVMAHEL 350
             YFLIRCGTWDNKILGMSWFNSYNVNTASDFEASGMSTTQLMYPGVMAHEL
             GHILGARHADDPKDLMYSKYTGYLFHLSEENMYRIAKNLGWEIADGD 397
             GHILGAEHTDNSKDLMYATFTGYLSHLSEKNMDIIAKNLGWEAA???
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FIG. 4. Comparison of the predicted protein sequence of BFT from strain 86-5443-2-2 with the partial sequence from strain VPI 13784 (underlined) reported by Moncrief et al. (17) and the protoxin sequence of strain VPI 13784 generated by PCR in this report. The mature BFT protein sequence is indicated in bold. Sequences were aligned by using the program BESTFIT.

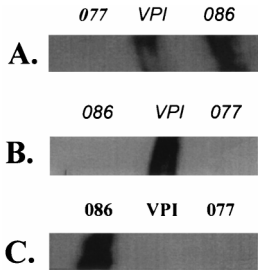

FIG. 5. Colony hybridization of *B. fragilis* strains. Toxigenic *B. fragilis* VPI 13784 (VPI) and 86-5443-2-2 (086) and nontoxigenic strain 077225-2 (077) were hybridized as described in Materials and Methods with either a *bft* fragment probe or oligonucleotides derived from region 1 (see Results) of the *bft* sequences of 86-5443-2-2 or VPI 13784. The sequences of the 86-5443-2-2 and VPI 13784 *bft* oligonucleotide probes are 5′-GGTGCTAGGCATGCGGATGATC-3′ and 5′-GGCGCTGAGCATACGGATAATT-3′, respectively. (A) Hybridization with *bft* fragment probe. (B) Hybridization with VPI 13784 oligonucleotide probe. (C) Hybridization with 86-5443-2-2 oligonucleotide probe.

the copy number of the *bft* gene, Southern analysis with the cloned *bft* gene (a 1.2-kb *Dra*III-*Sma*I fragment) was performed. A single copy of the *bft* gene was contained on a variety of restriction fragments in both strains, including 7-kb *Sma*I, 7.8-kb *Pst*I, and 3.8-kb *Bgl*II fragments (data not shown).

DISCUSSION

Although *B. fragilis* is a major cause of abdominal abscesses as well as the most common anaerobe recovered in bloodstream infections (4, 25), data implicating these organisms as causes of diarrheal disease are more recent. To date, ETBF is linked only to childhood diarrhea, with a notable absence of disease association in children less than 1 year of age, a result reminiscent of *Clostridium difficile* disease (27, 28, 32, 40). Although bloody diarrhea has been observed in experimental ETBF infections in rabbits (20, 22) and purified BFT stimulates hemorrhagic fluid accumulation in ligated intestinal segments in rats, rabbits, and lambs (24), illnesses in children have been nondescript without gross evidence of inflammatory diarrhea. However, more sensitive tests of intestinal damage and inflammation such as stool guiaics and lactoferrin (16) have yet to be reported. Although the pathogenetic mechanisms resulting in diarrhea in this infection are unknown, the ability of these strains to produce a 20-kDa heat-labile protein toxin identified to have protease activity has been proposed to be a critical virulence factor in disease.

TABLE 1. Identification of *bft* in human intestinal and extraintestinal strains of *B. fragilis*

Type and no. of strains	Biologic assays		No. of strains positive with indicated *bft* probe:		
	LLIL	HT29/C1 Cells	Fragment	VPI	86
Intestinal					
35	+	+	35	17	18
7	+	−	6	5	1
2	+	+	0	0	0
30	−	−	0	0	0
Extraintestinal					
4	ND[a]	+	4	4	0
59	ND	−	0	0	0
2	ND	−	2	0	2

[a] ND, not determined.

TABLE 2. HT29/C1 cellular activity of cell-free culture supernatants of ETBF strains

HT29/C1 cell titer	No. of strains hybridizing with indicated *bft* probe[a]:	
	VPI 13784	86-5443-2-2
1/16	1	1
1/64	1	1
1/128	3	2
1/256	3	3
1/1,024	1	2

[a] Nine VPI 13784 and nine (86-5443-2-2) probe-positive strains were tested.

A molecular genetic approach to assess the role of BFT in disease pathogenesis involves cloning the gene encoding BFT and ultimately constructing an isogenic mutant. In this study, we have cloned and sequenced the *B. fragilis* metalloprotease toxin gene from ETBF 86-5443-2-2. Despite isopropyl-β-D-thiogalactopyranoside (IPTG) induction and/or a >50-fold concentration of the culture supernatants, expression of the cloned *bft* gene in *E. coli* with the pBSII vector resulted in only low levels of transiently detectable toxin activity on HT29/C1 cells (data not shown). No consensus promoter or ribosomal binding-site information is available for *B. fragilis*; however, comparison with consensus *E. coli* sequences reveals a potential promoter sequence (Fig. 2) but no obvious ribosomal binding site. A weak ribosomal binding site offers one potential explanation why only low levels of biologically active BFT were detected in culture supernatants of *E. coli* clones containing the *bft* gene. Alternatively, processing and secretion of BFT may be inefficient in *E. coli*, BFT may be susceptible to other *E. coli* proteases, or full biologic activity in supernatants may be dependent on accessory virulence factors produced by ETBF strains.

The predicted protein sequence of BFT suggests that it is synthesized as a preproprotein. The amino-terminal sequence of the purified 20-kDa BFT has been determined for extracellular toxin purified from ETBF 86-5443-2-2 (this study) and VPI 13784 (37), and the sequences are nearly identical. However, the first amino acid residue of the extracellular toxin is residue 212 of the predicted 397-residue protein product of the *bft* gene. The initial 18 amino acids of the predicted BFT comprise a signal peptide with at least one charged amino acid in the first 5 residues (Lys-2, Lys-5) followed by a stretch of hydrophobic residues rich in Ala and Leu and devoid of charged residues and a potential cleavage site recognizable by a signal peptidase (Ala-18 to Cys-19) (26). This pattern is typical of a lipoprotein signal peptide (26), although we have no experimental evidence that BFT is a lipoprotein. As noted above, there is another in-frame ATG upstream of the ATG that we have designated the start codon, but the 8 residues added if this codon is chosen as the start codon would eliminate the predicted signal peptide. Our data predict that the initial BFT protein of 397 residues is first processed so that an 18-residue signal peptide is cleaved and then a second processing step results in cleavage of a 211-residue "pro" region to yield a final active product of 186 residues. The similarity in molecular mass of the final protein predicted from the gene sequence (20.7 kDa) and the size determined experimentally (20 kDa) (37, 43) is consistent with this interpretation. Based on recent data identifying the proprotein region of the *Pseudomonas aeruginosa* elastase as essential to both proper protein folding for biologic activity and secretion of the biologically active protein (14), we hypothesize that this region of the BFT protein may serve similar functions, resulting in the release of biologically active BFT into the culture supernatants or intestinal lumen. Consistent with this potential role in protein folding and secretion, the protoxin nucleotide and predicted protein sequences for ETBF VPI 13784 and 86-5433-2-2 are virtually identical whereas less homology is present in the nucleotide and predicted amino acid sequences of the mature toxin protein for these two strains. However, no homology was identified between the proprotein region of the *P. aeruginosa* elastase and BFT, indicating that additional studies such as site-directed mutagenesis will be necessary to assign a function to this region of the protein.

A major finding of this report is that the *bft* genes from ETBF VPI 13784 and 86-5433-2-2 can be distinguished by hybridization to specific oligonucleotide probes derived from an area of reduced homology in the sequences of their respective *bft* genes. Furthermore, hybridization of these oligonucleotide probes to a collection of previously characterized toxigenic and nontoxigenic human *B. fragilis* strains revealed that toxigenic strains contain one of these *bft* genes but not both. A small number of strains have previously been shown to be active in the LLIL and/or HT29/C1 cell assay but do not hybridize to either the *bft* fragment or oligonucleotide probes, suggesting that additional *B. fragilis* secretory toxins are yet to be identified. Based on these data and additional data from our laboratory indicating that these ETBF strains secrete BFTs which are also distinct by biochemical data (43), we have designated these alleles *bft-1* (VPI 13784) and *bft-2* (86-5443-2-2), which recognizes the order in which sequences for these two toxin genes were identified.

These data and a prior report by Moncrief et al. (17) indicate that the protein encoded by the *bft* gene has homology to a class of enzymes termed zinc metalloproteases as well as lesser homology to numerous other eukaryotic proteases (e.g., neutrophil, macrophage, and fibroblast proteases), particularly collagenases. The greatest homology has been found to a class of metalloproteases associated with snake venom, termed metzincins, although the pathogenetic importance of this homology is unclear. Of interest, however, this family of enzymes termed zinc metalloproteases includes both botulinum toxin and tetanus toxin (5). Recent data indicate that these toxins exhibit exquisite substrate selectivity with the ability to hydrolyze only a single specific protein within the target cell (6, 33). In contrast, BFT has been shown to hydrolyze in vitro one of the most abundant and ubiquitous cellular proteins, monomeric or G actin (17), and also has been shown to alter the distribution of F (or filamentous) actin in both unpolarized and polarized intestinal epithelial cells, consistent with the ability of this toxin to alter cell shape (1, 3, 11, 34). However, initial data obtained with inhibitors of pH-dependent receptor-mediated endocytosis or vesicular trafficking have not determined that BFT enters the cells by these mechanisms (3, 30); similarly, the predicted BFT sequence does not reveal sequences, such as K(R)DEL, suggesting Golgi localization of the toxin (12). In addition, recent data indicate that HT29/C1 cellular actin is not hydrolyzed after BFT treatment (29). Thus, the data suggest that the proteolytic activity of BFT on G actin in vitro is not related to its mechanism for altering F-actin arrangement in vivo.

Although hydrolysis of a nonactin cellular substrate is one plausible mechanism for the cellular activity of BFT, an alternative mechanism of action is suggested by the prediction of an amphipathic region at the carboxy terminus of the 86-5443-2-2 BFT. The amphipathic nature of this 20-residue region, in which nearly all of the hydrophobic residues are on one side of an alpha-helix, would be consistent with insertion of BFT mul-

timers in the eukaryotic membrane with the hydrophobic surfaces facing the lipid bilayer and the hydrophilic side facing inward to create an ion channel. This observation is similar to prior data on three other bacterial enteric toxins: the accessory cholera enterotoxin (Ace), the second *E. coli* heat-stable enterotoxin (STb), and the *Staphylococcus aureus* delta toxin (7, 35, 36). All three of these toxins, which stimulate intestinal secretion in one or more experimental models, have been identified to have either a predicted amphipathic region of the protein (Ace and STb) or have been shown to lead to channel formation in an artificial lipid bilayer model (delta toxin) (9, 15). However, there is, as yet, no experimental evidence indicating that pore formation in the apical membrane of the intestinal epithelial cell by an enteric bacterial toxin results in ion secretion by these cells and thus serves as a mechanism contributing to the development of diarrheal disease.

Interestingly, modeling of the carboxy terminus for the VPI 13784 BFT from the available sequences does not predict an amphipathic region for this protein. Since significant differences are detectable in the HT29/C1 cell biologic activity for crude and purified toxin preparations from these two strains (18, 43), genuine sequence differences may contribute to the different biologic activities detected. However, examination of a small number of ETBF strains in this report suggests that mechanisms other than *bft* subtype account for the variable toxigenicity of ETBF culture supernatants for HT29/C1 cells (8, 18, 38, 39, 41).

Our data also do not suggest that differences in gene copy number account for the enhanced HT29/C1 cell biologic activity of culture supernatants of ETBF 86-5443-2-2. Further studies to examine potential mechanisms accounting for the varying biological activity of culture supernatants of ETBF strains will further contribute to our understanding of the pathogenesis of this newly recognized enteropathogen.

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