J. Microbiol. Biotechnol. (2003), 13(2), 305–308

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Constructions of *bft-k* and *t-3* Deficient Mutants of *Bacteroides fragilis*: Possible Role for Metalloprotease in Pathogenesis

LEE, GUN YOUNG, GI-EUN RHIE, GYUNG TAE CHUNG, WON-KEUN SUNG, AND HEE-BOK OH*

Department of Microbiology, National Institute of Health, 5 Nokbun-Dong, Eunpyung-Gu, Seoul 122-701, Korea

Received: July 4, 2002
Accepted: September 25, 2002

Abstract We earlier reported the identification of *bft-k*, *t-3*, and a third *ORF* from an enterotoxigenic strain of *Bacteroides fragilis* 419, which was isolated from the blood of a Korean patient suffering from systemic infections. In the present study, the deleted fragments of the *t-3* and the *bft-k* genes from *B. fragilis* 419 were cloned into suicide vector pJST55 and used to create a mutant with chromosomal disruption of the *t-3* and *bft-k* genes. Structures of the selected mutants, DMP-2 and DBT-4, were found to be intermediate forms that integrated the suicide vector into the chromosome. *t-3* disrupted DMP-2 and *Bft-k* disrupted DBT-4 did not react with polyclonal antibodies against T-3 or BFT-K, and had no biological activity in HT29/C_1 cells.

Key words: *Bacteroides fragilis* 419, *bft-k*, *t-3*, knockout mutant

Bacteroides fragilis, a Gram-negative nonsporulating anaerobe, is most frequently isolated from infectious processes in humans and is a common component of the normal flora of the colon [4]. Some of these strains have been implicated as causes of watery diarrheal disease in livestock and humans, and are termed enterotoxigenic *Bacteroides fragilis* (ETBF) [15]. These strains are reported to stimulate fluid accumulation in lamb ligated ileal loops (LILL), and this secretory response has been attributed to a 20-kDa enterotoxin protein, which alters the morphology of human intestinal epithelial cells *in vitro*, especially HT29/C_1 cells [13].

Previously, we found a new isoform of BFT, termed BFT-K, which was more closely related to BFT-2 than BFT-1 [7]. The gene for BFT-K protein was cloned and sequenced from *B. fragilis* 419, which was isolated from the blood of a patient suffering from systemic infections [3]. In recent years, the virulent genes of pathogenic bacteria are often shown to be clustered within definable genetic elements termed pathogenicity islands [5]. The enterotoxin pathogenicity islet of *B. fragilis* 419 was cloned and sequenced, and found to be 6,045 bp in length and to contain 12-bp direct repeats near its end. Besides BFT-K, two ORFs have been identified in this pathogenicity islet; T-3 and a third ORF [16]. The *t-3* gene and a third ORF encode a 396-amino acid protein of a putative metalloprotease and a 59-amino acid protein, respectively.

Among the proteins encoded by the genes in the BFT pathogenicity islet, the T-3 protein appeared to be more conserved than the other gene products in the islet [10]. This suggests that T-3 protein plays a more important role in the pathogenicity of ETBF than BFT. However, little is known of how the T-3 protein contributes to ETBF pathogenesis. The present study was undertaken to analyze the functional role of *t-3* and *bft-k* using a gene knockout system [8].

Bacterial Strains, Plasmids, and Culture Condition

B. fragilis 419 and 661 were grown anaerobically on BHIS medium [3.7 g of brain heart infusion (BHI) base (Difco Laboratories, Detroit, MI, U.S.A.) per liter supplemented with 0.1 mg of vitamin K, 0.5 mg of hemin, and 50 mg of L-cysteine (all from Sigma, St. Louis, MO, U.S.A.)], and *E. coli* strains were grown on Luria-Bertani (LB) medium (Difco) at 37°C. The enterotoxigenic strain of *B. fragilis* 661, isolated from the blood of a Korean patient who suffered from systemic infections, was used as the recipient strain. *B. fragilis* 661 is inherently sensitive to clindamycin and resistant to ciprofloxacin, and therefore 100 μg of ciprofloxacin per ml was added to BHIS agar plates in mating experiments for selection purposes. *E. coli* DH5α was used as a host for plasmid maintenance and *E. coli* HB101 was used as a donor strain for transformation. RK231, a broad-host-range mobilizing IncP plasmid, was obtained from M. Malamy (Tufts University, Boston, MA,

*Corresponding author
Phone: 82-2-380-1460; Fax: 82-2-382-4891;
E-mail: hboh@nih.go.kr

Table 1. Strains & plasmids used in this study.

Strains	Relevant characteristics	Reference or source
B. fragilis		
Isolates		
419	Cln^r, Str^r, Gen^r, Cef^r, Cip^r	This study
661	Cln^r, Str^r, Gen^r, Cef^r, Cip^r	This study
Mutant		
DMP-2	*B. fragilis*, *t*-3 gene disrupted	This study
DBT-4	*B. fragilis*, *bft* gene disrupted	This study
E. coli		
HB101	*SupE*44*hsd* S20(*rB-mB-*)*rec*A13 *ara*-14 *pro*A2/*acY*1 *galk*2 *rpsL*20 *xyl*-5 *mtl*-1	Gibco/BRL
DH5α	*SupE*44Δ*laucu* 169(ϕ80*lac*ΔZM 15) *hsd* R17 *rec*A1 *end* A1 *gry*A96this-1 *rel*A1	Gibco/BRL
Plasmid		
PRK 231	RP 4 derivative Kan^r, Tc^r	
pJST55	Cln^r, Amp^r *B. fragilis* suicide vector	[17]
pTp-P419	pCR® 2.1 *bft* (1.2 kb *EcoRI*/ *Xho*I)of 419	This study
pBD1	pTp-P419 with 480 bp *SSPI*, *AccI* deletion	This study
pJSTBD1	Fragment of *Bam*HI, *Xba*I from pBDI cloned into pJST55	This study
pTMP419	pCR® 2.1 *t-3*(1.136 kb *EcoRI*/ *Xho*I) of 419	This study
pMPD	pTMP419 with 500 bp *Bgl*II, *Eco*RV deletion of *t*-3	This study
pJSTMPD	Fragment of *Bam*HI, *Xba*I from pMPD cloned into pJST55	This study

U.S.A.), and was used to provide mobilization functions in triparental filter matings. The antibiotics added for plasmid selection were ampicillin (100 μg/ml) for pTMP419 and pTp-P419, and kanamycin (50 μg/ml) for RK231. pTOPO (Invitrogen) and pJST55, obtained from C. Jeffrey Smith (East Carolina University, Greenville, NC, U.S.A.), were used to construct pJSTMPD and pJSTBD1. Plasmid RK231 was used to provide mobilization in triparental filter matings.

Construction of the *t-3* Mutant and the *bft-k* Mutant of *B. fragilis*

To construct pJSTMPD, a putative *t-3* gene fragment of 1,136 bp was amplified by PCR and subcloned into pTOPO (Invitrogen), creating pTMP419. An internal 0.5 kb *Kpn*I-*Xho*I fragment from pTMP419 was removed and cloned into the suicide vector, pJST55, creating pJSTMPD. In addition, a 1.2 kb *bft-k* gene fragment was amplified by PCR and subcloned into the pTOPO vector, creating pTp-P419. pTp-419 was then digested with *Ssp*I and *Acc*I, yielding a 460 bp fragment, which was cloned into suicide vector pJST55, creating pJSTBD1.

To obtain donor strains, subclones were obtained after transforming *E. coli* HB101 with pJSTMPD or pJSTBD1, and the obtained plasmid structures were confirmed by restriction endonuclease analysis. All donor strains were resistant to clindamycin (cln^r) and sensitive to ciprofloxacin (cip^s). These two plasmids, pJSTMPD and pJSTBD1, were mobilized into *Bacteroides* cells by a parental filter mating procedure [6, 9]. Thus, donor and recipient were mixed in a 1:2 volume ratio, centrifuged, and resuspended in 0.5 ml of BHIS. Twenty μl of this mixture was then spread on sterile nitrocellulose filters placed on the surface of a BHIS agar plate. After overnight aerobic incubation at 37°C, transconjugants were selected using BHIS agar containing clindamycin and ciprofloxacin. To confirm the insertion derivatives in the *t-3* and *bft-k* genes, chromosomal DNA was prepared [11] from each of 14

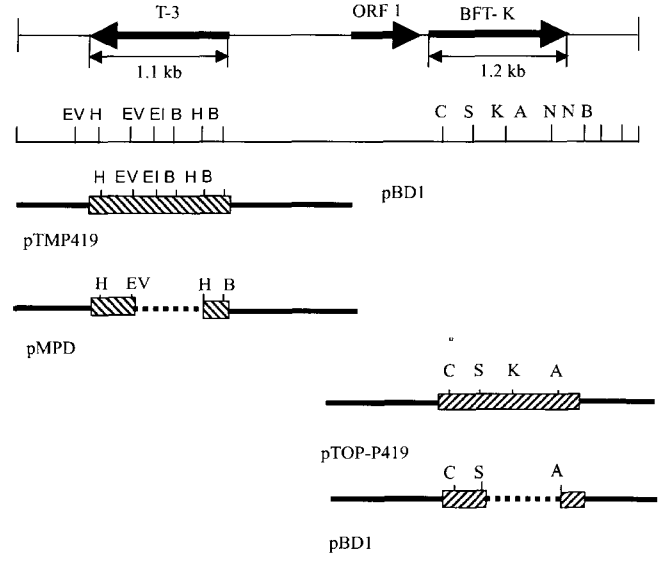

Fig. 1. Schematics of pTMP419, pMPD, pTop-P419, and pBD1, which carry the cloned *t-3*, *bft-k* genes from *B. fragilis* 419, respectively.
Restriction endonuclease abbreviations are as follow: EV, *Eco*RV; H, *Hpa*II; EI, *Eco*RI; B, *Bgl*II; C, *Cfo*I; S, *Ssp*I; K, *Kpn*I; A, *ACC*I; N, *Nde*I.

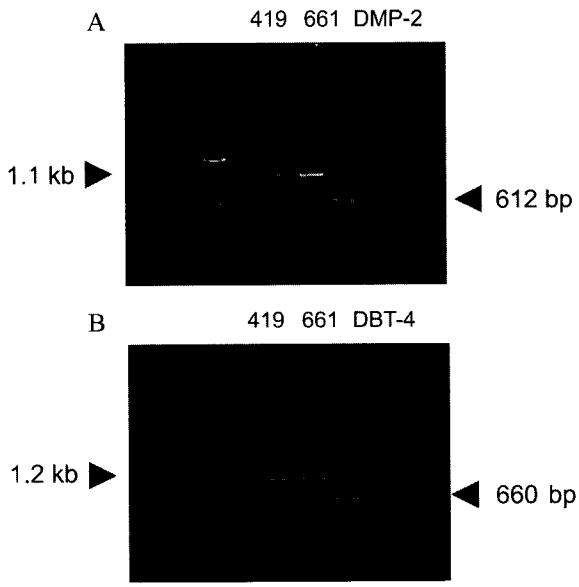

Fig. 2. Agarose gel electrophoresis of PCR product of ETBF 419 and 661 and transconjugants DMP-2 and DBT-4.
(A) The detection of *t-3* gene using primers MA and MB. (B) The detection of *bft* gene using primers BFT1 and BFT5.

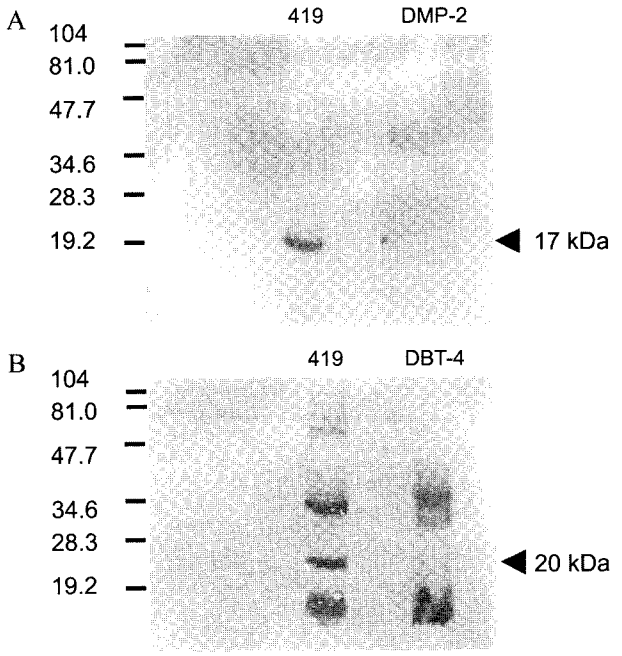

Fig. 3. Immunoblot analysis of culture supernatants obtained from ETBF and mutants.
(A) *B. fragilis* 661 (lane 1) and *t-3* disrupted DMP-2 (lane 2). Polyclonal antibodies raised in rabbit, instead of the mature form of the recombinant protein of T-3 produced in *E. coli*, were used. (B) *B. fragilis* 661 (lane 1) and *bft-k* disrupted DBT-4 (lane 2). Polyclonal antibodies raised in rabbit in response to recombinant BFT were used as the primary antibody.

isolated Cln^r colonies and the *t-3* and *bft-k* genes amplified by using primer [primer MA (forward primer, 5'-TTTTT-GTATGCATGCATTCCAAC-3'), MB (reverse primer, 5'-TTCTTCTAGCAGTCGTGTGTAC-3') and BFT1 (forward primer, 5'-GCGGAATTCATGTTCTAATGAAGCTGAT-3', BFT5 (reverse primer, 5'-TGGTCTCGAGATCGAGA-TCGCCATCTGCTATTTCC-3')] respectively. As a result of PCR, DMP-2, a mutant, was obtained that contained 612 bp of the partially deleted *t-3* gene instead of the 1.1 kb PCR product containing a complete *t-3* gene (Fig. 2A). A second mutant DBT-4, containing a 660 bp PCR product of the partially deleted *bft-k* gene, was isolated by PCR (Fig. 2B).

Functional Properties of *t-3* Mutant (DMP-2) and *bft-k* Mutant (DBT-4) of *B. fragilis*

To confirm that mutant DMP-2 and DBT-4 were defective in terms of the expressions of the T-3 and BFT-K proteins, respectively, concentrated culture supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot analysis [1, 2], using polyclonal anti-T-3 and BFT-K rabbit antibodies as a probe. Thus, the culture supernatants of DMP-2 and DBT-4 were concentrated by precipitation with trichloroacetic acid at a final concentration of 10%, resuspended in 0.1 N NaOH, and dialyzed against 50 mM Tris-HCl (pH 7.5) at 4°C. The fact that T-3 was produced from the mutant DMP-2 was confirmed by immunoblot analysis using a polyclonal anti-T-3 mouse antibody as a probe (Fig. 3A). The result showed that the ~17-kDa T-3 protein present in the wild-type cells was missing from the cell-free supernatant of the mutant strain, DMP-2. The ~20-kDa BFT-K protein did not react with the BFT-K of DBT-4, but it did react with the concentrated culture supernatant of wild-type *B. fragilis* strain 661 (Fig. 3B).

The cell-free supernatants of the strains DMP-2 and DBT-4 were tested for biological activity in HT29/C_1 cells, as previously described [12, 14]. Thus, HT29/C_1 cells were grown in 5% CO_2 in a humidified 37°C incubator in Dulbecco's modified Eagles medium. For experimental purposes, the cells were suspended 1:10 or 1:15 in 0.005% trypsin-0.053 mM EDTA, and plated onto covered 35 mm diameter dishes. HT29/C_1 cells were treated with the supernatant filtrates of DMP-2 and DBT-4 for 16 h at 37°C. BFT from ETBF 419 was used as a positive control, and the negative control included medium alone and crude supernatant filtrate of the nontoxigenic *B. fragilis* strain 077225-2. The results showed that culture filtrates prepared from *t-3* disrupted DMP-2 and *bft-k* disrupted DBT-4 had no effect on HT29/C_1 cell morphology and the filtrates prepared from 077225-2 also had no effect (Fig. 4).

In summary, this is the first study of the functional properties of the *t-3* gene in the *B. fragilis* pathogenicity islet using a suicide plasmid-based gene deletion system. *t-3* or *bft-k* gene disrupted mutants were constructed by

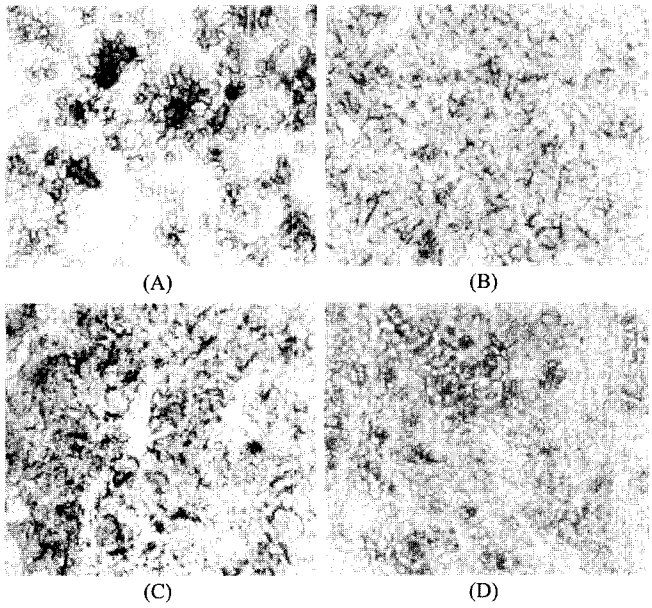

Fig. 4. Biological activities of the culture supernatants of enterotoxigenic *B. fragilis* 661 and mutants using HT29/C_1 cells. (A) *B. fragilis* 661. (B) Mutant DBT-4, *bft* disrupted. (C) Mutant DMP-2, *t-3* disrupted. (D) Negative control (nontoxigenic *B. fragilis* strain 077225-2).

using a suicide vector, and the function of the *t-3* and *bft-k* genes in the pathogenicity islet was analyzed. The result of PCR revealed that DMP-2 contained the complete *bft-k* gene and BFT-4 contained the complete *t-3* gene (data not shown), but these mutants had no biological activity on HT29/C_1 cells. We are now in the process of studying the gene structure of mutants by sequencing, and the role of the BFT-K and T-3 proteins in order to elucidate whether *t-3* and *bft-k* genes are harmonized in pathogenicity *in vivo*.

Acknowledgments

We appreciate the assistance of C. Jeffrey Smith and Michael H. Malamy for generously donating the vector and for helpful discussions concerning the protocol of conjugation. This work was supported by a research grant (2000) from the National Institute of Health (Korea) and from the Korean Ministry of Health and Welfare.

References

1. Choi, E. A., E. K. Yoon, K. S. Shin, and H. S. Kim. 2002. Expression of rotavirus capsid proteins VP6 and VP7 in mammalian cells using Semliki Forest Virus-based expression system. *J. Microbiol. Biotechnol.* **12:** 463-469.
2. Chung, C. C., H. H. Lee, and M. H. Cho. 2000. Immunoelectron microscopic localization and analysis of Herpes simplex virus type 1 antigenes. *J. Microbiol. Biotechnol.* **10:** 714-720.
3. Chung, G. T., A. A. Franco, S. Wu, G. E. Rhie, R. Cheng, H. B. Oh, and C. L. Sears. 1999. Identification of a third metalloprotease toxin gene in extraintestinal isolates of *Bacteroides fragilis*. *Infect. Immun.* **67:** 4945-4949.
4. Hardie, J. M. 1991. Dental and oral infection, pp. 245-267. *In* B. I. Duerden and B. S. Drasar (eds.), Anaerobes in Human Deasease, Edward Arnold, London, U.K.
5. Finlay, B. B. and S. Falkow. 1997. Common themes in microbial pathogenicity revisited. *Microbiol. Mol. Biol. Rev.* **61:** 136-169.
6. Franco, A. A., L. M. Mundy, M. Trucksis, S. Wu, J. B. Kaper, and C. L. Sears. 1997. Cloning and characterization of the *Bacteroides fragilis* metallo-protease toxin gene. *Infect. Immun.* **65:** 1007-1013.
7. Franco, A. A., R. K. Cheng, G. T. Chung, S. Wu, H. B. Oh, and C. L. Sears. 1999. Molecular evolution of the pathogenicity island of enterotoxigenic *Bacteroides fragilis* strains. *J. Bacteriol.* **181:** 6623-6633.
8. Godoy, V. G., M. M. Dallas, T. A. Russo, and M. H. Malamy. 1993. A role for *Bacteroides fragilis* neuraminidase in bacterial growth in two model system. *Infect. Immun.* **61:** 4415-4426.
9. Kling, J. J., R. L. Wright, J. S. Moncrief, and T. D. Wilkins. 1997. Cloning and characterization of the gene for the metalloprotease enterotoxin of *Bacteroides fragilis*. FEMS *Microbiol. Lett.* **146:** 279-284.
10. Koh, D. H., C. Y. Jeong, and J. K. Lee. 2001. Expression analysis of β-ketothiolase and acetyl-CoA reductase of *Rhodobacter sphaeroides*. *J. Microbiol. Biotechnol.* **11:** 1031-1037.
11. Lee, H. W., W. Y. Choi, K. S. Cho, and W. J. Choi. 2001. 16S/23S intergenic spacer region as a genetic marker for *Thiobacillus thiooxidans* and *T. ferrooxidans*. *J. Microbiol. Biotechnol.* **11:** 1046-1054.
12. Lee, M. J., Z. Zang, E. Y. Choi, and H. K. Shin. 2002. Cytoskeleton reorganization and cytokine production of macrophage by *Bifidobacterial* cells and cell-free extracts. *J. Microbiol. Biotechnol.* **12:** 398-405.
13. Moncrief, J. S., Obiso Jr., L. A. Barroso, J. J. Kling, R. L. Wright, R. L. Van Tassel, D. M. Lyerly, and T. D. Wilkins. 1995. The enterotoxin of *Bacteroides fragilis* is a metalloprotease. *Infect. Immun.* **63:** 175-181.
14. Mundy, L. M. and C. L. Sears. 1996. Detection of toxin production by *Bacteroides fragilis*: Assay development and screening of extraintestinal clinical isolates. *Clin. Infect. Dis.* **23:** 269-276.
15. Myers, L. L., D. S. Shoop, and M. M. Border. 1984. *Bacteroides fragilis*: A possible cause of acute diarrheal disease in newborn lambs. *Infect. Immun.* **44:** 241-244.
16. Rhie, G. E., G. T. Chung, Y. J. Lee, W. K. Sung, and H. B. Oh. 2000. Cloning, sequencing, and characterization of enterotoxin pathogenicity islet from *Bacteroides fragilis* 419. *J. Microbiol. Biotechnol.* **10:** 86-90.
17. Thompson, J. S. and M. H. Malamy. 1990. Sequencing the gene for an imipenem-efoxitin-hydrolyzing enzyme (*cfi* A) from *Bacteroides fragilis* TAL2480 reveals strong similarity between cfi A and *Bacillus cereus* β-lactamase II. *J. Bacterol.* **172:** 2584-2593.