Cloning of a DNA fragment encoding the 5'-terminus of the botulinum type E toxin gene from *Clostridium butyricum* strain BL6340

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Chromosomal DNA was extracted from toxigenic *Clostridium butyricum* strain BL6340 isolated from a case of infant botulism. After digestion by *EcoRI*, a DNA fragment of about 1 kbp was cloned into *Escherichia coli* using *λ*gt11, and was subcloned into pUC118. The *E. coli* cells transformed with this cloned fragment produced a 33 kDa protein which reacted with monoclonal antibodies recognizing the light chain (Lc) component of botulinum type E toxin. The nucleotide sequence of the cloned fragment was determined. The sequence was similar to that from botulinum type E toxin gene fragments previously determined by our laboratory (strains Mashike, Otaru and Iwanai). Several highly homologous sequences among the botulinum type A, C, E, butyricum and tetanus toxin genes were found in both translated and untranslated regions. These results suggest that the toxin gene of *C. butyricum* may have evolved by transfer from *C. botulinum*.

Introduction

Clostridium botulinum is the causative agent of food-borne botulism or infant botulism. Neurotoxins produced by the bacteria are classified into seven groups (A to G) based on their antigenicity. Recently, however, *Clostridium* species other than *C. botulinum* have been found to produce botulinum neurotoxin. *C. butyricum* and *C. barati* produce type E and type F neurotoxins, respectively, which are implicated in the onset of human infant botulism (Hall *et al.*, 1985; McCroskey *et al.*, 1986). The DNA extracted from a type E toxin-producing *C. butyricum* strain showed high homology to that obtained from a typical nontoxigenic *C. butyricum* strain (Suen *et al.*, 1988). It is therefore important to establish whether toxin genes exist in toxigenic and nontoxigenic *C. butyricum* strains, and to clarify the mechanism of transfer of toxin genes from *C. botulinum* to other clostridia. The N-terminal amino acid sequences of botulinum type A, B, C, D and E toxins, and of the toxin produced by toxigenic *C. butyricum*, have been determined (Gimenez *et al.*, 1988; Moriishi *et al.*, 1989;

Sathyamoorthy *et al.*, 1985; Tsuzuki *et al.*, 1988). The 5'-terminus and complete nucleotide sequence of type A toxin gene has been reported (Betlay *et al.*, 1989; Binz *et al.*, 1990; Thompson *et al.*, 1990). Recently, the complete nucleotide sequence of the structural gene of botulinum type C toxin and the 5'-terminal nucleotide sequence of the type E toxin gene (strains Mashike, Otaru, Iwanai) have been determined in our laboratory (Fujii *et al.*, 1990; Kimura *et al.*, 1990). The nucleotide sequences obtained from three type E toxin genes were identical. In this paper, we report the cloning, sequencing and expression of a toxin gene fragment from a toxigenic *C. butyricum* strain, and compare the 5'-terminal nucleotide sequence (and the deduced amino acid sequence) to those of botulinum toxins type A, C and E, and tetanus toxin.

Methods

Extraction of DNA from cells. One toxigenic strain of *C. butyricum* (BL6340, isolated from a case of infant botulism and kindly provided by Dr Hatheway, Centers for Disease Control, Atlanta, USA) and nine nontoxigenic strains (KZ145 and KZ589, provided by Dr Hatheway; IFO14810, IFO20064, Y-M-1a, Y-M-2a, Y-M-3a, Y-B-1a and IAM19001, provided by Dr Arai, Biotherapy Research Institute, Tokyo, Japan) were used. These strains were cultured in 250 ml LYG medium (1% (w/v) lactalbumin hydrolysate, 2% (w/v) yeast extract,

Abbreviation: mAb, monoclonal antibody.

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0.5% (w/v) glucose, 0.15% (w/v) cysteine hydrochloride, pH 7.2) at 30 °C for 12 h. The cells were collected by centrifugation at 6000 r.p.m. (11000 g) for 10 min at room temperature, and resuspended in 20 ml LYG medium containing 8% (w/v) polyethylene glycol 4000, 10 U penicillin G ml⁻¹ and 10 µg lysozyme ml⁻¹, and then incubated at 30 °C for 60–90 min. After centrifugation, the cells were lysed using 2–3% (w/v) of sodium dodecyl sulphate (SDS). Extraction and purification of chromosomal DNA were performed by the procedures described by Marmur (Marmur, 1961).

Monoclonal antibodies. Three monoclonal antibodies (mAbs) against light chain (Lc) component of type E toxin, EL161-38, EL211-3, EL219-15, were those prepared previously (Tsuzuki *et al.*, 1988). EL211-3 and EL219-15 react only with type E toxin, but EL161-38 also reacts with Lc components of botulinum type B, C, D and tetanus toxins.

Cloning and sub-cloning of the toxin gene. Cellular DNA from toxigenic strain BL6340 was digested by *Eco*RI (Takara Shuzo Co., Kyoto, Japan), ligated to λ gt11 *Eco*RI arms (Promega Corporation), and then packed *in vitro* by using Packagene (Promega Corporation). The gene library was plated on *E. coli* Y1090 (Promega Corporation), and positive clones were immunoscreened using mAb EL161-38 and the ProtoBlot Immunoscreening System (Promega Corporation). The isolated positive clones were purified, and the recombinant phage DNA was extracted. After digestion with *Eco*RI, the inserts separated from phage DNA were then sub-cloned into the *Eco*RI site on the polylinker of pUC118 (Takara Shuzo Co.).

DNA sequencing and Western blot analysis. DNA sequence analysis was performed by the dideoxy termination method using [α -³⁵S]dATP (NEN Products) and a T7 DNA sequencing kit (Pharmacia).

Gene products from *E. coli* strain Y1090 infected with recombinant phage, and from *E. coli* strain MV1184 transformed by recombinant plasmids, were analysed by Western blots with mAbs and with alkaline-phosphatase-labelled anti-mouse immunoglobulin G conjugate (Promega Corporation), nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. The transformed cells were incubated in 10 ml 2 × TY medium [1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 7.6] at 37 °C for 12 h. After centrifugation, the cells were suspended in 1 ml TEP buffer (100 mM-Tris/HCl, pH 7.4, 10 mM-EDTA, 1 mM-PMSF), sonicated for about 2 min at 0 °C, and centrifuged at 12000 r.p.m. for 10 min at 4 °C. The resulting supernatants were electrophoresed on 12% (w/v) SDS-polyacrylamide gel, and Western blot analysis was carried out as described previously (Tsuzuki *et al.*, 1988).

Dot blot hybridization. DNA samples (2, 20 and 40 µg) from toxigenic and nontoxigenic strains of *C. butyricum* were spotted on a nylon membrane (Pall Ultrafine Filtration Corp., Glen Cove, NY, USA). After denaturation and neutralization, the membrane was prehybridized in 50% (v/v) formamide hybridization buffer at 42 °C for 12 h, and then hybridized with ³⁵S-labelled toxin probe using a DNA labelling kit (Nippon Gene, Toyama, Japan) at 42 °C for 24 h according to the procedures described previously (Fujii *et al.*, 1988). The membrane was washed at 68 °C and autoradiographed.

Results and Discussion

Cloning and gene products

Chromosomal DNA isolated from the toxigenic *C. butyricum* strain BL 6340 was digested with *Eco*RI and ligated to λ gt11 *Eco*RI arms. After *in vitro* packaging,

Fig. 1. Western blot analysis of gene products by recombinant phage and plasmid. Cellular extracts from *E. coli* Y1090 infected with (1) λ gt11 containing a 1 kbp *Eco*RI fragment of the butyricum toxin gene (λ BU-L1); (2) λ gt11 containing a type E toxin gene *Eco*RI fragment (λ EM-L1); (3) control λ gt11; and from *E. coli* MV1184 transformed by pUC118 containing butyricum toxin gene *Eco*RI fragment (pBU-L1) (4 and 6) or botulinum type E toxin gene *Eco*RI fragment (pEM-L1) (5 and 7). The extracts were characterized by Western blots using mAb EL161-38 against botulinum type E toxin light chain. The cells of lanes 1–5, and lanes 6–7 were incubated in the presence and absence of IPTG, respectively.

and infection into *E. coli*, the recombinant plaques were screened for the production of toxin fragments using mAb EL161-38. Several positive clones were detected and one, λ BU-L1, was chosen for further study. λ BU-L1 DNA was prepared, and found to contain a single 1 kbp *Eco*RI insert. *E. coli* Y1090 cells infected with phage λ BU-L1 produced a 33 kDa protein which reacted with mAb EL161-38 both in the presence and in the absence of IPTG (Fig. 1). In addition, the infected cells reacted with mAb EL211-3, but not with mAb EL219-45 (data not shown). The 1.0 kbp *Eco*RI fragment was isolated from λ BU-L1, and then recloned into the *Eco*RI site of pUC118. *E. coli* strain MV1184, when transformed by the recombinant plasmid (pBU-L1) produced a protein of similar size (34 kDa) as that obtained from the phage clone (Fig. 1). These gene products migrated slightly faster than those produced by *E. coli* cells that had been transformed with phage or plasmid expressing an analogous fragment (about 1 kbp) which encodes the botulinum type E toxin gene (Fujii *et al.*, 1990).

Susceptibility of insert to endonuclease

The 1.0 kbp *Eco*RI fragment from pBU-L1 was digested with several restriction enzymes, and the restriction map

Fig. 2. Restriction endonuclease recognition sites in the fragments from *C. butyricum* (a) and *C. botulinum* type E (b). The map was determined by 4% NuSieve agarose gel electrophoresis of the plasmid DNAs cleaved by *EcoRI* (E), *DnaI* (D) and *HincII* (H).

was found to be similar, but not identical, to that of the analogous 1.0 kbp fragment from the *C. botulinum* type E toxin gene (Fig. 2).

Nucleotide and derived amino acid sequences of butyricum toxin gene EcoRI fragment

The 5'-terminal nucleotide sequence (756 bp) of the butyricum toxin gene and 227 bp of the 5'-untranslated region was determined. The number of nucleotides of the coding and noncoding regions was the same as that of the cloned type E toxin gene fragment (Fujii *et al.*, 1990). However, the nucleotide sequences of the butyricum and botulinum type E toxin genes were not identical; eight bases in the untranslated region, and 27 bases in the open reading frame were different (Fig. 3). A Shine-Dalgarno consensus sequence (AGGAGA) was found 14 nucleotides upstream from the translation initiation start codon (ATG). A base sequence corresponding to the -10 consensus region of *E. coli* (TATAAT) was identified at position 87, but no sequence corresponding to the -35 consensus sequence of *E. coli* (TTGACA) was detected. Two stem-loop structures, at bases 17 to 48 and 121 to 155, were also detected in the region upstream of the -10 region and the Shine-Dalgarno sequence, respectively. The deduced N-terminal amino acid sequence of butyricum toxin was identical to that previously reported by protein analysis of the toxin (Gimenez *et al.*, 1988). It was also similar to the protein sequence of the botulinum type E toxin (strains Mashike, Otaru and Iwanai) except for 17 amino acid residues, which were altered by the nucleotide changes described above (Fig. 4).

Comparison of nucleotide and amino acid sequences of the putative toxin gene with those of botulinum type A, C and tetanus toxins

As described above, the nucleotide sequences of butyricum and type E toxin gene fragments were similar in both the noncoding and coding regions. Since the nucleotide sequences of types A, C and E, and tetanus toxin gene have been determined, the sequences of these five toxin genes were compared. There exist several regions of homologous sequence in both untranslated and translated regions (Fig. 3). In the untranslated region, it was very easy to find sequences corresponding to a Shine-Dalgarno sequence which is presumably involved in the initiation of translation of the toxin genes. In contrast, the sequence corresponding to the -10 and -35 regions was difficult to identify, due to the high A-T content of the DNA. However, one potential -10 region was identified 121 bp upstream from a Shine-Dalgarno region.

In addition to these probable -10 and Shine-Dalgarno regions, we identified highly homologous sequences of ATGATAAAT and TATAAAAAATCA at about 2 and 21 bp downstream of the probable -10 region of the *C. butyricum*, *C. botulinum* type E and tetanus toxin genes, and the sequence ATATTAAA was found between the probable promoter and Shine-Dalgarno regions in all of the *C. butyricum*, type A, type C, type E and tetanus toxin genes. We propose that these homologous sequences may play a role in regulation of expression of the toxin genes.

It has been reported that tetanus toxin and the different types of botulinum toxins possess several consensus amino acid sequences of between 7 and 20 residues in length (Tsuzuki *et al.*, 1988). In addition to these sequences, we found three other highly homologous regions in botulinum type A, C, E, butyricum and tetanus toxins (Fig. 4). The nucleotide sequences corresponding to these three homologous regions were also quite similar to each other (Fig. 3). One region (amino acid residues 65 to 85 in butyricum toxin) was hydrophilic, and another, the region 201-223, was hydrophobic. This hydrophobic region might conceivably play a role in spanning the mammalian cell membrane, as suggested for tetanus toxin by Eisel *et al.* (1986).

Detection of toxin gene in toxigenic and nontoxigenic strains of C. butyricum

DNA samples (2, 20, and 40 µg) from one toxigenic and nine nontoxigenic strains of *C. butyricum* were spotted on a nylon membrane, and the membrane was hybridized with the 1 kbp *EcoRI* probe from the *C. butyricum* toxin

Butyricum (BL6340)	GAATTCAATT AGTAGATAAC AAAAAATAATG CAAAGACTTT TATTATTAAT AATGATATAT TTATTTCTAA	70
Type E (Mashike)	-----C- -----T -----G-----T----- -----C-----	
Type A (62A)	ACTAATAGAT AACAAAAATA ACGCAAAGAA GATGATAATT AGTAATGATA TATTTATTTT CAATTGTTTA	
Type C (c-st)	TCCTGAAAAAT AATAGAATAC AATTAGTAAG TTCCAAAGAT AATGCAAAAA AGATTACAGT TAATACTGAT	
Tetanus	TTATAATTTA ATTATGAATA ATATTCTTAA GATAAAAAAGT AAATTTTTTAA AAATTTAAAT TTT <u>CAGTT</u> TA	
Butyricum	CTGTTTAACT TTA <u>ACTTATA</u> ATAATGTAAA TGTATATTTA TCTATAAAAA ATCAAGATTA CAATTGGGTT	140
Type E	----- -C----- -G----- ←-----6.3-----	
Type A	ACCCTATCTT ATAACGGTAA ATATATATGT TTATCTATGA AAGATGAAAA CCATAATTGG ATCATATGTA	
Type C	TTATTTAGAC CTGATTGTAT AACATTTTCA TATAATGATA AATATTTTTC TCTATCACTT ACAGATGGAC	
Tetanus	CAAAAAATAA CCTGATTATG TTATATGTAA TTGTAAAAAA CATATAAAAA ATCAGAAAAA TTTAGGAGGT	
Butyricum	ATATCTGATC TTAATCATGA TATACCCAAA AAGTCATATC TATGGATATT AAAAAATATA TAAATTTAAA	210
Type E	----- -A----- -----	
Type A	ATAATGATAT GTCAAAGTAT TTGTATTTAT GGTCATTTAA ATAATTAATA ATTTAATTAA TTTAAATAT	
Type C	ATTATAATTG GATGATATGT AATGACAATA ACAAGGTGCT AAAGGTGCAC ATTTGTGGAT ATTAGAAAGT	
Tetanus	ATATTATTA TCGATTAAAT AATAATTTT TAATTTACTT TTGATTAATA AATATTAAT GTTTATTTTA	
Butyricum	ATTAGGAGAT GCTGTATATG <u>ORF</u> CCAACAATTA ATAGTTTTAA TTATAATGAT CCTGTTAATA ATAGAACAAT	280
Type E	----- -A----- -----G-----	
Type A	TATAGAGCT GTTAAATATG CCATTTGTTA ATAAACAATT TAATTATAAA GATCCTGTAA ATGGTCTTGA	
Type C	TAGGAGATGCT TAGTATTATG CCAATAACAA TTAACAACCT TAATTATTCA GATCCTGTTG ATAATAAAAA	
Tetanus	ATTAGGAGAT GATACCTATG CCAATAACCA TAAATAATTT TAGATATAGT GATCCTGTTA ATAATGATAC	
	SD	
Butyricum	TTTATATATT AAACCAGGCG GTTGTCAACA ATTTTATAAA TCATTTAATA TTATCAAAAA TATTTGGATA	350
Type E	----- -G----- -----	
Type A	TATTGCTTAT ATAAAAATTC CAAATGCAGG ACAAATGCAA CCAGTAAAAG CTTTTAAAAT TCATAATAAA	
Type C	TATTTTATAT TTAGATACTC ATTTAAATAC ACTAGCTAAT GAGCCTGAAA AAGCCTTTG CATTACAGGA	
Tetanus	AATTATTATG ATGGAGCCAC CATACTGTAA GGTCTAGAT ATCTATTATA AGGCTTTCAA AATAACAGAT	
Butyricum	ATTCCAGAGA GAAATGTAAT TGGTACAATT CCCCAGATT TTCTTCCGCC TACTTCATTG AAAAAATGGAG	420
Type E	----- -CC----- -A----- -A-----	
Type A	ATATGGGTTA TTCCAGAAAG AGATACATTT ACAAATCCTG AACAAGGAGA TTAAATCCA CCACCAGAAG	
Type C	AATATATGGG TAATACCTGA TAGATTTTCA AGAAATTCTA ATCCAAATTT AAATAAACCT CCTCGAGTTA	
Tetanus	CGTATTTGGA TAGTGCCGGA AAGGTATGAA TTTGGGACAA AACCTGAAGA TTTTAACCCA CCATCTTCAT	
Butyricum	ATAGTAGTTA TTATGACCCT AATTATTTAC AAAGTGATCA AGAAAAGGAT AAATTTTTAA AAATAGTCAC	490
Type E	----- -G----- -G-----	
Type A	CAAAACAAGT TCCAGTTTCA TATTATGATT CAACATATTT AAGTACAGAT AATGAAAAAG ATAATTATTT	
Type C	CAAGCCCTAA AAGTGGTTAT TATGATCCTA ATTATTTGAG TACTGATTCT GACAAAGATA CATTTTTAAA	
Tetanus	TAATAGAAGG TGCATCTGAG TATTACGATC CAAATTTATTT AAGGACTGAT TCTGATAAAG ATAGATTTTT	
Butyricum	AAAAATATTT AATAGAATAA ATCATAATCT TTCAGGAAGG ATTTTATTAG AAGAACTGTC AAAAGCTAAT	560
Type E	----- -A----- -G-----	

Type A	AAAGGGAGTT	ACAAAATTAT	TTGAGAGAAT	TTATTCAACT	GATCTTGCAA	CAATGTTGTT	AACATCAATA	
Type C	AGAAATTATA	AAGTTATTTA	AAAGAATTAA	TTCTAGAGAA	ATAGGAGAAG	AATTAATATA	TAGACTTTCC	
Tetanus	ACAAACCATC	GTAAGACTGT	TTAACAGAAT	TAAAAACAAT	GTAGCAGGTG	AAGCCTTATT	AGATAAGATA	
Butyricum	CCATATTTAG	GAAATGATAA	TACTCCAGAT	GGTGACTTCA	TTATTAATGA	TGCATCAGCA	GTTCCAATTC	630
Type E	-----	-G-----	-----	AA-C-A---C	A---GG---	-----	---GAG---A	
Type A	GTAAGGGGAA	TACCATTTTG	GGGTGGAAGT	ACAATAGATA	CAGAATTAAA	AGTTATTGAT	ACTAATTGTA	
Type C	ACACATATAC	CCTTTCCTGG	GAATAACAAT	ACTCCAATTA	ATACTTTTGA	TTTTCATGTA	GATTTTAAAC	
Tetanus	ATAAATGCCA	TACCTTACCT	TGGAAATTCA	TATTCCTTAC	TAGACAAGTT	TGATACAAAC	TCTAATTCAG	
Butyricum	AATTCTCAAA	TGGTAGCCAA	AGCATACTAT	TACCTAATGT	TATTATAATG	GGAGCAGAGC	CTGATTTTATT	700
Type E	-----	-----	GA-----	-----	-----	-----	-----	
Type A	TTAATGTGAT	ACAACCAGAT	GGTAGTTATA	GATCAGAAGA	ACTTAATCTA	GTAATAATAG	GACCCCTCAGC	
Type C	GTGTTGATGT	TAAACTAGCA	CAAGGTAACA	ACTGGGTAA	AACCTGGTAGC	ATAAATCCTA	GTGTTATAAT	
Tetanus	TATCTTTTAA	TTTATTAGAA	CAAGACCCCA	GTGAGCAAC	TACAAAATCA	GCAATGCTGA	CAAATTTAAT	
Butyricum	TGAAACTAAC	AGTTCCAATA	TTTCTCTAAG	AAATAATTAT	ATGCCAAGCA	ATCACGGTTT	TGGATCAATA	770
Type E	-----	-----	-----	-----	-----	-----	-----	
Type A	TGATATTATA	CAGTTTGAAT	GTAAGGCTT	TGCACATCAA	GTTTTGAATC	TTACGCCAAA	TGGTTATGGC	
Type C	AACCTGGACCT	AGAGAAAACA	TTATAGATCC	ACAACTTCT	ACGTTTAAAT	TAACTAACAA	TACTTTTGGC	
Tetanus	AATATTTGGA	CCTGGGCTG	TTTTAAATAA	AAATGAGGTT	AGAGGTATTG	TATTGAGGGT	AGATAATAAA	
Butyricum	GCTATAGTAA	CATTCTCACC	TGAATATTCT	TTTACATTTA	AAGATAATAG	TATGAATCAA	TTTATTCAAG	840
Type E	-----	-----	-----	-----	-T-----	-----	-----	
Type A	TCTACTCAAT	ACATTAGATT	TAGCCAGAT	TTTACATTTG	GTTTTGAGCA	GTCACTTGAA	GTTGATACAA	
Type C	GCACAAGAAG	GATTTGGTGC	TTTATCAATA	ATTCAATAT	CACCTAGATT	TATGCTAACA	TATAGTAATG	
Tetanus	AATTACTTCC	CATGTAGAGA	TGGTTTTGGC	TCAATAATGC	AAATGGCATT	TTGCCAGAA	TATGTACCTA	
Butyricum	ATCCTGCTCT	TACATTAATG	CATGAATTAA	TACATTCATT	ACATCGACTA	TATCGGGCTA	AAGGGATTAC	910
Type E	-----	-----	-----	-----	-----	-----	-----	
Type A	ATCCTCTTTT	AGGTGCAGGC	AAATTTGCTA	CAGATCCAGC	AGTAACATTA	GCACATGAAC	TTATACATGC	
Type C	CAACTAATGA	TGTAGGAGAC	GGTAGATTTT	CTAAGTCTGA	ATTTTGCATG	GATCCAATAC	TAATTTTAAT	
Tetanus	CCTTTGATAA	TGTAATAGAA	AATATTACGT	CACTCACTAT	TGCCAAAAGC	AAATATTTTC	AAGATCCAGC	
Butyricum	TACAATGTAT	ACTATAACAC	AAAAACAATA	TCCCCTAATA	ACAAATATAA	GAGGTACAAA	TATTGAAGAA	983
Type E	-----	-----	-----	-----	-----	-----	---	
Type A	TGGACATAGA	TTATATGGAA	TAGCAATTAA	TCCAAATAGG	GTTTTTAAAG	TAAATACTAA	TGCCTATTAT	GAA
Type C	GCATGAACCT	AATCATGCAA	TGCATAATTT	ATATGGAATA	GCTATACCAA	ATGATCAAAC	AATTTTCATCT	GTA
Tetanus	ATTACTATTA	ATGCACGAAC	TTATACATGT	ACTACATGCT	TTATACGGAA	TGCAGGTATC	AAGCCATGAA	ATT

Fig. 3. Nucleotide sequences of the 5'-translated and 5'-untranslated regions of butyricum, botulinum and tetanus toxin genes. The DNA sequence of the butyricum toxin gene *EcoRI* fragment is compared to the sequence of botulinum type A (Binz *et al.*, 1990), type C (Kimura *et al.*, 1990), type E (Fujii *et al.*, 1990) and tetanus (Eisel *et al.*, 1986) toxin genes. The putative Shine-Dalgarno sequence is indicated by SD. The sequences homologous to the -10 region of the *E. coli* promoter (TATAAT) are marked with double underlines. A sequence with similar dyad symmetry is underlined by divergent arrows. Numbers represent free energy values in kcal mol⁻¹ (1 kcal = 4.2 kJ). Dashes in the botulinum type E sequence represent bases identical to the *C. butyricum* sequence. The highly homologous sequences are underlined, and the start site of homology is indicated on the butyricum sequence by A (type A), C (type C) and T (tetanus). ORF is the translation initiation site of ATG.

Butyricum (BL6340)	<u>↓ ↓ ○ ○ ○ ↓ ○ ↓ ↓ ↓ ↓ ○ ↓ ○</u>	<u>M P T I N S F N Y N D P V N N R T I L Y</u>	I K P G G C Q Q F Y K S F N I M K N	<u>↓ ↓</u>	40
Type E (Mashike)	- - K - - - - - D - - - - -	- - - - - E - - - - -	- - - - -	- -	
Type A (62A)	<u>M P F V N K Q F N Y K D P V N G V D I A Y</u>	<u>I K I P N A G Q M Q P V K A F K I H N</u>			
Type C (c-st)	<u>M P I T I N N F N Y S D P V D N K N I L Y</u>	<u>L D T H L N T L A N E P E K A F R I T</u>			
Tetanus	<u>M P I T I N N F R Y S D P V N N D T I I M</u>	<u>M E P P Y C K G L D I Y Y K A F K I T</u>			
Butyricum	<u>↓ ↓ ○ ↓ ↓</u>	<u>I I P E R N V I G T I P Q D F L P P T S L K N G D S S</u>	<u>↓ ↓ ↓ ↓ ○ ↓ ↓ ↓ ↓ ↓</u>	<u>Y Y D P N Y L Q S D Q E K</u>	80
Type E	- - - - -	- - - - - T - - - - - H - - - - -	- - - - -	- - - - - E - -	
Type A	<u>K I W V I P E R D T</u>	<u>F T N P E E G D L N P P P E A K Q V P V</u>	<u>S Y Y D S T Y L S T</u>		
Type C	<u>G N I W V I P D R F</u>	<u>S R N S N P N L N K P P R V T S P K S G</u>	<u>Y Y D P N Y L S T D</u>		
Tetanus	<u>D R I W I V P E R Y</u>	<u>E F G T K P E D F N P P S S L I E G A S</u>	<u>E Y Y D P N Y L R T</u>		
Butyricum	<u>↓ ↓ ○ ↓ ↓</u>	<u>D K F L K I V T K I F N R I N D N L S G R I L L E E L S K A N P Y L G N D N T P</u>			120
Type E	- R - - - - -	- - - - - N - - - - - G - - - - -	- - - - -	- - - - -	
Type A	<u>D N E K D N Y L K G</u>	<u>V T K L F E R I Y S T D L G R M L L T S I V R G I P F W G G</u>			
Type C	<u>S D K D T F L K E I</u>	<u>I K L F K R I N S R E I G E E L I Y R L S T D I P F P G N N</u>			
Tetanus	<u>D S D K D R F L Q T</u>	<u>M V K L F N R I K N N V A G E A L L D K I I N A I P Y L G N</u>			
Butyricum		<u>D G D F I I N D A S A V P I Q F S N G S Q S I L L P N V I I M G A E P D L F E T</u>			160
Type E		- N Q - H - G - - - - - E - K - - - - - D - - - - -			
Type A		<u>S T I D T E L K V I D T N C I N V I Q P D G S Y R S E E L N L V I I G P S A D I</u>			
Type C		<u>N T P I N T F D F D V D F N S V D V K T R Q G N N W V K T G S I N P S V I I T G</u>			
Tetanus		<u>S Y S L L D K F D T N S N S V S F N L L E Q D P S G A T T K S A M L T N L I I F</u>			
Butyricum		<u>N S S N I S L R N N Y M P S N H G F G S I A I V T F S P E Y S F R F K D N S M N</u>			200
Type E		- - - - -	- - - - -	- - - - - N - - - - -	
Type A		<u>I Q F E C K S F G H E V L N L T R N G Y G S T Q Y I R F S P D F T F G F E E S L</u>			
Type C		<u>P R E N I I D P E T S T F K L T N N T F A A Q E G F G A L S I I S I S P R F M L</u>			
Tetanus		<u>G P G P V L N K N E V R G I V L R V D N K N Y F P C R D G F G S I M Q M A F C P</u>			
Butyricum		<u>↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓ ↓</u>	<u>E F I Q D P A L T L M H E L I H S L H G L Y G A K G I T T M Y T I T Q K Q N P L</u>		240
Type E		- - - - -	- - - - -	- - - - -	
Type A		<u>E V D T N P L L G A G K F A T D P A V T L A H E L I H A G H R L Y G I A I N P N</u>			
Type C		<u>T Y S N A T N D V G E G R F S K S E F C M D P I L I L M H E L N H A M H N L Y G</u>			
Tetanus		<u>E Y V P T F D N V I E N I T S L T I G K S K Y F Q D P A L L L M H E L I H V L H</u>			
Butyricum		<u>I T N I R G T N I E E F</u>			
Type E		- - - - -			
Type A		<u>R V F K V N T N A Y Y E</u>			
Type C		<u>I A I P N D Q T I S S V</u>			
Tetanus		<u>G L Y G M Q V S S H E I</u>			

Fig. 4. Amino acid sequences derived from the base sequence of butyricum toxin gene *Eco*RI fragment. Dashes in the botulinum type E sequence represent amino acid residues identical to the *C. butyricum* sequence. The highly conserved homologous sequences are underlined. Arrows indicate highly conserved amino acid residues among the five toxins, and open circles indicate common amino acid residues in four out of five toxins.

gene. A positive reaction was observed only with DNA from the toxigenic strain, BL6340 (data not shown). The nucleotide sequences of the toxin genes from *C. butyricum* and *C. botulinum* strains were quite similar in both coding and noncoding regions. Therefore, it is quite possible that a nontoxigenic *C. butyricum* strain has become toxigenic by acquiring the toxin gene from a *C. botulinum* type E strain; the toxin gene could perhaps have been transmitted from *C. botulinum* type E to *C. butyricum* cells through some transmissible vector, e.g. a bacteriophage or a plasmid.

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