Applied and Environmental Microbiology, Feb. 1996, p. 662–667 Vol. 62, No. 2
0099-2240/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Molecular Cloning of the Gene Encoding the Mosaic Neurotoxin, Composed of Parts of Botulinum Neurotoxin Types C1 and D, and PCR Detection of This Gene from *Clostridium botulinum* Type C Organisms

KOHJI MORIISHI,[1]* MINAKO KOURA,[1] NOBUHIRO FUJII,[2] YUKAKO FUJINAGA,[3]
KAORU INOUE,[3] BUNEI SYUTO,[4] AND KEIJI OGUMA[3]

Department of Veterinary Science, National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162,[1] Department of Microbiology, Sapporo Medical College, Sapporo 060,[2] Department of Bacteriology, Faculty of Medicine, University of Okayama, Okayama 700,[3] and Department of Agriculture, University of Iwate, Morioka,[4] Japan

Received 19 June 1995/Accepted 5 December 1995

The DNA fragment common to the genes encoding botulinum neurotoxin types C1 (BN/C1) and D (BN/D) was amplified by PCR from the culture supernatant of *Clostridium botulinum* type C strain 6813 (C6813) that was treated with either DNase I or proteinase K but not from the supernatant that was treated with both DNase I and proteinase K, suggesting the neurotoxin gene is located on a certain bacteriophage DNA. Thus, to isolate the neurotoxin gene, we performed PCR with the culture supernatant of C6813 and seven primer pairs designed from the genes encoding BN/C1 and BN/D. The coding region in the connected sequence encodes a neurotoxin composed of 1,280 amino acids with a molecular weight of 147,817. The neurotoxin from C6813 has 95% amino acid identity to BN/C1, except for its C-terminal one-third, which is quite similar to the C-terminal one-third of BN/D (95% identity). When we performed PCRs with four primer pairs designed from the 5′-terminal two-thirds of the BN/C1 gene and two primers from the 3′-terminal one-third of the BN/D gene, DNA fragments of the expected sizes (0.5 to 1.3 kbp) could be amplified from *C. botulinum* type C strains 6812 and 6814. These results suggest that some strains of *C. botulinum* type C contain the gene encoding the mosaic neurotoxin composed of parts of BN/C1 and BN/D.

One strain of *Clostridium botulinum* produces one of seven potent but serologically distinct neurotoxins (types A, B, C1, D, E, F, and G), which have been implicated in both human and animal botulism (25). Each of the seven neurotoxins is synthesized as a single-chain molecule with a molecular mass of about 150 kDa, which is subsequently cleaved and becomes the dichain form. The dichain-form toxin is composed of a light chain (about 50 kDa) and a heavy chain (about 100 kDa) located on the N-terminal and the C-terminal sides, respectively, of a whole toxin (25).

These seven types of neurotoxins, which all have common zinc-binding motifs in their light chains, belong to zinc-dependent proteases (9, 14). These reduced toxins, or their light chains, degrade with high specificity the synaptic proteins that are involved in the fusion of synaptic vesicles with the plasma membranes, thus inhibiting release of several neurotransmitters (26). Types A and E degrade with SNAP-25 (1); types B, D, F, and G degrade with VAMP/synaptobrevin (21–23, 31); and type C1 degrades with HPC-1/syntaxin (4). Although the proteolytic activity is located on their light chains, the channel-forming and the receptor-binding activities are located on the N-terminal and C-terminal halves, respectively, of their heavy chains (5, 13, 24).

Recent studies have elucidated the entire sequences of seven botulinum neurotoxin genes (2, 3, 6, 8, 10, 12, 27, 29, 30). The neurotoxin genes isolated from *C. botulinum* type C strains 468 and Stockholm are quite similar (99.9% identity) and have been reported to be the gene encoding botulinum neurotoxin type C1 (BN/C1) (10, 12). On the other hand, the neurotoxin genes isolated from *C. botulinum* type D strains 1873 and CB-16 are quite similar (99.9% identity) and have been reported to be the gene encoding botulinum neurotoxin type D (BN/D) (2, 27). However, some strains of *C. botulinum* types C and D have been known to produce neurotoxins that are not completely consistent with BN/C1 and BN/D on the basis of antigenicity (18, 28). The neurotoxin purified from strain 6813 of *C. botulinum* type C (BN/C6813) has antigenic structures for the BN/C1 light chain and the BN/D heavy chain, suggesting that the neurotoxins from *C. botulinum* type C strains could be separated into at least two isoforms. However, the difference between the primary structures of both isoforms has not yet been clarified in detail, because the entire amino acid sequence of BN/C6813 has been not determined.

In this report, we determined the entire nucleotide sequence of the gene encoding BN/C6813 and compared its deduced amino acid sequence with that of BN/C1 or BN/D. In addition, we detected the gene encoding the neurotoxin-like BN/C6813 from two other strains.

MATERIALS AND METHODS

Organisms. *C. botulinum* type C strains 6812 (C6812), 6813 (C6813), and 6814 (C6814), and type D strains South African (Dsa) and 4947 (D4947) were grown at 30°C for 4 days in cooked meat medium (18). The culture media of those organisms were centrifuged at 10,000 × *g*. The resulting supernatants were kept at −20°C until use.

Treatment of culture supernatant with DNase I and proteinase K. Culture medium was centrifuged at 15,000 rpm for 5 min. The resulting supernatant was filtered through a 0.22-μm-pore-diameter cellulose nitrate filter. Twenty micro-

* Corresponding author. Mailing address: Department of Veterinary Science, National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162, Japan. Phone: 03-5285-1111, ext. 2622. Fax: 03-5285-1150.

TABLE 1. Primers for PCR

Primer	Sequence	Nucleotide position[a]	
		BN/C1	BN/D
C-1[b]	5′tactgatttatttagacctg3′	−167–−146	
C0[c]	3′attgacctggatctcttttg5′	492–473	
C1[b]	5′gaaagttaggagatgttagt3′	−23–−4	
C2[c]	3′ccgtgttcttcctaaaccac5′	544–563	
C3[b]	5′tggtagcataaatcctagtg3′	447–466	
C4[c]	3′ccacttcaatgtcatttagc5′	1031–1012	
C5[b]	5′agattcgtagtagaatcttc3′	991–1010	
C6[c]	3′atggtcccctcttagttcag5′	1596–1577	
C8[c]	3′agtccttcactatttcttt5′	2266–2247	2255–2236
C9[b]	5′cttgttcagtatccagatag3′		3358–3377
C10[c]	3′agcatattttacaacttaag5′		3906–3887
YQA[b]	5′tatcaggcagatgcaatcaaagcta3′	2197–2221	2185–2209
LDV[c]	3′gtagtcctatattacgtcttcactc5′	2681–2657	2669–2645
LDV2[b]	5′caggatataatgcagaagtg3′	2660–2679	2648–2667
SVS[c]	3′agtcatagactattcttagg5′		3434–3415
RGN[c]	3′ttactatgatctcatgctcc5′	3491–3479	

[a] Nucleotide numbering is from A of the initiation codon (2, 12).
[b] Sense.
[c] Antisense.

liters of the filtered supernatant was mixed with 1 μl of chloroform, and then the mixture was centrifuged at 15,000 rpm for 5 min. Either 7 U of DNase I or 10 μg of proteinase K was added to the centrifuged supernatant. The mixture was incubated at 37°C for 1 h. After treatment with chloroform, the mixture was centrifuged at 15,000 rpm for 5 min. The resulting supernatant was used as a template for PCR. When the supernatant was treated with DNase I and proteinase K, DNase I was added to the supernatant after treatment with proteinase K and chloroform.

PCR. The degenerate oligonucleotide primers for PCR were synthesized on an Applied Biosystems, Inc., model 380B synthesizer. The nucleotide sequences of primers (designated C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, YQA, LDV, LDV2, SVS, and RGN) were derived from BN/C1 and BN/D genes (Table 1). The PCR amplifications were carried out in a reaction mixture (50 μl) containing 0.5 μl of culture supernatant, 100 pmol (each) of the two primers, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim, Tokyo, Japan) in Tris-HCl (pH 8.0), 0.2 mM (each) the deoxynucleoside triphosphates, 50 mM KCl, 2.0 mM $MgCl_2$, and 0.01% bovine serum albumin. Twenty-five PCR cycles (at 92°C for 1 min, 45°C for 1 min, and then 74°C for 2 min) were performed. The amplified fragments were purified with SUPREC-01 (Takara, Otsu, Japan) and subcloned into the "T-vector" constructed from pBluescript SK+ (16). The sequences of three clones from different DNA amplification reaction mixtures were determined to detect possible errors made by *Taq* polymerase. Nucleotide sequences were established by the dideoxy chain termination method with [α-^{35}S]dATP and Sequenase version 2.0 DNA polymerase (Amersham, Tokyo, Japan). Nucleotide numbers in BN/C1 and BN/D genes indicate numbering from A of the initiation codon ATG.

DNA-DNA hybridization. Amplified DNA fragments were transferred from agarose gels to Hybond N+ membranes (Amersham) (15). The amplified DNA products were labeled with [α-^{35}S]dATP with the random primer DNA labeling kit, version 2 (Takara). Hybridizations were performed at 65°C. Autoradiograms were analyzed with a Bio-Image analyzer, model BAS2000 (Fuji Film, Tokyo, Japan).

Nucleotide sequence accession number. The nucleotide sequence data reported in this article will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under accession number D49440.

RESULTS AND DISCUSSION

The BN/C1 gene has been shown to exist in toxigenic bacteriophage DNA from type C strains 468 (10) and Stockholm (12), whereas a toxigenic bacteriophage has not been isolated from type C strain 6813 (C6813). PCR with the primer pair YQA/LDV could amplify the DNA fragment of the expected size of 0.5 kbp from culture supernatant of C6813 that was treated with either DNase I or proteinase K (Fig. 1). However, PCR with the primer pair YQA/LDV could not amplify any DNA fragment from the culture supernatant of C6813 that was treated with both DNase I and proteinase K (Fig. 1). These

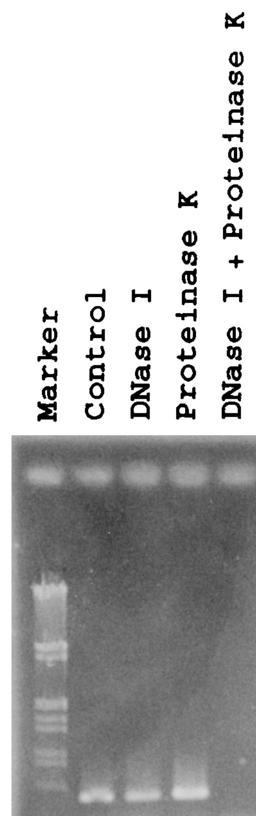

FIG. 1. Amplification of the part of the BN/C1 gene from the culture supernatant of C6813. Lamda phage genomic DNA digested with *Hin*dIII and *Eco*RI was used as a molecular weight marker.

results suggest that the BN/C6813 gene is located on a certain bacteriophage.

The sequencing strategy used in this study is shown in Fig. 2. The DNA fragments of the expected sizes of 0.7, 0.6, 0.6, 0.6, 1.3, and 0.5 kbp were amplified with the primer pairs C-1/C0, C1/C2, C3/C4, C5/C6, C5/C8, and YQA/LDV, respectively, which were designed from the 5′-terminal two-thirds of the BN/C1 gene and its upstream region. However, no DNA fragment could be amplified from C6813 by the primer pair LDV2/RGN, which targets the region extending from C-2660 to G-3491 in the BN/C1 gene. On the other hand, the DNA fragment of the expected size of 0.8 kbp was amplified by the primer pair LDV2/SVS, which targets the region extending from G-2648 to C-3434 in the BN/D gene (Fig. 3A and B), suggesting that the 3′-terminal one-third of the BN/C6813 gene is similar to that of the BN/D gene rather than that of the

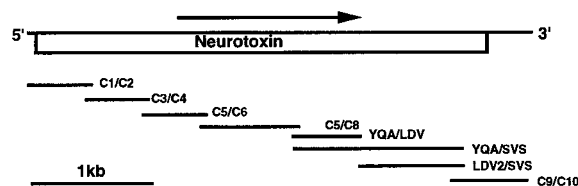

FIG. 2. Strategy for cloning the BN/C6813 gene. The open box and arrow indicate the coding region of BN/C6813 and the transcription orientation, respectively. Lines indicate PCR products created by amplification with primer pairs C1/C2, C3/C4/, C5/C6, C5/C8, YQA/LDV, YQA/SVS, LDV2/SVS, and C9/C10.

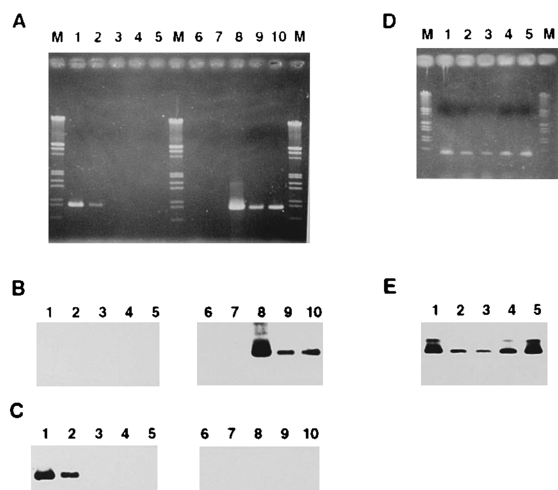

FIG. 3. Amplification of DNA fragments from *C. botulinum* type C and D strains. PCR was performed with the culture supernatants of Dsa (lanes 1 and 6), D4947 (lanes 2 and 7), C6812 (lanes 3 and 8), C6813 (lanes 4 and 9), and C6814 (lanes 5 and 10) and the primer pairs LDV2/RGN (lanes 1 to 5 in panels A, B, and C), LDV2/SVS (lanes 6 to 10 in panels A, B, and C), and YQA/LDV (lanes 1 to 5 in panels D and E). The reaction mixtures were subjected to agarose electrophoresis. The resulting gels were stained with ethidium bromide (A and D). PCR products in gels were transferred to sheets of nylon membrane and subjected to DNA-DNA hybridization. The PCR product from Dsa amplified by LDV2/RGN (C) or YQA/LDV (E) was used as a probe. The PCR product from C6813 amplified by primer pair LDV2/SVS (B) was also used as a probe. Autoradiograms are shown in panels B, C, and E.

BN/C1 gene. The DNA fragment of the expected size of 0.5 kbp was amplified from C6813 by the primer pair C9/C10, which targets the region extending from C-3358 to C-3906 in the BN/D gene. Amplified fragments were ligated to the T-vector and then sequenced.

The coding region in the connected sequence encodes a neurotoxin composed of 1,280 amino acids and with a molecular weight of 147,813 (Fig. 4). The deduced amino acid sequence has 77 and 71% identity to BN/C1 and BN/D, respectively (Fig. 5). The putative nicking site is located at the C terminus of Lys-449, as expected from the amino acid homology to BN/C1. The light chain (Met-1 to Lys-449) of the deduced neurotoxin has 97 and 48% identity to those of BN/C1 and BN/D, respectively, whereas the heavy chain (Thr-450 to Glu-1280) of the deduced protein has 66 and 85% identity to those of BN/C1 and BN/D, respectively. These comparative analyses are consistent with the finding reported by Terajima et al. (28) that the light and heavy chains of the purified BN/C6813 are similar to the BN/C1 light chain and the BN/D heavy chain, respectively.

The comparative analyses of BN/C6813, BN/C1, and BN/D (Fig. 5) indicate that BN/C6813, BN/C1, and BN/D are each composed of three regions: N-terminal (Met-1 to Leu-526 in BN/C6813 or BN/C1, Met-1 to Asn-521 in BN/D), core (Pro-527 to Val-901 in BN/C6813, Pro-527 to Leu-901 in BN/C1, Pro-522 to Leu-897 in BN/D), and C-terminal (Asn-902 to Glu-1280 in BN/C6813, Asn-902 to Glu-1291 in BN/C1, Asn-902 to Glu-1276 in BN/D) regions. The N-terminal region of BN/C6813 has 96 and 45% identity to those of BN/C1 and BN/D, respectively. The core region is conserved among three toxins (81 to 93% identity). The C-terminal region of BN/C6813 has 36 and 95% identity to those of BN/C1 and BN/D, respectively. Thus, BN/C6813 is the mosaic molecule composed of parts of BN/C1 and BN/D.

The neurotoxin from C6812 has antigenic structures similar to those of the BN/C1 light chain and the BN/D heavy chain, because antibody to the BN/C1 light chain or BN/D heavy chain could neutralize the toxicity of the neurotoxin from C6812 (20). The DNA fragments of the expected size of 0.8 kbp that were amplified from C6812, C6814, and C6813 with the primer pair LDV2/SVS but not with LDV2/RGN could hybridize with the labeled product from C6813 (Fig. 3A, B, and C), suggesting their nucleotide sequences are quite similar. On the other hand, the DNA fragments of the expected size of 0.5 kbp that were amplified from C6812, C6813, C6814, Dsa, and D4947 by the primer pair YQA/LVD could hybridize with the labeled product from Dsa (Fig. 3D and E). In addition, the DNA fragments of the expected sizes were amplified from the strains of *C. botulinum* type C, but not from Dsa, by the primer pairs C1/C2, C3/C4, C5/C6, and C9/C10 (Table 2). These results suggest that some strains of *C. botulinum* type C contain the gene encoding the mosaic neurotoxin-like BN/C6813.

The DNA fragment of the expected size of 0.8 kbp that was amplified from Dsa or D4947 by primer pair LDV2/RGN could not hybridize with the product from C6813 amplified by primer pair LDV2/SVS (Fig. 3B and C). The nucleotide sequence of the product from Dsa amplified by primer pair LDV2/RGN has 99 and 65% identity to corresponding regions of BN/C1 and BN/D, respectively (data not shown). However, the DNA fragment of the expected size of 0.5 kbp that was amplified from Dsa by primer pair YQA/LDV could hybridize with the product from C6813 or D4947 amplified by primer pair YQA/LDV. The nucleotide sequence of the product from Dsa amplified by primer pair YQA/LDV was conserved in BN/C1 (99% identity) and BN/D (98% identity). Our previous study (18) has suggested that the neurotoxin from Dsa (BN/Dsa) has similar antigenic structures for the BN/D light chain and for the BN/C1 heavy chain. Oguma et al. (20) reported that the antibody to the BN/D light chain or the BN/C1 heavy chain neutralized the toxicity of the neurotoxin from D4947 (BN/D4947). Thus, BN/Dsa or BN/D4947 might be composed of an N-terminal region that is quite similar to that of BN/D, the core region that is conserved between BN/C1 and BN/D, and a C-terminal region that is quite similar to that of BN/C1.

All types of botulinum neurotoxin are zinc-dependent endopeptidases, the intracellular substrates of which play an important role in the fusion of synaptic vesicles to plasma membrane (1, 4, 21–23, 31). Light chains of neurotoxins from strains 468, Stockholm, and 6813 of *C. botulinum* type C are composed of same sequence, suggesting their functions are quite similar. Thus, BN/C1 and BN/C6813 should act specifically on the same substrate, HPC-1/syntaxin.

The N-terminal halves of the heavy chains of BN/C6813, BN/C1, and BN/D may exhibit similar properties related to a specific function, because the N-terminal halves of their heavy chains correspond to the core regions that are conserved in BN/C6813, BN/C1, and BN/D. Botulinum neurotoxins have been known to form channels in lipid membranes and vesicles (5, 7, 17). The N-terminal half of the heavy chain of botulinum neurotoxin type A could form channels in lipid membrane (5). The hydrophobic amino acid sequences that exist within core regions of BN/C6813, BN/C1, and BN/D (Gly-655 to Glu-682 in BN/C1, or BN/C6813, Gly-651 to Glu-678 in BN/D) have 91% similarity (50 to 51% identity) to the ion channel-forming motif of botulinum neurotoxin type A, Gly-Ala-Val-Ile-Leu-Leu-Glu-Phe-Ile-Pro-Glu-Ile-Ala-Ile-Pro-Val-Leu-Gly-Thr-Phe-Ala-Leu-Val (17). The N-terminal half of the heavy chain of BN/Dsa might also be quite similar to those of BN/C1, BN/D, and BN/C6813, because the nucleotide sequence of the PCR product from Dsa amplified by primer pair YQA/LDV has 99% identity to that of the product from C6813 amplified

```
                                    (-145)attgtataacattttcatataatga
taaatatttttctctatcacttagagatggagattataattggatgatatgtaatgacaa
taacaaggtgcctaaaggcgcacatttgtggatattagaaagttaggagatgttagtatt
          *

       10        20        30        40        50        60
atgccaataacaattaacaactttaattattcagatcctgttgataataaaaatatttta
M  P  I  T  I  N  N  F  N  Y  S  D  P  V  D  N  K  N  I  L
       70        80        90       100       110       120
tatttagatactcatttaaatacattagctaatgagcctgaaaaagcctttcgcattata
Y  L  D  T  H  L  N  T  L  A  N  E  P  E  K  A  F  R  I  I
      130       140       150       160       170       180
gggaatatatgggtaatacccgatagattttcaagagattctaatccaaatttaaataaa
G  N  I  W  V  I  P  D  R  F  S  R  D  S  N  P  N  L  N  K
      190       200       210       220       230       240
cctcctcgagttacaagccctaaaagtggttattatgatcctaattatttgagtactgat
P  P  R  V  T  S  P  K  S  G  Y  Y  D  P  N  Y  L  S  T  D
      250       260       270       280       290       300
tctgaaaaagatacatttttaaaagaaattataaagttatttaaaagaattaactctaga
S  E  K  D  T  F  L  K  E  I  I  K  L  F  K  R  I  N  S  R
      310       320       330       340       350       360
gaaataggagaagaattaatatatagacttgcaacagacatacccttttcctgggaataac
E  I  G  E  E  L  I  Y  R  L  A  T  D  I  P  F  P  G  N  N
      370       380       390       400       410       420
aatactccaattaatacttttgattttgatgtagattttaacagtgttgatgttaaaact
N  T  P  I  N  T  F  D  F  D  V  D  F  N  S  V  D  V  K  T
      430       440       450       460       470       480
agacaaggtaacaactgggttaaaactggtagtataaatcctagtgttataataactgga
R  Q  G  N  N  W  V  K  T  G  S  I  N  P  S  V  I  I  T  G
      490       500       510       520       530       540
cctagagaaaacattatagacccagaaacttctacgtttaaattaactaacaatactttt
P  R  E  N  I  I  D  P  E  T  S  T  F  K  L  T  N  N  T  F
      550       560       570       580       590       600
gcggcacaagaaggatttggtgctttatcaataatttcaatatcacctagatttatgcta
A  A  Q  E  G  F  G  A  L  S  I  I  S  I  S  P  R  F  M  L
      610       620       630       640       650       660
acatatagtaatgcaactaataatgtaggagagggtagatttctaagtctgaattttgc
T  Y  S  N  A  T  N  N  V  G  E  G  R  F  S  K  S  E  F  C
      670       680       690       700       710       720
atggatccaatactaattttaatgcatgaacttaatcatacaatgcataatttatatgga
M  D  P  I  L  I  L  M  H  E  L  N  H  T  M  H  N  L  Y  G
      730       740       750       760       770       780
atagctataccaaatgatcaaagaatttcatctgtaactagtaatatttttattctcaa
I  A  I  P  N  D  Q  R  I  S  S  V  T  S  N  I  F  Y  S  Q
      790       800       810       820       830       840
tataaggtgaaattagagtatgcagaaatatatgcatttggaggtccaactatagacctt
Y  K  V  K  L  E  Y  A  E  I  Y  A  F  G  G  P  T  I  D  L
      850       860       870       880       890       900
attcctaaaagtggaaggaaatattttgaggaaaaggcattggattattatagatccata
I  P  K  S  G  R  K  Y  F  E  E  K  A  L  D  Y  Y  R  S  I
      910       920       930       940       950       960
gctaaaagacttaatagtataactactgcaaatccttcaagctttaataaatatatagga
A  K  R  L  N  S  I  T  T  A  N  P  S  S  F  N  K  Y  I  G
      970       980       990      1000      1010      1020
gaatataaacagaaacttattagaaagtatagattcgtagtagaatcttcaggtgaagtt
E  Y  K  Q  K  L  I  R  K  Y  R  F  V  V  E  S  S  G  E  V
     1030      1040      1050      1060      1070      1080
gcagtagatcgtaataagtttgctgagttatataaagaacttacacaaatatttacagaa
A  V  D  R  N  K  F  A  E  L  Y  K  E  L  T  Q  I  F  T  E
     1090      1100      1110      1120      1130      1140
tttaactacgctaaaatatataatgtacaaaataggaaaatatatctttcaaatgtatat
F  N  Y  A  K  I  Y  N  V  Q  N  R  K  I  Y  L  S  N  V  Y
     1150      1160      1170      1180      1190      1200
actccggttacggcaaatatattagacgataatgtttatgatatacaaaatggatttaac
T  P  V  T  A  N  I  L  D  D  N  V  Y  D  I  Q  N  G  F  N
     1210      1220      1230      1240      1250      1260
atacctaaaagtaatttaaatgtactatttatgggtcaaaatttatctcgaaatccagca
I  P  K  S  N  L  N  V  L  F  M  G  Q  N  L  S  R  N  P  A
     1270      1280      1290      1300      1310      1320
ttaagaaaagtcaatcctgaaaatatgctttatttatttacaaaattttgccataaagca
L  R  K  V  N  P  E  N  M  L  Y  L  F  T  K  F  C  H  K  A
     1330      1340      1350      1360      1370      1380
atagatggtagatcattatataataaaacattagattgtagagagcttttagttaaaaat
I  D  G  R  S  L  Y  N  K  T  L  D  C  R  E  L  L  V  K  N
     1390      1400      1410      1420      1430      1440
actgacttaccctttataggtgatattagtgatatcaaaactgatatatttttaagcaaa
T  D  L  P  F  I  G  D  I  S  D  I  K  T  D  I  F  L  S  K
     1450      1460      1470      1480      1490      1500
gatattaatgttgaaactgaagttatagactatccggacaatgtttcagtggatcaagtt
D  I  N  V  E  T  E  V  I  D  Y  P  D  N  V  S  V  D  Q  V
     1510      1520      1530      1540      1550      1560
attctcagtaagaatacctcagaacatggacaactagatttattataccctattattgaa
I  L  S  K  N  T  S  E  H  G  Q  L  D  L  L  Y  P  I  I  E
     1570      1580      1590      1600      1610      1620
ggtgagagtcaagtattaccgggagagaatcaagtcttttatgataatagaactcaaaat
G  E  S  Q  V  L  P  G  E  N  Q  V  F  Y  D  N  R  T  Q  N
     1630      1640      1650      1660      1670      1680
gttgattatttgaattcttattattacctagaatctcaaaaactaagtgataatgttgaa
V  D  Y  L  N  S  Y  Y  Y  L  E  S  Q  K  L  S  D  N  V  E
     1690      1700      1710      1720      1730      1740
gattttacttttacgacatcaattgaggaagctttggataatagtggaaaagtatatact
D  F  T  F  T  T  S  I  E  E  A  L  D  N  S  G  K  V  Y  T
     1750      1760      1770      1780      1790      1800
tactttcctaaactagctgataaagtaaatacgggtgttcaaggtggtttatttttaatg
Y  F  P  K  L  A  D  K  V  N  T  G  V  Q  G  G  L  F  L  M
     1810      1820      1830      1840      1850      1860
tgggcaaatgatgtagttgaagattttactacaaatattctaagaaaagatacattagat
W  A  N  D  V  V  E  D  F  T  T  N  I  L  R  K  D  T  L  D
     1870      1880      1890      1900      1910      1920
aaaatatcagatgtatcagctattattccctatataggacctgcattaaatataagtaat
K  I  S  D  V  S  A  I  I  P  Y  I  G  P  A  L  N  I  S  N
     1930      1940      1950      1960      1970      1980
tctgtaagaagggaaaattttactgaagcatttgcagttaccggtgtaactattttatta
S  V  R  R  E  N  F  T  E  A  F  A  V  T  G  V  T  I  L  L
     1990      2000      2010      2020      2030      2040
gaagcgtttcaagaatttacaatacctgcacttggtgcatttgtgatttatagtaaggtt
E  A  F  Q  E  F  T  I  P  A  L  G  A  F  V  I  Y  S  K  V
     2050      2060      2070      2080      2090      2100
caagaaagaaacgagattattaaaactatagataattgtttagaacaaaggattaaaaga
Q  E  R  N  E  I  I  K  T  I  D  N  C  L  E  Q  R  I  K  R
     2110      2120      2130      2140      2150      2160
tggaaagattcatatgaatggatgataggaacgtggttatccaggattactactcaattt
W  K  D  S  Y  E  W  M  I  G  T  W  L  S  R  I  T  T  Q  F
     2170      2180      2190      2200      2210      2220
aataatataagttatcaaatgtatgattctttaaattatcaggcagatgcaatcaaagat
N  N  I  S  Y  Q  M  Y  D  S  L  N  Y  Q  A  D  A  I  K  D
     2230      2240      2250      2260      2270      2280
aaaatagatttagaatataaaaaatactcaggaagtgataaagaaaatataaaaagtcaa
K  I  D  L  E  Y  K  K  Y  S  G  S  D  K  E  N  I  K  S  Q
     2290      2300      2310      2320      2330      2340
gttgaaaatttaaaaaatagtttagatataaaaatctcggaagcaatgaataatataaat
V  E  N  L  K  N  S  L  D  I  K  I  S  E  A  M  N  N  I  N
     2350      2360      2370      2380      2390      2400
aaatttatacgagaatgttctgtaacatacttatttaaaaatatgctccctaaagtaatt
K  F  I  R  E  C  S  V  T  Y  L  F  K  N  M  L  P  K  V  I
     2410      2420      2430      2440      2450      2460
gatgaattaaataagtttgatttaaaaactaaaacagaattaattaatcttatagatagt
D  E  L  N  K  F  D  L  K  T  K  T  E  L  I  N  L  I  D  S
     2470      2480      2490      2500      2510      2520
cataatatcattctagttggtgaagtagatagattaaaagcaaaagtaaatgagagtttt
H  N  I  I  L  V  G  E  V  D  R  L  K  A  K  V  N  E  S  F
     2530      2540      2550      2560      2570      2580
gaaaatacaataccctttaatatttttcatatactaataattctttattaaaagatata
E  N  T  I  P  F  N  I  F  S  Y  T  N  N  S  L  L  K  D  I
     2590      2600      2610      2620      2630      2640
attaatgaatatttcaatagtattaatgattcaaaaattttgagcttacaaaacaaaaaa
I  N  E  Y  F  N  S  I  N  D  S  K  I  L  S  L  Q  N  K  K
     2650      2660      2670      2680      2690      2700
aatgctttagtggatacatcaggatataatgcagaagtgaggctagaaggtgatgttcaa
N  A  L  V  D  T  S  G  Y  N  A  E  V  R  L  E  G  D  V  Q
     2710      2720      2730      2740      2750      2760
gttaatacgatatatacaaatgattttaaattaagtagttcaggagataaaattatagta
V  N  T  I  Y  T  N  D  F  K  L  S  S  S  G  D  K  I  I  V
     2770      2780      2790      2800      2810      2820
aatttaaataataatatttttatatagcgctatttatgagaactctagtgttagtttttgg
N  L  N  N  N  I  L  Y  S  A  I  Y  E  N  S  S  V  S  F  W
     2830      2840      2850      2860      2870      2880
attaagatatctaaagatttaactaattctcataatgaatatacaataattaatagtata
I  K  I  S  K  D  L  T  N  S  H  N  E  Y  T  I  I  N  S  I
     2890      2900      2910      2920      2930      2940
aaacaaaattctgggtggaaattatgtattaggaatggcaatatagaatggattttacaa
K  Q  N  S  G  W  K  L  C  I  R  N  G  N  I  E  W  I  L  Q
     2950      2960      2970      2980      2990      3000
gatattaatagaaagtataaaagtttaattttgattatagtgaatcattaagtcataca
D  I  N  R  K  Y  K  S  L  I  F  D  Y  S  E  S  L  S  H  T
     3010      3020      3030      3040      3050      3060
ggatatacaaataaatggtttttgttactataactaataatataatggggtatatgaaa
G  Y  T  N  K  W  F  F  V  T  I  T  N  N  I  M  G  Y  M  K
     3070      3080      3090      3100      3110      3120
ctttatataaatggagaattaaagcagagtgaaagaattgaagatttaaatgaggttaag
L  Y  I  N  G  E  L  K  Q  S  E  R  I  E  D  L  N  E  V  K
     3130      3140      3150      3160      3170      3180
ttagataaaaccatagtatttggaatagatgagaatatagatgagaatcagatgctttgg
L  D  K  T  I  V  F  G  I  D  E  N  I  D  E  N  Q  M  L  W
     3190      3200      3210      3220      3230      3240
attagagattttaatatttttctaaagaattaagcaatgaagatattaatattgtatat
I  R  D  F  N  I  F  S  K  E  L  S  N  E  D  I  N  I  V  Y
     3250      3260      3270      3280      3290      3300
gagggacaaatattaagaaatgttattaaagattattggggaaatcctttgaagtttgat
E  G  Q  I  L  R  N  V  I  K  D  Y  W  G  N  P  L  K  F  D
     3310      3320      3330      3340      3350      3360
acagaatattatattattaatgataattatatagataggtatatagcacctaaaagtaat
T  E  Y  Y  I  I  N  D  N  Y  I  D  R  Y  I  A  P  K  S  N
     3370      3380      3390      3400      3410      3420
atacttgtacttgttcagtatccagatagatctaaattatatactggaaatcctattact
I  L  V  L  V  Q  Y  P  D  R  S  K  L  Y  T  G  N  P  I  T
     3430      3440      3450      3460      3470      3480
attaaatcagtatctgataagaatccttatagtagaattttaaatggagataatataatg
I  K  S  V  S  D  K  N  P  Y  S  R  I  L  N  G  D  N  I  M
     3490      3500      3510      3520      3530      3540
tttcatatgttatataatagtgggaaatatatgataataagagatactgatacaatatat
F  H  M  L  Y  N  S  G  K  Y  M  I  I  R  D  T  D  T  I  Y
     3550      3560      3570      3580      3590      3600
gcaatagaaggaagagagtgttcaaaaaattgtgtatatgcattaaaattacagagtaat
A  I  E  G  R  E  C  S  K  N  C  V  Y  A  L  K  L  Q  S  N
     3610      3620      3630      3640      3650      3660
ttaggtaattatggtataggtatatttagtataaaaaatattgtatctcaaaataaatat
L  G  N  Y  G  I  G  I  F  S  I  K  N  I  V  S  Q  N  K  Y
     3670      3680      3690      3700      3710      3720
tgtagtcaaattttctctagttttatgaaaaatacaatgcttctagcagatatatataaa
C  S  Q  I  F  S  S  F  M  K  N  T  M  L  L  A  D  I  Y  K
     3730      3740      3750      3760      3770      3780
ccttggagattttcttttgaaaatgcatacacgccagttgcagtaactaattatgagaca
P  W  R  F  S  F  E  N  A  Y  T  P  V  A  V  T  N  Y  E  T
     3790      3800      3810      3820      3830      3840
aaactattatcaacttcatcttttggaaatttatttctagggatccaggatgggtagag
K  L  L  S  T  S  S  F  W  K  F  I  S  R  D  P  G  W  V  E
     3850      3860      3870      3880      3890
taatacaataaaaatttaatataaactattaaattatattacaagttttggaaattta
*
```

FIG. 4. Nucleotide sequence and deduced amino acid sequence of the BN/C6813 gene. The deduced amino acid sequence is shown under the nucleotide sequence. The dot underline (aggaga near the top, left column) and asterisks indicate a ribosome binding site and the termination codon, respectively.

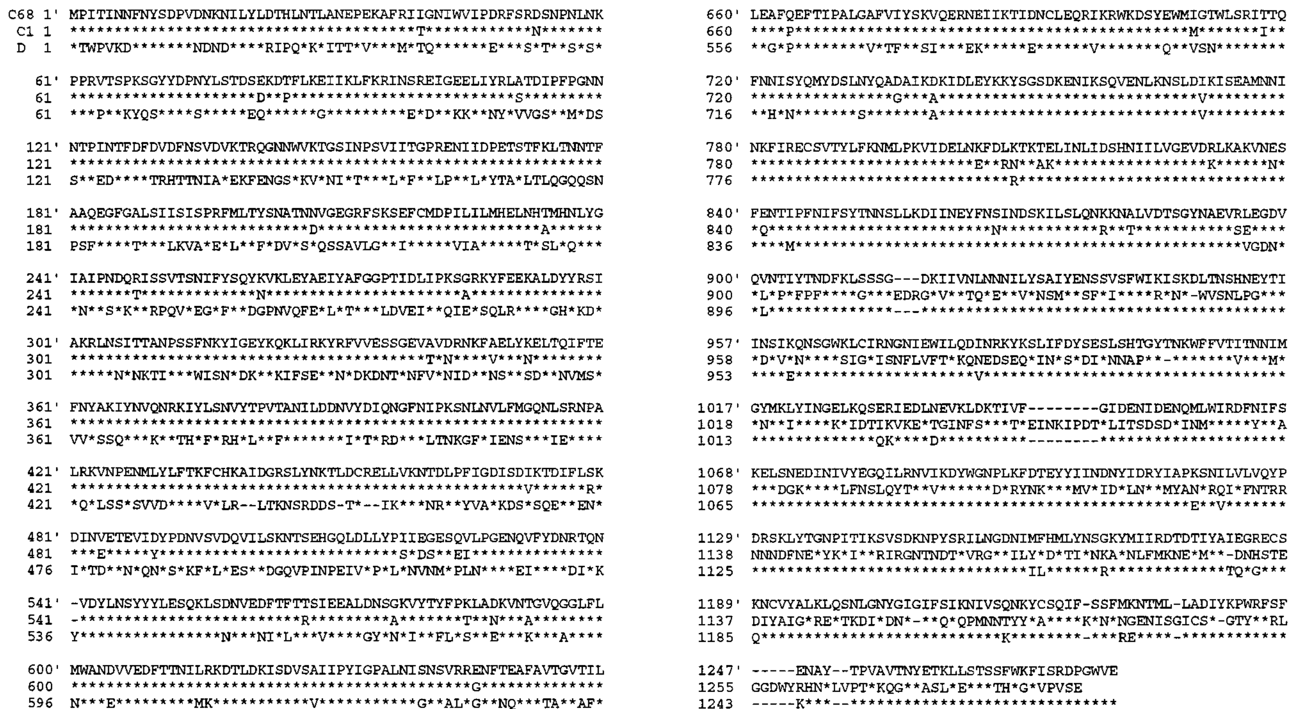

FIG. 5. Comparison of the amino acid sequence of BN/C6813 with that of BN/C1 or BN/D. Dashes and asterisks denote gaps and homologous residues, respectively. The sequences of BN/C1 and BN/D are as cited in reports by Kimura et al. (12) and Binz et al. (2), respectively.

by primer pair YQA/LDV (data not shown). The core regions of neurotoxins from strains of *C. botulinum* types C and D may exhibit similar channel-forming abilities.

BN/C1 could bind to a receptor different from the receptor for BN/D on the target cells (32). A monoclonal antibody recognizing the sequence within the C-terminal 50 amino acids of the BN/C1 heavy chain could inhibit the binding of the whole toxin to neuroblastoma cells, suggesting that the binding domain of the target cell is located on the C-terminal 50 amino acids of the heavy chain (13). BN/C6813 might not bind to the receptor for BN/C1, because the C-terminal 50 amino acids of BN/C6813 were quite different from those of BN/C1 but were the same as those of BN/D. Thus, BN/C6813 might bind to the receptor for BN/D.

A *C. botulinum* type C organism has been known to produce either of two types of ADP-ribosyltransferase C3: the D1873 type or the Dsa type (19). both isoforms have 66% amino acid homology. Although type C strains 468 and Stockholm could produce BN/C1 and D1873-type C3, type C strains 6812, 6813, and 6814 could produce BN/C6813 and Dsa-type C3. A C3 gene and a neurotoxin gene from a type C organism have been shown to be located on a toxigenic bacteriophage (19). Hauser et al. (11) suggested that a 21.5-kbp DNA fragment carrying the C3 gene in C468, Cst, and D1873 phage might be a putative transposable element. However, the mechanisms of the construction of mosaic neurotoxin genes from type C and D organisms have not been clarified. Further analyses of several genetic events involved in the evolution and construction of the botulinum neurotoxin gene are necessary for exact diagnosis and effective treatment of botulism.

TABLE 2. Detection of PCR products from *C. botulinum* type C and D organisms

Template	Detection of PCR product after amplification with primer pair[a]						
	C1/C2	C3/C4	C5/C6	YQA/LDV	LVD2/RGN	LDV2/SVS	C9/C10
C6812	+	+	+	+	−	+	+
C6813	+	+	+	+	−	+	+
C6814	+	+	+	+	−	+	+
Dsa	−	−	−	+	+	−	−

[a] +, a DNA fragment of the expected size in the reaction mixture after the PCR could be detected on an agarose gel stained with ethidium bromide; −, no DNA fragment of the expected size in the PCR mixture could be detected on an agarose gel stained with ethidium bromide, even if the PCR cycle was repeated after addition of *Taq* DNA polymerase to the mixture.

ACKNOWLEDGMENTS

We thank Masashi Tatsumi for helpful discussion.

This work was supported by a grant-in-aid (no. 06760270) for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. **Binz, T., J. Blasi, S. Yamasaki, A. Baumeister, E. Link, T. C. Sudhof, R. Jahn, and H. Niemann.** 1994. Proteolysis of SNAP-25 by types E and A botulinal neurotoxins. J. Biol. Chem. **269:**1617–1620.
2. **Binz, T., H. Kurazono, M. R. Popoff, M. W. Eklund, G. Sakaguchi, S. Kozaki, K. Krieglstein, A. Henschen, D. M. Gill, and H. Niemann.** 1990. Nucleotide sequence of the gene encoding *Clostridium botulinum* neurotoxin type D. Nucleic Acids Res. **18:**5556.
3. **Binz, T., H. Kurazono, M. Wille, J. Frevert, K. Wernars, and H. Niemann.** 1990. The complete sequence of botulinum neurotoxin type A and comparison with other clostridial neurotoxins. J. Biol. Chem. **265:**9153–9158.
4. **Blasi, J., E. R. Chapman, S. Yamasaki, T. Binz, H. Niemann, and R. Jahn.** 1993. Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. EMBO J. **12:**4821–4828.
5. **Blaustein, R. O., W. J. Germann, A. Finkelstein, and B. R. DasGupta.** 1987. The N-terminal half of the heavy chain of botulinum type A neurotoxin forms channels in planar phospholipid bilayers. FEBS Lett. **226:**115–120.
6. **Campbell, K., M. D. Collins, and A. K. East.** 1993. Nucleotide sequence of the gene coding for *Clostridium botulinum* (*Clostridium argentinense*) type G neurotoxin: genealogical comparison with other clostridial neurotoxins. Biochim. Biophys. Acta **1216:**487–491.

7. **Donovan, J. J., and J. L. Middlebrook.** 1986. Ion-conducting channels produced by botulinum toxin in planar lipid membranes. Biochemistry **25:**2872–2876.
8. **East, A. K., P. T. Richardson, D. Allaway, M. D. Collins, T. A. Roberts, and D. E. Thompson.** 1992. Sequence of the gene encoding type F neurotoxin of *Clostridium botulinum*. FEMS Microbiol. Lett. **75:**225–230.
9. **Fujii, N., K. Kimura, N. Yokosawa, K. Tsuzuki, and K. Oguma.** 1992. A zinc-protease specific domain in botulinum and tetanus neurotoxins. Toxicon **30:**1486–1488.
10. **Hauser, D., M. W. Eklund, H. Kurazono, T. Binz, H. Niemann, D. M. Gill, P. Boquet, and M. R. Popoff.** 1990. Nucleotide sequence of *Clostridium botulinum* C1 neurotoxin. Nucleic Acids Res. **18:**4924.
11. **Hauser, D., M. Gibert, M. W. Eklund, P. Boquet, and M. R. Popoff.** 1993. Comparative analysis of C3 and botulinal neurotoxin genes and their environment in *Clostridium botulinum* types C and D. J. Bacteriol. **175:**7260–7268.
12. **Kimura, K., N. Fujii, K. Tsuzuki, T. Murakami, T. Indoh, N. Yokosawa, K. Takeshi, B. Syuto, and K. Oguma.** 1990. The complete nucleotide sequence of the gene coding for botulinum type C1 toxin in the C-ST phage genome. Biochem. Biophys. Res. Commun. **171:**1304–1311.
13. **Kimura, K., N. Fujii, K. Tsuzuki, N. Yokozawa, and K. Oguma.** 1991. The functional domains of *Clostridium botulinum* type C neurotoxin, p. 375–385. *In* 10th World Congress on Animal, Plant and Microbial Toxins 1991. International University of Singapore, Singapore.
14. **Kurazono, H., S. Mochida, T. Binz, U. Eisel, M. Quanz, O. Grebenstein, K. Wernars, B. Poulain, L. Tauc, and H. Niemann.** 1992. Minimal essential domains specifying toxicity of the light chains of tetanus toxin and botulinum neurotoxin type A. J. Biol. Chem. **267:**14721–14729.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
16. **Marchuk, D., M. Drumm, A. Saulino, and S. Collins.** 1991. Construction of T-vector, a rapid and general system for direct cloning of unmodified PCR products. Nucleic Acids Res. **19:**1154.
17. **Montal, M. S., R. Blewitt, J. M. Tomich, and M. Montal.** 1992. Identification of an ion channel-forming motif in the primary structure of tetanus and botulinum neurotoxins. FEBS Lett. **313:**12–18.
18. **Moriishi, K., B. Syuto, S. Kubo, and K. Oguma.** 1989. Molecular diversity of neurotoxins from *Clostridium botulinum* type D strains. Infect. Immun. **57:**2886–2891.
19. **Moriishi, K., B. Syuto, M. Saito, K. Oguma, N. Fujii, N. Abe, and M. Naiki.** 1993. Two different types of ADP-ribosyltransferase C3 from *Clostridium botulinum* type D lysogenized organisms. Infect. Immun. **61:**5309–5314.
20. **Oguma, K., S. Murayama, B. Syuto, H. Iida, and S. Kubo.** 1984. Analysis of antigenicity of *Clostridium botulinum* type C_1 and D toxins by polyclonal and monoclonal antibodies. Infect. Immun. **43:**584–588.
21. **Schiavo, G., F. Benfenati, B. Poulain, O. Rossetto, P. Polverino-de-Laureto, B. R. DasGupta, and C. Montecucco.** 1992. Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature (London) **359:**832–835.
22. **Schiavo, G., C. Malizio, W. S. Trimble, P. Polverino-de-Laureto, G. Milan, H. Sugiyama, E. A. Johnson, and C. Montecucco.** 1994. Botulinum G neurotoxin cleaves VAMP/synaptobrevin at a single Ala-Ala peptide bond. J. Biol. Chem. **269:**20213–20216.
23. **Schiavo, G., O. Rossetto, A. Santucci, B. R. DasGupta, and C. Montecucco.** 1992. Botulinum neurotoxins are zinc proteins. J. Biol. Chem. **267:**23479–23483.
24. **Shone, C. C., P. Hambleton, and J. Melling.** 1987. A 50-kDa fragment from the NH_2-terminus of the heavy subunit of *Clostridium botulinum* type A neurotoxin forms channels in lipid vesicles. Eur. J. Biochem. **167:**175–180.
25. **Simpson, L. L.** 1986. Molecular pharmacology of botulinum toxin and tetanus toxin. Annu. Rev. Pharmacol. Toxicol. **26:**427–453.
26. **Sudhof, T. C., P. D. Camilli, H. Niemann, and R. Jahn.** 1993. Membrane fusion machinery: insights from synaptic proteins. Cell **75:**1–4.
27. **Sunagawa, H., T. Ohyama, T. Watanabe, and K. Inoue.** 1992. The complete amino acid sequence of the *Clostridium botulinum* type D neurotoxin, deduced by nucleotide sequence analysis of the encoding phage d-16 phi genome. J. Vet. Med. Sci. **54:**905–913.
28. **Terajima, J., B. Syuto, J. O. Ochanda, and S. Kubo.** 1985. Purification and characterization of neurotoxin produced by *Clostridium botulinum* type C 6813. Infect. Immun. **48:**312–317.
29. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, T. Atkinson, and N. P. Minton.** 1992. The complete amino acid sequence of the *Clostridium botulinum* type-E neurotoxin, derived by nucleotide-sequence analysis of the encoding gene. Eur. J. Biochem. **204:**657–667.
30. **Whelan, S. M., M. J. Elmore, N. J. Bodsworth, J. K. Brehm, T. Atkinson, and N. P. Minton.** 1992. Molecular cloning of the *Clostridium botulinum* structural gene encoding the type B neurotoxin and determination of its entire nucleotide sequence. Appl. Environ. Microbiol. **58:**2345–2354.
31. **Yamasaki, S., A. Baumeister, T. Binz, J. Blasi, E. Link, F. Cornille, B. Roques, E. M. Fykse, T. C. Sudhof, R. Jahn, et al.** 1994. Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. J. Biol. Chem. **269:**12764–12772.
32. **Yokosawa, N., K. Tsuzuki, B. Syuto, N. Fujii, K. Kimura, and K. Oguma.** 1991. Binding of botulinum type C1, D and E neurotoxins to neuronal cell lines and synaptosomes. Toxicon **29:**261–264.