APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2004, p. 7192–7199 Vol. 70, No. 12
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.12.7192–7199.2004

Differentiation of the Gene Clusters Encoding Botulinum Neurotoxin Type A Complexes in *Clostridium botulinum* Type A, Ab, and A(B) Strains

Giovanna Franciosa, Francesca Floridi, Antonella Maugliani, and Paolo Aureli*

Centro Nazionale per la Qualità degli Alimenti e per i Rischi Alimentari, Reparto Pericoli Microbiologici Connessi agli Alimenti, Istituto Superiore della Sanità, Rome, Italy

Received 16 April 2004/Accepted 12 July 2004

We describe a strategy to identify the clusters of genes encoding components of the botulinum toxin type A (boNT/A) complexes in 57 strains of *Clostridium botulinum* types A, Ab, and A(B) isolated in Italy and in the United States from different sources. Specifically, we combined the results of PCR for detecting the *ha33* and/or *p47* genes with those of *boNT/A* PCR-restriction fragment length polymorphism analysis. Three different type A toxin gene clusters were revealed; type A1 was predominant among the strains from the United States, whereas type A2 predominated among the Italian strains, suggesting a geographic distinction between strains. By contrast, no relationship between the toxin gene clusters and the clinical or food source of strains was evident. In two *C. botulinum* type A isolates from the United States, we recognized a third type A toxin gene cluster (designated type A3) which was similar to that previously described only for *C. botulinum* type A(B) and Ab strains. Total genomic DNA from the strains was subjected to pulsed-filed gel electrophoresis and randomly amplified polymorphic DNA analyses, and the results were consistent with the *boNT/A* gene clusters obtained.

Botulinum neurotoxin (boNT) type A (boNT/A) is one of the seven (A to G) immunologically distinct toxins produced by *Clostridium botulinum*. The toxin may be synthesized by the microorganism in foods, under peculiar conditions, or directly in the human body. The ingestion of foods contaminated with preformed boNT causes food-borne botulism, whereas the absorption of boNT from the body site of synthesis causes either intestinal toxemia botulism (referred to as infant botulism in infants and infant-like botulism in adults) or wound botulism, depending on the route of entry of the boNT-producing clostridia.

All forms of botulism disease are characterized by flaccid paralysis of peripheral muscles resulting from the specific action of boNT at the neuromuscular junctions (16). boNT/A is the serotype most frequently involved in human botulism in the United States (3) and the second most common cause of botulism in Italy and Europe (36). boNT/A is also an effective therapeutic drug against a wide variety of involuntary muscle disorders and for pain relief, and it has been successfully used for aesthetic treatments (20).

The high potency and oral toxicity of boNT have been attributed to different factors, including the fact that it is synthesized by botulinum neurotoxigenic clostridia in combination with one or more associated nontoxic proteins as stable complexes (27). The nontoxic proteins include a nontoxic nonhemagglutinating (NTNH) protein and four hemagglutinin (HA) proteins with different molecular masses (~54, 33, 20, and 14 kDa) (21). Three forms of boNT complexes are produced by clostridia, with the form depending on the serotype or strain (27): the medium (M) complex (~270 kDa), which consists of the boNT protein (~150 kDa) and NTNH protein (~120 kDa); the large (L) complex (~400 kDa), consisting of boNT, NTNH, and all four HA proteins; and the extra-large (LL) complex (~900 kDa), consisting of 2 L subunits. Certain strains of *C. botulinum* type A produce the LL complex together with L and M complexes (19, 31); these strains are referred to as type A1. Other strains of *C. botulinum* type A that secrete the M complex only, with no HA proteins, are designated type A2; these strains have been predominantly described in Japan (5, 29, 35).

Genes encoding components of the boNT/A1 and boNT/A2 complexes are arranged in clusters which vary in structure and organization (Fig. 1) (21); three genes (*ha33*, *ha17*, and *ha70*) encoding the HA proteins are present in type A1 genetic clusters, whereas these genes are absent from type A2 gene clusters. On the other hand, a *p47* gene, whose product plays an as-yet-undetermined role, although it is believed to be a regulatory one (23), is present only in type A2 gene clusters. Furthermore, the comparison of DNA sequences between *boNT/A1* genes from various sources (GenBank accession numbers AF488749, AF461540, M30196, and X52066) (2, 6, 37, 39) and the *boNT/A2* gene from the Kyoto-F strain isolated in Japan (GenBank accession number X73423) (38) has revealed a 94% sequence homology between *boNT/A1* and *boNT/A2* genes, which is lower than the 99% sequence homology observed among *boNT/A1* gene sequences. Other differences between A1 and A2 genetic clusters include the orientation and position of the positive regulatory gene *botR/A* (26), and the sequence of the NTNH gene (24).

The structure of the toxin gene clusters encoding components of the boNT complexes has also been elucidated for several strains of *C. botulinum* type A harboring a *bont/B* gene in their genome which is either expressed (*C. botulinum* Ab) or unexpressed [*C. botulinum* A(B)] (18, 28). These strains share

* Corresponding author. Mailing address: Centro Nazionale per la Qualità degli Alimenti e per i Rischi Alimentari, Reparto Pericoli Microbiologici Connessi agli Alimenti, Istituto Superiore della Sanità, Viale Regina Elena, 299, 00161 Rome, Italy. Phone: 39 06 4990 3420. Fax: 39 06 4938 7101. E-mail: p.aureli@iss.it.

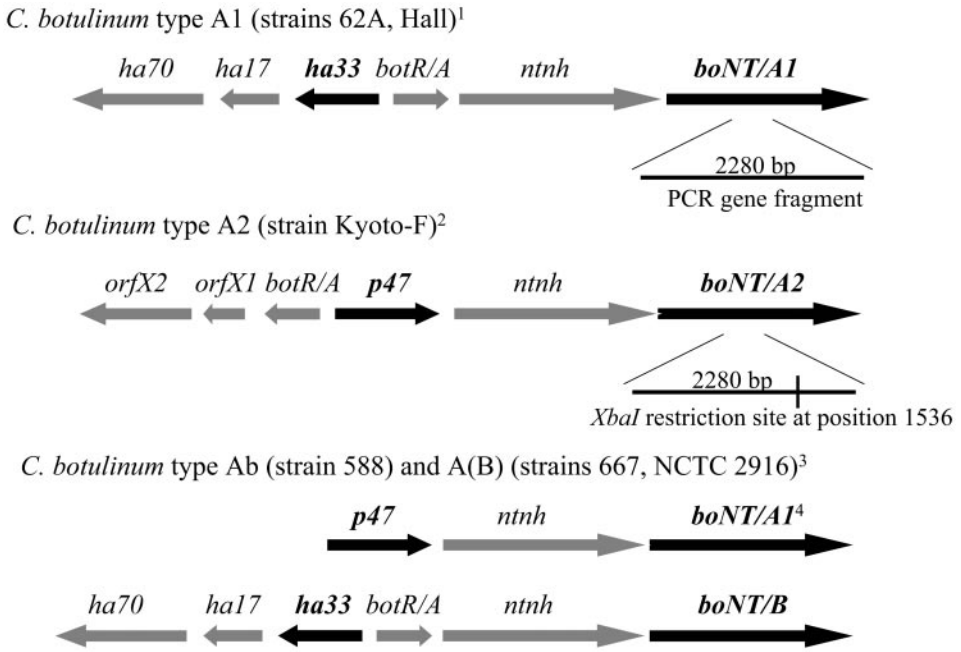

FIG. 1. Schematic representation of type A toxin gene clusters of *C. botulinum* types A, A(B), and Ab. Genes in boldface type are those selected for PCR amplification in our strategy to identify the type A gene clusters. The computer-predicted XbaI restriction site in the *boNT/A2* PCR gene fragment is shown. [1]See references 6 and 39; [2]see reference 6; [3]see references 7, 18, and 28; [4]strain of *C. botulinum* type Ab from Japan, for which only a few segments of the *boNT/A* gene have been sequenced, has shown a *boNT/A2* gene sequence (14).

the same *boNT* gene cluster organization (Fig. 1); the HA genes are in the *bont/B* gene cluster, whereas the *p47* gene is in the *bont/A* gene cluster, as in type A2 strains. However, the *C. botulinum* Ab and A(B) strains studied to date display a *bont/A1* gene sequence (5, 7, 18, 28), with the possible exception of an Ab strain from Japan for which three segments of the *boNT/A* gene have been sequenced and found to be closely related to the *boNT/A2* gene from the Kyoto-F strain (14).

Information on *boNT/A* gene clusters is available for only a limited number of strains. In the present work, we describe the identification of *boNT/A* gene clusters in 57 strains of *C. botulinum* types A, Ab, and A(B) with different origins through the PCR detection of *ha33* and *p47* genes and analysis of *boNT/A* gene polymorphisms. Differentiation of the *boNT/A* gene clusters correlated well with the results of strain genotyping through pulsed-field gel electrophoresis (PFGE) and randomly amplified polymorphic DNA (RAPD) analysis.

MATERIALS AND METHODS

***Clostridium* isolates and growth conditions.** A total of 57 *C. botulinum* strains were used in this study (Table 1). Thirty-seven strains were from the culture collection of the Istituto Superiore della Sanità (Italy's National Health Institute), Rome, Italy, including 17 clinical isolates representing all of the clinical isolates from type A botulism in Italy since 1993 and 20 food isolates. Seven of the clinical isolates were from patients involved in an outbreak of food-borne botulism in Italy caused by the consumption of contaminated "mascarpone" cheese (1); 18 of the food isolates were from mascarpone cheese samples and other cheese products analyzed during that outbreak (12). The remaining 20 strains, including reference strain 62A, had been kindly provided by C. L. Hatheway of the Botulism Laboratory of the Centers for Disease Control and Prevention, Atlanta, Ga.

All strains had been shown to produce boNT/A at the time of isolation. Some of the isolates had also been previously characterized for the presence of *boNT* genes through PCR; of these strains, nine had been shown to contain, in addition to the *boNT/A* gene, a *boNT/B* gene in their genome which was either silent [*C. botulinum* A(B)] (CDC-2171, CDC-4893, CDC-5277, CDC-5178, CDC-3517, and CDC-1727) (9) or expressed (*C. botulinum* Ab) (CDC-588, CDC-1436, and CL87) (8, 9, 10).

Clostridium stock cultures were checked for purity on egg yolk agar plates (Oxoid, Basingstoke, England). Single colonies from the plates were transferred to TPGY broth (5% Trypticase, 0.5% peptone, 0.4% glucose, 2% yeast extract, 0.1% sodium thioglycollate [pH 7.0]) and grown overnight at 37°C under conditions of anaerobiosis (GasPack jars; Oxoid). One milliliter from each broth culture was centrifuged and washed with 1× TE buffer (10 mM Tris [pH 7.4], 1 mM EDTA), and the pellets were resuspended in 1 ml of sterile distilled water.

PCR assays. *boNT/A* and *boNT/B* gene fragments of 2,280 and 1,284 bp, respectively, were detected through specific PCRs, as reported elsewhere previously (9). The primers H1 and H2 for the amplification of a 774-bp region of the *ha33* gene were the same as those described by East et al. (7). Primers p47-A (5′-ACTTATGGTTGGGATATTGTTT-3′) and p47-B (5′-TATATTCTCACC TTCTTTAATTTGC-3′) were selected on the basis of the *p47* gene sequence from the Japanese strain Kyoto-F (GenBank accession number X96493) to obtain a 1,236-bp PCR product with the aid of the Primer 3 program, which is available online (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi).

PCRs with each primer pair were carried out separately in a programmable thermal cycler (model PTC100; MJ Research, Waltham, Mass.). For *ha33* and *p47* gene fragment amplifications, a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.2 mM each deoxyribonucleoside triphosphate (100 mM) (Amersham Pharmacia Biotech, Piscataway, N.J.), 0.5 μM primers, and 5 μl of cell suspensions was used; mixtures were preheated in the thermal cyclers at 99°C for 10 min to improve the cell lysis, and then 1.25 U of *Taq* polymerase (Applied Biosystems, Foster City, Calif.) was added. The cycling conditions were as follows: 35 cycles at 95°C for 45 s, 58°C for 45 s, and 72°C for 45 s, followed by a final extension phase at 72°C for 10 min for the amplification of the *ha33* gene fragment and 35 cycles consisting of 1 min at 95°C, 1 min at 60°C, and 1 min at 72°C and a final cycle at 72°C for 10 min for the amplification of the *p47* gene fragment.

Ten microliters of the PCR products was resolved by gel electrophoresis by using 1% agarose gels containing ethidium bromide and visualized under UV light. All experiments were performed at least twice.

Restriction fragment length polymorphism (RFLP) analysis of *boNT/A* gene

TABLE 1. Strains of *C. botulinum* analyzed in this study and total results of PCR, PCR-RFLP, PFGE and RAPD experiments

Strain	Toxin type	Source[c]	*ha33*[d]	*p47*[d]	*boNT/A* RFLP type	*boNT/A* gene cluster type	No. of clusters generated by[e]:		
							PFGE (XhoI)	PFGE (SmaI)	RAPD (OPG5)
CL1F[a]	A	c-FB, 1996, Naples	−	+	A2	2	NT	NT	1
CL206[a]	A	c-FB, 1996, Catanzaro	−	+	A2	2	NT	NT	1
CL3N[a]	A	c-FB, 1996, Naples	−	+	A2	2	NT	NT	1
CL4G[a]	A	c-FB, 1996, Naples	−	+	A2	2	NT	NT	1
CL5C[a]	A	c-FB, 1996, Naples	−	+	A2	2	NT	NT	1
CL6P[a]	A	c-FB, 1996, Catanzaro	−	+	A2	2	NT	NT	1
CL7Z[a]	A	c-FB, 1996, Catanzaro	−	+	A2	2	NT	NT	1
CL129	A	c-FB, 1997, Potenza	−	+	A2	2	NT	NT	1
CL130	A	c-FB, 1998, Verona	−	+	A2	2	1	1	ND
CL29	A	c-INF, 1998, Rome	−	+	A2	2	1	1	ND
CL103	A	c-INF, 1996, Brescia	−	+	A2	2	1	1	2
CL186	A	c-INF, 2001, Cosenza	−	+	A2	2	1	1	ND
CL260	A	c-INF, 2003, Rome	−	+	A2	2	1	1	ND
CL104	A	c-INF-LIKE, 1997, Novara	−	+	A2	2	2	2	3
CL127	A	F, honey, 1997, Padua	−	+	A2	2	1	1	ND
M 42[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 52[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 106[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 184[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 244[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 309[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 354[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 478[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 529[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 537[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 545[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 568[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 633[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 649[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 664[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 1001[b]	A	F, mascarpone, 1996	−	+	A2	2	NT	NT	1
M 867[b]	A	F, mozzarella cheese, 1996	−	+	A2	2	NT	NT	1
M 949[b]	A	F, cream cheese, 1996	−	+	A2	2	NT	NT	1
CL91	Ab	c-FB, 1993, Milan	+	+	A2	2	3	3	4
CL92	Ab	c-FB, 1993, Nuoro	+	+	A2	2	3	3	ND
CL87	Ab	c-FB, 1995, Catania	+	+	A2	2	4	4	5
S 157	Ab	F, salt, 1999, Bari	+	+	A2	2	3	3	ND
CDC-62A	A	c-FB (reference)	+	−	A1	1	5	5	6
CDC-1757	A	c-FB, 1977, Oreg.	+	−	A1	1	6	6	7
CDC-1839	A	c-FB, 1978, Tex.	+	−	A1	1	7	7	7
CDC-1903	A	c-FB, 1978, Oreg.	−	+	A1	3	8	8	8
CDC-1882	A	c-INF, 1978, Ariz.	−	+	A1	3	8	8	ND
CDC-2712	A	c-INF, 1979, Ariz.	+	−	A1	1	5	5	ND
CDC-2776	A	c-INF, 1979, Utah	+	−	A1	1	5	5	ND
CDC-3680	A	c-INF, 1981, Kans.	+	−	A1	1	6	6	ND
CDC-5196	A	c-INF, 1984, Mont.	+	−	A1	1	9	9	9
CDC-8213	A	c-INF, 1992, Alaska	+	−	A1	1	9	10	9
CDC-2153	A	c-INF-LIKE, 1978, Tex.	+	−	A1	1	10	11	9
CDC-3675	A	c-Unknown, 1980, Oreg.	+	−	A1	1	11	12	10
CDC-588	Ab	c-FB, 1976, Ohio	+	+	A1	3	12	13	11
CDC-1436	Ab	c-INF, 1977, Utah	+	+	A2	2	13	14	8
CDC-2171	A(B)	c-FB, 1978, P.R.	+	+	A2	2	14	15	12
CDC-4893	A(B)	c-FB, 1983, Ill.	+	+	A1	3	15	16	9
CDC-5277	A(B)	c-FB, 1984, W. Va.	+	+	A1	3	15	16	ND
CDC-5178	A(B)	c-FB, 1984, La.	+	+	A1	3	16	17	12
CDC-3517	A(B)	c-INF, 1980, Ariz.	+	+	A1	3	16	17	ND
CDC-1727	A(B)	F, unspecified, 1977, Alaska	+	+	A1	3	17	18	13

[a] See reference 1.
[b] See reference 12.
[c] c-FB, clinical (food-borne botulism); c-INF, clinical (infant botulism); c-INF-LIKE, clinical (infant-like botulism); c-Unknown, clinical (form of botulism not known); F, food.
[d] +, strains positive for indicated gene by PCR; −, strains negative for indicated gene by PCR.
[e] NT, not typeable; ND, not done.

PCR products. The remaining *boNT/A* PCR product volume reactions were purified (QIAquick PCR purification kit; QIAGEN, Crawley, United Kingdom), and 15 μl was incubated with 10 U of XbaI (Roche Diagnostics, Mannheim, Germany) in a final volume of 20 μl containing the appropriate enzyme buffer at 37°C overnight. To verify the patterns obtained, all restriction analyses were performed at least twice.

DNA fragment length polymorphisms were distinguished by electrophoresis on a 2.5% agarose gel.

PFGE experiments. Genomic DNA extraction was performed as described previously by Hielm et al. (17). DNA was digested with 20 U of either XhoI or SmaI enzyme (Roche Diagnostics) as recommended by the manufacturer. DNA fragments were separated by PFGE in a CHEF Mapper apparatus (Bio-Rad

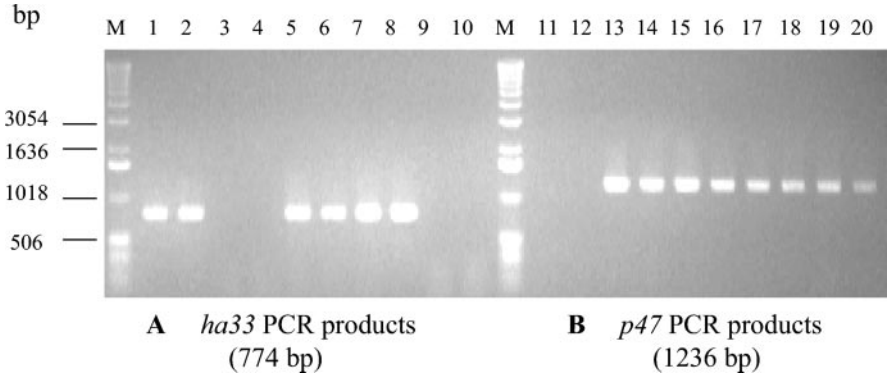

FIG. 2. *ha33* PCR products (A) and *p47* PCR products (B) amplified from *C. botulinum* type A strains 62A (lanes 1 and 11), CDC-2776 (lanes 2 and 12), CDC-1882 (lanes 3 and 13), CDC-1903 (lanes 4 and 14), CL130 (lanes 9 and 19), and CL29 (lanes 10 and 20); from *C. botulinum* type A(B) strains CDC-3517 (lanes 5 and 15) and CDC-5277 (lanes 6 and 16); and from *C. botulinum* type Ab strains CDC-588 (lanes 7 and 17) and CL91 (lanes 8 and 18). M, molecular size standard (1-kb DNA Ladder; Life Technologies, Gaithersburg, Md.).

Laboratories, Hercules, Calif.). A constant temperature of 14°C was used, and the electrophoresis parameters were as follows: voltage of 6 V/cm, an angle of 120°, and switch times of 4 to 40 s (linear ramping factor) for 22 h. Gels were stained with ethidium bromide and visualized with a GelDoc 2000 apparatus (Bio-Rad Laboratories). The similarity between macrorestriction patterns was determined by using the Diversity Database software (Bio-Rad Laboratories). Clustering was performed by applying the Dice coefficient and the unweighted pair-group method using arithmetic averages; an intralinkage homology level of ≥80% (2% position tolerance) was chosen as the cutoff for defining the clusters. The results of the clustering analysis were confirmed by visual comparison. PFGE clusters were arbitrarily numbered.

RAPD analysis. Forty-four selected strains of *C. botulinum* (Table 1) were subjected to RAPD analysis. The random primer OPG5 (5′–CTGAGACGGA–3′), previously shown to discriminate among *C. botulinum* strains, was used (11). RAPD amplification was performed with a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, and 0.4 μM primer. Five microliters of each cell suspension, prepared as described above, was used as a template. A negative control in which cells were replaced with 5 μl of sterile distilled water was included. Mixtures were heated at 99°C for 10 min in the thermal cycler, and 1.25 U of *Taq* polymerase was added. The cycling program was as follows: 1 cycle of 94°C for 8 min; 40 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min; and l cycle of 72°C for 5 min. RAPD products were separated by electrophoresis on a 2.5% agarose gel. RAPD patterns were evaluated visually; only intense bands were accepted for the analysis, and minor variations (very faint bands) were neglected. One band difference was considered sufficient to discriminate between profiles. To ensure reproducibility, RAPD experiments were performed at least twice.

Mouse neutralization assay. Three strains (CL91, CL92, and S157) yielding positive *boNT/A* and *boNT/B* PCR products were subjected to a mouse neutralization assay for detecting the concomitant production of neurotoxin types A and B, as previously described (8).

RESULTS

PCR detection of *boNT/A* and *boNT/B* genes and toxin production. The presence of the *boNT/A* gene was confirmed in all 57 clostridial strains. Nine of the strains (eight from the United States and one from Italy) were also confirmed to be positive for the *boNT/B* gene (data not shown). We also found that an additional three strains from Italy (CL91, CL92, and S157), previously classified as *C. botulinum* type A, possessed both *boNT/A* and *boNT/B* genes (data not shown). These strains were demonstrated by the mouse neutralization assay to actually produce minor amounts of boNT/B (100 minimum lethal dose units/ml) together with boNT/A (8,000 minimum lethal dose units/ml) (A-to-B ratio, 80:1) and were thus reclassified as *C. botulinum* Ab (Table 1).

PCR amplification of *ha33* and *p47* gene fragments. According to the scheme depicted in Fig. 1, we expected to amplify the *ha33* gene from *C. botulinum* type A1 strains, the *p47* gene from type A2 strains, and both the *ha33* and *p47* genes from the *C. botulinum* strains possessing both the *boNT/A* gene and the *boNT/B* gene (either expressed or unexpressed). To check the effectiveness of our approach, we carried out preliminary PCR experiments with primers specific for *ha33* and *p47* genes using the strains 62A (*C. botulinum* type A1 [6, 7]) and CDC-588 (*C. botulinum* Ab), whose *boNT/A* and *boNT/B* gene cluster organization has already been established (18), as templates for the amplification (Fig. 1). An *ha33* amplification product of the expected size was obtained from strain 62A, whereas two products whose sizes were consistent with the *ha33* and *p47* gene fragments were amplified from strain 588 (Fig. 2), as expected.

The results of the amplification of the *ha33* and *p47* gene fragments for the remaining strains are reported in Table 1. Thirty-three *C. botulinum* type A strains from Italy were negative for *ha33*, while they yielded a positive *p47* PCR gene product of the predicted size (about 1,236 bp). The remaining four strains of *C. botulinum* type Ab from Italy (Table 1) were positive for both the *ha33* and *p47* genes, as expected.

Ten of the *C. botulinum* type A strains from the United States, including reference strain 62A, were negative for the *p47* gene and positive for the *ha33* gene; the *ha33* PCR products were 774 bp in length, as expected. The remaining two strains (CDC-1882 and CDC-1903) exhibited an *ha33* and *p47* gene pattern similar to that observed for the Italian strains (i.e., *ha33* negative and *p47* positive) (Fig. 2). Both genes were amplified from the eight strains of *C. botulinum* Ab and A(B) isolated in the United States (Table 1).

PCR-RFLP identification of *boNT/A* gene sequence type. For restriction endonuclease selection, *boNT/A1* and *boNT/A2* gene sequences of *C. botulinum* type A strains available from the GenBank database (accession numbers AF488749, AF461540, M30196, X52066, and X73423) (2, 6, 37–39) were aligned (GCG software package) and analyzed with the Webcutter 2 restriction program (http://www.firstmarket.com/cutter/cut2.html). The theoretical analysis revealed a single XbaI restriction site at position 1536 of the 2,280-bp *boNT/A2* gene region amplified by our *boNT/A* primer set which was absent from the analogous region of the *boNT/A1* gene sequences. Restriction analyses of *boNT/A* gene PCR products with XbaI endonuclease would be expected

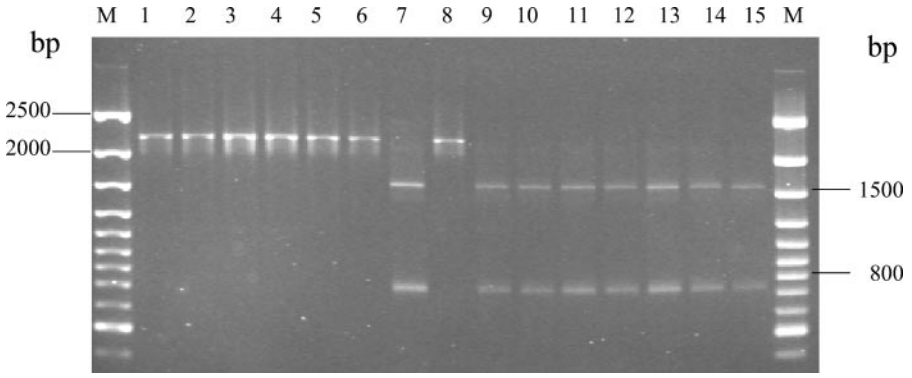

FIG. 3. Results of XbaI restriction of *boNT/A* PCR products from *C. botulinum* type A strains 62A (lane 1), CDC-1757 (lane 2), CDC-1839 (lane 3), CDC-1882 (lane 4), CDC-1903 (lane 5), CL130 (lane 11), CL186 (lane 12), CL127 (lane 13), CL29 (lane 14), and CL104 (lane 15); from *C. botulinum* type A(B) strains CDC-3517 (lane 6) and CDC-2171 (lane 7); and from *C. botulinum* type Ab strains CDC-588 (lane 8), CL91 (lane 9), and S157 (lane 10). M, molecular size standard (DNA Ladder Mix; Genenco, Bakersfield, Calif.).

to produce two restriction fragments of 1,536 and 734 bp from *boNT/A2* gene sequences (*boNT/A2* PCR-RFLP type) and no restriction products from *boNT/A1* gene sequences (*boNT/A1* PCR-RFLP type) (Fig. 1), thus allowing the *boNT/A* gene sequence type to be identified. The PCR-RFLP method to characterize the *boNT/A* gene sequence was validated on strains 62A and CDC-588, both of which are known to possess a *boNT/A1* gene type (6, 18) (Fig. 3).

The results of the RFLP analyses of the *boNT/A* gene PCR products obtained from the clostridial strains are summarized in Table 1. The *boNT/A* products amplified from all of the *C. botulinum* strains isolated in Italy, including the four strains of *C. botulinum* Ab, yielded two restriction fragments of the predicted sizes upon restriction with XbaI, in accordance with a *boNT/A2* gene sequence type. No XbaI restriction fragments were obtained from the *boNT/A* amplification products of any of the *C. botulinum* type A strains isolated in the United States, including the reference strain 62A (Fig. 3). The *boNT/A* PCR products from strains CDC-1903 and CDC-1882 were not cleaved by XbaI, nor were the *boNT/A* gene amplification products from six of the remaining *C. botulinum* type Ab and A(B) strains from the United States (*boNT/A1* PCR-RFLP type) (Fig. 3). The *boNT/A* gene PCR products from the last two strains [*C. botulinum* type Ab strain CDC-1436 and *C. botulinum* type A(B) strain CDC-2171] were the only ones among the strains from the United States to yield the two-band XbaI restriction profile of the *boNT/A2* PCR-RFLP type.

PFGE analyses. Of the 57 *C. botulinum* strains, only 31 were able to be characterized by PFGE (Table 1). Smeared DNA profiles were repeatedly obtained from the remaining 26 strains, all from Italy (data not shown); 25 of these 26 strains were the clinical and food isolates related to the mascarpone outbreak, and the other strain (CL129) was a clinical isolate from a single case of food-borne botulism. No heat and/or chemical (formaldehyde) treatment, as described by Hielm et al. (17), was sufficient to inactivate the DNases (data not shown).

Of the 31 typeable strains, 18 different clusters with a similarity level of ≥80% were generated by SmaI, and 17 were generated by XhoI (Table 1); PFGE clusters within the strains were consistent for the two enzymes, except for strain CDC-8213, which clustered with another strain (CDC-5196) when analyzed with XhoI and produced a unique profile when restricted by SmaI.

The 11 typeable strains from Italy produced four different PFGE clusters (two among six *C. botulinum* type A strains and the other two among four *C. botulinum* type Ab strains). Interestingly, of the latter strains, S157 from a sea salt sample produced SmaI and XhoI profiles that were indistinguishable from those of *C. botulinum* Ab clinical strains CL91 and CL92 (Table 1).

Thirteen and 14 different PFGE clusters were obtained with XhoI and SmaI, respectively, among the 20 strains from the United States. Eight clusters with XhoI and nine clusters with SmaI were recognized among the 12 *C. botulinum* type A strains, four with both enzymes among six *C. botulinum* type A(B) strains and two with both enzymes for the two type Ab strains CDC-588 and CDC-1436 (Table 1).

Representative PFGE profiles obtained with the XhoI enzyme from the clostridial strains are shown in Fig. 4.

RAPD analyses. The 26 strains of *C. botulinum* type A which were nontypeable by PFGE were subjected to RAPD typing. Eighteen additional strains (4 from Italy and 14 from the United States) representative of the different PFGE clusters were included in the analysis. Thirteen distinct RAPD patterns were detected among the 44 total strains analyzed (Table 1). All 26 PFGE nontypeable strains from Italy shared the same RAPD profile. Another 12 patterns were recognized among the 18 additional strains analyzed. In general, RAPD profiles correlated well with PFGE profiles; however, in some cases, strains with different PFGE profiles shared the same RAPD profile. This result was in accordance with the higher discrimination capability of PFGE compared to RAPD, as is generally accepted (15). Some RAPD profiles are shown in Fig. 5.

DISCUSSION

We developed a strategy to identify the *boNT/A* gene clusters of 57 *C. botulinum* type A, Ab, and A(B) strains based on the combination of the *ha33* and *p47* genes and the RFLP analysis of the *boNT/A* gene sequence. A similar approach had previously been used by Cordoba et al. (5) to determine the presence or absence of HA and NTNH genes and the *boNT/A*

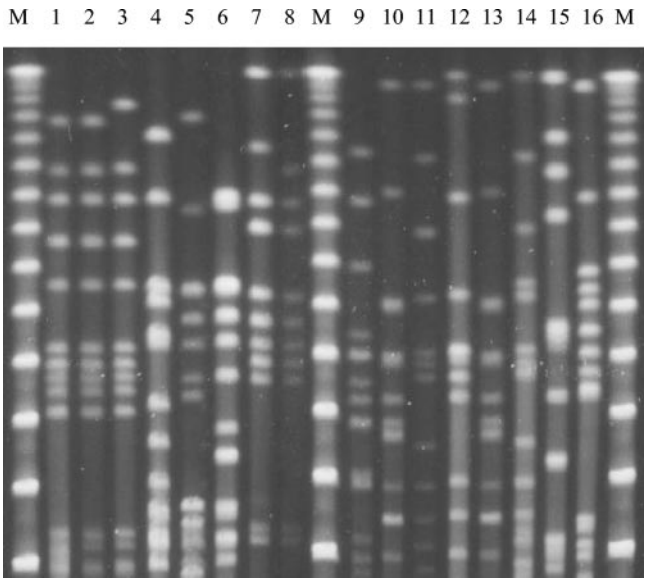

FIG. 4. XhoI PFGE profiles of *C. botulinum* type A strains CL103 (lane 1), CL186 (lane 2), CL260 (lane 3), 62A (lane 4), CDC-1839 (lane 5), CDC-5196 (lane 6), CDC-1882 (lane 7), and CDC-1903 (lane 8); *C. botulinum* type A(B) strains CDC-2171 (lane 9), CDC-5277 (lane 10), CDC-1727 (lane 11), CDC-3517 (lane 12), and CDC-4893 (lane 13); and *C. botulinum* type Ab strains CDC-588 (lane 14), CL91 (lane 15), and CL87 (lane 16). M, molecular size standard (Lambda Ladder PFG marker; New England Biolabs, Hitchin, United Kingdom).

gene polymorphisms in 25 *C. botulinum* strains. In our study, the *ha33* and *p47* genes were detected by PCR. For *boNT/A* gene sequence type identification, we performed computer-assisted alignment and restriction analysis of the available sequences of the *boNT/A* gene to select enzymes discriminating between the sequences of the *boNT/A* gene fragment amplified by our primer set. This fragment spans a region of the gene encoding part of the boNT/A heavy chain, where most of the amino acid differences between toxins A1 and A2 have been observed (38). *boNT/A1* and *boNT/A2* genes had previously been distinguished from each other by RFLP and DNA probes (5).

Amplification of the *ha33* and *p47* genes and *boNT/A* PCR-RFLP analyses were applied to strains of *C. botulinum* types A, Ab, and A(B) from various sources and with different geographical origins. All 33 strains of *C. botulinum* type A isolated in Italy displayed a combination consistent with the type A2 gene cluster; they were negative for the *ha33* gene and positive for the *p47* gene and revealed a *boNT/A2* gene sequence type. Strains of *C. botulinum* type A2 were first described in Japan (29, 34); two additional strains were later identified by Cordoba et al. (one from the United Kingdom and one from Mauritius) (5). Considering that type A2 strains have rarely been described worldwide, the fact that all of the strains of *C. botulinum* type A isolated to date in Italy are type A2 is outstanding.

Ten of the 12 strains of *C. botulinum* type A from the United States showed a combination presumptive of the type A1 gene cluster (i.e., they were positive for the *ha33* gene and negative for the *p47* gene and yielded a *boNT/A1* PCR-RFLP type). However, the two remaining strains of *C. botulinum* type A (strains CDC-1882 from infant botulism and CDC-1903 from food-borne botulism) were negative for *ha33* and positive for *p47* genes (like type A2 strains), whereas they showed a *boNT/A1* PCR-RFLP type. This result was surprising since this combination has been reported only for the *boNT/A* gene clusters of *C. botulinum* type A(B) and Ab strains (5, 7, 18, 28) (Fig. 1). Based on these considerations, we hypothesize that a third *boNT/A* gene cluster exists among strains of *C. botulinum* type A which we have designated type A3. The fact that only 2 of the 45 *C. botulinum* type A strains investigated showed a type A3 *boNT/A* gene cluster suggests that this variant is rare.

The 12 strains of *C. botulinum* types Ab and A(B) were positive for both the *ha33* and the *p47* genes, as predicted on the basis of the *boNT/A* and *boNT/B* gene clusters (Fig. 1). Although our approach did not allow us to locate the positions of *ha33* and *p47* genes on the different *boNT* clusters, based on previous knowledge, only the *p47* gene may be assumed to be present in the *boNT/A* gene cluster (7, 18, 28) (Fig. 1).

All type Ab strains from Italy revealed a *boNT/A2* PCR-RFLP type. Among the *C. botulinum* strains possessing A and B toxin genes studied to date, only one strain from Japan has revealed a *boNT/A2* gene sequence (14). Based on the combination *p47*-positive and *boNT/A2* PCR-RFLP type, the type Ab strains from Italy were assigned to the type A2 gene cluster.

Five type A(B) strains and one Ab strain from the United States showed a *boNT/A1* PCR-RFLP type; based on the combination *p47*-positive and *boNT/A1* RFLP type, these strains were assigned to the type A3 gene cluster. The two remaining strains [strain CDC-1436 of type Ab and strain CDC-2171 of type A(B)] were the only strains from the United States that displayed a *boNT/A2* PCR-RFLP type (type A2 gene cluster).

The results of our analyses showed that the *boNT/A* genetic clusters differed when the strains from Italy were compared to those from the United States; the type A2 gene cluster predominates among the isolates from Italy, whereas the type A1 gene cluster is predominant among the isolates from the United States. Previously, a similar distinction had been observed between *C. botulinum* type A strains isolated in Japan and those isolated in the United States based on the molecular sizes of toxin complexes produced by the strains (35). This result suggests that the population in Italy resembles that of Japan, and it might reflect different evolutionary lineages of the clostridia; although this possibility remains to be demonstrated, a similar geographic separation has been hypothesized

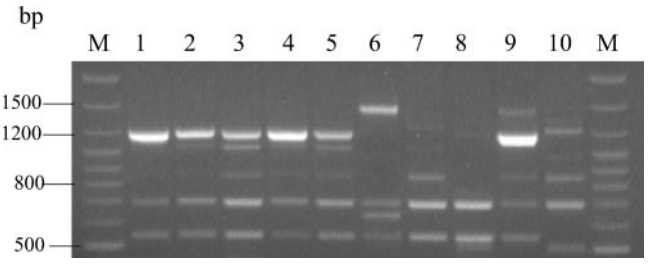

FIG. 5. RAPD profiles obtained with primer OPG5 from strains CL1F (lane 1), M354 (lane 2), M949 (lane 3), M867 (lane 4), CL129 (lane 5), CL103 (lane 6), CL104 (lane 7), CL91 (lane 8), CL87 (lane 9), and reference strain 62A (lane 10). M, molecular size standard (DNA Ladder Mix; Genenco).

for strains of neurotoxigenic *Clostridium butyricum*, which have been isolated only in Italy and China (30).

On the other hand, no correlation between the *boNT/A* gene clusters and the source of isolation (different clinical forms of botulism and diverse foods) was found, in accordance with findings by Cordoba et al. (5). This result is in contrast with previous observations that HA-negative (type A2) strains were more frequently associated with infant botulism (34).

The PFGE profiles of strains were generally consistent with the *boNT/A* gene clusters obtained. In particular, the two strains of *C. botulinum* type A exhibiting a type A3 gene cluster (CDC-1882 and CDC-1903) shared very similar profiles with each restriction enzyme used in the analyses (Fig. 4), indicating that the two strains are strictly related; profiles with both enzymes were different from those of all the remaining strains. The profiles of the Italian strains were distinguished from those of the United States, substantiating the geographic distinction between strains. Some strains of different clinical origins shared the same PFGE profiles, confirming the previous conclusion of no existing relationship between the genetic clones and the outcome of the disease. Twenty-five of the strains from Italy were not typeable by PFGE. Problems related to DNA smearing in PFGE analyses are due to the overproduction of DNases by some microbial strains and have specifically been reported for nonproteolytic *C. botulinum* strains (type E and some type B and F strains) (17) and for *Clostridium* spp. other than *C. botulinum* (33). We experienced the same problems with *C. botulinum* type A (proteolytic) strains. The strains that were not typeable by PFGE were thus subjected to RAPD analysis, a less discriminatory technique (15) which, however, has the advantage of overcoming DNase overproduction; strains representative of the different PFGE profiles obtained were included in the analyses. We found that all nontypeable strains produced the same RAPD profile, which was distinguished from the profiles of the other strains, suggesting a strict genetic relationship between them. This finding was interesting since most of the nontypeable strains had been isolated from an outbreak of botulism due to contaminated mascarpone consumption and from different samples of mascarpone and other cheeses analyzed during that outbreak (12). In total, the 37 Italian *C. botulinum* strains yielded a few banding patterns with both PFGE and RAPD techniques; therefore, since they had been isolated in different places and years, a low genetic diversity among strains distributed in Italy can be deduced.

In summary, we identified three different *boNT/A* gene clusters among 57 strains of *C. botulinum* which were consistent with PFGE and RAPD genotyping. These clusters potentially encode three different boNT/A complexes. It is already known that boNT/A1 and boNT/A2 complexes differ from each other in terms of molecular size, antigenicity of components, and boNT/A amino acid sequence; in fact, the neurotoxin moieties of the two complexes display a 10% amino acid difference, restricted to the heavy chain of proteins (29, 38), which is involved in the receptor recognition at the neuromuscular junctions (32). The third cluster that we identified theoretically leads to the production of an HA-negative medium-sized (M) complex similar to that produced by type A2 strains, except for the presence of boNT/A1 toxin.

The importance of the different complexes in pathology is still unclear. A number of roles have been attributed to the hemagglutinins (predominantly to the HA33 protein) of boNT complexes, specifically, (i) the linking of two L boNT/A complexes to form the LL complex (19), (ii) protection of boNTs from acidity and proteases of the stomach (31), (iii) the binding to the intestinal cells and translocation of boNTs through the intestinal mucosa (13, 19), and (iv) interaction with sugar moieties on red blood cells and delivery of boNTs into the bloodstream (4, 13). The latter two roles are still controversial since it has been demonstrated that both HA-positive and HA-negative complexes of the toxin can be absorbed from the gastrointestinal tract of mice and enter circulation (25). Moreover, the nontoxic proteins of complexes contribute to the stability and effectiveness of the boNT/A therapeutic agent; in fact, the larger boNT/A complex has been reported to be more effective as a therapeutic reagent, given its higher stability (20). Hence, variation in the composition of hemagglutinins in the boNT/A complexes might affect both the functionality and the potency of the neurotoxin itself.

On the other hand, differences between *boNT/A1* and *boNT/A2* gene sequences imply that the related boNTs have different amino acid composition and antigenic properties (22), with important implications for the production of specific antibodies against boNT/A; the receptor-binding abilities of boNT/A1 and boNT/A2 toxins should also be investigated.

Additional studies are being performed to sequence the different *boNT/A* gene clusters from some representative strains in order to confirm them and to identify any additional nucleotide differences that may exist between them. Comparisons of the activities of the related boNT/A toxin complexes in animal models and in cell culture systems will also be needed to eventually differentiate their mechanisms and roles.

ACKNOWLEDGMENTS

This work was supported in part by the National Center for Botulism grant OA/C from the Italian Ministry of Health.

We thank Fabrizio Anniballi for help with mouse experiments.

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