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Diversity of Proteolytic *Clostridium botulinum* Strains, Determined by a Pulsed-Field Gel Electrophoresis Approach

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Pulsed-field gel electrophoresis (PFGE) was applied to the study of the similarity of 55 strains of proteolytic *Clostridium botulinum* (*C. botulinum* group I) types A, AB, B, and F. Rare-cutting restriction enzymes ApaI, AscI, MluI, NruI, PmeI, RsrII, SacII, SmaI, and XhoI were tested for their suitability for the cleavage of DNA of five proteolytic *C. botulinum* strains. Of these enzymes, SacII, followed by SmaI and XhoI, produced the most convenient number of fragments for genetic typing and were selected for analysis of the 55 strains. The proteolytic *C. botulinum* species was found to be heterogeneous. In the majority of cases, PFGE enabled discrimination between individual strains of proteolytic *C. botulinum* types A and B. The different toxin types were discriminated at an 86% similarity level with both SacII and SmaI and at an 83% similarity level with XhoI. Despite the high heterogeneity, three clusters at a 95% similarity level consisting of more than three strains of different origin were noted. The strains of types A and B showed higher diversity than the type F organisms which formed a single cluster. According to this survey, PFGE is to be considered a useful tool for molecular epidemiological analysis of proteolytic *C. botulinum* types A and B. However, epidemiological conclusions based on PFGE data only should be made with discretion, since highly similar PFGE patterns were noticed, especially within the type B strains.

The most commonly reported forms of botulism are foodborne botulism, wound botulism, and infant botulism. Foodborne botulism is an intoxication caused by consumption of preformed botulinum neurotoxin, while wound and infant botulism are both infections in which neurotoxin is formed in vivo (13). Proteolytic *Clostridium botulinum* (*C. botulinum* group I) is one of six phylogenetically and physiologically distinct clostridia that forms botulinum neurotoxin. It is responsible for a majority of the cases of infant botulism and wound botulism as well as many cases of classical food-borne botulism (1, 2, 4, 16, 17). Nonproteolytic *C. botulinum* (*C. botulinum* group II) is responsible for most other cases of food-borne botulism (13). The confirmation of food-borne botulism outbreaks has been based on the detection of botulinum toxin or *C. botulinum* organisms of the same toxin type from both the patient and the suspected food item. In order to be able to confirm the epidemiological relatedness of the strains isolated from patients and from food, a method for genotyping proteolytic *C. botulinum* isolates is needed. Such a method would also be of utility when investigating cases of wound or infant botulism.

Since the first paper on pulsed-field gel electrophoresis (PFGE) typing of *C. botulinum* type A strains Hall A and 62 A was published (10), no reports on the genetic diversity of strains of proteolytic of *C. botulinum* as determined by PFGE have been published. With nonproteolytic *C. botulinum*, a high genetic diversity determined by PFGE and randomly amplified polymorphic DNA (RAPD) analysis has been reported (5, 6, 8, 9). These methods have been used as tools for epidemiological analysis of strains of nonproteolytic *C. botulinum* (6, 9, 11). However, RAPD analysis could not separate strains of proteolytic *C. botulinum* types A and F below the serotype level (8). Furthermore, the discriminatory power of repetitive element sequence-based PCR with proteolytic *C. botulinum* was even lower than that of RAPD (8). Based on these results, it was concluded that proteolytic *C. botulinum* might be a more homogeneous species than nonproteolytic *C. botulinum* (8).

To extend our knowledge of the biodiversity of proteolytic *C. botulinum* and to evaluate the usefulness of PFGE in the genotyping of this organism, strains of proteolytic *C. botulinum* from various origins were studied using an optimized PFGE protocol.

MATERIALS AND METHODS

***C. botulinum* strains.** A total of 55 *C. botulinum* strains from culture collections of the Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland, and the Institute of Food Research, Norwich, United Kingdom, were used to determine the genetic diversity of proteolytic *C. botulinum*. Of these strains, 19 were of type A, 28 were of type B, and 3 were of type F, whereas 5 strains contained the toxin gene for both types A and B. The strains included both European and American isolates. The sources of the strains are shown in Table 1. Each of the strains, ATCC 3502, ATCC 19397, ATCC 7948, NCTC 2012, ATCC 25763, ATCC 25764, and ATCC 35415, were initially obtained from more than one laboratory (Table 1). The proteolytic activity of the strains was confirmed by plating on reinforced *Clostridium* medium containing 5% (vol/wt) skim milk (15).

DNA preparations. After being incubated anaerobically at 37°C for 2 days, single colonies of pure cultures were picked from blood agar plates and inoculated into 10 ml of tryptone-peptone-glucose-yeast extract broth (Difco Laboratories, Detroit, Mich.) and incubated for 16 h at 37°C. DNA isolation was performed according to a previously described method (5), with slight modifications. A 4-ml volume of the 16-h culture was chilled on ice, and cells were harvested by centrifugation (1,100 × *g*) at 4°C and suspended in PIV buffer (10 mM Tris [pH 7.5], 1 M NaCl) supplemented with 10% (vol/vol) formaldehyde

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TABLE 1. Proteolytic *Clostridium botulinum* strains included in the study

Strain	Type detected by PCR	Initial source (year)[b]	Present source
ATCC 3502 (1)[a]	A	ATCC (1996)	DFEH
ATCC 3502 (2)[a]	A	N. Minton, CAMR (2000)	IFR
ATCC 19397 (1)[a]	A	M. Wictome, CAMR (1997)	IFR
ATCC 19397 (2)[a]	A	A. East, IFR (1997)	IFR
ATCC 19397 (3)[a]	A	ATCC (1996)	DEFH
ATCC 7948 (1)[a]	A	J. Crowther, Unilever (1981)	IFR
ATCC 7948 (2)[a]	A	T. Roberts, IFR (1993)	IFR
ATCC 7948 (3)[a]	A	NCTC (1981) (NCTC 3805)	IFR
ATCC 7948 (4)[a]	A	S. Lindroth, Univ. of Calif. (1989)	DEFH
ATCC 7948 (5)[a]	A	L. Taylor, TRS (1993)	IFR
NCTC 2012 (1)[a]	A	NCTC (1993)	IFR
NCTC 2012 (2)[a]	A	L. Taylor, TRS (1993)	IFR
ATCC 25763^T (1)[a]	A	ATCC (1996)	DEFH
ATCC 25763^T (2)[a]	A	L. Taylor, TRS (1993)	IFR
BL81/17	A	J. Crowther, Unilever (1981)	IFR
BL81/18A	A	J. Crowther, Unilever (1981)	IFR
16037	A	LFRA (1981)	IFR
96 A	A	T. Roberts (1993)	IFR
CDC1690	A	A. East, IFR (1997)	IFR
NCTC 3806	A	NCTC (1993)	IFR
Eyemouth	A	L. Taylor, TRS (1993)	IFR
FT 53	A	L. Taylor, TRS (1993)	IFR
SL-2	A	S. Lindroth, Univ. of Calif. (1989)	DEFH
SL-3	A	S. Lindroth, Univ. of Calif. (1989)	DEFH
SL-4	A	S. Lindroth, Univ. of Calif. (1989)	DEFH
RS-3	A	S. Lindroth, Univ. of Calif. (1989)	DEFH
69 A	A	S. Lindroth, Univ. of Calif. (1989)	DEFH
Hall A (CDC)	A	S. Lindroth, Univ. of Calif. (1989)	DEFH
CDC13280	AB	A. East, IFR (1997)	IFR
NCTC2916	AB	M. Wictome, CAMR (1997)	IFR
CDC5001	AB	C. Hatheway, CDC (1987)	IFR
MDa10	AB	A. East, IFR (1997)	IFR
CDC588	AB	A. East, IFR (1993)	IFR
ATCC 7949	B	ATCC (1996)	DEFH
ATCC 17841	B	ATCC (1996)	DFEH
McClung 133-4803	B	S. Lindroth, Univ. of Calif. (1989)	DFEH
126 B	B	Institut Pasteur (1962)	DFEH
SL-5	B	S. Lindroth, Univ. of Calif. (1989)	DEFH
SL-1	B	S. Lindroth, Univ. of Calif. (1989)	DEFH
4 B	B	T. Roberts (1993)	IFR
FT 239	B	L. Taylor, TRS (1993)	IFR
BL 150	B	Unilever (1986)	IFR
BL81/18B	B	J. Crowther, Unilever (1981)	IFR
2345	B	J. Crowther, Unilever (1981)	IFR
NCTC 3815	B	J. Crowther, Unilever (1981)	IFR
BL81/25	B	LFRA (1981)	IFR
ATCC 2743	B	ATCC (1981)	IFR
BL 143	B	Unilever (1986)	IFR
CDC15044	B	C. Hatheway, CDC (1987)	IFR
CDC7827	B	C. Hatheway, CDC (1993)	IFR
FT 243	B	L. Taylor, TRS (1993)	IFR
NCIB 4301	B	L. Taylor, TRS (1993)	IFR
M65/16	B	DFEH (soil, 2002)	DFEH
M1/3	B	DFEH (soil, 2002)	DFEH
M170/4	B	DFEH (soil, 2003)	DFEH
M153/1	B	DFEH (soil, 2003)	DFEH
M43/15	B	DFEH (soil, 2002)	DFEH
M193/15	B	DFEH (soil, 2003)	DFEH
M197/17	B	DFEH (soil, 2003)	DFEH
He3396	B	DFEH (horse feces, 2002)	DFEH
He3323	B	DFEH (horse feces, 2002)	DFEH
ATCC 25764 (1)[a]	F	H. Tranter, CAMR (1993)	IFR
ATCC 25764 (2)[a]	F	ATCC (1996)	DFEH
ATCC 35415 (1)[a]	F	NCTC (1993) (NCTC 10281)	IFR
ATCC 35415 (2)[a]	F	P. McClure, Unilever (1993)	IFR
F4VI	F	S. Lindroth, Univ. of Calif. (1989)	DFEH

[a] The same strain was obtained from more than one laboratory, marked 1 to 5.

[b] ATCC, American Type Culture Collection (Rockville, Md.); DFEH, Department of Food and Environmental Hygiene (Helsinki University); CAMR, Centre for Applied Microbiology and Research (Salisbury, United Kingdom); IFR, Institute of Food Research (Norwich, United Kingdom); NCTC, National Collection of Type Cultures (London, United Kingdom); TRS, Torry Research Station; LFRA, Leatherhead Food Research Association; Univ. of Calif., University of California; CDC, Centers for Disease Control and Prevention.

solution. The cell suspensions were kept on ice for 1 h, with gentle shaking every 15 min. The cells were then washed twice with PIV buffer and resuspended in 1 ml of double-strength lysis solution (12 mM Tris [pH 7.5], 2 M NaCl, 200 mM EDTA [pH 8.0], 1% Brij 58, 0.4% deoxycholate, 1% sodium lauroyl sarcosine, 40 μl of RNase/ml, 2 mg of lysozyme/ml, 40 U of mutanolysin/ml). A volume of 500 μl of each cell suspension was mixed with an equal amount of 2% (wt/vol) low-melting-point agarose (InCert agarose; Cambrex Bio Science, Rockland, Maine) and cast in GelSyringe dispensers (New England Biolabs, Beverly, Mass.). The formed gel plugs were then incubated in lysis solution (6 mM Tris [pH 7.5], 1 M NaCl, 100 mM EDTA [pH 8.0], 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium lauroyl sarcosine, 20 μl of RNase/ml, 1 mg of lysozyme/ml, 20 U of mutanolysin/ml) with gentle shaking at 37°C overnight. The next day, the plugs were rinsed with Tris-EDTA buffer, and the DNA isolation was completed by washing the plugs twice with ESP (0.5 M EDTA [pH 8.0], 10% sodium lauroyl sarcosine, 100 μg of proteinase K/ml) at 50°C for 3 h. The inactivation of proteinase K was performed with Pefablock SC (Roche Diagnostics GmbH, Mannheim, Germany) at 37°C overnight. On the third day, the plugs were washed with Tris-EDTA buffer twice at 37°C for 2 h with gentle shaking.

The toxin types of the pure cultures were confirmed with multiplex PCR for *C. botulinum*, specific for botulinum neurotoxin genes of types A, B, E, and F (12), and/or using the PCR protocol and primers described previously by Franciosa et al. (3).

Restriction enzyme digestions and PFGE. Nine rare-cutting restriction enzymes, ApaI, AscI, MluI, NruI, PmeI, RsrII, SacII, SmaI, and XhoI (New England Biolabs), were chosen for testing the cleavage of DNA of proteolytic *C. botulinum*. The suitability of the nine enzymes for a molecular epidemiological analysis was tested by using five strains of proteolytic *C. botulinum*: two of type A (ATCC 3502 and ATCC 19397), two of type B (ATCC 2743 and FT 243), and one of type F (ATCC 25764). Samples were electrophoresed at 8°C through a 1% (wt/vol) agarose gel (Seakem Gold agarose; BMA, Rockland, Maine) in 0.5× Tris-borate-EDTA buffer (Amresco, Solon, Ohio) at 200 V for 20 to 22 h with a Gene Navigator system (Pharmacia, Uppsala, Sweden) with a hexagonal electrode. Different pulse time ramps were tested, when necessary, to find the optimal running conditions. Low Range PFG marker (New England Biolabs) was used for fragment size evaluation. After the electrophoresis, the gel was stained for 30 to 45 min in distilled water containing 0.5 μg of ethidium bromide/ml. The gel was rinsed with distilled water and destained in water for at least 2 h. The DNA fragment sizes were estimated by comparing their running lengths to those of the molecular weight markers. The gels were photographed by using the Alpha Imager 2000 documentation system (Alpha Innotech, San Leandro, Calif.). Each strain was run at least twice with each enzyme.

PFGE pattern analysis. The fingerprint patterns of the 55 strains obtained with SacII, SmaI, and XhoI were analyzed with BioNumerics software, version 3.5 (Applied Maths, Sint-Martens-Latem, Belgium). The similarity between all pairs was expressed by the Dice coefficient correlation, and unweighted-pair group method using average linkages clustering was used for the construction of the dendrograms. The position tolerance was set at 2.0%, with no increase towards the end of the pattern, and the optimization value was 0.5%. The enzyme combinations were formed by numerical analysis combining the unweighted pattern information of single restriction enzymes. The discriminatory ability of different enzymes was calculated by using the discriminatory index (7).

RESULTS

Of the nine enzymes tested, AscI, MluI, and RsrI created only a few fragments, whereas the majority of the fragments generated by ApaI and PmeI were too small to allow a reliable genomic analysis (<100 kb) (Fig. 1). NruI showed a varying performance producing a convenient pattern of fragments with some strains, but with other strains, there were problems in interpreting bands that were very close to each other, suggesting duplicate bands. In addition, NruI performed digestions of the genome of certain strains too frequently, producing several fragments that were too poorly separated to be reliably analyzed. Based on the suitable numbers of fragments produced, SacII, SmaI, and XhoI were chosen for PFGE and cluster analysis of the 55 strains. With SacII and SmaI, the preferable pulse time ramp was 1 to 26 s, and the run was completed in 22 h. With XhoI, the optimal pulse time ramp was 1 to 15 s in 18 h.

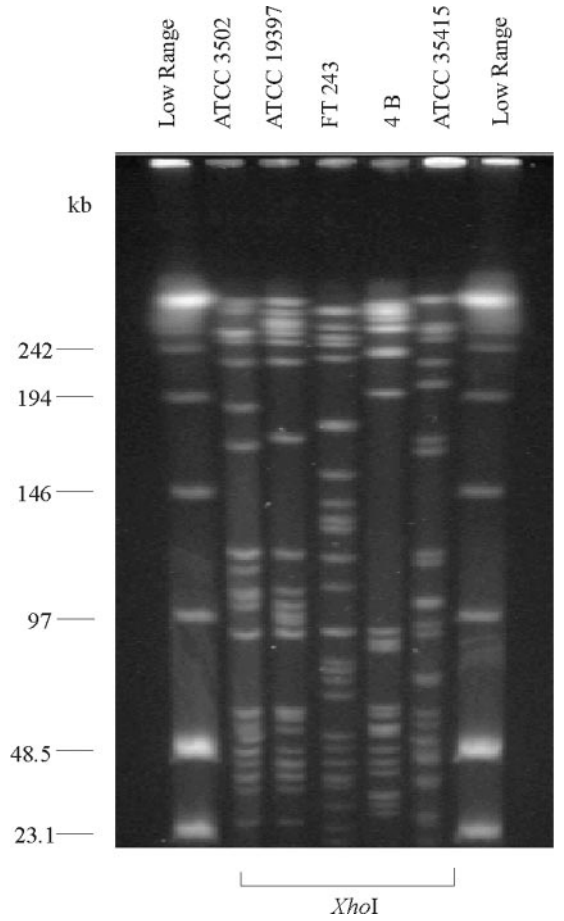

FIG. 1. Digestion patterns of type A (ATCC 3502) and type B (FT 243) proteolytic *C. botulinum* strains using the rare-cutting restriction enzymes ApaI, AscI, MluI, NruI, PmeI, and RsrII. The pulse time ramp was 1 to 22 s, and the running time was 20 h. The outermost lanes and the lane in the middle contain the Low Range PFG marker.

All the *C. botulinum* strains investigated resulted in clear macrorestriction patterns by PFGE. The enzymes SacII, SmaI, and XhoI produced a suitable fingerprint pattern for a molecular epidemiological analysis, with the number of fragments ranging from 10 to 22, from 9 to 21, and from 14 to 25, respectively (Fig. 2 and 3). The smallest fragments included in the analysis were 48 kb when SacII and SmaI were used and 24 kb when XhoI was used, due to the shorter pulse time ramp which separated the smaller bands more clearly. The median number of fragments was 17, 13, and 19 with SacII, SmaI, and XhoI, respectively.

The similarity levels to differentiate the proteolytic *C. botulinum* serotypes A, B, and F into distinct clusters were 86% with both SacII and SmaI and 83% with XhoI. The type F strains were shown to be similar with both SacII and XhoI. Only with SmaI could the strains be divided into two subgroups with one band (<97 bp) difference. However, according to the analysis performed with the BioNumerics software, the similarity between the subgroups was higher than 95%; thus, the strains are considered to form only one cluster (Fig. 4, cluster

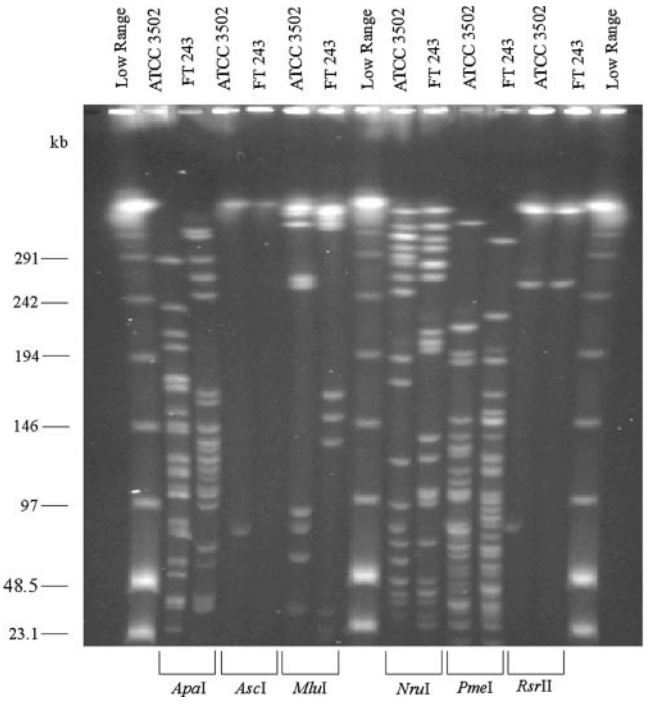

FIG. 2. Digestion patterns of five proteolytic *C. botulinum* strains, two of type A (ATCC 3502 and ATCC 19397), two of type B (FT 243 and 4B), and one of type F (ATCC 35415), using the rare-cutting restriction enzymes SacII and SmaI. The pulse time ramp was 1 to 22 s, and the running time was 20 h. The outermost lanes and the lane in the middle contain the Low Range PFG marker.

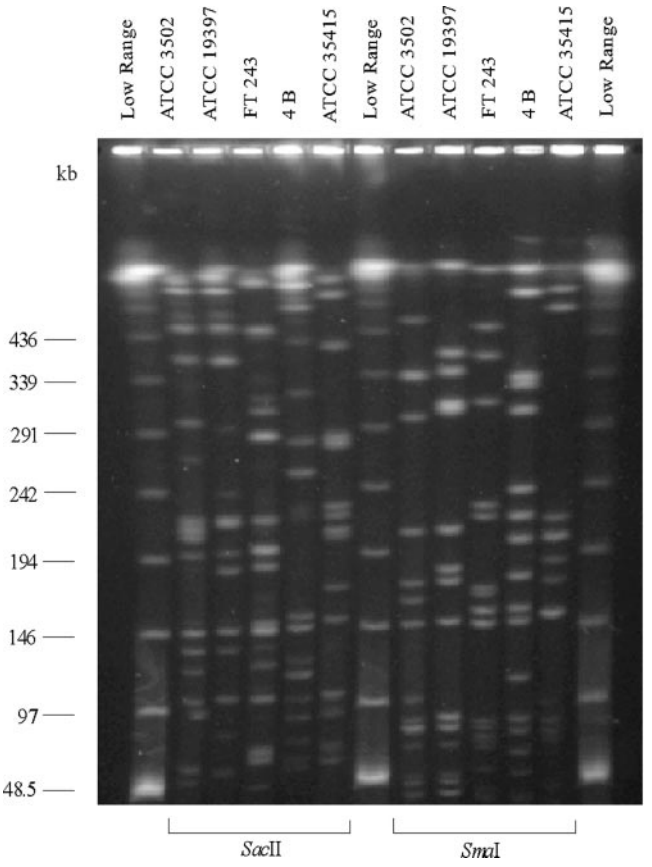

FIG. 3. Digestion patterns of five proteolytic *C. botulinum* strains, two of type A (ATCC 3502 and ATCC 19397), two of type B (FT 243 and 4B), and one of type F (ATCC 35415), using the rare-cutting restriction enzyme XhoI. The pulse time ramp was 1 to 15 s, and the running time was 18 h. The outermost lanes contain the Low Range PFG marker.

10). The same strains of type A and type F, which originated from different laboratories, were shown to be similar with all three enzymes.

The overall dendrogram showed a high heterogeneity (Fig. 4). With SacII, a total of 33 different PFGE patterns were produced, and with SmaI and XhoI, the numbers of patterns were 29 and 32, respectively (Table 2). By combining SacII with SmaI or with XhoI, an increase in the discriminatory index compared to the discriminatory index of SacII was noted, and the number of different PFGE types produced was increased to 38 with XhoI and to 37 with SmaI. When all three enzymes were combined, the number of PFGE types was 37.

As a whole, the clusters created by the three enzymes overlapped significantly. Clusters 1, 2, 3, 5, 9, and 12, created by using SacII (Fig. 4), were also present in the dendrograms created by using SmaI and XhoI. Clusters 6 and 7, produced by SacII (Fig. 4), were also created by using SmaI but combined to form a single cluster with XhoI. Cluster 11, formed with SacII, was also created with SmaI, while XhoI differentiated strain CDC15044 from other strains in this cluster. With SmaI and XhoI, all the type AB strains were included in a single cluster, while only two of these strains clustered together when SacII was used (Fig. 4, cluster 10). However, cluster 4, formed by SacII, was missed when SmaI or XhoI was used. Also, instead of cluster 8, as formed by SacII, composed of 126B and BL81/18B, there was a cluster composed of BL81/18B and BL81/20 (Fig. 4). In addition, 4B and McClung 133-4803, which were clearly differentiated with SacII digestion, were grouped in a cluster with SmaI or XhoI.

Despite the overall heterogeneity, three clusters at the 95% similarity level consisting of more than three strains of different origin were formed, one of the clusters representing type A strains and two clusters representing type B strains (Fig. 4, clusters 1, 7, and 11, respectively). Cluster 1 (Fig. 4) comprises six type A strains, including ATCC 7948, obtained from five sources. This strain was originally isolated from virgin soil in the United States in the early part of the 20th century, while RS-3 and BL81/18A were isolated in the latter part of the 20th century from Pacific red snapper in the United States and ham in Europe, respectively. Cluster 11 (Fig. 4) comprises 10 type B strains from diverse locations. Strains BL150, BL143, and FT239 are of European origin, while strains CDC15044, NCIB 4301, and ATCC 7949 are of American origin. These strains are also from different sources, including fish, fruit, and mud. The other type B cluster was composed of five Finnish soil samples.

DISCUSSION

Of the nine enzymes tested, SacII, SmaI, and XhoI resulted in the best fingerprint patterns and may thus be recommended for the differentiation of proteolytic *C. botulinum* strains. SacII produced the highest number of patterns as a single enzyme,

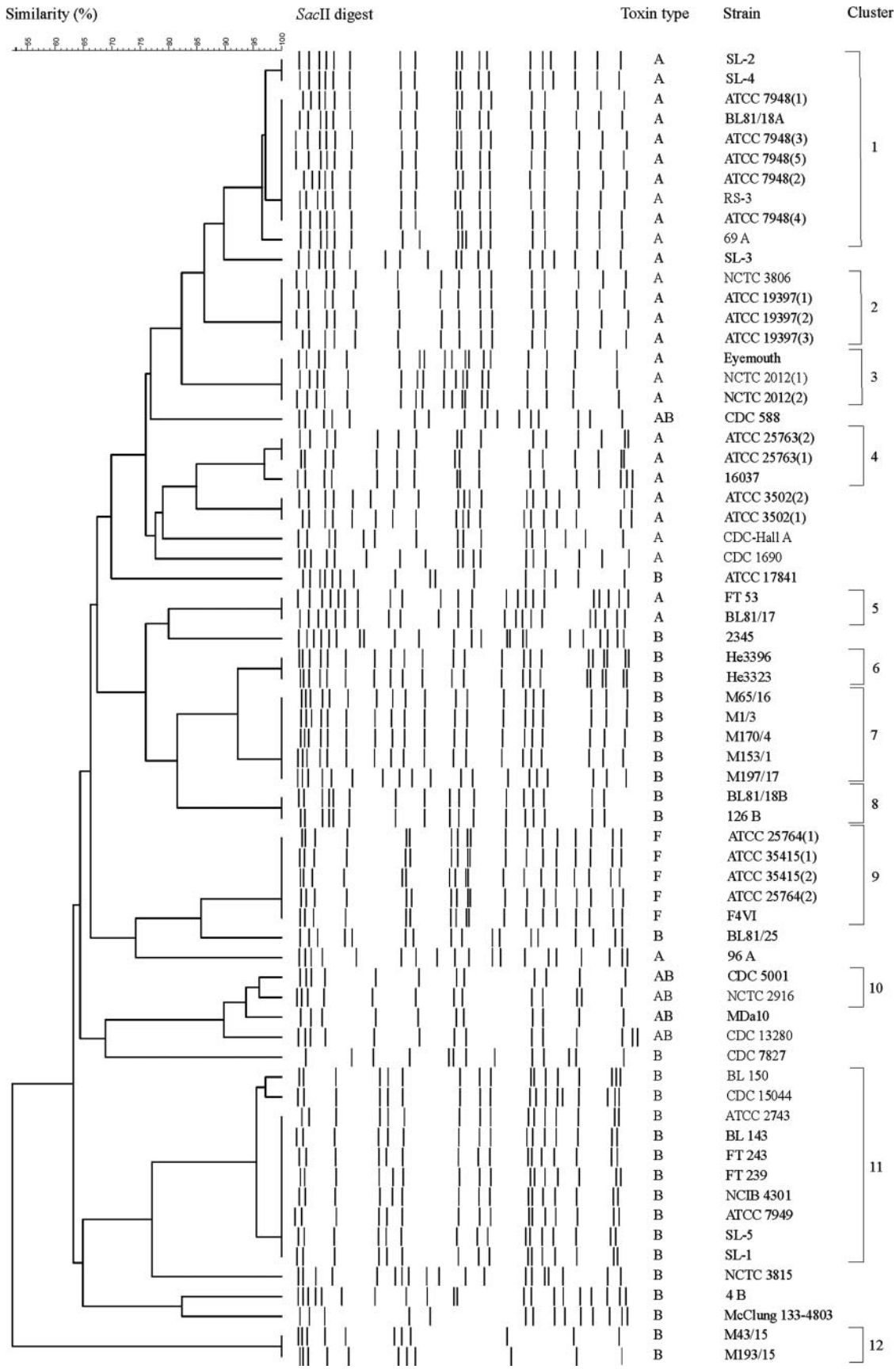

FIG. 4. Dendrogram of 55 proteolytic *C. botulinum* strains based on digestion patterns obtained with the SacII restriction enzyme.

TABLE 2. Discriminatory indices and numbers of clusters and PFGE profiles of 55 strains[a]

Enzymes used	No. of PFGE patterns	No. of clusters[b]	Discriminatory index
SacII	33	12	0.945
SmaI	29	12	0.941
XhoI	32	11	0.941
SacII + SmaI	37	10	0.943
SacII + XhoI	38	10	0.952
SmaI + XhoI	36	11	0.941
SacII + SmaI + XhoI	37	10	0.949

[a] Produced with different enzymes alone and combined data sets based on an analysis with BioNumerics software.
[b] Cluster refers to a group of strains at a 95% similarity level.

and thus, it seems that the use of SacII alone in epidemiological analyses would be an adequate and cost-effective choice for genomic analysis of proteolytic *C. botulinum* strains. If further discrimination of the strains or confirmation of strain similarity is needed, the number of different PFGE types and the discriminatory power of the method can be increased by using a combination of SacII and XhoI.

The PFGE method separated all the different toxin types of proteolytic *C. botulinum* into distinct clusters or individual branches at an 86% similarity level with both SacII and SmaI and at an 83% similarity level with XhoI. There were no specific fragments associated with type A, type B, or type F strains that could be used to determine toxin type. In the majority of cases, PFGE enabled discrimination between individual strains of proteolytic *C. botulinum* types A and B.

A total of seven out of nine Finnish isolates possessed similar patterns, even though the samples from which the organism was isolated were from different parts of Finland. They form only one cluster when XhoI is used and are divided into two clusters sharing a visually very similar fingerprint pattern with both SacII (Fig. 4, clusters 6 and 7) and SmaI. Despite the low number of the isolates studied, this finding might suggest a higher similarity between strains isolated from a restricted area, but further studies are needed to confirm this observation. Less variation in the PFGE patterns of the type B strains as a whole is noted compared to the patterns of the type A strains. While 54% of the type B strains were grouped into clusters containing more than three strains (Fig. 4, clusters 7 and 11), only 32% of type A strains were grouped in this way (Fig. 4, cluster 1). Interestingly, within clusters 1 and 11 (Fig. 4), there were strains which have been isolated from different types of materials from two different continents over an extend period of time. Within the type F strains, a high similarity between the patterns was observed, but since the number of the type F isolates available is small, it is not possible to make any conclusions concerning the pattern homology of type F strains as a whole.

On seven occasions, a strain which, according to the name, represented the same isolate was originally derived from more than one laboratory. These strains have previously been available in commercial culture collections and, dating back several decades, they have been exchanged between laboratories and gone through cycles of sporulation and germination. Even though these same strains may be considered similar, the fingerprint patterns have slight differences when looked at by the naked eye due to factors that have an effect on the electrophoretic migration of DNA bands (Fig. 4). The appropriate levels of optimization and tolerance in the numerical analysis guarantee that these intergel-related differences do not reflect the pairwise comparison result and dendrogram construction. The similarity between the fingerprint patterns of the same strains may suggest a high constancy of the genotype.

Compared to the method described previously for typing nonproteolytic *C. botulinum* (5), certain modifications were necessary when proteolytic *C. botulinum* strains were typed. When a volume of 8 ml of culture grown overnight was used for the DNA isolation, as suggested for nonproteolytic *C. botulinum* (5), the amount of DNA was too high to produce clear fragments, thus complicating the interpretation of the fingerprints. To decrease the DNA yield, cells were washed with only 4 ml of tryptone-peptone-glucose-yeast extract broth instead of 8 ml. In addition, a doubled volume of double-strength lysis solution was used for casting the plugs compared to the previous protocol. The gels were stained with a solution made in distilled water, as described previously (14), which was noted to produce a good resolution of the small fragments. With nonproteolytic *C. botulinum*, it has been noted that there are number of strains, indigestible by SmaI especially, possibly resulting either from CG methylation or to a lesser extent from the DNA degradation by endonucleases, as discussed previously (5, 9). This was not observed for strains of proteolytic *C. botulinum*, as all the strains included in this study were typeable with the three enzymes.

According to a previous report, SacII is not the best alternative for strain identification of nonproteolytic *C. botulinum* strains, and with certain strains, it has to be replaced by its isoschizomer, KspI (5). SmaI and XhoI were both among the five enzymes recommended for the differentiation of the nonproteolytic *C. botulinum* strains, but typing of the nonproteolytic *C. botulinum* using different enzyme combinations has not been previously reported. As nonproteolytic *C. botulinum* and proteolytic *C. botulinum* represent phylogenetically and physiologically distinct clostridia, it could be expected that the suitable enzymes for PFGE typing may differ between these two organisms.

This is the first description of the genetic diversity of proteolytic *C. botulinum*. Apart from the three distinct clusters observed, it may be concluded that, as studied by PFGE, proteolytic *C. botulinum* is a heterogeneous species. This finding differs from previous observations based on the analysis of 15 strains by RAPD (8). According to the survey (8), five type A strains (ATCC 3502, ATCC 19397, ATCC 25763, ATCC 7948, and SL-3) as well as two type B strains (McClung 133-4803 and ATCC 7949) were confirmed to be similar at the toxin type level by RAPD, but as demonstrated in our study, these strains may clearly be discriminated from each other by PFGE. Our results suggest that the PFGE method is to be considered a useful tool for studying the epidemiological relatedness of proteolytic *C. botulinum* isolate types A and B obtained from patients and from food. However, the possible occurrence of clusters that share a highly similar PFGE pattern, especially within the type B strains, should be noted, and therefore, epidemiological conclusions based on PFGE data only should be made with discretion. According to our results as well as those from previous research on the diversity of nonproteolytic

C. botulinum (5, 10), it may be concluded that establishing a worldwide PulseNet-like databank of *C. botulinum* could be of use for the purposes of molecular epidemiological research.

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