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Efficient DNA Fingerprinting of *Clostridium botulinum* Types A, B, E, and F by Amplified Fragment Length Polymorphism Analysis

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Amplified fragment length polymorphism (AFLP) analysis was applied to characterize 33 group I and 37 group II *Clostridium botulinum* strains. Four restriction enzyme and 30 primer combinations were screened to tailor the AFLP technique for optimal characterization of *C. botulinum*. The enzyme combination HindIII and HpyCH4IV, with primers having one selective nucleotide apiece (Hind-C and Hpy-A), was selected. AFLP clearly differentiated between *C. botulinum* groups I and II; group-specific clusters showed <10% similarity between proteolytic and nonproteolytic *C. botulinum* strains. In addition, group-specific fragments were detected in both groups. All strains studied were typeable by AFLP, and a total of 42 AFLP types were identified. Extensive diversity was observed among strains of *C. botulinum* type E, whereas group I had lower genetic biodiversity. These results indicate that AFLP is a fast, highly discriminating, and reproducible DNA fingerprinting method with excellent typeability, which, in addition to its suitability for typing at strain level, can be used for *C. botulinum* group identification.

Clostridium botulinum is the causative agent of botulism, a rare disease, but one that is life threatening if left untreated. Human botulism is mainly caused by *C. botulinum* strains belonging to groups I (proteolytic) and II (nonproteolytic). Classical food-borne botulism is generally the predominant disease form. However, in the United States, the most common form is infant botulism (30). Recently, wound botulism among injecting drug users has also become a problem (26, 27, 29). Despite its clinical importance, relatively little is known about the biodiversity of *C. botulinum*.

DNA-based typing methods, such as pulsed-field gel electrophoresis (PFGE), randomly amplified polymorphic DNA (RAPD), and ribotyping, have been applied to genotype *C. botulinum* (9, 11, 14, 20, 31). However, methods with good discriminatory power, typeability, and reproducibility are lacking. Although PFGE has proved to be highly discriminating for typing *C. botulinum* type E and other clostridia, it is not suitable for all clostridial strains due to the degradation of DNA as a result of extracellular DNase production (9, 15, 19). The use of RAPD in database creation is hindered by its inadequate reproducibility (7) and, finally, whereas ribotyping can be fully automated (Riboprinter), it is more suitable for subtyping bacterial species than strain typing because of its lower discriminating ability (11).

Amplified fragment length polymorphism (AFLP) analysis, a PCR-based DNA fingerprinting method initially described by Vos et al. (33), has gained notable interest as a genotyping method for bacterial species. AFLP analysis consists of three steps: the purified total DNA is digested with two restriction enzymes, which is followed by ligation of restriction site-specific adapters and amplification by PCR of a subset of fragments (33). AFLP analyses whole-genome DNA for polymorphism, and no prior sequence information about the target DNA is needed. Klaassen et al. (18) have also reported that, unlike PFGE, AFLP analysis is not affected by partial degradation of DNA.

AFLP has been shown to be highly reproducible and discriminative (17, 28). In addition to epidemiological and outbreak studies (1–4, 32), AFLP can also be used for differentiation and identification of bacteria at the species level (6, 16, 17, 28). Although AFLP has been used to characterize related species, such as *Clostridium difficile* (18), *Clostridium novyi* (25), and *Clostridium perfringens* (24), it has not, to our knowledge, been used in the molecular typing of *C. botulinum*.

The aim of the present study was to assess the applicability of AFLP analysis in characterization of *C. botulinum* groups I and II and to study the genetic biodiversity of *C. botulinum* types A, B, E, and F. In addition, various enzyme and primer combinations were screened to tailor the AFLP technique for optimal characterization of *C. botulinum*.

MATERIALS AND METHODS

Bacterial strains and PCR. A total of 33 group I and 37 group II *C. botulinum* strains from the 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 studied (Table 1). Multiplex PCR assay for the simultaneous detection of *C. botulinum* types A, B, E, and F, as previously described by Lindström et al. (22), or PCR assay for separate detection of type A, B, and E neurotoxin genes in *C. botulinum* (8) were used to confirm the serotype of each strain.

DNA extraction. DNA was extracted according to the method of Hyytiä et al. (14), with slight modifications. Strains were grown in a tryptose-peptone-glucose-yeast medium (Difco Laboratories, Detroit, Mich.) under anaerobic conditions at 37°C (*C. botulinum* group I) or 30°C (*C. botulinum* group II) for 14 to 16 h. The cells were lysed in TE (10 mM Tris-HCl, 1 mM EDTA) containing lysozyme (8.3 mg/ml; Sigma, St. Louis, Mo.) and mutanolysin (167 IU/ml; Sigma) at 37°C for 15 min (*C. botulinum* group I) or 2 h (*C. botulinum* group II) with gentle shaking. Lysis was completed by adding proteinase K (54 μg/ml; Finnzymes, Espoo, Finland), 0.24 M NaCl, 9.5 mM EDTA, and 0.8% (vol/vol) sodium dodecyl sulfate. The mixture was incubated at 60°C for 1 h with gentle shaking. Phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol]) and chloroform–2-pentanol (24:1 [vol/vol]) extractions were performed, and DNA was ethanol (95% [vol/vol]) precipitated and resuspended in TE overnight. RNA was removed by RNase (475 μg/ml; Sigma) at 37°C for 40 min with gentle shaking, followed by

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TABLE 1. *C. botulinum* strains used in this study

Serotype	Group	Strain[a]	Origin	Source[b]	PCR result for botulinum neurotoxin
A	I	ATCC 25763	Type strain	ATCC	A
	I	69A	Human stool	DFEH	A
	I	62A	Cow liver	DFEH	A
	I	93/2 (FT 30)	ND[c]	IFR	A
	I	RS-3A (Lindroth RS-3A)	Pacific red snapper	DFEH	A
	I	RS-4A (Lindroth RS-4A)	Pacific red snapper	DFEH	A
	I	ATCC 3502 (Hall 174A)	ND	ATCC	A
	I	93/21 (Eyemouth)*	ND	IFR	A
	I	93/33 (NCTC 2012)*	Wild duck paste	IFR	A
	I	93/42 (NCTC 3806)	Peas	IFR	A
	I	97/13 (CDC 1690)	Botulism outbreak	IFR	A
	I	BL 81/19 (16037)*	Botulism outbreak	IFR	A
AB	I	97/11 (MDa10)	Infant	IFR	A, B
	I	97/12 (CDC 588)	Botulism outbreak	IFR	A, B
	I	97/15 (NCTC 2916)	Corn	IFR	A, B
B	I	KS 30/6*	Pollen	DFEH	B
	I	KS 35/5*	Pollen	DFEH	B
	I	BL 81/21 (NCTC 3815)	Cheese	IFR	B
	I	BL 81/25	Asparagus	IFR	B
	I	126B	ND	DFEH	B
	I	BL81/29 (NCIB 10657)	ND	IFR	B
	I	BL 87/5B (CDC 15044)	Blackberry	IFR	B
	I	93/36 (4B)	ND	IFR	B
	I	KV40/10	Beeswax	DFEH	B
	I	BL 86/7A (BL150)	Fish	IFR	B
	I	KH57/1	Honey	DFEH	B
	I	93/37 (3807/1)	ND	IFR	B
	I	ATCC 17841 (1347B)	ND	ATCC	B
	I	93/3 (CDC 7827)	Human stool	IFR	B
	II	2B (Eklund 2)	Marine sediment	DFEH	B
	II	17B (Eklund 17, ATCC 25765)	Marine sediment	ATCC	B
	II	2217B (ATCC 17844)	ND	ATCC	B
	II	BL93/6 (CDC 5900)	Human	IFR	B
	II	BL 87/7B (5900 A-T-A-3)	Human	IFR	B
	II	BL 90/4 (Prevot 59)	ND	IFR	B
	II	BL 93/10 (Kapchunka B2)	Fish	IFR	B
E	II	K-3	Rainbow trout	DFEH	E
	II	K-7	Rainbow trout	DFEH	E
	II	K-8	Rainbow trout	DFEH	E
	II	K-22	Burbot	DFEH	E
	II	K-23	Burbot	DFEH	E
	II	K-25	Burbot	DFEH	E
	II	K-28	Burbot	DFEH	E
	II	K-29	Burbot	DFEH	E
	II	K-31	Vendace	DFEH	E
	II	K-32	Vendace	DFEH	E
	II	K-35	Vendace	DFEH	E
	II	K-37	Whitefish	DFEH	E
	II	K-44	Rainbow trout	DFEH	E
	II	K-45	Rainbow trout	DFEH	E
	II	K-46[c]	Rainbow trout roe	DFEH	E
	II	K-47	Rainbow trout	DFEH	E
	II	K-51[c]	Rainbow trout	DFEH	E
	II	K-53[c]	Rainbow trout	DFEH	E
	II	K-61[c]	Baltic herring	DFEH	E
	II	K-76	Vendace	DFEH	E
	II	K-125	Rainbow trout roe	DFEH	E
	II	K-126	Salmon	DFEH	E
	II	BL 93/7 (CDC 7854)	Fish	IFR	E
	II	BL 93/8 (CDC 8073)	Human	IFR	E
	II	31-2570E	ND	DFEH	E
	II	BL 81/26 (Beluga)	Beluga flipper	IFR	E
F	I	93/26 (ATCC 25764)	Crab	IFR	F
	I	93/29 (FT 15, Langeland)	ND	IFR	F
	I	93/30 (FT 42, ATCC 25764)	ND	IFR	F
	I	93/31 (FT 14, Langeland)	ND	IFR	F
	II	202 F (ATCC 23387)	Marine sediment	ATCC	F
	II	BL 86/32 (Colworth 47)	ND	IFR	F
	II	BL 86/33 (Colworth 187)	ND	IFR	F
	II	BL 86/34 (Colworth 195)	ND	IFR	F

[a] That is, the strain code; original name is in parentheses. *, strains used in initial testing.

[b] ATCC, American Type Culture Collection, Manassas, Va.; DFEH, Culture Collection of the Department of Food and Environmental Hygiene, University of Helsinki, Helsinki, Finland; IFR, Culture Collection of the Institute of Food Research, Norwich, England.

[c] ND, no data available.

TABLE 2. Thirty primer combinations screened during initial testing to determine optimal primer sets for AFLP typing of *C. botulinum*

Primer specific to rare-cutting restriction site sequence[a,b]	Primer specific to frequent-cutting restriction site sequence[a]
Eco-C	Hpy-C
Eco-C	Hpy-G
Apa-0	Hpy-A
Apa-0	Hpy-C
Apa-0	Hpy-G
Apa-A	Hpy-A
Apa-C	Hpy-0
Apa-C	Hpy-A
Apa-G	Hpy-0
Apa-G	Hpy-A
Hind-0	Mse-A
Hind-0	Mse-C
Hind-0	Mse-G
Hind-A	Mse-A
Hind-A	Mse-G
Hind-C	Mse-A
Hind-C	Mse-C
Hind-C	Mse-G
Hind-0	Hpy-A
Hind-0	Hpy-C
Hind-0	Hpy-G
Hind-A	Hpy-A
Hind-A	Hpy-C
Hind-A	Hpy-G
Hind-C	Hpy-A
Hind-C	Hpy-C
Hind-C	Hpy-G
Hind-G	Hpy-0
Hind-G	Hpy-A
Hind-G	Hpy-C

[a] 0, no selective extension; A, C, or G, selective nucleotide at the 3′ end of the primer.
[b] IRD800-labeled primer.

the addition of 0.2 M NaCl, chloroform–2-pentanol extraction, and ethanol precipitation. DNA concentrations were determined with a BioPhotometer (Eppendorf, Hamburg, Germany). DNA was stored at −70°C.

AFLP analysis. In initial testing, four enzyme combinations and 30 primer couplings (Table 2) were screened by using DNA extracted from *C. botulinum* type A ($n = 3$), B ($n = 2$), and E ($n = 4$) strains (Table 1).

An AFLP protocol described previously by Keto-Timonen et al. (17) was used. Briefly, DNA (400 ng) was digested with either 15 U of HindIII (New England Biolabs, Beverly, Mass.), 15 U of EcoRI (New England Biolabs), or 15 U of ApaI (Roche Diagnostics, Basel, Switzerland) and 15 U of HpyCH4IV (New England Biolabs) or 15 U of MseI (New England Biolabs) in 1× One-Phor-All Buffer Plus (Amersham Biosciences, Buckinghamshire, United Kingdom), bovine serum albumin (0.1 mg/ml), and dithiothreitol (5 mM). Restriction site-specific adapters (0.4 and 0.04 μM; MWG-Biotech AG, Ebersberg, Germany) (Table 3) were ligated with 1.1 U of T4 DNA ligase (New England Biolabs) in 1× One-Phor-All Buffer Plus, 200 μM ATP, 5 mM dithiothreitol, and bovine serum albumin at 0.1 mg/ml. Samples were stored at −20°C prior to PCR amplification.

Restriction fragments with specific adapters were diluted with sterile, distilled deionized water (1:10) and amplified by preselective PCR (72°C for 2 min, followed by 20 cycles of 94°C for 20 s, 56°C for 2 min, and 72°C for 2 min) by using primers without selective extension in a 20-μl reaction mixture containing 4 μl of diluted template DNA, 15 μl of Amplification Core Mix (Applied Biosystems, Foster City, Calif.), 25 nM primer specific to rare-cutting restriction site sequence (MWG-Biotech AG), and 125 nM primer specific to frequent-cutting restriction site sequence (MWG-Biotech AG) (Table 3). Primer combinations used during subsequent selective amplification are listed in Table 2. The templates were diluted with sterile, distilled deionized water (1:20) prior to selective amplification, which was performed in a 10-μl reaction mixture containing 1.5 μl of diluted template, a 50 nM concentration of IRD800-labeled primer specific to rare-cutting restriction site sequence (MWG-Biotech AG), a 250 nM concentration of primer specific to frequent-cutting restriction site sequence, and 7.5 μl of Amplification Core Mix. Treatment consisted of 94°C for 2 min, followed by 1 cycle of 94°C for 20 s, 66°C for 30 s, and 72°C for 2 min, followed by lowering the annealing temperature by 1°C each cycle to 56°C for 10 cycles, followed in turn by an additional 19 cycles at a 56°C annealing temperature and a final 30-min extension at 60°C. All PCR amplifications were performed with a PTC-200 Peltier thermal cycler (MJ Research, Inc., Waltham, Mass.).

Selective amplification products (10 μl) were mixed with 5 μl of stop solution (LI-COR, Lincoln, Nebr.), denatured at 95°C for 3 min, and placed on ice. Denatured fragments were separated on a 7% denaturing polyacrylamide gel. Electrophoresis was performed in 1× Tris-borate-EDTA buffer at 2,000 V, 35 mA, and 40 W at 50°C for 140 min on an automatic DNA sequencer (LI-COR Global IR2 4200LI-1 Sequencing System; LI-COR). A molecular weight marker (IRDye800 50- to 700-bp sizing standard; LI-COR) used for gel normalization was loaded onto every third lane. The level of reproducibility was determined by using *C. botulinum* type E strain K-51 as an internal reference, which underwent each step of the DNA extraction, AFLP analysis, and electrophoresis, thereby also providing a standard for comparison among different data sets. In addition, independent duplicate experiments were performed with a total of 38 *C. botulinum* strains representing different serotypes of groups I and II.

AFLP pattern analysis. AFLP patterns were analyzed by using BioNumerics software 3.0 (Applied Maths, Kortrijk, Belgium). The similarities between normalized AFLP patterns (range, 50 to 350 bp) were calculated with the Pearson product-moment correlation coefficient (1.0% optimization). Clustering and construction of dendrograms were performed by using the unweighted pair-group method with arithmetic averages.

RESULTS

To obtain the optimal distribution of DNA fragments, four different enzyme combinations were initially tested. The results

TABLE 3. Adapter and primer oligonucleotides used in AFLP

Oligonucleotide	Sequence(s)
AFLP adapters	
Apa adapter	5′-TCGTAGACTGCGTACAGGCC-3′ and 3′-CATCTGACGCATGT-5′
Eco adapter	5′-CTCGTAGACTGCGTACC-3′ and 3′-CTGACGCATGGTTAA-5′
Hind adapter	5′-CTCGTAGACTGCGTACC-3′ and 3′-CTGACGCATGGTCGA-5′
Hpy adapter	5′-GACGATGAGTCCTGAC-3′ and 3′-TACTCAGGACTGGC-5′
Mse adapter	5′-GACGATGAGTCCTGAG-3′ and 3′-CTACTCAGGACTCAT-5′
Core sequences of AFLP primers[a]	
Apa primer	5′-GACTGCGTACAGGCCC-3′
Eco primer	5′-GACTGCGTACCAATTC-3′
Hind primer	5′-GACTGCGTACCAGCTT-3′
Hpy primer	5′-CGATGAGTCCTGACCGT-3′
Mse primer	5′-GATGAGTCCTGAGTAA-3′

[a] No selective bases or an adjacent A, C, or G selective nucleotide at the 3′ end of the primer.

obtained with the enzyme combinations HindIII-HpyCH4IV, EcoRI-HpyCH4IV, or HindIII-MseI yielded high-quality fingerprints, with the HindIII-HpyCH4IV combination having the most uniform distribution of DNA fragments. The enzyme coupling ApaI and HpyCH4IV produced only a few fragments ranging from 50 to 350 bp with the eight primer combinations tested and thus was unsuitable for AFLP analysis. All primer combinations containing the Hind primer without selective extension generated complex banding patterns with numerous fragments and were deemed inappropriate for numerical analysis. The selective primers Hind-C and Hpy-A amplified DNA fragments evenly in the 50- to 350-bp range and consistently displayed strong signals on gels. In addition, strains described as identical by PFGE profiling yielded comparable results with AFLP analysis, and thus primers Hind-C and Hpy-A were selected for further analysis.

All strains studied were typeable by AFLP. The numerical analysis of AFLP profiles yielded two distinct group-specific clusters, with <10% similarity between proteolytic and nonproteolytic *C. botulinum* strains (Fig. 1). Strains belonging to groups I and II clustered together with a similarity value of 45 and 26%, respectively (Fig. 2 and 3). Group II was further divided into three main clusters. *C. botulinum* types B and F clustered together with a similarity value of 62%. The other two clusters consisted of *C. botulinum* type E strains.

All group I strains showed the group-specific fragments sizes of 129, 145, and 336 bp. (Fig. 2). Fragments of 114 and 315 bp were specific to *C. botulinum* group II strains (Fig. 3). However, no *C. botulinum* species- or serotype-specific fragments were observed. The PCR analysis of type A, B, E, and F neurotoxin genes yielded the expected amplification products and thus confirmed the serotype of each strain included in the study (Table 1).

In reproducibility testing, the banding pattern, measured based on fragment sizes, of the internal reference *C. botulinum* strain K-51 remained constant during each AFLP analysis. In addition, independent duplicate experiments of 38 *C. botulinum* strains resulted in identical AFLP banding profiles. Despite identical patterns, the internal reference sample showed 89% or higher similarity between different gels due to small differences in lane or background intensities. The 89% cutoff value was therefore used to define the AFLP type. By this criterion, 19 and 23 different AFLP types of *C. botulinum* groups I and II, respectively, were identified (Fig. 1).

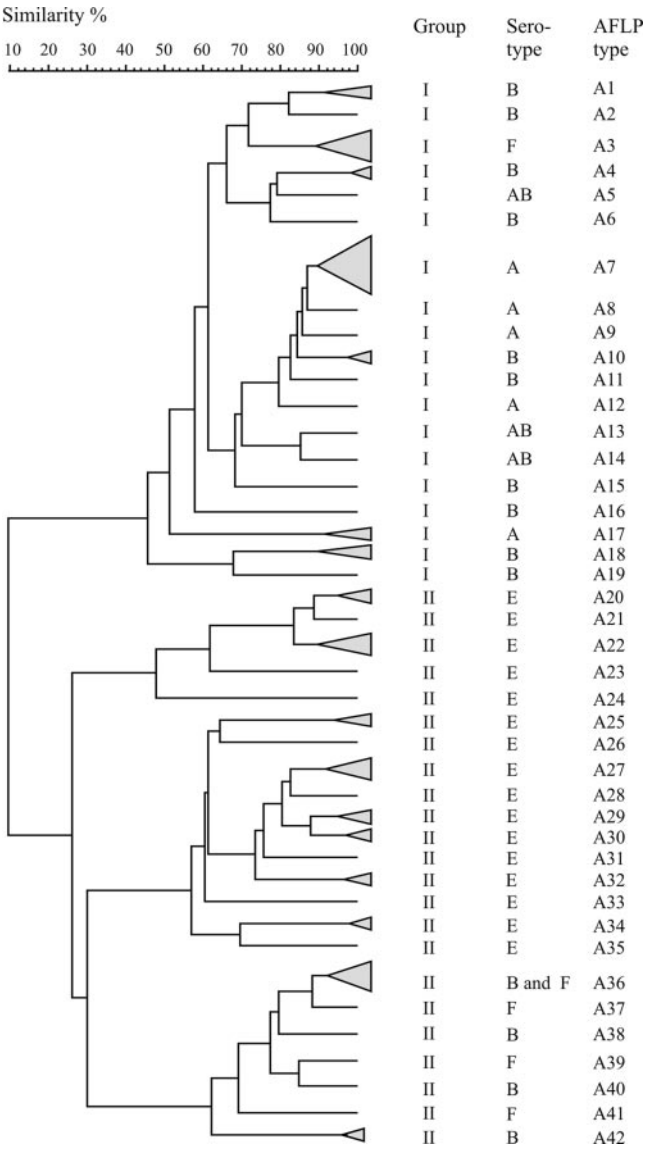

FIG. 1. Simplified dendrogram of AFLP profiles of *C. botulinum* group I (n = 33) and group II (n = 37) strains. Clusters containing AFLP patterns showing >89% similarity are shaded. A similarity analysis was performed by using the Pearson product-moment correlation coefficient, and clustering was performed by using the unweighted pair-group method with arithmetic averages. The cophenetic correlation of the dendrogram is 97%.

DISCUSSION

AFLP clearly differentiated between strains of *C. botulinum* in groups I and II. This is in accord with previous analyses of 16S rRNA sequences demonstrating that strains of group I *C. botulinum* types A, B, and F are phylogenetically distant from group II *C. botulinum* types B, E, and F (12, 13). Since group-specific AFLP fragments were also detected in both groups I and II, AFLP analysis may be suitable for group identification as well. Although some serotype-specific subclustering was revealed in both genomic groups, the AFLP method was deemed unsuitable for definitive serotype determination of *C. botulinum* types A, B, and F. Despite the fact that no serotype-specific fragments were observed, the two distinct serotype-specific clusters of *C. botulinum* type E suggest that AFLP databases can be used to define serotype E of *C. botulinum*.

In group II, extensive diversity was observed among strains of *C. botulinum* type E, which were divided into two main clusters, whereas type B and F strains showed highly similar AFLP profiles and clustered together with a similarity value of 62%. Extensive biodiversity between strains of type E was demonstrated earlier by PFGE (10, 15). However, no high similarity of fingerprints among type B and F strains has been revealed by ribotyping, PFGE, or RAPD, which might be due to the limited number of nonproteolytic *C. botulinum* type B and F strains included in these studies (9, 11, 14). In group I,

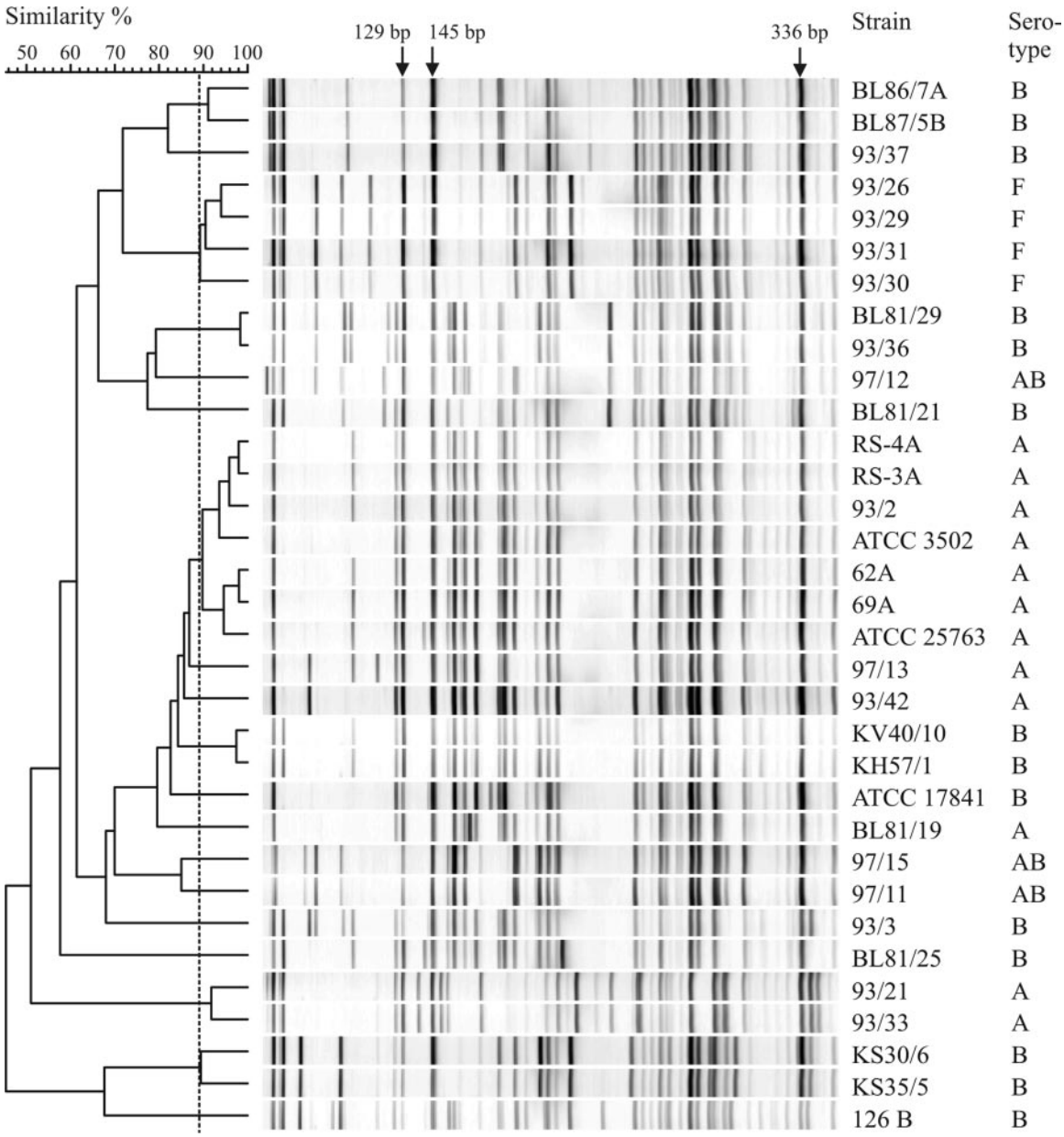

FIG. 2. Normalized banding patterns and dendrogram of 33 *C. botulinum* group I (proteolytic) strains based on AFLP analysis. A similarity analysis was performed by using the Pearson product-moment correlation coefficient, and clustering was performed by using the unweighted pair-group method with arithmetic averages. The dashed line shows the cutoff similarity value (89%). Arrows indicate the group-specific fragments.

both visual examination and numerical analysis of band patterns revealed less diversity than in group II. This is either due to true lower genetic biodiversity of the strains studied or due to the selected enzyme and primer combination being more suitable for discriminating group II strains. However, previous analyses by using RAPD, repetitive element sequence-based PCR, and ribotyping have also suggested less genetic variation among *C. botulinum* group I strains (11, 14).

A total of 42 different AFLP types were detected, indicating that AFLP is also an efficient tool for typing at the strain level. In groups I and II, indistinguishable or highly similar AFLP profiles were observed in strains that had no known common origin, a phenomenon also described by Hielm et al. (9) and Hyytiä et al. (15).

AFLP was found to be a highly reproducible method for DNA fingerprinting of *C. botulinum*. Since all strains studied were typeable by AFLP, the extracellular DNase production detected in some clostridial strains did not appear to affect the outcome of AFLP analysis. AFLP also proved to be a fast method; the analysis, including numerical data analysis, could be done within two working days when the analysis was begun with pure DNA. Moreover, less hands-on time is required than with methods such as PFGE, and partial automation enables high throughput of samples.

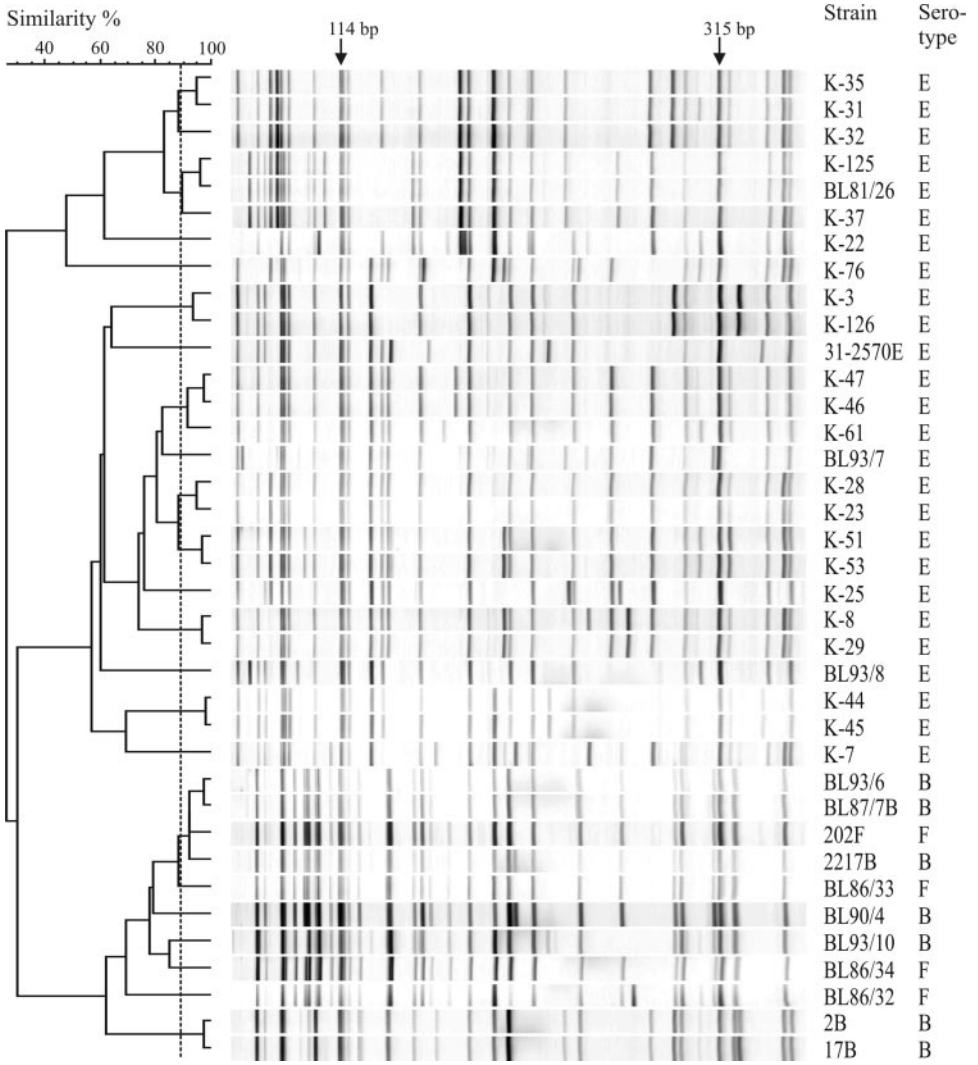

FIG. 3. Normalized banding patterns and dendrogram of 37 *Clostridium botulinum* group II (nonproteolytic) strains based on AFLP analysis. A similarity analysis was performed by using the Pearson product-moment correlation coefficient, and clustering was performed by using the unweighted pair-group method with arithmetic averages. The dashed line shows the cutoff similarity value (89%). Arrows indicate the group-specific fragments.

AFLP patterns can easily be collected in databases, and the electronic fingerprinting data, generated by an automated sequencer, facilitate the transmission of results between different laboratories. Nevertheless, standardization of protocols, reagents, and equipment is necessary before interlaboratory databases can be created. Due to the inevitable variation in lane or background intensities, it is also useful to use an internal reference strain to determine the cutoff value used for AFLP type definition. The similarity level of ≥89% for internal reference strains is in agreement with earlier findings (5, 28). Lindstedt et al. (21) suggested that variation in lane or background intensities might be due to differences in the effectiveness of digestion-ligation or PCR amplification steps. It is also worthwhile to ascertain visually that the banding patterns on the gel are of uniform quality prior to numerical analysis.

In the evaluation of a typing system, several criteria must be assessed. The most important of these are typeability, ease of interpretation and performance, reproducibility, and discriminatory power (23). The results of the present study indicate that AFLP is a fast, reproducible, and highly discriminating genotyping method with excellent typeability for characterization of *C. botulinum* group I and II strains. These features make AFLP an attractive alternative to other genotyping methods in outbreak situations. In addition to typing at strain level, AFLP may be a suitable tool for *C. botulinum* group identification.

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