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Identification of *Clostridium* Species and DNA Fingerprinting of *Clostridium perfringens* by Amplified Fragment Length Polymorphism Analysis[▽]

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An amplified fragment length polymorphism (AFLP) method was applied to 129 strains representing 24 different *Clostridium* species, with special emphasis on pathogenic clostridia of medical or veterinary interest, to assess the potential of AFLP for identification of clostridia. In addition, the ability of the same AFLP protocol to type clostridia at the strain level was assessed by focusing on *Clostridium perfringens* strains. All strains were typeable by AFLP, so the method seemed to overcome the problem of extracellular DNase production. AFLP differentiated all *Clostridium* species tested, except for *Clostridium ramosum* and *Clostridium limosum*, which clustered together with a 45% similarity level. Other *Clostridium* species were divided into species-specific clusters or occupied separate positions. Wide genetic diversity was observed among *Clostridium botulinum* strains, which were divided into seven species-specific clusters. The same AFLP protocol was also suitable for typing *C. perfringens* at the strain level. A total of 29 different AFLP types were identified for 37 strains of *C. perfringens*; strains initially originating from the same isolate showed identical fingerprinting patterns and were distinguished from unrelated strains. AFLP proved to be a highly reproducible, easy-to-perform, and relatively fast method which enables high throughput of samples and can serve in the generation of identification libraries. These results indicate that the AFLP method provides a promising tool for the identification and characterization of *Clostridium* species.

The genus *Clostridium* is a heterogeneous group of bacteria which currently consists of 181 described species. Clostridia are widely distributed in the environment as well as in the intestinal tract of humans and of many animals. Several *Clostridium* species are pathogenic to humans, domestic animals, or wildlife and are responsible for well-known clostridial diseases such as tetanus, gas gangrene, botulism, pseudomembranous colitis, and food-borne illness (10). In addition, clostridia can be involved in a variety of human infections, such as cholecystitis, pneumonia, bacteremia, empyema, and abscesses, and can thus be isolated from various clinical specimens. However, many of the isolates can be occasional contaminants, or nonpathogenic clostridia, and may not be involved in the disease process. Therefore, the reliable identification of clostridia, isolated from clinical specimens, is important. In addition, a link must be established between isolated clostridia and pathological changes.

Despite the clinical significance of clostridia, reliable, practical, and fast identification methods are few. Although simple tests can serve to identify most commonly isolated *Clostridium* species, the identification of other clostridia by conventional biochemical testing and gas-liquid chromatography is still laborious, expensive, and time-consuming. Furthermore, several commercial identification systems for anaerobic bacteria have failed to accurately identify *Clostridium* species (23, 29, 30, 43). Due to these evident drawbacks of conventional methods, there is a growing trend toward molecular diagnostics of bacteria that are difficult to identify by phenotypic characters (28, 42).

Amplified fragment length polymorphism (AFLP) is a DNA fingerprinting method based on the digestion of total DNA with two restriction enzymes, followed by the ligation of restriction site-specific adapters and the amplification of a subset of fragments by PCR (46). The AFLP approach, using a fluorescently labeled primer and detection of the fragments with an automated DNA sequencer, allows the use of internal size markers for accurate band sizing as well as partial automation and straightforward computer-assisted analysis, which make AFLP advantageous for the generation of extensive fingerprinting databases.

AFLP has proven to be a useful tool in epidemiological and outbreak studies (1, 5, 9, 20). In addition to strain typing, AFLP has been utilized in taxonomic studies. AFLP has been used to differentiate *Aeromonas* (16), *Acinetobacter* (19), *Arcobacter* (36), avian mycoplasma (13), *Burkholderia* (3), *Campylobacter* (7, 35), and *Xanthomonas* (18), and the results appear to agree closely with results obtained by DNA-DNA hybridization experiments (2, 3, 16, 38).

AFLP has been used to genotype *Clostridium botulinum* (21), *Clostridium difficile* (24), *Clostridium novyi* (32), and *Clostridium perfringens* (31), but the ability of AFLP analysis in differentiating *Clostridium* species remains unevaluated. Therefore, we applied an AFLP protocol previously used to characterize *C. botulinum* on 24 different *Clostridium* species, with special emphasis on pathogenic clostridia of medical or veterinary interest, to examine whether AFLP is a suitable tool for

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TABLE 1. List of *Clostridium* strains used in the study

Species	Strain code	Origin	Source[a]
C. aurantibutyricum	ATCC 17777	Type strain	DMM
C. baratii	CCUG 24033	Type strain	DMM
C. bifermentans	Cbif patient	Patient	DMM
	1213/00	ND[b]	EVIRA
	Cbif 101[c]	ND	DFEH
	271	ND	DFEH
	Cbif 9855/69	ND	DFEH
C. botulinum type A	ATCC 25763	Type strain	DFEH
	ATCC 3502	ND	DFEH
	ATCC 7948[d]	Virgin soil	IFR
	62A (ATCC 7948)[d]	Virgin soil	DFEH
	NCTC 3806	Peas	IFR
C. botulinum type AB	NCTC 2916	Home-canned corn	IFR
	NCFB 3037[c]	Infant botulism	IFR
C. botulinum type B	ATCC 17841 (p)[e]	ND	DFEH
	ATCC 17844 (np)[e]	ND	DFEH
	126 B (p)	ND	DFEH
	CDC 7827 (p)	Human stool	IFR
	KV 40/10 (p)	Beeswax	DFEH
	Eklund 2B (np)	Marine sediment	DFEH
	Prevot 59 (np)	ND	IFR
	Kapchunka B2 (np)	Fish	IFR
C. botulinum type C	ATCC 17782[c]	ND	DMM
	Cbot 106C [c]	Horse	DFEH
	Stockholm C	ND	DFEH
	1672 C	ND	DFEH
C. botulinum type D	ATCC 9633	Cattle botulism (lamziekte)	DFEH
	Gunisson D	ND	DFEH
C. botulinum type E	K-8	Rainbow trout intestines	DFEH
	K-22	Burbot intestines	DFEH
	K-25	Burbot surface	DFEH
	K-35	Vendace	DFEH
	K-37	Hot smoked whitefish	DFEH
	K-51	Rainbow trout surface	DFEH
	K-126	Hot smoked salmon	DFEH
	31-2570E	ND	DFEH
C. botulinum type F	ATCC 25764 (p)[c]	Crab	IFR
	ATCC 23387 (np)	Marine sediment	DFEH
	FT 14 (p)	ND	IFR
	Colworth 47 (np)	ND	IFR
	Colworth 195 (np)	ND	IFR
C. butyricum	ATCC 19398	Type strain	DMM
	Cbutyr 102	ND	DFEH
	BL-5262-9RE[c]	Human	IFR
	1204	Silage	DFEH
C. cadaveris	CCUG 24035	Type strain	DMM
C. chauvoei	41	ND	DFEH
	95/1147[c]	ND	EVIRA
C. difficile	ATCC 9689	Type strain	DMM
	ATCC 17858	ND	DMM
	CCUG 19126	Human abdominal wound	DMM
C. hastiforme	ATCC 33268	Type strain	DMM
C. histolyticum	ATCC 8034	ND	DMM
	105009	ND	DMM
C. innocuum	ATCC 14501	Type strain	DMM
C. limosum	CCUG 24037	Type strain	DMM
C. novyi	LV 5536	ND	DMM
	139/99	ND	EVIRA
C. paraputrificum	Cparap patient	Patient	DMM
C. perfringens	ATCC 13124[d]	Type strain	DMM
	ATCC 3624[c]	ND	DFEH
	ATCC 3626[c]	Intestinal contents of lamb	DFEH
	NCTC 8237 (ATCC 13124)[d]	Type strain	EVIRA
	NCTC 3110	ND	EVIRA
	NCTC 3180[d]	Ovine enterotoxaemia (struck)	EVIRA
	NCTC 8239[c]	Salt beef	DFEH
	NCTC 8798	Meat rissole, food poisoning outbreak	DFEH

Continued on facing page

TABLE 1—*Continued*

Species	Strain code	Origin	Source[a]
	NCTC 8346[d]	Sheep	EVIRA
	NCTC 8084	ND	EVIRA
	NCTC 10239	ND	DFEH
	CCUG 44727	ND	DFEH
	CCUG 2036 (NCTC 3180)[d]	Ovine enterotoxaemia (struck)	DFEH
	CCUG 2037 (NCTC 8346)[c,d]	Sheep	DFEH
	CCUG 2038	ND	DFEH
	88V10-8	Human	DFEH
	42	ND	DFEH
	106	ND	DFEH
	AAD 1527e	Antibiotic-associated diarrhea	DFEH
	SIDS 5-14	Infant intestines	DFEH
	Cperf 606	ND	DFEH
	10204	Food	DFEH
	D9030[f]	Human, food poisoning outbreak	DFEH
	D903[f]	Human, food poisoning outbreak	DFEH
	D9032[f]	Human, food poisoning outbreak	DFEH
	Perf pat	Patient	DMM
	VELL 35/3	ND	DMM
	SB 3992[c]	ND	DMM
	4732[c]	Game fry	DFEH
	108	ND	DFEH
	15525/88	Human	DFEH
	15563/88	Human	DFEH
	F-3686	ND	DFEH
	88V10-9	Human	DFEH
	15394/88	Human	DFEH
	Cperf 103A	ND	DFEH
	Cperf 104D	ND	DFEH
C. ramosum	CCUG 24038	Type strain	DMM
C. rectum	DSMZ 1295	Type strain	DMM
C. septicum	ATCC 12464[c]	Type strain	DMM
	105	ND	DFEH
	43[c]	ND	DFEH
	105011[c]	ND	DMM
	501/96[c]	ND	EVIRA
	105020	ND	DMM
C. sporogenes	ATCC 3584	Type strain	DMM
	ATCC 19404[c]	Gas gangrene	DMM
	ATCC 25784	Reference strain	DMM
	AMRI/96	ND	DFEH
	KL8	Cheese	DFEH
	KL4	Cheese	DFEH
	M2843	ND	DFEH
	M66-7-1	Cheese	DFEH
	105008	ND	DMM
	NINF 45	ND	DMM
	Patient −88	Patient	DMM
C. sordellii	ATCC 9714[c]	Type strain	DMM
	NCTC 8780	ND	DMM
	LV1765/1988	ND	DMM
	105013[c]	ND	DMM
	64/97[c]	ND	EVIRA
	Csord patient	Patient	DMM
	Csord 105	ND	DFEH
C. subterminale	ATCC 25774[c]	Type strain	DMM
C. tertium	ATCC 14573[c]	Type strain	DMM
	ATCC 19405	ND	DMM
C. tetani	ATCC 19406[c]	Type strain	DMM
	105012[c]	ND	DMM
C. tyrobutyricum	1193	Cheese	DFEH
	1194	Cheese	DFEH

[a] DFEH, Department of Food and Environmental Hygiene, University of Helsinki, Finland; DMM, Department of Medical Microbiology, University of Turku, Finland; EVIRA, Finnish Food Safety Authority, Kuopio Research Unit, Finland; IFR, Institute of Food Research, Norwich, England.
[b] ND, no data available.
[c] Strains used in reproducibility testing.
[d] The same strain was also obtained from another culture collection with a different strain code (given in parentheses).
[e] np, nonproteolytic; p, proteolytic.
[f] Isolates originating from the same outbreak.

the identification of clostridia. In addition, we assessed the ability of the same AFLP protocol to type clostridia at the strain level by focusing on *C. perfringens* strains of all five toxin types.

MATERIALS AND METHODS

Bacterial strains. A total of 129 strains of 24 different *Clostridium* species from the Culture Collections of the Department of Food and Environmental Hygiene, University of Helsinki, Finland; the Department of Medical Microbiology, University of Turku, Finland; the Institute of Food Research, Norwich, United Kingdom; and the Finnish Food Safety Authority, Kuopio Research Unit, Kuopio, Finland, were studied (Table 1). Strains consisted of type strains ($n = 18$) and strains originating from diverse sources and locations, including clinical, environmental, and food samples. Three *C. perfringens* isolates originated from the same outbreak and three of the *C. perfringens* NCTC reference strains (NCTC 3180, NCTC 8237, and NCTC 8346) were also obtained from another culture collection with different strain codes (CCUG 2036, ATCC 13124, and CCUG 2037, respectively).

DNA extraction. Total DNA was extracted as previously described by Hyytiä et al. (17), with slight modifications. Strains were grown in a tryptose-peptone-glucose-yeast medium (Difco Laboratories, Detroit, MI) under anaerobic conditions at the optimal growth temperature for each strain for 14 to 16 h. The cells were resuspended with 400 μl TE (10 mM Tris-HCl, 1 mM EDTA) and incubated with 7.9 mg/ml lysozyme (Sigma, St. Louis, MO), 159 IU/ml mutanolysin (Sigma), and 476 μg/ml RNase (Sigma) at 37°C with gentle shaking for 15 min (*C. botulinum* group I), 2 h (*C. botulinum* group II), or 1 h (other *Clostridium* species). Lysis was completed by adding 52 μg/ml proteinase K (Finnzymes, Espoo, Finland), 0.23 M NaCl, 9.1 mM EDTA, and 0.8% (vol/vol) sodium dodecyl sulfate. After thorough mixing, 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 the DNA was precipitated with ethanol (95% [vol/vol]), rinsed with 70% ethanol, and resuspended with 100 μl sterile, distilled, deionized water; DNA was stored at −70°C. DNA concentrations were determined using a BioPhotometer (Eppendorf, Hamburg, Germany).

AFLP analysis. An AFLP protocol previously used to characterize *C. botulinum* strains (21) was used with some modifications. Total DNA (400 ng) was digested with 15 U HindIII (New England Biolabs, Beverly, MA) and 15 U HpyCH4IV (New England Biolabs) in 1× One-Phor-All buffer plus (Amersham Biosciences, Buckinghamshire, United Kingdom), 5 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin. Restriction site-specific HindIII adapter (0.04 μM; Oligomer, Helsinki, Finland) and HpyCH4IV adapter (0.4 μM; Oligomer) (Table 2) were ligated with 1.1 U T4 DNA ligase (New England Biolabs) in 1× One-Phor-All buffer plus (Amersham Biosciences), 5 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 200 μM ATP. Samples were stored at −20°C prior to PCR amplification.

The digested and ligated DNA samples were diluted with sterile, distilled, deionized water (1:2) and amplified by preselective PCR (72°C for 2 min; 20 cycles of 94°C for 20 s, 56°C for 2 min, and 72°C for 2 min) in a 20-μl reaction mixture containing 4 μl of diluted template DNA, 15 μl amplification core mix (Applied Biosystems, Foster City, CA), 25 nM Hind-0 primer (Oligomer), and 125 nM Hpy-0 primer (Oligomer) (Table 2). After preselective amplification, the templates were diluted with sterile, distilled, deionized water 1:20. Selective amplification was performed in a 10-μl reaction mixture containing 1.5 μl of diluted template, 50 nM 6-carboxyfluorescein (FAM)-labeled Hind-C primer (Oligomer), 250 nM Hpy-A primer (Oligomer) (Table 2), and 7.5 μl amplification core mix (Applied Biosystems) (94°C for 2 min; 1 cycle of 94°C for 20 s, 66°C for 30 s, 72°C for 2 min; then the annealing temperature was lowered by 1°C each cycle to 56°C [10 cycles], followed 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, MA).

Selective amplification products (1 μl) were mixed with 0.5 μl of internal size standard (GS-500 LIZ; Applied Biosystems) to enable accurate band sizing and 11.5 μl Hi-Di formamide (Applied Biosystems) and denatured at 95°C for 2 min. Denatured fragments were electrophoresed on POP-4 polymer (Applied Biosystems) on an ABI PRISM 310 genetic analyzer (Applied Biosystems) in 1× genetic analyzer buffer with EDTA (Applied Biosystems). The electrophoresis conditions were 15 kV at 60°C for 28 min. Data preprocessing was performed using GeneScan 3.7 fragment analysis software (Applied Biosystems).

TABLE 2. Adapter and primer oligonucleotides used in AFLP

Oligonucleotide	Sequence
Adapters	
HindIII	5′-CTCGTAGACTGCGTACC-3′
	3′-CTGACGCATGGTCGA-5′
HpyCH4IV	5′-GACGATGAGTCCTGAC-3′
	3′-TACTCAGGACTGGC-5′
Primers	
Hind-0	5′-GACTGCGTACCAGCTT-3′
Hpy-0	5′-CGATGAGTCCTGACCGT-3′
Hind-C (FAM labeled)	5′-GACTGCGTACCAGCTTC-3′
Hpy-A	5′-CGATGAGTCCTGACCGTA-3′

Reproducibility testing. Reproducibility of the method was determined by performing duplicate ($n = 17$), triplicate ($n = 5$), fivefold ($n = 2$), or sixfold ($n = 1$) experiments, including DNA extraction, AFLP analysis, electrophoresis, and numerical data analysis, with a total of 25 strains representing different *Clostridium* species (Table 1). In addition, reproducibility among different data sets was assessed using *C. botulinum* type E strain K-51 as an internal reference, which underwent each step of the DNA extraction and AFLP analysis a total of 11 times, thereby providing a standard for comparison among different data sets.

AFLP pattern analysis. AFLP patterns were analyzed using BioNumerics software, version 4.5 (Applied Maths, Kortrijk, Belgium), and similarity between normalized AFLP patterns (range 75 to 450 bp) was calculated with the Pearson product-moment correlation coefficient. Clustering and construction of dendrograms were performed by using the unweighted pair-group method with arithmetic averages.

RESULTS

All clostridial strains studied were typeable by AFLP. The enzyme and primer combination used in AFLP analysis generated evenly distributed banding patterns in the range of 75 to 450 bp. Based on the cluster analysis, the 45% similarity level served to differentiate *Clostridium* strains at the species level (Fig. 1). By this criterion, AFLP analysis divided *Clostridium* strains into 21 clusters (I to XXI); 20 of the clusters consisted of strains belonging to a single species. In addition, eight strains, which were the only representatives of the particular species studied, occupied separate positions. AFLP failed, however, to discriminate between *Clostridium ramosum* and *Clostridium limosum*, which clustered together with a 45% similarity level (cluster XIV).

In reproducibility testing among different data sets, the internal reference *C. botulinum* strain K-51 showed 93% or higher similarity due to small variations in peak heights. However, the resulting AFLP banding patterns, measured based on fragment sizes, remained constant during each separate run. In addition, after independent repeated experiments in the 25 different *Clostridium* strains tested, none of the AFLP banding profiles changed. The 93% cutoff value served to define the AFLP type of *C. perfringens* strains.

C. botulinum strains were divided into seven distinct clusters. Group I (proteolytic) *C. botulinum* strains were linked together at a similarity value of 56% (cluster III). Group II (nonproteolytic) *C. botulinum* strains formed three clusters; cluster XV consisted of strains of *C. botulinum* types B and F, whereas *C. botulinum* type E strains were divided into clusters XVI and XVII. *C. novyi* and group III *C. botulinum* types C and D clustered together with a similarity value of 22%. *C. novyi* and *C. botulinum* type D formed single clusters XI and XIII,

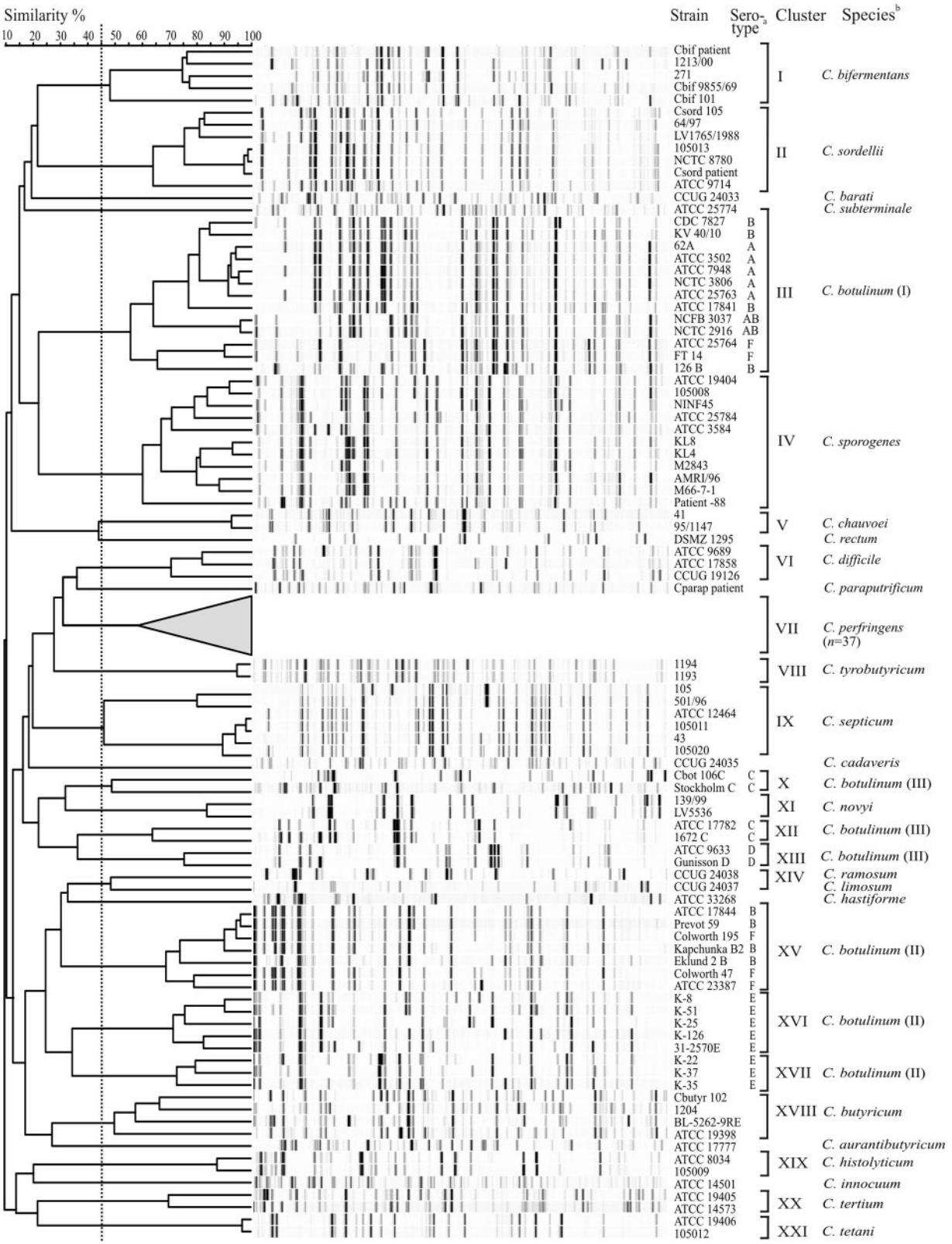

FIG. 1. Reconstructed AFLP banding patterns and a dendrogram of 24 *Clostridium* species based on AFLP analysis. The cluster containing 37 *C. perfringens* strains is shaded. A similarity analysis was performed using the Pearson product-moment correlation coefficient, and clustering was performed by using the unweighted pair-group method with arithmetic averages. *Clostridium* clusters generated at the 45% similarity level (dashed line) are marked with Roman numerals (I to XXI). a, serotype of *C. botulinum* strains; b, the *C. botulinum* group definition is in parentheses.

respectively, whereas *C. botulinum* type C strains were divided into two separate clusters (X and XII). The AFLP analysis clearly differentiated between *C. botulinum* group I and *Clostridium sporogenes*. However, *C. sporogenes* and *C. botulinum* group I strains were linked together at a similarity value of 22%.

C. perfringens strains ($n = 37$) clustered together with a similarity value of 58% (Fig. 1 and 2). Two subclusters of *C. perfringens* strains were observed. Strains belonging to subclusters 1 and 2 clustered together with a similarity value of 62% and 85%, respectively. In subcluster 1, toxin types A, B, C, D, and E were detected, whereas subcluster 2 consisted only of strains of toxin type A (Fig. 2). With a 93% cutoff value, *C. perfringens* strains were divided into 29 different AFLP types. However, visual examination of AFLP patterns of *C. perfringens* revealed minor fragment differences in strains of AFLP types aflp3 and aflp5. In three events, the same *C. perfringens* strain was obtained from two different commercial culture collections. The AFLP analysis of these pairs that initially originate from the same isolate resulted in identical fingerprinting patterns. Identical AFLP profiles were also observed for isolates originating from the same outbreak.

DISCUSSION

In this present study, we applied AFLP to 24 different *Clostridium* species and a total of 129 strains. AFLP differentiated all species tested, except for *C. ramosum* and *C. limosum*. Thirteen species were separated into single species-specific clusters, and eight strains, which were the only representatives of those particular species, also occupied separate positions. Furthermore, *C. botulinum* strains were divided into seven species-specific clusters. Although AFLP failed to discriminate between *C. ramosum* and *C. limosum* at the 45% similarity level, these species were linked together at a relatively low similarity level of 49%, and the differences between AFLP patterns were evident in visual examination. Since it is necessary to extend the identification library with several AFLP patterns of well-defined strains for each species to obtain reliable species identification (7), further AFLP analysis on larger numbers of strains of *C. ramosum* and *C. limosum* may facilitate differentiation between these species as well.

Some *Clostridium* strains are known to produce extracellular DNase, which may limit the use of DNA fingerprinting methods such as pulsed-field gel electrophoresis (PFGE) (11, 25, 41, 44, 45). Since all strains studied were typeable by AFLP, the AFLP method seemed to overcome the problem of extracellular DNase production. Furthermore, the AFLP method proved to be highly reproducible and the similarity level of ≥93% for internal reference strains is in accordance with earlier studies (3, 4, 6, 22, 36). The slight variation seen in peak heights of AFLP patterns during reproducibility testing may result from differences in the effectiveness of digestion-ligation or PCR amplification steps (27). The 93% cutoff value used for defining the AFLP type of *C. perfringens* and the 45% similarity level, which served to differentiate *Clostridium* strains at the species level, are in agreement with previous AFLP studies of different bacteria (6, 22, 39). AFLP also proved to be a relatively fast, easy-to-use method. The AFLP analysis, including numerical data analysis, can be completed within two working days when initiated with pure DNA.

The results of the AFLP analysis confirmed the phylogenetic finding based on 16S rRNA sequencing of three distinct lineages of *C. botulinum* groups I, II, and III (15). The distribution of *C. botulinum* strains in more than one cluster may also stem from the wide genetic diversity observed among group II *C. botulinum* strains. This is in accordance with previous studies based on PFGE (12) and AFLP (21).

C. sporogenes, which displays high 16S rRNA sequence homology (15) and DNA relatedness (26) with group I *C. botulinum* types A, B, and F, is considered a nontoxigenic counterpart of group I *C. botulinum*. Ghanem et al. (8) also reported that 17% of *C. sporogenes* strains were incorrectly identified as *C. botulinum* type A or B by cellular fatty acid analysis. The AFLP results are in agreement with those of previous studies on the close relationships of *C. botulinum* group I and *C. sporogenes*, since these species were linked together with AFLP, albeit with a relatively low similarity value. With AFLP, however, a clear distinction emerged between *C. botulinum* group I and *C. sporogenes* species-specific clusters, indicating that AFLP analysis is capable of discriminating between these *Clostridium* species.

C. novyi type A and *C. botulinum* types C and D cannot be differentiated from each other by their phenotypic properties, and *C. novyi* type A is considered a nontoxigenic variant of group III *C. botulinum* (14). That group III *C. botulinum* and *C. novyi* clustered together with AFLP suggests that they are closely related. This is in accordance with previous studies based on 16S rRNA gene sequence analysis, which revealed that *C. novyi* type A and *C. botulinum* types C and D are grouped as a separate phylogenetic lineage (15). With AFLP, single species-specific subclusters were observed for *C. novyi* and *C. botulinum* type D, while *C. botulinum* type C strains were divided in two separate subclusters. Wide diversity among *C. botulinum* type C has also been reported by Nakamura et al. (34), who found that based on DNA-DNA homology studies, one strain of *C. botulinum* type C (Stockholm strain) was more closely related to a group consisting of *C. novyi* type B and *Clostridium haemolyticum* strains than to the other strains of *C. botulinum* type C examined.

Phenotypically, *C. bifermentans* closely resembles *C. sordellii*. The main criterion for the differentiation of these species is the lack of urease production by *C. bifermentans*, although researchers have reported urease-negative strains of *C. sordellii* (33). In this present study, one of the *C. sordellii* strains (LV1765/1988) was urease negative, which could hamper identification based on phenotypic testing. However, AFLP clearly differentiated between *C. sordellii* and *C. bifermentans* strains.

The AFLP method previously described for the characterization of *C. botulinum* (21) also proved suitable for the characterization of *C. perfringens*. Although some toxinotype-related subclustering was observed, in general, AFLP was deemed unsuitable for differentiation between various toxinotypes of *C. perfringens*. This finding was expected, since genes encoding three major toxins of *C. perfringens* (β, ε, and ι toxins) are located on plasmids, thus enabling even toxinotype change of a strain by loss or acquisition of plasmids (37). The existence of strains of different toxin types in the same cluster has also been revealed by multiple-locus variable-number tandem-re-

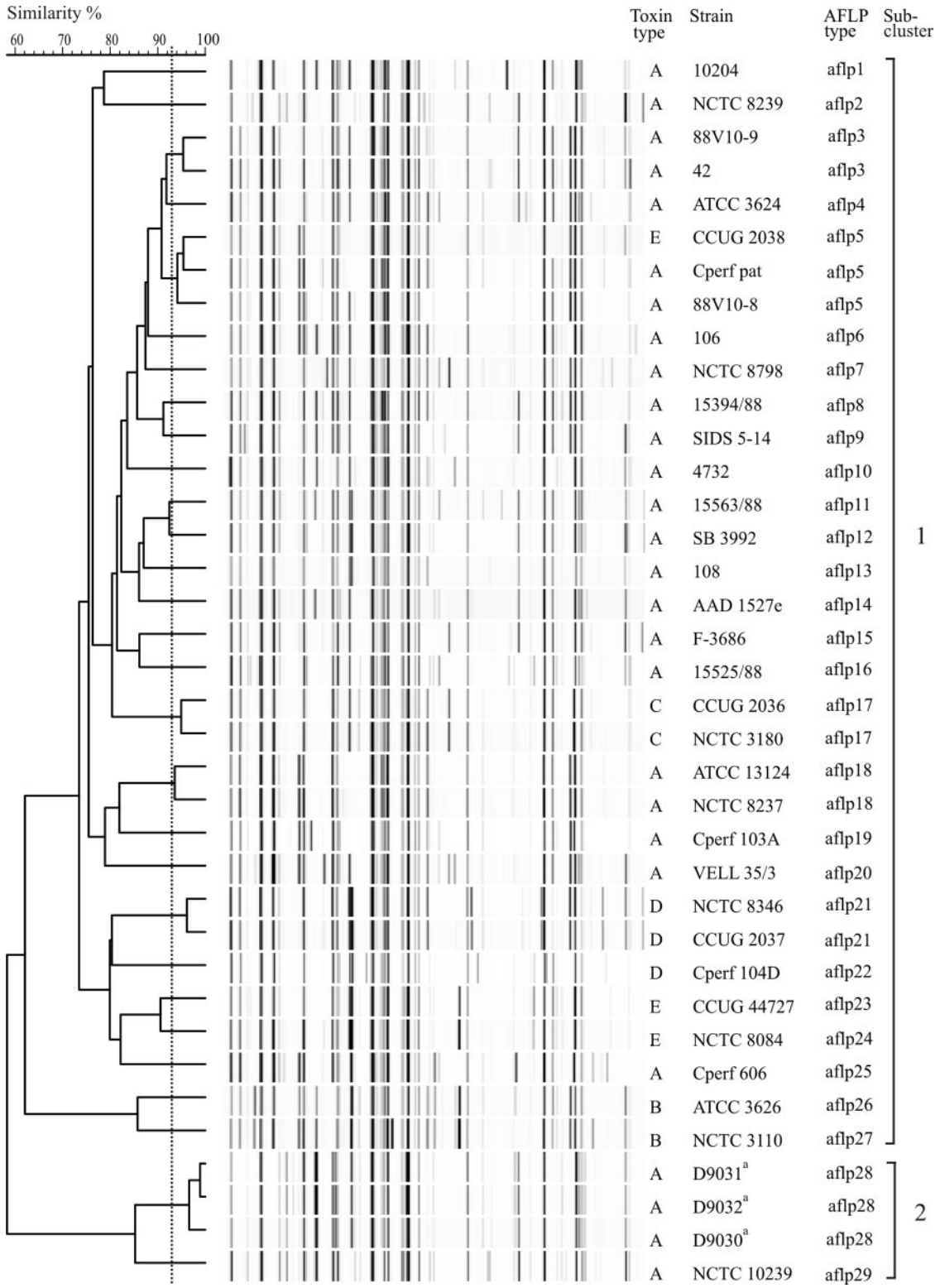

FIG. 2. Reconstructed AFLP banding patterns and a dendrogram of 37 *Clostridium perfringens* strains based on AFLP analysis. A similarity analysis was performed using the Pearson product-moment correlation coefficient, and clustering was performed by using the unweighted pair-group method with arithmetic averages. The dashed line shows the cutoff similarity value (93%). a, isolates originating from the same outbreak.

peat analysis, suggesting that the acquisition of plasmid-borne major toxin genes is a rather recent event and that strains of different toxin types may not have distinct evolutionary histories (40). Unrelated *C. perfringens* strains resulted in divergent AFLP banding patterns, whereas *C. perfringens* strains initially originating from the same isolate or from the same outbreak showed identical fingerprinting patterns, thus indicating that AFLP is a highly discriminative genotyping method. With AFLP, we observed excellent typeability; thus, AFLP is an attractive alternative to PFGE in outbreak situations. In addition to the high diversity of AFLP profiles of *C. perfringens* and *C. botulinum*, the AFLP patterns of strains of *C. bifermentans*, *C. septicum*, *C. sordellii*, and *C. sporogenes* showed substantial diversity, suggesting that AFLP may be able to subtype these species at the strain level. Further research is warranted to evaluate the usefulness of AFLP in epidemiological studies of these *Clostridium* species.

We conclude that AFLP is a highly reproducible, easy-to-use, and relatively fast method which can be applied to different clostridia and used for the generation of identification libraries. Therefore, libraries of AFLP profiles of well-defined *Clostridium* strains provide a valuable additional tool in the identification of *Clostridium* species. Due to partial automation, which enables high throughput of samples, AFLP seems particularly well suited for screening large numbers of isolates. Moreover, the same protocol can be used in typing at the strain level. This present study demonstrated the value of AFLP for distinguishing between strains of *C. perfringens*, and AFLP can thus be utilized in epidemiological and outbreak studies of *C. perfringens*.

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