Identification of Type A, B, E, and F Botulinum Neurotoxin Genes and of Botulinum Neurotoxigenic Clostridia by Denaturing High-Performance Liquid Chromatography

Giovanna Franciosa,¹ Manoocheher Pourshaban,¹ Alessandro De Luca,² Anna Buccino,²
Bruno Dallapiccola,^{2,3} and Paolo Aureli^{1*}

National Reference Center for Botulism, National Center for Food Quality and Risk Assessment, Istituto Superiore della Sanità, 00161 Rome,¹ IRCCS-CSS, San Giovanni Rotondo, and CSS-Mendel Institute, 00198 Rome,² and Department of Experimental Medicine and Pathology, University of Rome "La Sapienza," 00185 Rome,³ Italy

Received 31 July 2003/Accepted 22 March 2004

Denaturing high-performance liquid chromatography (DHPLC) is a recently developed technique for rapid screening of nucleotide polymorphisms in PCR products. We used this technique for the identification of type A, B, E, and F botulinum neurotoxin genes. PCR products amplified from a conserved region of the type A, B, E, and F botulinum toxin genes from *Clostridium botulinum*, neurotoxigenic *C. butyricum* type E, and *C. baratii* type F strains were subjected to both DHPLC analysis and sequencing. Unique DHPLC peak profiles were obtained with each different type of botulinum toxin gene fragment, consistent with nucleotide differences observed in the related sequences. We then evaluated the ability of this technique to identify botulinum neurotoxigenic organisms at the genus and species level. A specific short region of the 16S rRNA gene which contains genus-specific and in some cases species-specific heterogeneity was amplified from botulinum neurotoxigenic clostridia and from different food-borne pathogens and subjected to DHPLC analysis. Different peak profiles were obtained for each genus and species, demonstrating that the technique could be a reliable alternative to sequencing for the rapid identification of food-borne pathogens, specifically of botulinum neurotoxigenic clostridia most frequently implicated in human botulism.

Botulism is a severe neuromuscular disease affecting humans and animals which results from the blockage of acetylcholine release from the synaptic vesicles at the neuromuscular junctions due to the specific action of botulinum neurotoxins. Seven (A to G) antigenically distinct botulinum neurotoxins are known, but only types A, B, E, and F cause most human botulism cases. Neurotoxigenic *Clostridium butyricum* and *C. botulinum* can produce type E botulinum neurotoxin. Neurotoxigenic *C. baratii* and *C. botulinum* can produce type F botulinum neurotoxin. Only *C. botulinum* strains have been proven to produce type A or B botulinum neurotoxin (15). Except for the ability to produce botulinum toxins, other clostridia species share the phenotypic and genotypic characteristics of these botulinum toxin-producing clostridial species: the close relationship between neurotoxigenic and nonneurotoxigenic clostridia has been shown by DNA-DNA hybridization studies and sequencing of rRNA genes (19, 20, 22). As a consequence, the demonstration of botulinum toxin production by in vivo or in vitro tests and/or of the gene encoding the botulinum toxin in the microbial genome by PCR are the most conclusive tests for identification of botulinum neurotoxigenic strains (16). After the botulinum toxin and/or the presence of the neurotoxin gene in the genomic DNA has been demonstrated, the species of the neurotoxigenic strain remains to be determined; biochemical characterization of strains and/or se-

quencing of rRNA genes commonly serves this purpose (17, 19, 20). Although identification of microbial species is not a priority in botulism outbreak investigations, rapid identification could be crucial in tracing the source of neurotoxigenic clostridia causing botulism and to evaluate contamination of food production chains and other environments.

Rapid identification of bacteria has recently been achieved by detection of DNA sequence variation in a conserved region of the 16S rRNA genes through denaturing high-performance liquid chromatography (DHPLC) (18). In this technique, two PCR products (a reference and a test product) are first denatured and then allowed to reanneal as DNA heteroduplex molecules. Any mismatches between the two strands cause a differential retention of the heteroduplex compared to the reference (homoduplex) product during separation in an ion-pair reverse-phase liquid chromatography system under partial denaturation temperatures and are revealed as different peak profiles (29, 30). Identification of 36 of the 39 bacterial species tested was achieved through DHPLC analysis by Hurtle et al. (18); *C. botulinum* was among the three species that could not be identified because the 16S ribosomal DNA (rDNA) target sequence from the one strain tested was not amplified.

Here, we describe the application of two different DHPLC experiments, one for detecting sequence variation in conserved regions of the botulinum neurotoxin A, B, E, and F genes, and another for the 16S rRNA gene. Sequence differences in toxin genes have proven useful for distinguishing between botulinum neurotoxin types with PCR (8) and in 16S rRNA genes for bacterial genera and species (3). The aim of this study was to evaluate whether DHPLC can be used to rapidly identify the

* Corresponding author. Mailing address: National Reference Center for Botulism, National Center for Food Quality and Risk Assessment, Istituto Superiore della Sanità, Viale Regina Elena 299, 00161 Rome, Italy. Phone: 39 06 49903420. Fax: 39 06 49387101. E-mail: p.aureli@iss.it.

TABLE 1. Bacterial strains used in this study

Bacteria	Strain no.	Reference
Botulinum neurotoxicogenic clostridia		
<i>Clostridium botulinum</i> type A	13	
	129	
	42N	
	137	
	138	
<i>Clostridium botulinum</i> type B		
Proteolytic	177	
	196	
	215	
	216	
Nonproteolytic	CDC/4848	
<i>Clostridium botulinum</i> type Ab	87	
<i>Clostridium botulinum</i> type E	37	
	47	
<i>Clostridium botulinum</i> type F (proteolytic)	CDC/BL2821	
<i>Clostridium butyricum</i> type E	20	2
	21	2
	86	10
	109	10
	145/1	1
	146	1
	190	1
	JP/KZ1890	24
	JP/LCL155	25
	CDC/6366	23
<i>Clostridium baratii</i> type F		
Other food-borne pathogens		
<i>Salmonella enteritidis</i>	25/2	
<i>Bacillus cereus</i>	B-179	
<i>Listeria monocytogenes</i>	148	
<i>Clostridium perfringens</i>	222	
<i>Aeromonas hydrophila</i>	198/37	
<i>Escherichia coli</i> O157	ED503	
<i>Shigella dysenteriae</i>	12	
<i>Enterococcus faecalis</i>	JH2-2	

^a All strains were from the Istituto Superiore della Sanità collection except for the CDC and JP strains (see text).

neurotoxicogenic organisms most frequently involved in human botulism and their toxin gene types.

MATERIALS AND METHODS

Bacterial strains. Thirty-two bacterial strains were included in this study (Table 1). Of them, 14 strains were *C. botulinum* (five type A, five type B, one type Ab, two type E, and one type F), nine strains were neurotoxicogenic *C. butyricum* type E, and one strain was neurotoxicogenic *C. baratii* type F.

All *C. botulinum* strains were from the Istituto Superiore di Sanità collection except for the nonproteolytic type B strain CDC/4848 and the type F strain CDC/BL2821, which were a kind gift from Charles Hatheway, Centers for Disease Control, Atlanta, Ga. They had been isolated either from clinical specimens or from food samples during investigations of botulism outbreaks and surveys of food products for contamination with *C. botulinum*.

Neurotoxicogenic *C. butyricum* type E strains JP/LCL155 and JP/KZ1890 were kindly provided by Shinichi Nakamura, Kanazawa University, Japan: they had been isolated from food (JP/LCL155) and soil (JP/KZ1890) samples isolated during investigation of two different outbreaks of food-borne botulism in China (25, 26). The remaining *C. butyricum* type E strains had been isolated in Italy from clinical samples from patients suffering from botulism (1, 2, 10).

Strain CDC/6366 of neurotoxicogenic *C. baratii* type F was from the Centers for Disease Control collection and had been isolated from a case of botulism in an adult (24).

Additionally, some food-borne bacterial pathogens from the Istituto Superiore di Sanità collection of genera and species other than *C. botulinum*, *C. butyricum*, and *C. baratii* were included in the study.

All bacterial isolates were identified and characterized at the time of isolation. The purity of stock cultures was checked on appropriate agar media (Oxoid,

Basingstoke, United Kingdom): egg yolk agar for clostridia, aeromonas medium base for *Aeromonas hydrophila*, mannitol egg yolk polymyxin agar for *Bacillus cereus*, brilliant green agar for *Salmonella enteritidis*, listeria selective agar base (Oxford) for *Listeria monocytogenes*, and MacConkey sorbitol agar for *Shigella dysenteriae* and *Escherichia coli* O157. Single colonies were picked from the agar plates, inoculated into 9-ml brain heart infusion (BHI) broth tubes, and incubated overnight at 37°C; only clostridia were grown anaerobically in GasPak jars (Oxoid).

PCR experiments. One milliliter of the overnight broth cultures was centrifuged, washed with TE buffer (10 mM Tris, pH 7.4, 1 mM EDTA), and resuspended in 200 µl of TE. Suspensions were heated at 94°C for 10 min in a thermal cycler (model PT100 Minicycler; M. J. Research, Inc., Watertown, Mass.) and centrifuged. Two microliters of each supernatant was used as the template in each amplification reaction.

Two sets of primer pairs (Primm, Milan, Italy) were used in separate PCR experiments. The first set of primers, P260 and P261, served to amplify a common 260-bp fragment from the neurotoxin genes (8). Another primer pair was used to amplify a specific region of approximately 400 bp within the highly conserved 16S rDNA (3, 28).

The reaction mixtures and cycling conditions used for the separate amplification reactions were as described elsewhere (3, 8). AmpliTaq Gold polymerase (Applied Biosystems, Foster City, Calif.) was used in all experiments, which required an activation step at 94°C for 11 min prior to the amplification reactions.

All PCRs were performed in a programmable thermal cycler (M. J. Research, model PT100). PCR products were checked by agarose gel electrophoresis to make sure that only the specific product was amplified and no other PCR products that might cause artificial heteroduplex formation and affect the DHPLC analysis were amplified.

DNA sequencing. PCR products amplified from the neurotoxin genes of nine *C. botulinum* strains (five type A, three type B, and one type E) and three *C. butyricum* type E strains were sequenced with either primer P260 or P261 (5 pmol) with the Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems). The sequence reactions were analyzed with an ABI Prism DNA sequencer (model 377; Applied Biosystems). Analysis of sequences was performed with the software package from GCG, Madison, Wis.

DHPLC analysis. DHPLC was carried out on a 3500 HT WAVE DNA fragment analysis system (Transgenomic, Crewe, United Kingdom) equipped with a DNA 3500 HT column (Transgenomic). Before DHPLC analysis, 10 nl (≈300 ng of DNA) of the botulinum toxin gene PCR product for each strain was mixed with an equal volume of PCR product from *C. botulinum* type A strain 13, which was randomly selected as a reference strain for neurotoxin gene type characterization. In the same way, 10 nl (≈300 ng of DNA) of the 16S rDNA PCR product for each strain was mixed with an equal volume of PCR product from *C. butyricum* strain 21, which was used as a reference strain for species discrimination. Hybridization conditions included a 5-min preincubation at 95°C, followed by a 45-min cooling period at 25°C.

The PCR products were separated (flow rate of 1.5 ml/min) through a 5% linear acetonitrile gradient. The standard buffers were prepared from concentrated triethylammonium acetate (TEAA, 100 ml; Transgenomic 553301) to give buffer A 0.1 M TEAA and buffer B 0.1 M TEAA plus 25% acetonitrile. The wash buffer was 8% acetonitrile. Analysis for each amplified sample took 2.5 min, including column regeneration and equilibration. The oven temperatures and the start concentrations of buffer B for optimal heteroduplex separation were determined with WAVEmaker software version 4.1.40 (Transgenomic), which gives a computer-assisted determination of the melting profile and analytical conditions for each fragment. The temperature giving 70 to 80% double-helical fraction of the *C. botulinum* type A toxin gene and *C. butyricum* 16S rDNA PCRs was defined for the analyses. *C. botulinum* type A toxin gene DHPLC analysis was performed at 51.9°C and 56% buffer B starting concentration. DHPLC data analysis was based on subjective comparison of sample and reference chromatograms.

Nucleotide sequence accession numbers. Botulinum neurotoxin A, B, and E partial sequences from some of the neurotoxicogenic strains used in this study have been deposited in the GenBank database under accession numbers AY327850 through AY327861 and AY555068 through AY555070.

RESULTS AND DISCUSSION

No PCR product was obtained from the nonneurotoxicogenic strains tested in this study with primers P260 and P261 (results not shown). A PCR product of about 260 bp was amplified

A-129	AAAGGACGATCGTGTATATCATCCTCTATGTTAAATTTATTTGATCCAAATAAATATGTC	60
A-138	AAAGGACGATCGTGTATATCATCCTCTATGTTAAATTTATTTGATCCAAATAAATATGTC	60
A-42N	AAAGGACGATCGGATATATCATACTATATGTTAAATTTATTTGATCCAAATAAATATGTC	60
A-13	AAAGGACGATCGGATATATCATACTATATGTTAAATTTATTTGATCCAAATAAATATGTC	60
A-137	AAAGGACGATCGGATATATCATACTATATGTTAAATTTATTTGATCCAAATAAATATGTC	60
B-216	CCTTTAATGTACAATAAAGAATATTATATGTTTAAATGCGGGGAATAAAAAATTCATATATT	60
B-CDC/4848	CCTTTAATGTATAATAAAGAATATTATATGTTTAAATGCGGGGAATAAAAAATTCATATATT	60
B-215	CCTTTAATGTACAATAAAGAATATTATATGTTTAAATGCGGGGAATAAAAAATTCATATATT	60
B-196	CCTTTAATGTACAATAAAGAATATTATATGTTTAAATGCGGGGAATAAAAAATTCATATATT	60
E-37	TATTTGCTTTATGACAAAGAGCTCTATTTATTAATGTGTTAAACCCAAATAACTTTATT	60
E-109	TATTTGCTTTATGACAAAGAATATTATTTATTAATGTGTTAAACCCAAATAACTTTATT	60
E-JP/LCL155	TATTTGCTTTATGACAAAGAATATTATTTATTAATGTGTTAAACCCAAATAACTTTATT	60
E-21	TATTTGCTTTATGACAAAGAATATTATTTATTAATGTGTTAAACCCAAATAACTTTATT	60
F-CDC/BL2821	TATTTGTTATATAATAAAGATATTATTTATTTGAATTTACTAAGAACAGATAAGTCTATT	60
F-CDC/6366	TACCTATTATATAATAAAGAAATATTACTTTATTAATTTATTAACCCAAATATGCTCTGTT	60
	* * * * *	
A-129	GATGTAAATAATATAGGTATTAGAGGTTATATGTATCTTAAAGGGCCTAGAGGTAGCGTA	120
A-138	GATGTAAATAATATAGGTATTAGAGGTTATATGTATCTTAAAGGGCCTAGAGGTAGCGTA	120
A-42N	GATGTAAATAATATAGGTATTAGAGGTTATATGTATCTTAAAGGGCCTAGAGGTAGCGTA	120
A-13	GATGTAAATAATATAGGTATTAGAGGTTATATGTATCTTAAAGGGCCTAGAGGTAGCGTA	120
A-137	GATGTAAATAATATAGGTATTAGAGGTTATATGTATCTTAAAGGGCCTAGAGGTAGCGTA	120
B-216	AAACTAAAGAA-AGATTCATCTGTAGGTGAA---ATTTTAACACGTA-GCAAATATAATC	115
B-CDC/4848	AAACTAGTGAA-AGATTCATCTGTAGGTGAA---ATTTTAACACGTA-GCAAATATAATC	115
B-215	AAACTAAAGAA-AGATTCATCTGTAGGTGAA---ATTTTAACACGTA-GCAAATATAATC	115
B-196	AAACTAAAGAA-AGATTCATCTGTAGGTGAA---ATTTTAACACGTA-GCAAATATAATC	115
E-37	GATGGGAGAAA---AGATTCT-----ACTTTACGCATTAATAA-----TAT	98
E-109	AATAGGAGAAC---AGATTCT-----ACTTTAAGCATTAAATAA-----TAT	98
E-JP/LCL155	AATAGGAGAAC---AGATTCT-----ACTTTAAGCATTAAATAA-----TAT	98
E-21	AATAGGAGAAC---AGATTCT-----ACTTTAAGCATTAAATAA-----TAT	98
F-CDC/BL2821	ACTCAGAATTC---AACTTTTCTAA-----ATATTAATCAACAAAGAGGTGTTTAT	108
F-CDC/6366	ACTAAAAATTC---CGATATTCTTA-----ATATTAATCAACAAAGAGGTGTTTAT	108
	* * * *	
A-129	GTGACTACAAACATTTATTTAAATTCACCTTTGTATGAGGGGACAAAATTTATTATAAAA	180
A-138	GTGACTACAAACATTTATTTAAATTCACCTTTGTATGAGGGGACAAAATTTATTATAAAA	180
A-42N	GTGACTACAAACATTTATTTAAATTCACCTTTGTATGAGGGGACAAAATTTATTATAAAA	180
A-13	GTGACTACAAACATTTATTTAAATTCACCTTTGTATGAGGGGACAAAATTTATTATAAAA	180
A-137	GTGACTACAAACATTTATTTAAATTCACCTTTGTATGAGGGGACAAAATTTATTATAAAA	180
B-216	AAAATCCAATTATATAAATTA-TAGAAATTTATATATCGGAGAAAAATTTATTATAAGA	174
B-CDC/4848	AGAATTCCAATTATATAAATTA-TAGAAATTTATATATTGGAGAAAAATTTATTATAAGA	174
B-215	AAAATCCAATTATATAAATTA-TAGAAATTTATATATCGGAGAAAAATTTATTATAAGA	174
B-196	AAAATCCAATTATATAAATTA-TAGAAATTTATATATCGGAGAAAAATTTATTATAAGA	174
E-37	AAGACGC--ACTATTCTTTTAGCTAATAGATTATATAGGGGATAAAGGTTAAAAATACAA	156
E-109	AAGAAGC--ACTATTCTTTTAGCTAATAGATTATATAGTGAATAAAAGTTAAAAATACAA	156
E-JP/LCL155	AAGAAGC--ACTATTCTTTTAGCTAATAGATTATATAGTGAATAAAAGTTAAAAATACAA	156
E-21	AAGAAGC--ACTATTCTTTTAGCTAATAGATTATATAGTGAATAAAAGTTAAAAATACAA	156
F-CDC/BL2821	CAGAAACCAATATTTTTTCCAACACTAGATGGTATACAGGAGTAGAAGTTATTATAAGA	168
F-CDC/6366	CAGAAACCAATATTTTTTCCAACACTAGATGGTATACAGGAGTAGAAGTTATTATAAGA	168
	* * * * *	

FIG. 1. Aligned sequences of botulinum toxin gene fragments amplified by primers P260 and P261 from *C. botulinum* type A (strains 129, 138, 42N, 13, and 137), type B proteolytic (strains 216, 215, and 196) and nonproteolytic (strain CDC/4848), type E (strain 37), type F (strain CDC/BL2821), and *C. butyricum* type E (strains 109, 21, and JP/LCL155) and *C. baratii* type F (CDC/6366). Asterisks indicate identical nucleotides.

from all botulinum neurotoxic clostridial strains investigated, including *C. botulinum* type Ab (strain 87), which possesses both A and B genes and produces both neurotoxin types (12). The primer set that we used had originally been selected in the most conserved regions of the botulinum neurotoxin genes to amplify a common 260-bp fragment. Specific toxin types are revealed by hybridization with individual internal probes (8).

We sequenced the positive PCR products from some strains of *C. botulinum* type A, B, E, and F and from botulinum neurotoxic *C. butyricum* type E and *C. baratii* type F to more precisely define the differences in the nucleotide sequences between botulinum neurotoxin types. The aligned sequences of the neurotoxin gene fragments analyzed are shown in Fig. 1. Pairwise comparisons of these sequences showed high homology between fragments from genes for the same neuro-

A-129	AAATATGC-----TTCTGGAA--ATGAA-GATAATATTGTTAGAAATGATGA----TCG	227
A-138	AAATATGC-----TTCTGGAA--ATGAA-GATAATATTGTTAGAAATGATGA----TCG	227
A-42N	AAATATGC-----TTCTGGAA--ATGAA-GATAATATTGTTAGAAATGATGA----TCG	227
A-13	AAATATGC-----TTCTGGAA--ATGAA-GATAATATTGTTAGAAATGATGA----TCG	227
A-137	AAATATGC-----TTCTGGAA--ATGAA-GATAATATTGTTAGAAATGATGA----TCG	227
B-216	AGAAAGTCA---AATTCTCAATCTATAAATGATGATATAGTTAGAAATGATGA----TCG	227
B-CDC/4848	AGAGAGTCA---AATTCTCAATCTATAAATGATGATATAGTTAGAAAAGAAGA----TTA	227
B-215	AGAAAGTCA---AATTCTCAATCTATAAATGATGATATAGTTAGAAATGATGA----TCG	227
196-B	AGAAAGTCA---AATTCTCAATCTATAAATGATGATATAGTTAGAAATGATGA----TCG	227
E-37	AGGGTTA----AGAGAAGTAG-TCCTACCGATAATTGTGTTAGAGAAAGTGAGAGATCG	210
E-109	AGAGTTA----ATAATAGTAG-TACTAACGATAATCTTGTTAGAAATGATGA----TCG	206
E-JP/LCL155	AGAGTTA----ATAATAGTAG-TACTAACGATAATCTTGTTAGAAATGATGA----TCG	206
E-21	AGAGTTA----ATAATAGTAG-TACTAACGATAATCTTGTTAGAAATGATGA----TCA	206
F-CDC/BL2821	AAAAATGGATCTACAGATATATCTAATACAGATAATTTTGTTAGAAAAAATGATCTGGCA	228
F-CDC/6366	AAAGTTGGTTCTACAGATACATCTAATACAGATAATTTTGTAAGAAAAAATGATACAGTA	228
	* * * * *	

Accession numbers		
A-129	TGTAT 232	AY327852
A-138	TGTAT 232	AY327854
A-42N	TGTTT 232	AY327850
A-13	TGTAT 232	AY327851
A-137	TGTAT 232	AY327853
B-216	TGTAT 232	AY327857
B-CDC/4848	TATAC 232	AY555068
B-215	TGTAT 232	AY327856
B-196	TGTAT 232	AY327855
E-37	TGTAT 215	AY327858
E-109	TGTAT 211	AY327860
E-JP/LCL155	TGTAT 211	AY327861
E-21	TGTAT 211	AY327859
F-CDC/BL2821	TATAT 233	AY555069
F-CDC/6366	TATAT 233	AY555070
	* *	

FIG. 1—Continued.

toxin type. Depending on the strains being compared, the levels of similarity were at least 97.8% between neurotoxin A gene fragments, 99.5% between neurotoxin B gene fragments from the proteolytic strains, which decreased to 95% compared to the nonproteolytic type B strain CDC/4848, and 98.1% between neurotoxin E gene fragments derived from neurotoxicogenic *C. butyricum* strains. These sequences were 84.5% similar to the sequence of the neurotoxin E gene fragment from *C. botulinum* type E strain 37. Sequences of botulinum neurotoxin F gene fragments amplified from *C. botulinum* type F and *C. baratii* type F displayed an 88.5% homology level.

The similarity level between the sequences of fragments amplified from different neurotoxin type genes was 44 to 54% between types A and B, 52 to 62% between types B and E, 42 to 59% between types A and E, 55 to 58% between types A and F, 51 to 58% between types B and F, and 55 to 63% between types E and F. These results confirm previous works that showed differences in neurotoxin gene sequences relative to different toxin types; rapid DHPLC identification of botulinum neurotoxin types was tested to compare neurotoxin amplicons for nucleotide differences.

In our experiments, the reference toxin gene PCR product from *C. botulinum* type A (strain 13) was first mixed with each amplicon obtained from the remaining four strains of *C. bot-*

ulinum type A (Table 1). DHPLC analyses, carried out at a column temperature of 51.9°C, showed peak profiles nearly identical to that produced by the homoduplex reference DNA molecule from strain 13 (Fig. 2). At the same temperature, heteroduplexes formed between the reference botulinum neurotoxin A PCR product and botulinum neurotoxin B PCR products from the proteolytic strains produced similar DHPLC patterns; the heteroduplex formed with the neurotoxin B PCR product from the nonproteolytic strain CDC/4848 yielded a different profile (Fig. 2), in accordance with nucleotide differences between neurotoxin B sequences from proteolytic and nonproteolytic strains (Fig. 1). The heteroduplex formed with the PCR product from strain 87 (*C. botulinum* Ab) yielded a unique peak profile, different from that of both neurotoxin A and neurotoxin B (Fig. 2). This result could be explained by the fact that a common segment of both the neurotoxin A and B genes was concomitantly amplified by our primer set from this strain, which could not be separated by agarose gel electrophoresis; sequencing of such overlapping PCR products would be inconclusive.

All A-E heteroduplexes formed with neurotoxin E gene fragments from *C. butyricum* strains were similar; however, different peak profiles were observed among mixtures of the reference PCR product and the neurotoxin E PCR products,

FIG. 2. DHPLC analysis of botulinum neurotoxin gene amplicons. Comparison between *C. botulinum* type A strains 129, 13 (reference), 137, 138, and 42N, type Ab strain 87, proteolytic type B strains 177, 196, 215, and 216, nonproteolytic type B strain CDC/4848, type E strains 37 and 47, *C. butyricum* type E strains 20 and 190, *C. botulinum* type F strain CDC/BL2821, and *C. baratii* type F strain CDC/6366.

depending on the strains from which the fragments had been amplified, i.e., either *C. botulinum* type E or neurotoxicogenic *C. butyricum* type E (Fig. 2). Likewise, the heteroduplexes formed with the botulinum neurotoxin F gene products amplified from *C. botulinum* type F and neurotoxicogenic *C. baratii* type F produced different peak profiles (Fig. 2). These results were consistent with the nucleotide variation observed between the sequences of botulinum neurotoxin E gene fragments amplified from *C. botulinum* type E and *C. butyricum* type E and between the sequences of botulinum neurotoxin F segments from *C. botulinum* type F and *C. baratii* type F (Fig. 1).

C. botulinum and neurotoxicogenic *C. butyricum* share the ability to produce botulinum neurotoxin E, while both *C. botulinum* and neurotoxicogenic *C. baratii* can produce botulinum neurotoxin F; the differentiation of the three species by conventional methods is rather difficult (13, 23). Neurotoxicogenic *C. butyricum* type E has been implicated in infectious botulism (2, 10) and has recently been shown to be an emergent food-borne pathogen (1, 25, 26, 28); indeed, like *C. botulinum*, it can grow and produce toxin in food under favorable conditions (1). In Italy, *C. butyricum* type E appears to be more widespread than *C. botulinum* type E, based on isolation from several cases of botulism (9). *C. baratii* type F has only been isolated in the United States, where it has been implicated in two cases of infant botulism (13, 27) and at least 9 of 12 botulism type F cases in adults between 1981 and 2002 (J. Sobel, personal communication). An easy test to distinguish between the three clostridial species, such as the DHPLC protocol described here, would represent a good alternative to the standard methods of identification.

Peak profiles of heteroduplexes formed between the reference botulinum neurotoxin A product and the gene products from different botulinum neurotoxin types were clearly distinguishable from one another according to the toxin type (Fig. 2). Elution times for peaks ranged from 1 to 2 min at the temperature of the analyses. Both retention time and peak

numbers varied, although the peak number was mainly assumed to be conducive for sequence variation.

In total, eight distinct DHPLC profiles were detected between the different botulinum neurotoxin gene fragments amplified from *C. botulinum* type A, proteolytic B, nonproteolytic B, Ab, E, and F, *C. butyricum* type E, and *C. baratii* type F (Fig. 2); these results correlated well with the nucleotide differences observed in the respective sequences (Fig. 1).

Although the combination of PCR with primers P260 and P261 and DHPLC would be sufficient to identify the botulinum neurotoxin gene types and to discriminate between the neurotoxicogenic clostridia, we attempted another DHPLC approach to more generally distinguish *C. botulinum* and neurotoxicogenic *C. butyricum* and *C. baratii* from other common food-borne pathogens through the analysis of 16S rDNA gene products. Hurtle et al. (18) used a similar approach for identifying *Bacillus anthracis* and *Yersinia pestis*; however, *C. butyricum* was not among the species tested, and the primer set used in that study could not amplify the conserved 16S rDNA sequence of interest from *C. botulinum*. Here, we used the degenerate primers DA71 and DA72, which had previously proved useful for distinguishing among bacterial genera and species, including *C. botulinum* and *C. butyricum*, upon sequencing of the amplified products (3, 28).

An approximately 400-bp fragment was amplified by PCR with primers DA71 and DA72 from all bacterial strains included in this study (Table 1). The size of the amplicons was consistent with the length of fragments within the highly conserved bacterial 16S rRNA genes (3). For DHPLC analysis of 16S rDNA gene fragments, the product amplified from neurotoxicogenic *C. butyricum* type E strain 21 was used as a reference and first mixed with each rDNA PCR fragment from the remaining eight type E botulinum toxin-producing *C. butyricum* strains. All DHPLC profiles matched that produced by the reference homoduplex product from strain 21 (Fig. 3).

The 16S rDNA products from different types of *C. botulinum*

FIG. 3. DHPLC analysis of 16S rDNA gene fragments. Comparison between *C. butyricum* type E strains 109, 145/1, 146, 190, 20, 21 (reference), 86, 109, JP/KZ1890, and JP/LCL155, *C. botulinum* type A strain 42N, proteolytic B strain 216, nonproteolytic B strain CDC/4848, E strain 37, F strain CDC/BL2821, and *C. baratii* type F strain CDC/6366.

mixed with the reference 16S rDNA produced unique DHPLC profiles, in accordance with the nucleotide differences previously reported in these conserved DNA regions from *C. botulinum* strains of different types, as did those from proteolytic and nonproteolytic strains of *C. botulinum* type B (19, 20) (Fig. 3). The DHPLC chromatogram produced by *C. baratii* type F was different from those of the other clostridia (Fig. 3). Microbial strains belonging to the nonclostridial genera included in this study were also distinguished by DHPLC analysis of 16S rDNA fragments (Fig. 4). The elution times of the peaks varied from 1 to 2 min at the analysis temperature of 61.8°C. In conclusion, this 16S rDNA-directed DHPLC approach enabled the rapid identification of the 11 different bacterial food-borne pathogen species included in this study.

So far, DHPLC has mainly been applied for mutational analysis in the highly conserved human genome (4, 6, 11); however, it has recently been used specifically for detecting mutations in antibiotic resistance genes with microorganisms (5, 7, 14) as well as identifying bacterial genera and species (18). Although the genetic variability of microbial genomes is

higher than that of the human genome, the choice of conserved gene targets and, eventually, of degenerate primers may help in adapting DHPLC analysis to bacteria. The present study has shown that PCR with degenerate primers directed at either botulinum neurotoxin genes or 16S rDNA genes, followed by appropriate DHPLC analysis, allows the identification of neurotoxicogenic organisms and their differentiation from other food-borne pathogens. Several approaches to detect genetic variations are available, including DNA sequencing, which has high costs; single-strand conformation polymorphisms, which has limited sensitivity; DNA hybridization arrays, which can give significant false-positive results; and denaturing gradient gel electrophoresis, which is technically challenging (21). DHPLC analysis has advantages over these techniques in terms of time, ease of analysis, and reproducibility, especially when a large number of samples must be analyzed.

ACKNOWLEDGMENT

This work was supported in part by the Italian Ministry of Health.

REFERENCES

1. Anniballi, F., L. Fenicia, G. Franciosa, and P. Aureli. 2002. Influence of pH and temperature on the growth and toxin production of neurotoxicogenic strains of *Clostridium butyricum* type E. *J. Food Prot.* **65**:1267–1270.
2. Aureli, P., L. Fenicia, B. Pasolini, M. Gianfranceschi, L. M. McCroskey, and C. L. Hatheway. 1986. Two cases of type E infant botulism caused by neurotoxicogenic *C. butyricum* in Italy. *J. Infect. Dis.* **154**:207–211.
3. Boye, K., E. Hogdall, and M. Borre. 1999. Identification of bacteria using two degenerate 16S rDNA sequencing primers. *Microbiol. Res.* **154**:23–26.
4. Colosimo, A., V. Guida, A. De Luca, M. P. Cappabianca, I. Bianco, G. Palka, and B. Dallapiccola. 2002. Reliability of DHPLC in mutational screening of beta-globin (HBB) alleles. *Hum. Mutat.* **19**:287–295.
5. Cooskey, R. C., G. P. Morlock, B. P. Holloway, J. Limor, and M. Hepburn. 2002. Temperature-mediated heteroduplex analysis performed by using denaturing high-performance liquid chromatography to identify sequence polymorphisms in *Mycobacterium tuberculosis* complex organisms. *J. Clin. Microbiol.* **40**:1610–1616.
6. De Luca, A., A. Buccino, D. Gianni, M. Mangino, S. Giustini, A. Richetta, L. Divona, S. Calvieri, R. Mingarelli, and B. Dallapiccola. 2003. NF1 gene analysis based on DHPLC. *Hum. Mutat.* **21**:171–172.
7. Eaves, D. J., E. Liebana, M. J. Woodward, and L. J. Piddock. 2002. Detection of gyrA mutations in quinolone-resistant *Salmonella enterica* by denaturing high-performance liquid chromatography. *J. Clin. Microbiol.* **40**:4121–4125.

FIG. 4. DHPLC peak profiles of 16S rDNA gene fragments. A comparison between microbial food-borne pathogens belonging to different genera and species is shown.

8. Fach, P., M. Gibert, R. Griffais, J. P. Guillou, and M. R. Popoff. 1995. PCR and gene probe identification of botulinum neurotoxin A-, B-, E-, F-, and G-producing *Clostridium* spp. and evaluation in food samples. *Appl. Environ. Microbiol.* **61**:389–392.
9. Fenicia, L., G. Franciosa, and P. Aureli. 1999. Classic and emergent forms of botulism: the current status in Italy. *Eur. Surveill.* **4**:7–9.
10. Fenicia, L., G. Franciosa, M. Pourshaban, and P. Aureli. 1999. Intestinal toxemia botulism in two young people, caused by *Clostridium butyricum* type E. *Clin. Infect. Dis.* **29**:1381–1387.
11. Flex, E., A. De Luca, M. R. D'Apice, A. Buccino, B. Dallapiccola, and G. Novelli. 2002. Rapid scanning of myotubularin (MTM1) gene by denaturing high-performance liquid chromatography. *Neuromuscul. Disord.* **12**:501–505.
12. Franciosa, G., L. Fenicia, M. Pourshaban, and P. Aureli. 1997. Recovery of a strain of *Clostridium botulinum* producing both neurotoxin A and neurotoxin B from canned macrobiotic food. *Appl. Environ. Microbiol.* **63**:1148–1150.
13. Hall, J. D., L. M. McCroskey, B. J. Pincomb, and C. L. Hatheway. 1985. Isolation of an organism resembling *Clostridium baratii* which produces type F botulin toxin from an infant with botulism. *J. Clin. Microbiol.* **21**:654–655.
14. Hannachi-M'Zali, F., J. E. Ambler, C. F. Taylor, and P. M. Hawkey. 2002. Examination of single and multiple mutations involved in resistance to quinolones in *Staphylococcus aureus* by a combination of PCR and denaturing high-performance liquid chromatography (DHPLC). *J. Antimicrob. Chemother.* **50**:649–655.
15. Hatheway, C. L. 1995. Botulism: the present status of the disease. *Curr. Top. Microbiol. Immunol.* **195**:55–77.
16. Hatheway, C. L., and J. L. Ferreira. 1996. Detection and identification of *Clostridium botulinum* neurotoxins. *Adv. Exp. Med. Biol.* **391**:481–498.
17. Holdeman, L. V., E. P. Cato, and W. E. C. Moore. 1975. *Anaerobe laboratory manual*, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg, Va.
18. Hurtle, W., D. Shoemaker, E. Henchal, and D. Norwood. 2002. Denaturing HPLC for identifying bacteria. *BioTechniques* **33**:386–388, 390–391.
19. Hutson, R. A., D. E. Thompson, and M. D. Collins. 1993. Genetic interrelationships of saccharolytic *Clostridium botulinum* types B, E and F and related clostridia as revealed by small-subunit rRNA gene sequences. *FEMS Microbiol. Lett.* **108**:103–110.
20. Hutson, R. A., D. E. Thompson, P. A. Lawson, R. P. Schocken-Itturino, E. C. Bottger, and M. D. Collins. 1993. Genetic interrelationships of proteolytic *Clostridium botulinum* types A, B, and F and other members of the *Clostridium botulinum* complex as revealed by small-subunit rRNA gene sequences. *Antonie van Leeuwenhoek* **64**:273–283.
21. Kristensen, V. N., D. Kelefiotis, T. Kristensen, and A. L. Borresen-Dale. 2001. High-through-put methods for detection of genetic variation. *BioTechniques* **30**:318–332.
22. Lee, W. H., and H. Riemann. 1970. Correlation of toxic and nontoxic strains of *Clostridium botulinum* by DNA composition and homology. *J. Gen. Microbiol.* **60**:117–123.
23. McCroskey, L. M., C. L. Hatheway, L. Fenicia, B. Pasolini, and P. Aureli. 1986. Characterization of an organism that produces type E botulin toxin but which resembles *Clostridium butyricum* from the feces of an infant with type E botulism. *J. Clin. Microbiol.* **23**:201–202.
24. McCroskey, L. M., C. L. Hatheway, B. A. Woodruff, J. A. Greenberg, and P. Jurgenson. 1991. Type F botulism due to neurotoxicogenic *Clostridium baratii* from an unknown source in an adult. *J. Clin. Microbiol.* **29**:2618–2620.
25. Meng, X., T. Karasawa, K. Zou, X. Kuang, X. Wang, C. Lu, C. Wang, K. Yamakawa, and S. Nakamura. 1997. Characterization of a neurotoxicogenic *Clostridium butyricum* strain isolated from the food implicated in an outbreak of food-borne type E botulism. *J. Clin. Microbiol.* **35**:2160–2162.
26. Meng, X., K. Yamakawa, K. Zou, X. Wang, X. Kuang, C. Lu, C. Wang, T. Karasawa, and S. Nakamura. 1999. Isolation and characterisation of neurotoxicogenic *Clostridium butyricum* from soil in China. *J. Med. Microbiol.* **48**:133–137.
27. Paisley, J. W., B. A. Lauer, and S. S. Arnon. 1995. A second case of infant botulism type F caused by *Clostridium baratii*. *Pediatr. Infect. Dis. J.* **14**:912–914.
28. Pourshaban, M., G. Franciosa, L. Fenicia, and P. Aureli. 2002. Taxonomic identity of type E botulin toxin-producing *Clostridium butyricum* strains by sequencing of a short 16S rDNA region. *FEMS Microbiol. Lett.* **214**:119–125.
29. Spiegelman, J. L., M. N. Mindrinos, and P. J. Oefner. 2000. High-accuracy DNA sequence variation screening by DHPLC. *BioTechniques* **29**:1084–1090.
30. Wolford, J. K., D. Blunt, C. Ballecer, and M. Prochazka. 2000. High-throughput SNP detection by using DNA pooling and denaturing high performance liquid chromatography. *Hum. Genet.* **107**:483–497.