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Development and Validation of PCR Primers To Assess the Diversity of *Clostridium* spp. in Cheese by Temporal Temperature Gradient Gel Electrophoresis

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A nested-PCR temporal temperature gradient gel electrophoresis (TTGE) approach was developed for the detection of bacteria belonging to phylogenetic cluster I of the genus *Clostridium* (the largest clostridial group, which represents 25% of the currently cultured clostridial species) in cheese suspected of late blowing. Primers were designed based on the 16S rRNA gene sequence, and the specificity was confirmed in PCRs performed with DNAs from cluster I and non-cluster I species as the templates. TTGE profiles of the PCR products, comprising the V5-V6 region of the 16S rRNA gene, allowed us to distinguish the majority of cluster I species. PCR-TTGE was applied to analyze commercial cheeses with defects. All cheeses gave a signal after nested PCR, and on the basis of band comigration with TTGE profiles of reference strains, all the bands could be assigned to a clostridial species. The direct identification of *Clostridium* spp. was confirmed by sequencing of excised bands. *C. tyrobutyricum* and *C. beijerinckii* contaminated 15 and 14 of the 20 cheese samples tested, respectively, and *C. butyricum* and *C. sporogenes* were detected in one cheese sample. Most-probable-number counts and volatile fatty acid were determined for comparison purposes. Results obtained were in agreement, but only two species, *C. tyrobutyricum* and *C. sporogenes*, could be isolated by the plating method. In all cheeses with a high amount of butyric acid (>100 mg/100 g), the presence of *C. tyrobutyricum* DNA was confirmed by PCR-TTGE, suggesting the involvement of this species in butyric acid fermentation. These results demonstrated the efficacy of the PCR-TTGE method to identify *Clostridium* in cheeses. The sensitivity of the method was estimated to be 100 CFU/g.

The growth of spore-forming bacteria of the genus *Clostridium* causes severe defects in cheeses with long ripening times, such as Beaufort, Emmental, and Comté, as a consequence of late blowing or butyric blowing. The biochemical basis of this defect is the fermentative transformation of lactic acid to butyric acid (causing rancid flavor), acetic acid, carbon dioxide, and hydrogen (responsible for distension of the hole, which can in extreme cases result in the cheese bursting). Nevertheless, the defects found in cheeses (distribution and appearance of holes, splitting, taste, and smell) were in some cases not related to high butyric acid content (6). Almost all the commercial value of the contaminated cheese is lost, which represents a high economic impact in cheese production. The *Clostridium* species present in cheese originate from milk which is contaminated by these spores during milking. Silage feed of poor microbiological quality is one of the most important sources of spores in raw milk (9). In previous work, the species *C. tyrobutyricum* and *C. sporogenes* were the main organisms associated with late gas blowing in the cheese process because of their ability to ferment lactic acid (3, 17, 19, 22, 34). Other species of clostridia producing butyric acid, like *C. butyricum*, *C. beijerinckii*, *C. pasteurianum*, *C. tertium*, *C. perfringens*, and *C. tetanomorphum*, have also been detected in both milk and cheese (13, 17, 19). Conventional methods for detecting clostridia in foods involve heat shock of the samples to destroy vegetative cells, followed by a most-probable-number (MPN) enumeration based on gas production in anaerobically incubated medium with lactate as carbon and electron source. This method requires further tests on pure cultures obtained from gas-positive tubes to identify the species, including gas-liquid chromatographic analysis of volatile and nonvolatile fatty acids (16, 19). The latter techniques are too slow and not sufficiently reliable for results required in the food industry. Molecular methods would allow workers to overcome the inherent limitations of these cultivation-based approaches and to detect directly the vegetative cells that lead to cheese defects. 16S rRNA gene analysis has led to improved, expedient, and reliable methods for genus and species identification (36). Previous work has utilized PCR or nested-PCR approaches with species-specific primers, in order to specifically identify *C. tyrobutyricum*, *C. beijerinckii*, *C. sporogenes*, and *C. butyricum* (15, 20). Although reliable for the identification of these species, these primers have the disadvantage of being applicable to only a restricted set of microorganisms. Consequently, a set of specific primers must be available to identify all the agents responsible for alteration. Denaturing gradient gel electrophoresis (DGGE) (10), temperature gradient gel electrophoresis (28),

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or temporal temperature gradient gel electrophoresis (TTGE) (37) is suitable for the analysis of mixed microbial communities (7, 24) and can also be focused on the numerically subdominant bacteria by specific amplification of 16S rRNA genes (14). This tool leads to the separation of DNA fragments of the same length but with different base-pair sequences. The genus *Clostridium* is phylogenetically extremely heterogeneous. Comparison of 16S rRNA genes has allowed the division of this genus into subgroups. Cluster I is the largest clostridial group, representing 25% of the currently cultured clostridial species (8). All the previous organisms detected in cheese belong to cluster I. Members of this cluster exhibit relatively high levels of intracluster similarity (>90%) with some variations in 16S rRNA gene sequence among the strains which make this gene suitable for differentiation of clostridia (8). The objectives of the present study were to develop a PCR-TTGE-based method for specific and sensitive detection of clostridia of cluster I directly in cheese samples without culturing and to allow routine identification of the corresponding clostridial strains.

MATERIALS AND METHODS

Bacterial culture conditions and genomic DNA extraction from pure cultures. The clostridial and nonclostridial strains used are described in Table 1. All these strains may be found in milk ecosystems or from bovine dung. Clostridium strains were grown anaerobically in Trypticase-yeast extract-glucose broth at 37°C. Anaerobic conditions were created by boiling the medium and flushing with it with nitrogen gas during the cooling of broth. *Lactobacillus* strains were cultivated in MRS broth (Difco, Detroit, Mich.) at 37°C, *Streptococcus* spp were grown in M17 (Difco) broth at 37°C, *Propionibacterium freudenreichii* was grown in reinforced clostridial medium (Merck, Darmstadt, Germany), and *Bacillus cereus* and *Escherichia coli* were grown in nutrient broth (Difco) at 37°C.

For all strains, 2 ml of overnight cultures was collected and DNA was isolated with a DNA extraction kit (InstaGene Matrix; Bio-Rad Laboratories, Ivry sur Seine, France).

Collection and nucleic acid extraction from cheese samples. Samples of cheese from commercial sources (20 with and 1 without the presence of anomalous fermentation) were treated or stored as 250-mg aliquots in 2-ml screw-cap tubes at −80°C until processing. Total DNA was extracted as previously described (12). The concentration and integrity of the nucleic acids were determined visually by electrophoresis on a 1% agarose gel containing ethidium bromide.

Primer design. All primers used in this study are listed in Table 2. 16S rRNA sequences of the target group were obtained from the GenBank database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/) (1) and were aligned using the program Clustal W (33). After alignment, the primer sequences were visually searched. Potential target sites were further analyzed by BLAST to search for homologous nucleotide sequences in the database. Their specificities were theoretically evaluated by the RDP CHECK-PROBE program (21). Primers were synthesized commercially by Proligo France SAS. The appropriate annealing temperatures and primer specificities were determined using the genomic DNA of pure-culture control strains.

PCR amplification. The PCR was carried out by respectively amplifying the V5-V8 region of the 16S rRNA gene of phylogenetic cluster I. Primers S-*-Cbot-0691-a-S-23 (P930), specific for cluster I, and S-*-Clit-1338-a-A-22 (P932) amplified a fragment of 665 bp (Table 2). PCR amplifications were performed in a final volume of 25 μl which included 12 μl of premix (0.5 μM of forward and reverse primers, 4% final concentration of dimethyl sulfoxide), 12 μl of HotStar *Taq* Master Mix (Qiagen, Courtaboeuf, France), and 5 ng of genomic DNA. The samples were subjected to amplification in a PTC 100 Thermal Cycler (MJ Research, Inc.). The PCR program consisted of 15 min at 95°C to allow HotStar *Taq* activation and 30 or 40 cycles of 94°C for 20 s, 58°C for 30 s, and 72°C for 30 s. A 5-min final extension at 72°C was performed at the end of cycling steps, and reaction mixtures were stored at 4°C. The overall time used for this cycle was 120 min. Five microliters of the PCR products was analyzed on 2% agarose gels containing ethidium bromide. Negative controls (without DNA) were run in all amplifications.

Nested-PCR amplification. Serial dilutions of PCR products were tested to determine the optimal DNA concentrations for nested PCR which amplified 290 bp of the V5-V6 region of the 16S rRNA gene. From these dilutions 1 μl was used as template. The PCR was performed with primers S-D-Bact-1060-a-A-21 (P983) and S-D-Bact-0786-a-S-20 (P984) (Table 2) according to the complete protocol described above, but with 25 cycles run and with the annealing temperature set at 64°C. The GC clamp at the 5′ end of primer P984 was coamplified and prevented complete dissociation of the two DNA strands during TTGE (23). Amplification and analysis by agarose gel electrophoresis were performed as

TABLE 1. Bacterial strains and PCR results[a]

Cluster	Species	Strain	Source[b]	PCR result
I	*C. tyrobutyricum*	500	CNRZ	+
I	*C. tyrobutyricum*	502	CNRZ	+
I	*C. tyrobutyricum*	503	CNRZ	+
I	*C. tyrobutyricum*	505	CNRZ	+
I	*C. tyrobutyricum*	509	CNRZ	+
I	*C. tyrobutyricum*	608	CNRZ	+
I	*C. tyrobutyricum*	30L20	ADRIA	+
I	*C. tyrobutyricum*	32L25	ADRIA	+
I	*C. tyrobutyricum*	39L26	ADRIA	+
I	*C. tyrobutyricum*	105092	CIP	+
I	*C. butyricum*	B 269	CNR	+
I	*C. butyricum*	650	CNRZ	+
I	*C. butyricum*	NCTC 7423	INRA	+
I	*C. butyricum*	CB 1002	INRA	+
I	*C. butyricum*	CB 1003	INRA	+
I	*C. sporogenes*	73C179	ITFF	+
I	*C. sporogenes*	35CL13	ITFF	+
I	*C. sporogenes*	73C224	ITFF	+
I	*C. sporogenes*	CL18	ADRIA	+
I	*C. sporogenes*	106155	CIP	+
I	*C. beijerinckii*	528	CNRZ	+
I	*C. beijerinckii*	529	CNRZ	+
I	*C. beijerinckii*	530	CNRZ	+
I	*C. beijerinckii*	531	CNRZ	+
I	*C. beijerinckii*	104308	CIP	+
I	*C. pasteurianum*	ATCC 6013	CNR	+
I	*C. paraputrificum*	ATCC 25780	CNR	+
I	*C. perfringens*	ATCC 13124	INRA	+
I	*C. subterminale*	B 294	CNR	+
I	*C. tertium*	454	CNRZ	+
I	*C. tertium*	NCTC 541	INRA	+
I	*C. tetanomorphum*	DSM 4474	CNR	+
XI	*C. difficile*	198	CNR	−
XI	*C. glycolicum*	ATCC 14880	CNR	−
XI	*C. irregulare*	ATCC 25756	CNR	−
XI	*C. sticklandii*	NCIB 10654	CNR	−
XI	*C. sordellii*	NCIB 10717	CNR	−
XI	*C. bifermentans*	DSM 630	CNR	−
XI	*C. bifermentans*	TM	CNR	−
XI	*C. bifermentans*	744-83	CNR	−
XI	*C. bifermentans*	104309	CIP	−
XI	*C. bifermentans*	B 267	CNR	−
	Bacillus cereus	A30	CIP	−
	Lactobacillus delbrueckii	LL 51	ITFF	−
	Lactobacillus helveticus	LH 56	ITFF	−
	Streptococcus thermophilus	ST 85	ITFF	−
	Propionibacterium freudenreichii	P 9	ITFF	−
	Staphylococcus aureus	53.156	CIP	−
	Escherichia coli	54.127	CIP	−
	Enterococcus faecalis	EF 264	ITFF	−
	Enterococcus durans	SD 129	ITFF	−

[a] PCR was carried out by amplifying the V5-V8 region of the 16S rRNA gene of cluster I by using primers P930 and P932.

[b] ADRIA, Association pour le Développement de la Recherche appliquée aux Industries Agro-alimentaires; CIP, Collection de l'Institut Pasteur; CNR, Centre National de Référence des Bactéries Anaérobies; CNRZ, Collection du Centre National de la Recherche Zootechnique; INRA, Institut National de la Recherche Agronomique; ITFF, Institut Technique Français des Fromages.

TABLE 2. 16S rRNA gene target primers used in nested PCR for specific amplification of cluster I and for TTGE analysis

Primer name	OPD nomenclature[a]	Specificity[b]	Sequence (5′ to 3′)[c]	PCR annealing temp (°C)	Reference
P930	S-*-Cbot-0691-a-S-23	Cluster I	GTG AAA TGC GTA GAG ATT AGG AA	58	This work
P932	S-*-Clit-1338-a-A-22	Cluster I and XI	GAT YYG CGA TTA CTA GYA ACT C	58	This work
P984	S-D-Bact-0786-a-S-20	Bacteria	(GC clamp)[d] GAT TAG ATA CCC TGG TAG TC	64	36
P983	S-D-Bact-1060-a-A-21	Bacteria	TCA CGA CAC GAG CTG ACG ACA	64	36

[a] OPD, Oligonucleotide Probe Database
[b] Clusters as defined by Collins et al. (8).
[c] Y, T/C; R, A/G; S, G/C.
[d] GC clamp, CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC C.

described for PCR amplification. The overall time used for this cycle was 70 min. The dilution producing the cleanest PCR band was used for TTGE analysis.

Detection limit of PCR. In order to estimate the detection limit of PCR, a clostridium-free cheese sample of 250 mg was artificially contaminated with 250 μl of 10-fold dilutions of pure culture of *C. tyrobutyricum* at 10^9 cells · ml^{-1}. The sample from each tube of the different dilutions was subjected to DNA extraction as described previously.

Sensitivity of the TTGE technique. The sensitivity of TTGE for the detection of subdominant bacterial species in cheese was tested. Overnight liquid cultures of two strains (*C. tyrobutyricum* and *C. beijerinckii*, *C. tyrobutyricum* and *C. sporogenes*, and *C. beijerinckii* and *C. sporogenes*) were mixed in different ratios (1/1, 1/10, and 1/100) and added to clostridium-free cheese samples. The mixtures were then subjected to DNA extraction and PCR-TTGE as described previously.

Analysis of PCR products by TTGE. TTGE analysis of PCR amplicons was performed using a DCode Universal Mutation Detection System (Bio-Rad Laboratories) according to the procedures described previously (30) with the following modifications. The electrophoresis was performed in an 8% (wt/vol) polyacrylamide-bisacrylamide gel and 7 M urea with 1.25× TAE buffer (Tris-acetic acid-EDTA; Bio-Rad Laboratories) at a constant voltage of 70 V for 16 h. A temperature gradient from 63 to 70°C and a temperature ramp rate of 0.4°C/h were applied. Five-microliter samples of PCR products were loaded in wells. After electrophoresis, the gels were stained for 30 min with SYBR Green I nucleic acid gel stain (Roche Diagnostics, Mannheim, Germany), and their images were digitized using a Storm system (Molecular Dynamics, Sunnyvale, Calif.).

Clostridium identification ladder cluster I. TTGE gels were standardized by including an identification ladder obtained by mixing PCR products of the V5-V6 region of 16S rRNA from pure cultures of seven *Clostridium* reference strains (*C. perfringens*, *C. butyricum*, *C. pasteurianum*, *C. subterminale*, *C. beijerinckii*, *C. tyrobutyricum*, and *C. sporogenes*).

Gel analysis. For direct identification of bands in given TTGE profiles, a database was created by using the GelCompar II software package (Applied Maths, Kortrijk, Belgium). This database contained the V5-V6 bands of *Clostridium* species of cluster I listed in Table 1. The inclusion of a reference pattern consisting of amplicons from seven *Clostridium* reference strains every eight lanes on each TTGE gel allowed a digital normalization of patterns in the GelCompar database. After normalization, tentative identification of *Clostridium* strains was performed by comparing positions of bands in sample profiles with those of reference strains present in the database.

Sequencing of excised TTGE bands. The selected DNA bands were visualized on a UV-Transluminator and excised directly from the gels with a sterile pipette tip. They were allowed to diffuse overnight at 4°C in 100 μl of sterile water. Three microliters of eluate from individual bands was used to reamplify the PCR products. Before sequencing, the purity of each PCR-reamplified TTGE band was checked by running on a denaturing gradient gel in parallel to the original cheese sample. The PCR products were sent to a sequencing facility (Genome Express, Meylan, France). Recovered sequences were aligned with 16S rRNA gene fragments available from the National Center for Biotechnology Information database. The BLAST program was used to determine the closest known relatives.

MPN enumeration of *Clostridium* spp. Ten grams of cheese sample was homogenized in 90 ml of sodium citrate solution at 40°C with a stomacher. The samples were heat shocked at 75°C for 10 min to kill vegetative cells. With the use of 0.1% tryptone solution as diluent and BBB (Bryant and Burkey modified by Bergère) broth as the medium, the multiple dilution procedure was used for determination of spore concentration by MPN as follows: 1-ml aliquots of sample or dilution were inoculated into each of three tubes containing 9 ml of BBB broth. After inoculation, the tubes were sealed by addition of a layer, about 15 mm thick, of paraffin; incubated at 37°C for 7 days; and observed for gas production (5). For each sample, one positive tube was plated on RCM medium (Merck), and up to three colonies were isolated and tentatively identified by PCR-TTGE.

Volatile fatty acid analysis. Butyric acid fermentation in cheeses was detected by gas-liquid chromatography analysis of butyric acid. Twenty grams of cheese was mixed with 50 ml of sulfuric acid and homogenized with a stomacher for 5 min. The liquid phase was then neutralized by a 1% NaOH solution containing phenolphthalein. The soaps obtained were dried by vacuum (2). Samples were stored at 4°C before gas-liquid chromatography analysis. Five microliters of each of the supernatants was injected in a gas chromatograph equipped with a flame ionization detector (Intersmat IGC 121 DFL; Touzart et Matignon, Courtaboeuf, France) and connected to an integrator (model ICR 1B; Touzart et Matignon). A glass column (2 m by 4 mm) packed with 10% SP 1000 plus 1% H_3PO_4 on Chromosorb W AW (100/120 mesh) was used. The instrument was operated at 125°C for 6 min and then programmed at 20°C min^{-1} to 145°C. The operating conditions were as follows: injector temperature, 200°C; detector temperature, 250°C; carrier gas (nitrogen) flow rate, 30 ml min^{-1} (4).

RESULTS

Primer design. Details of the specific primer set developed are summarized in Table 2. Seventy cluster I 16S rRNA gene sequences and 17 other clostridial and 16 nonclostridial 16S rRNA gene sequences obtained from GenBank were analyzed to identify candidate primer sequences. The target region for the forward primer P930 was identified at locations 664 to 686 of the *C. butyricum* ATCC 19398 (type species and type strain) rRNA gene. Table 3 shows the target group of the primer, including all organisms possessing the target sequences and related species displaying one or more mismatches. Theoretical hybridization targets for the cluster I-specific primer were determined by using all 16S rRNA gene sequences deposited in the RDP. The results showed that 90.4% of the 145 hits obtained belonged to members of cluster I, 4.8% belonged to as-yet-unidentified bacteria, and 4.8% belonged to other groups (*Gloeothece* group, *Sporomusa* group, and *C. histolyticum* subgroup). The presence of these non-cluster I sequences may have only limited consequences since these species are generally not observed in milk. The primer showed a complete homology of sequence with all *Clostridium* species of cluster I. A reverse primer was also selected from the 16S rRNA gene sequences of cluster I and non-cluster I strains (Table 4). The analysis revealed a consensus region at positions 1303 to 1325 of the *C. butyricum* ATCC 19398 rRNA gene, mainly found in members of clusters I and XI despite some variations in the 4th, 5th, and 17th bases from the 3′ end (G-to-A conversions). The BLAST report revealed that 28% of the 533 16S rRNA

TABLE 3. Alignment of 16S rRNA gene sequences from *Clostridium* and non-*Clostridium* species corresponding to the primer sequence of P930[a]

Species	Strain	5′ 691	G	T	G	A	A	A	T	G	C	G	T	A	G	A	G	A	T	T	A	G	G	A	A	3′ 713
C. butyricum	ATCC 19398^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. beijerinckii	NCIMB 9362^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. tertium	ATCC 14573		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. paraputrificum	ATCC 25780^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. aurantibutyricum	NCIMB 10659^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens	ATCC 13124^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. acetobutylicum	ATCC 824^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. subterminale	ATCC 25774^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. tetanomorphum	DSM 528		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. scatologenes	ATCC 25775^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. tyrobutyricum	ATCC 25755^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. pasteurianum	ATCC 6013^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. sporogenes	ATCC 3584^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. sticklandii	ATCC 12662^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	G	.	.	.	G	
C. difficile	NCTC 11209^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	
C. irregulare	ATCC 25756^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	
C. glycolicum	DSM 1288^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	
C. bifermentans	NCIMB 10716^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	
C. sordellii	ATCC 9714^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	G	
Bacillus cereus	ATCC 27877		.	.	.	.			.	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	G	
Lactobacillus delbrueckii	ATCC 9649^T		.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	A	T	.	.	.	.	
Lactobacillus helveticus	NCDO 2712^T		.	.	.	G	.	.	.	.	.	.	.	.	.	.	T	.	.	A	T	.	.	.	.	
Streptococcus thermophilus	DSM 20617^T		.	.	.	.	.	.	.	.	.	.	.	.		.	T	.	.	A	T	.	.	.	G	
Propionibacterium freudenreichii	DSM 20271^T		.	.	.	G	.	.	.	.	.	.	C	.	.	.	T	.	.	C	G	.	.	.	.	
Staphylococcus aureus	ATCC 12600^T		.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	A	G	.	.	.	G	
Escherichia coli	MRE600		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.	.	.	G	
Enterococcus faecalis	JCM 5803^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	A	T	.	.	.	G	
Enteroccocus durans	NCFB 596		.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	A	T	.	.	.	G	

[a] Only the nucleotides different from the nucleotides in the target sequence are shown. Numbering is based on the *E. coli* 16S rRNA gene sequence.

gene sequences which were available in the database and contained that region belonged to cluster I, 8.6% belonged to cluster XI, 18.8% belonged to as-yet-unidentified bacteria, and 44.6% belonged to members of other genera or species, such as *Streptomyces* (45%), *C. purinolyticum* (20%), *Eubacterium* (10%), *Atopobium* (9%), *Thermoanaerobacter* (5%), *C. leptum* (4%), *Rubrobacter* (4%), *Caloramator* (2%), and *C. histolyticum* (1%). All the species of cluster I matched the primer, confirming the cluster I selectivity of the primer system.

Experimental specificity of oligonucleotide primers. The specificity of forward primer P930 in combination with reverse primer P932 was evaluated by PCR by using DNA isolated from the species of cluster I, *Clostridium* species belonging to cluster XI, and non-*Clostridium* strains. The optimal annealing temperature was empirically determined with 2°C increments from 56 to 70°C and with the use of DNA from 51 bacterial strains (Table 1). An annealing temperature of 58°C, slightly above the calculated optimal temperature of both primers, and 30 cycles of PCR were used. Agarose gel electrophoresis of the PCR products revealed an amplicon size of 665 bp. The latter was detected with all strains of *Clostridium* species of cluster I tested but with no non-cluster I species, indicating a 100% specificity of the primer system for cluster I. A higher number of cycles (40 cycles) led to a weak amplification of species of cluster XI. Negative controls with water instead of template DNA showed no amplification in all experiments.

Identification of reference strains by TTGE. The different migration of the amplicons allowed us to distinguish most of the *Clostridium* strains listed in Table 1, except for *C. tyrobutyricum* and *C. tetanomorphum*, despite the relatively low level of similarity in the 16S rRNA gene region amplified by the primers (95%). In most cases only one band was seen, indicating that there was no or little intragenomic variation for the sequence amplified. One or more additive weak bands were observed with *C. tertium* and *C. paraputrificum*. For several strains, the TTGE patterns contained two bands that migrated very closely together (Fig. 1). This observation indicates that there were DNA molecules with a slightly different melting behavior, likely due to incomplete extension reaction during PCR caused by the GC clamp (25) or by hairpin formation within the GC clamp of the forward primer. In addition, V5-V6 TTGE profiles from strains of the same species displayed the same electrophoretic mobility as verified by using three strains of each species (Fig. 1). The reproducibility of patterns obtained for each strain in a complex community was tested in order to check the possibility of preferential amplification. Therefore, DNAs of four strains (*C. tyrobutyricum* IP 105092, *C. butyricum* INRA CB1002, *C. beijerinckii* CNRZ 528, and *C. sporogenes* IP 106155) were mixed in a 1:1:1:1 ratio, and the mixture was used as a template in a PCR. The profile obtained was a combination of the patterns obtained for each strain analyzed separately, but not with the same intensities. The bands of *C. butyricum* and *C. tyrobutyricum* were more intense than those of *C. beijerinckii* and *C. sporogenes* (data not shown). This PCR bias could not be eliminated by performing PCR in the presence of cosolvents or by using special PCR procedures such as "touchdown" PCR but could be reduced in the presence of 4% dimethyl sulfoxide. The sensitivity of

TABLE 4. Alignment of 16S rRNA gene sequences from *Clostridium* and non-*Clostridium* species corresponding to the primer sequence of P932[a]

Species	Strain	5′ 1359	G	A	T	Y	Y	G	C	G	A	T	T	A	C	T	A	G	Y	A	A	C	T	C	3′ 1338
C. butyricum	ATCC 19398^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. beijerinckii	NCIMB 9362^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. tertium	ATCC 14573		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. paraputrificum	ATCC 25780^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. aurantibutyricum	NCIMB 10659^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. perfringens	ATCC 13124^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. acetobutylicum	ATCC 824^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. subterminale	ATCC 25774^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. tetanomorphum	DSM 528		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. scatologenes	ATCC 25775^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. tyrobutyricum	ATCC 25755^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. pasteurianum	ATCC 6013^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. sporogenes	ATCC 3584^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. sticklandii	ATCC 12662^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. difficile	NCTC 11209^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. irregulare	ATCC 25756^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. glycolicum	DSM 1288^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. bifermentans	NCIMB 10716^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
C. sordellii	ATCC 9714^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	
Bacillus cereus	ATCC 27877		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	
Lactobacillus delbrueckii	ATCC 9649^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	
Lactobacillus helveticus	NCDO 2712^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	
Streptococcus thermophilus	DSM 20617^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	
Propionibacterium freudenreichii	DSM 20271^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	
Staphylococcus aureus	ATCC 12600^T		.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	
Escherichia coli	MRE600		.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	
Enterococcus faecalis	JCM 5803^T		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	
Enteroccocus durans	NCFB 596		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	.	

[a] Only nucleotides different from the nucleotides in the target sequence are shown. Numbering is based on the *E. coli* 16S rRNA gene sequence.

TTGE for detection of a subdominant bacterial species was tested by comparing the band intensities of different species (*C. tyrobutyricum*, *C. beijerinckii*, and *C. sporogenes*) mixed in different ratios. The competitive-PCR approach indicated that the limit of detection of the less represented species was 10% of the total DNA (Fig. 2).

Application of PCR-TTGE to detection of *Clostridium* in cheese samples. Cluster-specific assays were applied to DNA extracted from 21 cheese samples. It was possible to obtain PCR-ready DNA from all samples. A 100-fold dilution was nevertheless necessary to overcome an inhibitory effect in DNA extracted from cheese samples. Up to three tests with different aliquots from each sample were performed. All the results obtained are summarized in Table 5. Experimental testing of the protocol on cheese samples revealed that the number of cycles of PCR needed to be increased to 40 cycles. The detection limits of *Clostridium* in cheese were investigated at different concentrations. After nested PCR the detection sensitivity was increased 10 times compared to the first PCR. It was possible to detect 100 CFU/g of cheese (data not shown). In all cheeses with gas production (holes in the cheese matrix) and elevated concentrations of butyric acid, nested PCR resulted in a specific signal. The TTGE analysis of 16S rRNA genes of *Clostridium* from cluster I demonstrated the presence of one or two detectable species in each cheese sample (Fig. 3). After normalization, band positions were compared with the GelCompar database of reference strains. For all samples, assignment of detectable *Clostridium* to reference species appeared possible. Figure 3 shows the resulting tentative identification of different *Clostridium* species in 20 cheese samples. Nine samples showed the presence of *C. tyrobutyricum* and *C. beijerinckii*, one showed the presence of *C. tyrobutyricum* and *C. sporogenes*, and one showed the presence of *C. beijerinckii* and *C. butyricum*. For all other samples, only one species was detected. Five samples showed the presence of *C. beijerinckii*, and four showed the presence of *C. tyrobutyricum*. Furthermore, there were no bands present in cheese sample lanes which could not be assigned to any of the reference clostridial species. As reported in Table 5, differences were observed when the PCR-TTGE results of two or three aliquots from the same sample were compared, underlining the presence of ecological microniches in cheese. The direct identification of *Clostridium* spp. in cheese samples was confirmed by sequencing of excised bands. All sequences obtained from the bands were ≥98% similar to the species assignment made on the base of band comigration. Results of TTGE analyses were compared with those of bacteriological culture. All the cultured strains of *Clostridium* were identified by PCR-TTGE analysis. The profiles of bacterial isolates matched those obtained from cheeses (data not shown).

DISCUSSION

This study was conducted to develop a 16S rRNA gene-based PCR protocol for the detection and identification of *Clostridium* of cluster I in cheese samples by using TTGE. A nested-PCR approach was applied, combining a first cluster-specific PCR step with a second bacterial PCR step. A specific

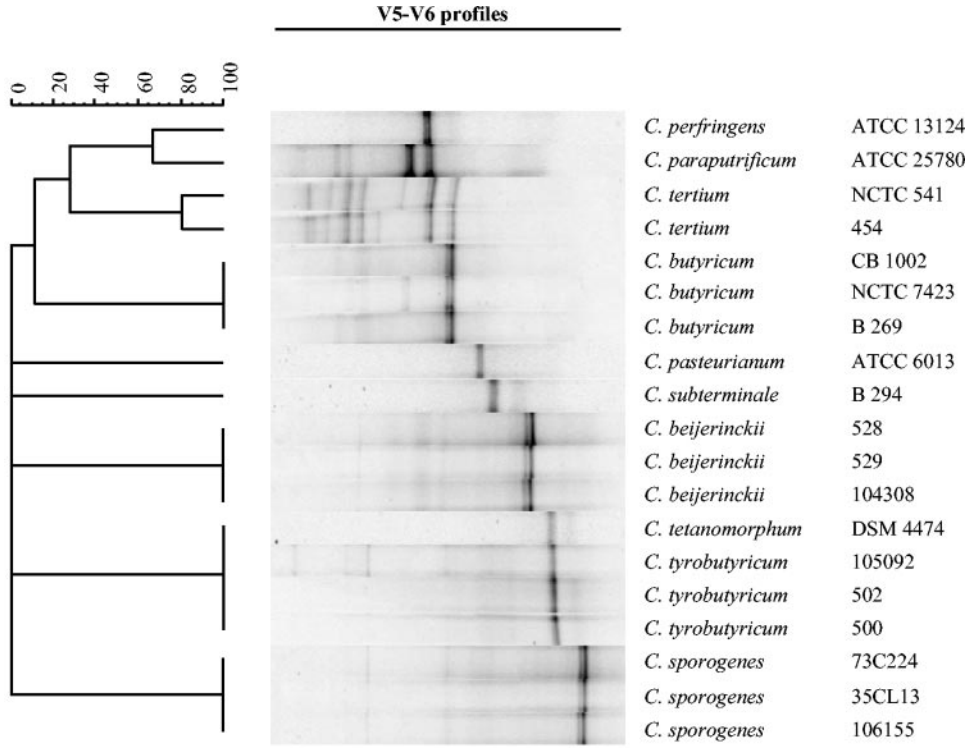

FIG. 1. Clustering of TTGE profiles of clostridial reference species. Similarity coefficients (Dice indices) were calculated for each pair of TTGE profiles in order to create a similarity matrix. This dendrogram was constructed using the UPMGA algorithm (unweighted pair group method using arithmetic averages). Numbers next to the species assignment are the strain numbers.

probe for *Clostridium* cluster I was developed by Franks et al. in 1998 for fluorescent in situ hybridization (11). Based on the multialignment data, we found other specific sequences from the 16S rRNA gene which were highly conserved in cluster I and which displayed complete homology with 100% of species of this cluster. Moreover, in contrast to probes, mismatches are most critical at the 3′ terminus of PCR primers, mainly the first three bases at the 3′ end, in order to enhance the specificity (18, 31). The PCR primers were subjected to theoretical testing with database information and experimental testing with pure cultures and DNA from cheese samples. The PCR system combining P930 and P932 was highly specific for *Clostridium*. In a nested setup, the DNA amplification system as designed in 1987 by Woese (36) was used in order to generate a clamped PCR fragment for TTGE separation. Using our primers, a fragment of 290 bp, suitable for TTGE analysis, was amplified.

We demonstrated the potential of TTGE fingerprints, based upon 16S rRNA gene fragments amplified via nested PCR from community DNA, to characterize the clostridial population in cheese. Our results showed that the separation of the V5-V6 amplicons in a TTGE gel resulted in an identification of all tested *Clostridium* species, except for *C. tyrobutyricum* and *C. tetanomorphum*, which displayed identical band positions for the two amplicons. A previous work had already shown that fragments of different sequences arising from different species might migrate at the same position on denaturing gradient gels (26). However, *C. tetanomorphum* is rarely found in cheese samples, and sequence analysis of TTGE bands can be used to differentiate between species. Another observation was that *C. tertium* and *C. paraputrificum* displayed additional weak bands, possibly as a result of operon heterogeneity, as previously observed for different genera (29, 32), or due to sequence-specific DNA polymerization artifacts. This heterogeneity does not impair the identification potential of the technique as long

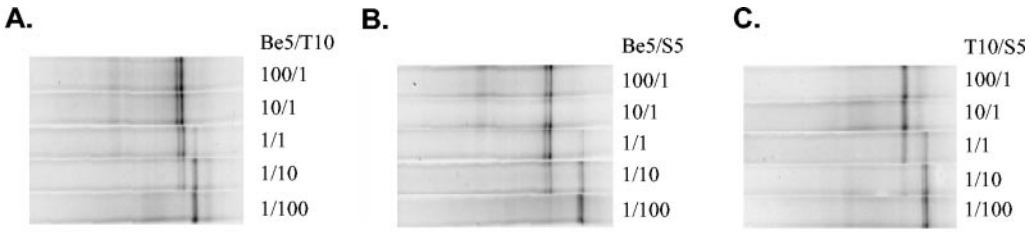

FIG. 2. TTGE profiles of V5-V6 amplicons of *C. tyrobutyricum* and *C. beijerinckii* (A), *C. beijerinckii* and *C. sporogenes* (B), and *C. tyrobutyricum* and *C. sporogenes* (C) DNAs mixed in different ratios. T10, *C. tyrobutyricum* (105092); Be5, *C. beijerinckii* (104308); S5, *C. sporogenes* (73C224).

TABLE 5. Results obtained by MPN, culture, chromatographic analysis of butyric acid, TTGE analysis of the cheese samples, and strain identification by sequencing

Cheese sample no.	Type	MPN value (s/g[a])	Culture[b]	Butyric acid concn[c]	Strain identification by TTGE	No. of identical results/no. analysed	Identification by sequencing
21	Emmental	$>1.1 \times 10^5$	*C. sporogenes*	76.0	*C. sporogenes*	3/3	*C. sporogenes*
					C. tyrobutyricum	2/3	ND[d]
22	Gruyère	$>1.1 \times 10^5$	*C. tyrobutyricum*	85.5	*C. tyrobutyricum*	3/3	ND
					C. beijerinckii	2/3	*C. beijerinckii*
23	Comté	4×10^3	*C. tyrobutyricum*	110.4	*C. tyrobutyricum*	3/3	*C. tyrobutyricum*
24	Comté	<30	−	118.4	*C. beijerinckii*	2/3	ND
					C. tyrobutyricum	3/3	*C. tyrobutyricum*
25	Comté	<30	−	178.7	*C. beijerinckii*	2/3	ND
					C. tyrobutyricum	3/3	*C. tyrobutyricum*
26	Comté	<30	−	39.7	*C. beijerinckii*	2/2	*C. beijerinckii*
					C. tyrobutyricum	1/2	ND
27	Comté	2.3×10^2	*C. tyrobutyricum*	227.9	*C. tyrobutyricum*	3/3	*C. tyrobutyricum*
29	Emmental	<30	−	20.2		2/2	
31	Emmental	$>1.1 \times 10^5$	*C. tyrobutyricum*	33.5	*C. tyrobutyricum*	3/3	*C. tyrobutyricum*
32	Beaufort	<30	−	30.5	*C. beijerinckii*	1/1	*C. beijerinckii*
34	Beaufort	<30	−	81.2	*C. beijerinckii*	1/1	*C. beijerinckii*
35	Beaufort	<30	−	35.8	*C. beijerinckii*	2/2	*C. beijerinckii*
					C. butyricum	1/2	*C. butyricum*
36	Ossau-Iraty	4×10^2	−	519.1	*C. beijerinckii*	1/2	*C. beijerinckii*
					C. tyrobutyricum	1/2	ND
37	Ossau-Iraty	1.5×10^3	*C. tyrobutyricum*	450.8	*C. tyrobutyricum*	2/2	*C. tyrobutyricum*
38	Ossau-Iraty	40	−	22.3	*C. beijerinckii*	1/1	*C. beijerinckii*
39	Ossau-Iraty	<30	−	15.4	*C. beijerinckii*	1/1	*C. beijerinckii*
40	Ossau-Iraty	<30	−	57.7	*C. beijerinckii*	2/2	*C. beijerinckii*
43	Comté	ND	−	16.1	*C. beijerinckii*	1/2	ND
					C. tyrobutyricum	1/2	ND
44	Comté	<30	−	52.0	*C. beijerinckii*	1/2	*C. beijerinckii*
					C. tyrobutyricum	2/2	ND
45	Comté	ND	−	47.9	*C. beijerinckii*	1/2	*C. beijerinckii*
					C. tyrobutyricum	2/2	*C. tyrobutyricum*
46	Comté	ND	−	39.3	*C. beijerinckii*	1/3	ND
					C. tyrobutyricum	2/3	ND

[a] s/g germinated spore concentration.
[b] Negative culture results means absence of clostridial colonies on the plates.
[c] The data are expressed in milligrams per 100 g of cheese.
[d] ND, not determined.

as the bands remain consistent among different strains of the same species.

Developed and optimized for analysis of potential mixtures of *Clostridium* in a cheese ecosystem, the technique was validated using artificial mixtures of cluster I species in *Clostridium*-free cheese samples. For these mixtures, all species present could be identified. A detection limit of 100 CFU/g of cheese was established, underlining the high sensitivity of the method. With a nonspecific primer for the *Clostridium* genus, the sensitivity of such a method was estimated to be 10^4 CFU/g. In this case, an enrichment period of cheese samples before DNA extraction is needed (7). Based on in vitro tests on pure cultures, we expect clostridial species to be detected even when they constitute only 10% of the clostridial population.

The applicability of the technique was demonstrated by the analysis of 21 cheese samples. Our results have shown that DNA extracted from cheese according to the protocol developed by Godon et al. can always be successfully used as template DNA in PCR (12). Furthermore, the extraction protocol used, including bead beating, provided sufficient DNA for amplification of *Clostridium*, as even spores were sufficiently lysed (27). It allows workers to check for the possible presence of *Clostridium* at very early stages of ripening, as outgrowth of cells is not required to obtain a signal.

All cheeses with splitting and/or rancid flavor gave a signal after nested PCR. After normalization, comparison of the amplicon migration patterns in TTGE gels with those of reference strains allowed us to identify the bacteria. All the bands present on the TTGE gel could be linked to a given clostridial species.

In all the 20 cheeses with defects, only four different species were detected: *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum*, and *C. sporogenes*. The most important species detected with PCR-TTGE were *C. beijerinckii* and *C. tyrobutyricum*, which contaminated 15 and 14 samples of the 20 tested, respectively. For 45% of cheeses only one population was found, and 55% were characterized by the detection of two clostridial species. In order to compare the direct detection of *Clostridium* in cheese with the traditional method for detecting spores, colonies isolated from gas-positive tubes were identified by TTGE. The profiles of the bacterial isolates matched those obtained from cheeses. Culture and direct molecular detection were consistent, but only the spores of two strains, *C. tyrobutyricum* and *C. sporogenes*, found conditions favorable for growth in

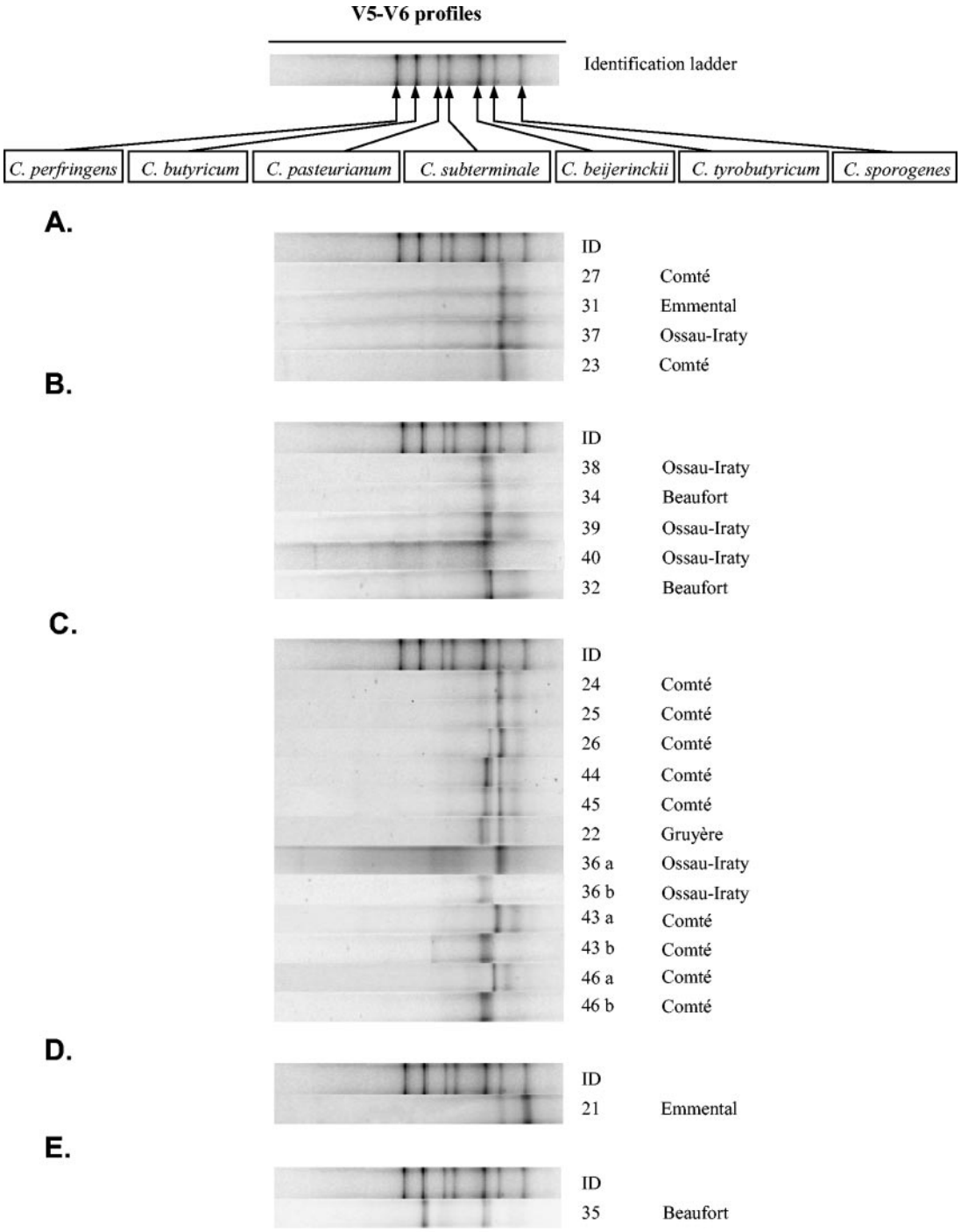

FIG. 3. TTGE profiles of PCR amplicons of the V5-V6 regions of 16S rRNA genes for the identification of *Clostridium* species present in cheese. Cheese samples contained detectable *C. tyrobutyricum* (A), *C. beijerinckii* (B), *C. beijerinckii* and *C. tyrobutyricum* (C), *C. tyrobutyricum* and *C. sporogenes* (D), and *C. beijerinckii* and *C. butyricum* (E). ID, identification ladder. For each dendrogram the Dice index was 100%. PCR-TTGE profiles a and b represent two different patterns from the same sample.

culture medium and could be isolated by the plating method. These results were in agreement with the ones obtained in previous work by the traditional plating method. Toyoda et al., studying Gouda cheese with defects, mainly detected *C. tyrobutyricum* and, in a lesser proportion, *C. sporogenes*, *C. butyricum*, and *C. beijerinckii* (34). Matteuzzi et al. found that, in only 15% of examined Grana cheese samples with defects, blowing was associated with detection of two species, *C. sporogenes* and *C. tyrobutyricum*, but *C. beijerinckii* was never detected (22). In our study, the molecular method allowed the detection of *C. beijerinckii* in cheese samples and underlined the limitation of cultivation, mainly due to the stress imposed

by cultivation steps, the necessity for strictly anoxic conditions, and the germination efficiency. When our results obtained by PCR-TTGE and by butyric acid analysis were compared, a good correlation was observed. In all cheeses with a high amount of butyric acid (>100 mg/100 g of cheese), the presence of *C. tyrobutyricum* DNA was confirmed by PCR-TTGE, strongly suggesting the involvement of this species in butyric acid fermentation. In most cheeses with hole formation and contamination with *C. beijerinckii*, a concentration of butyric acid slightly higher than usual was found (10 to 40 mg/100 g). These results suggest that *C. beijerinckii* did not produce large quantities of butyric acid during ripening, even though it was able to do so in culture media. The implication of this species in splitting of cheese could be due to its ability to produce hydrogen, but it is important that this defect could be caused by other gas-producing bacteria. It would be interesting to study the general metabolic activity of *C. beijerinckii* and *C. tyrobutyricum* in cheese to assess whether *C. beijerinckii* may have an impact on cheese defects and to better understand the relationship between these two species. Since the ratio of 16S rRNA genes and rRNA is dependent on cellular activity, the comparison of TTGE patterns derived from 16S rRNA and rRNA gene amplicons should indicate if the presence of *C. tyrobutyricum* is a prerequisite for the occurrence of butyric acid fermentation in cheese (35).

This is the first study allowing the detection and differentiation of all clostridial species present in cheese with a single test. It is noteworthy that clostridia such as *C. butyricum* and *C. beijerinckii* cannot easily be identified by normal culture and biochemical techniques but can readily be differentiated by PCR-TTGE with P930-P932 primers. Moreover, this technique is rapid since no cloning and sequencing of the TTGE bands are required, provided that an identification match is obtained with the database. The experimental procedure developed in this study can be implemented as a routine detection method in the industry in order to screen cheese during production and to have a complete study of the diversity of *Clostridium* species in defective cheeses.

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