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Identification of *Clostridium tyrobutyricum* as the Causative Agent of Late Blowing in Cheese by Species-Specific PCR Amplification

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Butyric acid fermentation, the late-blowing defect in cheese, caused by the outgrowth of clostridial spores present in raw milk, can create considerable loss of product, especially in the production of semihard cheeses like Gouda cheese, but also in grana and Gruyère cheeses. To demonstrate the causative relationship between *Clostridium tyrobutyricum* and late blowing in cheese, many cheesemaking experiments were performed to provoke this defect by using spores from several strains of the major dairy-related clostridia. A method of PCR amplification of a part of the 16S rRNA gene in combination with hybridization with species-specific DNA probes was developed to allow the specific detection of clostridial sequences in DNAs extracted from cheeses. The sensitivity was increased by using nested PCR. Late blowing was provoked in experimental cheeses with 28 of the 32 *C. tyrobutyricum* strains tested, whereas experimental cheeses made with spores from *C. beijerinckii*, *C. butyricum*, and *C. sporogenes* showed no signs of butyric acid fermentation. In all experimental and commercial cheeses with obvious signs of late blowing, DNA from *C. tyrobutyricum* was detected; in some cheeses, signals for *C. beijerinckii* were also found. It was concluded that only *C. tyrobutyricum* strains are able to cause butyric acid fermentation in cheese.

Butyric acid fermentation in cheese (late blowing), caused by the outgrowth of clostridial spores present in raw milk and most commonly originating from silage, can create considerable loss of product, especially in the production of semihard cheeses like Gouda cheese, but also in grana and Gruyère cheeses (2, 3, 5, 20). Although *Clostridium tyrobutyricum* is the most frequently isolated strain from late-blown cheeses (3, 20), spores of other clostridia, particularly *C. beijerinckii*, *C. butyricum*, and *C. sporogenes*, have also been isolated from natural and processed cheeses and raw milk (5, 6, 21). Since all of these clostridia are capable of forming butyric acid and hydrogen in various media (19), the correlation between the appearance of this defect and the presence of a specific clostridial species is difficult.

For the production of semihard cheeses like Gouda cheese, it is very important to limit the number of spores of bacteria capable of causing late blowing in the cheese milk to fewer than 1 spore per 10 ml. Currently available methods to detect and to enumerate these spores in milk are inaccurate and nonspecific and take at least several days before the results are available (2). To improve these methods, it is crucial to know whether this defect is caused by a single *Clostridium* species or whether several dairy-related clostridia are able to cause butyric acid fermentation in cheese. The aim of this work was to demonstrate the causal relationship between *C. tyrobutyricum* and butyric acid fermentation in semihard Gouda cheese. This was done by performing many cheesemaking experiments with spores from many different clostridial strains. In addition, the presence of specific clostridial cells in many cheeses with the late-blowing defect obtained from commercial sources was analyzed.

Conventional methods for the isolation of clostridial cells from cheeses with late-blowing symptoms are very complicated and usually result in a mixture of isolates belonging to different clostridial species (3, 13). The identification of isolates is problematic since specific media to discriminate between the clostridial species mentioned above do not exist and phenotypic discrimination is almost impossible (4, 9, 12). For this reason, we previously developed identification methods that use specific DNA probes (9). The use of these probes on the basis of sequence variability in specific regions of the 16S rRNA gene (1, 14) allows reliable identification of *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum*, *C. tyrobutyricum*, and *C. sporogenes*.

To overcome the problems associated with the isolation of clostridial cells from cheeses, we chose the strategy of directly detecting species-specific sequences in DNAs isolated from cheeses with specific probes. A method of PCR amplification (17) of a part of the 16S rRNA gene in combination with hybridization with species-specific probes (10) was developed. To further increase the sensitivity, this method was optimized by using specific probes in nested PCR. This nested PCR method was applied to study the relation between the presence of *C. tyrobutyricum* and other clostridial species and the occurrence of late blowing in commercial and experimental cheeses.

MATERIALS AND METHODS

Collection of strains. Sixty-seven clostridial isolates obtained from different culture collections were used in cheesemaking experiments and are listed in Table 1. Most of these strains were originally isolated in association with dairy products or from farm environments. All strains were classified by the methods described by Klijn et al. (9), which are based mainly on the API 20A system (15) and hybridization with species-specific DNA probes. Some strains were originally received as unidentified strains or appeared to have been classified incorrectly. Strains were maintained by inoculation in AC broth (Difco, Detroit, Mich.) in glass ampoules which were evacuated and sealed. Following pasteurization for 15 min at 75°C, ampoules were incubated for a minimum of 4 days at 30°C or until good growth was obtained and were then stored at 4°C. Clostridia were grown at 30°C in anaerobic jars equipped with Anaerocult A (Merck, Darmstadt, Germany) packs unless otherwise stated.

Production of spores. Spores were produced by growing cultures for 1 week at 30°C under anaerobic conditions in three liquid media, i.e., AC broth, PAN broth [tryptone, 5 g; L-(+)-sodium lactate syrup (60%; wt/wt), 25 g (or sodium lactate, 15 g); yeast extract, 10 g; sodium acetate 3.aq, 8 g; water, 1 liter; sterilized for 15 min at 121°C; final pH, 6.5 ± 0.2], and RCM (Merck). To produce sufficient

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TABLE 1. Strains used in cheesemaking experiments

Species	Strain(s)[a]
C. tyrobutyricum	ADRIA T860, ADRIA T932, ADRIA T103, ATCC 25755^T, DSM 663, BLVM 1519/A, CEGT01, CNRZ 500, CNRZ 505, CNRZ 564, CNRZ 569, CNRZ 596, CNRZ 611, EFAM 1528, EFAM 1553 EFAM 1554, EFAM 1556, EFAM 1558, EFAM 1559, EFAM 1600, EFAM 1602, EFAM 1519, EFAM 1527, IVVO V24, IVVO S42, NIZO BZ15, NIZO BZ18, NIZO S46, NIZO 51, NIZO FL104, NIZO BZ2, NIZO BZ6
C. butyricum	ATCC 19398^T
C. beijerinckii	ADRIA 6B3 ADRIA 25L17, ADRIA 27L17, ATCC 25752^T, ATCC 6014, ATCC 14823, ATCC 14949, ATCC 14950, ATCC 17791[b], BAM M1[b], BAM M2[b], CNRZ 530[b]
C. sporogenes	ADRIA SC25-4, ADRIA S882, ATCC 3584^T, BLVM 1363/A, BLVM 1527, EFAM 1356, EFAM 1534, EFAM 1552, EFAM 1601, IVVO V12A, IVVO V27, IVVO V30, IVVO V31, IVVO S39, IVVO V71, IVVO V80, IVVO V110, NIZO pII, NIZO S2, NIZO N2, NIZO 22.5, NIZO 24.1, NIZO 889

[a] Collections abbreviated as follows: ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany; EFAM, Eidgenössiche Forschungsanstalt für Milchwirtschaft, Bern, Switzerland; BLVM, Bundesanstalt für Milchwirtschaft, Wolfpassing, Austria; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-Josas, France; IVVO, Instituut voor Veevoedingsonderzoek, Lelystad, The Netherlands; ADRIA, Association pour le Dévelopement de la Recherche Appliquée, Quimper, France; NIZO, Netherlands Institute for Dairy Research.

[b] Received as *C. butyricum.*

quantities of spores, 100- to 1,000-ml cultures were centrifuged (15 min at about 3,000 × *g*), and spores were suspended in 10 ml of sterile skim milk and stored in small portions at −20°C.

After at least 1 week of storage, the number of CFU was estimated, both directly and after pasteurization for 15 min at 75°C, by plating on RCM agar and anaerobic incubation for 4 days at 30°C.

Cheesemaking. Gouda cheeses were made by standard procedures with bactofuged (spores eliminated by centrifugation) milk to reduce the initial levels of clostridial spores (22). From 800 kg of bactofuged cheese milk, 12 cheeses were produced in four cheese vats, one of which was used for the production of control cheeses. In the other three cheese vats, clostridial spores (100 to 1,000/ml) were added to the cheese milk. Spore suspensions were freshly thawed and used as such or pasteurized. To facilitate butyric acid fermentation, some adaptations of the standard procedures were applied. The pH was normal (5.3 at 14 days), the salt concentration (2.6% on dry matter at 14 days rather than 3.5%) and nitrate concentration (5 g of sodium nitrate per 100 liters of milk rather than 15 g) were lower, and the cheeses were ripened after being brined at 19°C instead of 13°C.

Detection of late blowing. During the ripening of cheeses, gas formation was regularly monitored by palpitation of cheeses by an experienced cheesemaker. After 6 and 12 weeks, cheeses were analyzed. Butyric acid fermentation in cheeses was detected both by visual inspection after the cheese had been cut and by high-performance liquid chromatography (HPLC) analysis of butyric acid. During visual inspections, the formation of holes by hydrogen gas produced during butyric acid fermentation (Fig. 1) and the presence of typical colonies of clostridia were observed. Such colonies are seen as dark round hard spots in cheese (standard diameter ± 1 mm). For butyric acid analysis, 10 g of ground cheese was mixed with 30 ml of 1 M perchloric acid and homogenized with a stomacher (Seward, London, United Kingdom) for 5 minutes. After settling for 1 h, the supernatant fluid was filtered through a Millex-GV membrane (0.22-μm pore size; Millipore). Samples were stored at 4°C before HPLC analysis. HPLC analysis (injection volume, 25 μl) was performed at 20°C on an Aminex ion-exclusion column (HPX-87H; 300 by 7.8 mm; Bio-Rad), with 0.01 N sulfuric acid (flow rate, 0.6 ml/min) as the eluent and a refractive index detector (Erma-7510). A butyric acid content of more than 100 mg per kg of cheese (in 12-week-old cheeses) was considered to be indicative of butyric acid fermentation.

DNA extraction from cheese. For DNA isolation from cheese, 5 g of ground cheese was homogenized in 50 ml of sodium citrate solution (2% [wt/vol] trisodium citrate dihydrate) at 45°C with a stomacher. From this suspension, 2 ml was incubated for 30 min at 37°C after the addition of 200 μl of proteinase K (final concentration, about 2 mg/ml). After centrifugation for 10 min at 2,750 × *g*, the fat layer was removed with a cotton tip and 1 ml of supernatant fluid was also removed with as much fat as possible. The remaining 1 ml, including the pellet, was incubated with 1 ml of THMS (30 mM Tris-HCl [pH 8.0], 3 mM $MgCl_2$, 25% [wt/vol] sucrose) and 100 μl of lysozyme (final concentration, 1 mg/ml) for 30 min at 37°C. Then 100 μl of 10% sodium dodecyl sulfate (SDS) solution (final concentration, 0.5%) was added and again incubated for 10 min at 60°C. Subsequently, 2 ml of phenol-TE (phenol equilibrated to TE [10 mM Tris-HCl, pH 8.0; 1 mM EDTA]) was added, as described by Sambrook et al. (18), mixed with a Vortex mixer, and centrifuged for 20 min at 2,750 × *g*. The supernatant fluid was transferred to a clean tube, and 1 ml of phenol-TE and 1 ml of chloroform were added. After being mixed with a Vortex mixer and centrifuged for 20 min at 2,750 × *g*, the top layer was transferred to a clean tube, and 2 ml of chloroform was added and mixed on a Vortex mixer. The tube was centrifuged for 3 min at 2,750 × *g*. The top layer was transferred to a clean tube, and 100 μl of 3 M sodium acetate (pH 5.5) and 5 ml of 96% ethanol (−20°C) were added. The tube was turned over a few times and stored overnight at −20°C. Then the tube was centrifuged for 20 min at 2,750 × *g*, and the liquid was removed as completely as possible. Two milliliters of 70% ethanol was added, briefly mixed with a Vortex mixer, and centrifuged for 10 min at 2,750 × *g*. The liquid was removed as completely as possible, and the pellet was dried in a vacuum exsiccator (about 15 min) and dissolved in 0.5 ml of TE.

PCR amplification. PCRs for specific amplification of the V6 region of the 16S rRNA gene were performed by using a Thermocycler 480 (Perkin-Elmer, Gouda, The Netherlands). The reactions were carried out in sterile 0.5-ml tubes which contained 50 μl of the following buffer: 10 mM Tris-HCl (pH 8.8), 3.0 mM $MgCl_2$, 50 mM NaCl, 2.5 mM (each) deoxynucleoside triphosphates, 1 U of *Taq* polymerase (Ampli-Taq; Perkin-Elmer), and 15 ng (each) of primers P3 and P4 (Table 2). After being heated to 95°C to eliminate all protease activity, 5 μl of template DNA was added. Amplification was done in 30 cycles of melting DNA at 94°C for 1 min, annealing at 55°C for 1.5 min, and elongation at 72°C for 2.5 min.

Nested PCR. Nested PCR was performed by first amplifying a part of the 16S rRNA gene (nucleotides 41 to 1114) with primers P1 and P2 according to the protocol described above for 45 cycles. The resulting PCR product was diluted 10-fold to decrease the remaining concentrations of primers P1 and P2. Then 5 μl of this dilution was used as the template for the second PCR amplification with one of the specific primers and P5 (Table 2). In this second step, the conditions were adapted with respect to the annealing temperature (63 or 72°C) and the number of cycles (20 or 25). When the annealing temperature was closer to the elongation temperature (72°C), the number of cycles was decreased (see Table 3).

Hybridization with specific DNA probes. For hybridization with specific probes, the PCR product (the amplified V6 region) was denatured by the addition of 100 μl of 0.5 M NaOH and then transferred to GeneScreen Plus membranes (DuPont, Boston, Mass.) by using a dot blot manifold (Minifold; Schleicher & Schuell, Inc., Keene, N.H.). After being blotted, membranes were neutralized in 0.5 M Tris-HCl (pH 8.0) and air dried. Prehybridization and hybridization were performed with 0.5 M sodium phosphate buffer (pH 7.2) containing 3% SDS and 1% bovine serum albumin. After 30 min of prehybridization at 55°C, the probe that had been 5′ end labelled with [γ-^{32}P]ATP (Amersham, Buckinghamshire, United Kingdom) was added, and incubation continued for 4 h. Blots were washed with 0.3 M NaCl and 0.03 M sodium citrate at 60°C until a clear signal was found and were then exposed to Kodak X-ray films.

RESULTS

Cheesemaking experiments. The strains used for contamination of experimental cheeses are listed in Table 1. None of the control cheeses showed any signs of late blowing after 12 weeks. During these experiments, it appeared that spores cultivated from pure cultures were less successful in provoking late blowing than were spores present in milk from natural contamination. Therefore, several cheese productions were carried out for a number of strains and large infective doses (1,000 spores per ml of cheese milk) were applied. The presence of late blowing was determined both by visual inspection (Fig. 1) and by analysis of butyric acid contents (more than 100 mg/kg of 12-week-old cheese). For all cheesemaking experiments, 28 of the 32 *C. tyrobutyricum* strains tested were shown to cause butyric acid fermentation in Gouda cheese. *C. tyrobutyricum* NIZO FL104, NIZO S46, EFAM 1527, and CNRZ 611 did not cause butyric acid fermentation in experimental cheeses. In parallel experiments with spores of 35 strains of *C. beijerinckii*, *C. butyricum*, and *C. sporogenes*, late blowing was never detected. Only one strain of *C. butyricum* was tested,

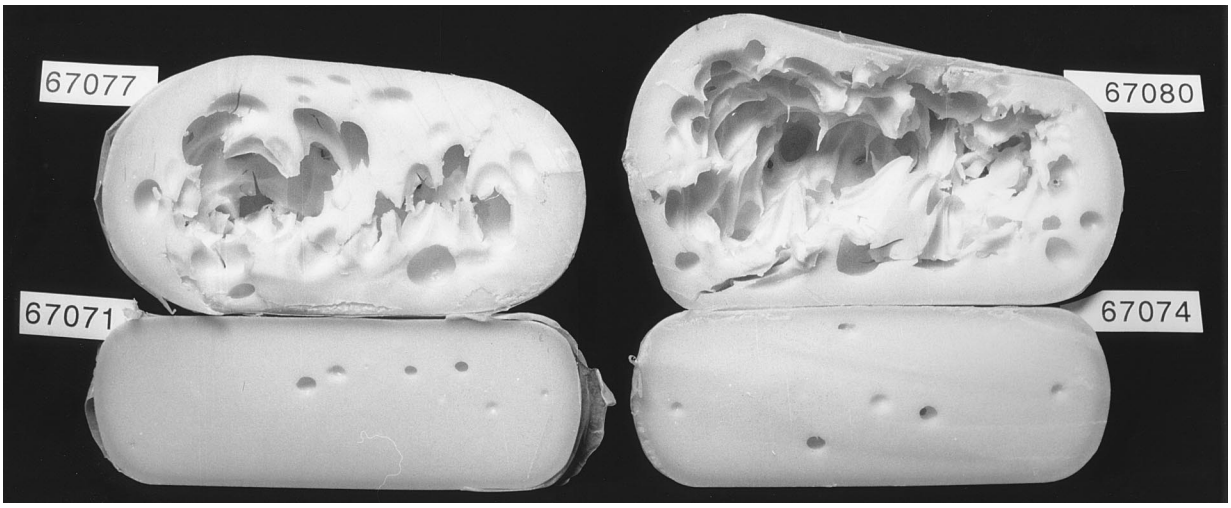

FIG. 1. Cheeses with (top) and without (bottom) butyric acid fermentation. Cheeses 67077 and 67080 were made of cheese milk contaminated with spores of *C. tyrobutyricum* NIZO 51 and ATCC 25755^T. Cheeses 67071 and 67074 were controls.

since it was not possible to obtain sufficient spores from the other *C. butyricum* strains in our collection.

PCR amplification and specific hybridization. Extraction from cheese of DNA that can be successfully used in PCR amplification is reported to be difficult (18, 19). The procedure developed here yielded relatively small amounts of DNA that were often not visible on agarose gels stained with ethidium bromide, especially from older cheeses. However, by using high magnesium concentrations in PCR mixtures together with the optimization of the dilution series, positive results were obtained in PCR amplifications.

The DNAs isolated from six experimental cheeses were used in PCR amplification of the V6 area of the 16S rRNA gene with general primers P3 and P4 (based on the conserved sequences flanking the V6 region) (Table 2). The reaction product was blotted on a nylon membrane and hybridized with P3 (as a control for PCR efficiency) and the species-specific DNA probes for *C. tyrobutyricum* and *C. sporogenes* (Table 2) (9). Specific signals were obtained with the *C. tyrobutyricum*-specific DNA probe for cheeses made with milk inoculated with *C. tyrobutyricum* spores and showing symptoms of late blowing (Table 3), as indicated by high levels of butyric acid content and excessive holes formed in the cheese matrix. No specific signals were found for control cheeses, cheeses not showing symptoms of late blowing although they were made of milk contaminated with spores of *C. tyrobutyricum* NIZO BZ18, or cheeses made of milk contaminated with spores of *C. sporogenes* IVVO V27. Amplified DNA from the last cheese also showed no signal when the *C. sporogenes*-specific DNA probe was used. To increase the sensitivity of this method, we developed a method involving nested PCR of isolated DNA.

Nested PCR. In the first step of nested PCR, part of the 16S rRNA gene (nucleotides 40 to 1114 [conforming to the *Escherichia coli* nomenclature]) was amplified with primers P1 and P2. The efficiency of this PCR amplification was checked on an agarose gel stained with ethidium bromide (Fig. 2A). After the first step was found to be successful, the second amplification was performed on the diluted product of the first step with a specific primer and P5 (Table 3). The specificities of the primers used in PCRs were validated with DNAs isolated from pure cultures of the different species used in this study (Fig. 2B to F). The products resulting from the second amplification step were analyzed on an agarose gel stained with ethidium bromide. This step can result in several amplification products because of the carryover of primers from the first PCR step. However, the concentration is relatively low, compared with that of the specific primer and P5 (15 ng/μl), as a result of incorporation into the product formed in the first PCR step and dilution between the two PCR steps. The largest fragment (1,070 bp) is the amplification product of P1 and P2 (the same product that is formed in the first PCR step), the next largest (750 bp) is the amplification product of P2 and P5, and the

TABLE 2. Primers used in this study

Primer and specificity (sense)[a]	Sequence	Annealing temp (°C)	*E. coli* numbering	Location
P1 (S)	GCGGCGTGCCTAATACATGC	55	41–60	Conserved region
P2 (A)	GGGTTGCGCTCGTTGCGGGA	55	1094–1114	Conserved region
P3 (S)	GCAACGCGAAGAACCTTACC	55	966–985	Conserved region
P4 (A)	ATCTCACGACACGAGCTGAC	55	1064–1083	Conserved region
P5 (S)	GGAATCTTCCACAATGGGCG	55–72	361–380	Conserved region
Pac, *C. acetobutylicum* (A)	GGACTTCATCCATTACGGACTAAC	63	≈1000–1050	V6 region
Pbe, *C. beijerinckii* (A)	CTTCCCCGATTAAGGGTAATTCAG	72	≈1000–1050	V6 region
Pbu, *C. butyricum* (A)	GTGGCTTGCTCCATTACAGAGTAA	72	≈1000–1050	V6 region
Pty, *C. tyrobutyricum* (A)	CGCCTATCTCTAGGTTATTCAG	63	≈1000–1050	V6 region
Psp, *C. sporogenes* (A)	CACCTATCTCTAGGCTATGCAA	63	≈1000–1050	V6 region

[a] S, sense; A, antisense.

TABLE 3. Comparison of detection methods for *C. tyrobutyricum* in cheese (amplification of the V6 region followed by hybridization with a specific DNA probe and nested PCR)

Inoculation[a]	Concn of butyric acid (mg/kg)[b]	Reaction result[c]				
		Amplification of V6 region	Hybridization		Nested PCR	
			C. tyrobutyricum[d]	*C. sporogenes*[e]	*C. tyrobutyricum*	*C. sporogenes*
No spores		+	−	−	−	−
C. tyrobutyricum NIZO BZ6	264	+	+	−	+	−
C. tyrobutyricum NIZO BZ6	953	+	++	−	+	−
C. tyrobutyricum NIZO BZ6	1,167	+	++	−	+	−
C. sporogenes IVVO V27	36	+	−	+	−	±
C. tyrobutyricum NIZO BZ18	66	+	−	−	+	−

[a] 1,000 spores per ml were added to the cheese milk as indicated.
[b] A concentration of >100 mg/kg is considered indicative of late blowing.
[c] ++, strongly positive reaction; +, positive reaction; ±, weakly positive reaction; −, no reaction.
[d] Hybridization probe, 5′-CCCCTGAATAACCTAGAGATAGGCG-3′.
[e] Hybridization probe, 5′-CCCTTGCATAGCCTAGAGATAAGG-3′.

smallest fragment (660 bp) is the amplification product of the specific primer and P5 (Fig. 2).

Specific detection of clostridia in cheese by nested PCR. The same cheeses used for direct hybridization of PCR products were analyzed by nested PCR (Table 3). This technique yielded positive reactions with the *C. tyrobutyricum*-specific primer in all cheeses contaminated with *C. tyrobutyricum* spores. In addition, nested PCR with the *C. sporogenes*-specific primer resulted in a specific signal in the cheese contaminated with *C. sporogenes* IVVO V27 spores, which had been negative by direct hybridization of the amplified V6 region from the 16S rRNA gene. To get an estimate of the relative sensitivity of this method, some cheeses contaminated with *C. tyrobutyricum* spores were monitored over time. After 3 weeks, clear signals were found by species-specific nested PCR (Table 4). At the same time, elevated concentrations of butyric acid were detected in these cheeses. The experiment showed that detection by nested PCR requires at least the germination and possibly outgrowth of spores, since no signals were found in cheeses during the first 2 weeks.

All experimental cheeses which showed no significant symptoms of late blowing were analyzed by species-specific nested PCR. In most cheeses inoculated with *C. tyrobutyricum*, weak signals were found, indicating that either the germination of spores or the growth of cells was retarded. In some cheeses contaminated with *C. beijerinckii*, *C. butyricum*, and *C. sporogenes*, clear signals were found by homologous species-specific nested PCR.

Nested PCR was subsequently used to analyze 23 cheeses from commercial sources (with and without late blowing symptoms) with primers specific for *C. acetobutylicum*, *C. beijerinckii*, *C. butyricum*, *C. tyrobutyricum*, and *C. sporogenes*. In the 11 commercial cheeses that did not show any symptoms of late blowing, no signal was obtained with any specific primer by species-specific nested PCR. In all commercial cheeses with

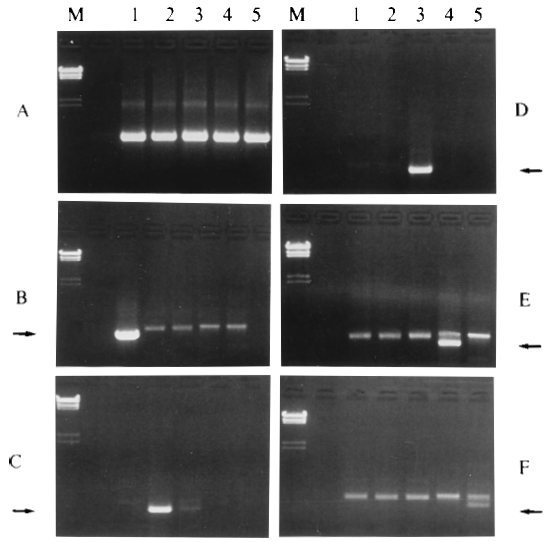

FIG. 2. Specificities of the species-specific primers used in nested PCR. DNAs extracted from pure cultures of the five type strains *C. acetobutylicum* LMG 5710 (lanes 1), *C. beijerinckii* ATCC 25752 (lanes 2), *C. butyricum* ATCC 19398 (lanes 3), *C. tyrobutyricum* ATCC 25755 (lanes 4), and *C. sporogenes* ATCC 3584 (lanes 5) were used in this nested PCR to demonstrate the specificities of the primers listed in Table 2. Lanes M, lambda *Hin*dIII. Shown are agarose (1.5%) gels stained with ethidium bromide and containing the products of PCR amplifications with primers P1 and P2 (A), Pac and P5 (B), Pbe and P5 (C), Pbu and P5 (D), Pty and P5 (E), and Psp and P5 (F). (B to F) Products of second PCR step. Arrows indicate the specific product (about 660 bp).

TABLE 4. Specific detection by nested PCR with the *C. tyrobutyricum*-specific primer in cheeses made of milk inoculated with *C. tyrobutyricum* NIZO 51[a]

Age of cheese (wk)	Concn of butyric acid (mg/kg)	Result	
		Visual inspection[b]	Specific PCR[c]
1	ND[d]	−	−
2	ND	−	−
3	267	+	+
4	677	+	+
5	970	+	+
6	969	+	+
7	956	++	+
8	1,226	++	+
9	1,893	++	+
10	1,676	++	+
11	1,841	++	+
6 (control)	ND	−	−
11 (control)	ND	−	−

[a] 1,000 spores per ml.
[b] −, no holes; +, isolated holes; ++, excessive hole formation and colonies present.
[c] −, negative reaction; +, positive reaction; ++, strongly positive reaction.
[d] ND, not detected (detection limit is about 50 mg/kg).

TABLE 5. Specific detection by nested PCR of five clostridial species in cheeses from commercial sources suspected of butyric acid fermentation[a]

Cheese code	Concn of butyric acid (mg/kg)	Reaction result[b]					
		General PCR	Specific PCR for:				
			C. tyrobutyricum	*C. sporogenes*	*C. butyricum*	*C. beijerinckii*	*C. acetobutylicum*
1	463	+	+	−	−	−	−
2	179	+	+	−	−	−	−
3	1,790	+	++	−	−	−	−
4	624	+	+	−	−	±	−
5	189	+	++	−	−	+	−
6	ND[c]	+	±	−	−	−	−
7	ND	+	±	−	−	−	−
8	109	+	+	−	−	−	−
9	96	+	±	−	−	−	−
10	119	+	+	−	−	±	−
11	195	+	+	−	−	±	−
12	307	+	+	−	−	−	−

[a] Eleven cheeses without symptoms of late blowing were negative in all specific PCR assays.
[b] ++, strongly positive; +, positive; ±, weakly positive; −, negative.
[c] ND, not detected (detection limit is about 50 mg/kg).

obvious gas production (holes in the cheese matrix) and elevated concentrations of butyric acid, specific nested PCR signals for *C. tyrobutyricum* were found, but some cheeses yielded an additional signal for *C. beijerinckii* (Table 5).

DISCUSSION

Extraction of DNA from cheese and other food matrices for subsequent use in PCRs is often considered to be a problem (16, 23, 24). Rossen et al. (16) have shown that cheese matrices, various food substances, and chemicals used to extract DNA from foodstuffs can inhibit PCR. Our work has shown that DNA extracted from cheese can be successfully used as template DNA in PCR. Potential problems were overcome by increasing the magnesium concentration in the PCR mix and optimizing the dilution of the cheese extract (generally 25- to 50-fold). Another method involving a two-phase extraction procedure to eliminate inhibitory substances from cheese extracts has recently been described by Lantz et al. (11).

A nested two-step PCR method was successfully developed to detect the presence of clostridial DNA in cheese. To obtain optimal performance in this nested PCR, previously described primers for *C. tyrobutyricum*, *C. sporogenes*, *C. butyricum*, and *C. beijerinckii* (9) had to be redesigned because the specificity of a PCR primer depends largely on the terminal bases at the 3′ end (7). These primers proved to be reliable in specific identifications of these various species (Fig. 2).

The procedure developed, which involves the extraction of DNA from cheese followed by nested PCR with specific primers, allowed the detection of *C. tyrobutyricum* in cheeses at adequate levels, since a clear signal was found in all cheeses with obvious symptoms of late blowing. The results of experiments in which the occurrence of specific DNA sequences in cheeses was monitored over time suggest that DNA is not extracted from spores and that germination and possibly outgrowth of cells are required to obtain a signal. Recently, a method of bead-bead extraction was reported to allow extraction of DNA from *Bacillus anthracis* spores (8). Adaptation of such a method to clostridial spores may provide additional information.

Our results show that with pure isolates, only *C. tyrobutyricum* is able to provoke late blowing; this species is present in all naturally contaminated cheeses that show this defect. This strongly suggests that the presence of *C. tyrobutyricum* is a prerequisite for the occurrence of butyric acid fermentation in cheese. However, it appeared that in cheeses artificially contaminated with cultivated spores of this species, late blowing was not always easily provoked. This could have been due to impaired germination of cultivated spores or to a decrease in the ability of germinated spores to form butyric acid and hydrogen gas, compared with that of natural milk contaminants.

In some experimental cheeses contaminated with spores of *C. sporogenes* or *C. beijerinckii*, specific signals were obtained by nested PCR, and signals were also found for *C. beijerinckii* in some commercial cheeses with late blowing. This shows that these clostridia are at least able to germinate and possibly to grow in cheese. Nevertheless, even if they do grow, these species apparently do not produce hydrogen gas and butyric acid under these conditions, even though they have the ability to do so in various culture media (19). Most anaerobic spores found in raw milk belong to the species *C. beijerinckii* (6). Since commercial cheeses with late blowing are obviously made of cheese milk with a high level of natural contamination of spores, the presence of *C. beijerinckii* can be expected.

To improve currently used methods for routine detection of clostridial spores in milk, the causative agent of late blowing in cheese had to be identified. This study unequivocally proves the causal relationship between *C. tyrobutyricum* and butyric acid fermentation in cheese and rules out the involvement of other dairy-related clostridial species. Currently, PCR amplification is not a technique that can be implemented as a routine detection method in the cheese industry. However, the results of this study indicate that a routine molecular detection method for *C. tyrobutyricum* spores in cheese milk with a species-specific DNA probe would be of great value to the dairy industry to avoid considerable losses during cheese production.

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