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A Direct PCR Detection Method for *Clostridium tyrobutyricum* Spores in up to 100 Milliliters of Raw Milk

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A direct detection method for *Clostridium tyrobutyricum* spores in up to 100 ml of raw milk is presented. The bacterial spores are concentrated by centrifugation after chemical extraction of the milk components. The vegetative cells are selectively lysed, and their DNA is digested and washed away. Afterwards, the DNA is liberated from the spores by microwave treatment. For the identification of the *C. tyrobutyricum* DNA, a two-step PCR method with two nested pairs of primers is used. The primers were derived from the 16S-23S rRNA spacer region of *C. tyrobutyricum*, and the specificity of each of them for *C. tyrobutyricum* is demonstrated. The detection limit can be estimated to be between 3 and 30 spores in 100 ml of raw milk.

Clostridium tyrobutyricum is a gram-positive, spore-forming, anaerobic bacterium which is considered to be the principal organism responsible for the late spoilage of brine-salted semi-hard and hard cheeses (e.g., Gouda, Edam, or Emmental cheese) by gas formation. The number of spores which causes the defect varies according to the production parameters from five (with no bactofugation or nitrate addition) to 10^6 (with bactofugation and nitrate addition) spores per liter of cheese milk (15, 18). Because the causative organism is a sporeformer, late blowing is difficult to control at the production level. A possible way to deal with the problem is to screen the cheese milk before production by determining the *C. tyrobutyricum* spore content.

Most methods for detecting spores of *C. tyrobutyricum* are based on spore germination and vegetative growth. The commonly used most-probable-number method takes 4 to 7 days (16) and needs an identification step to confirm the presence of *C. tyrobutyricum* (13).

An alternative method is based on membrane filtration of the milk followed by growing of the bacteria on the membrane (1). The identity of *C. tyrobutyricum* may be confirmed by immunological methods (12) or by a DNA hybridization method (13).

Direct detection of the spores instead of the vegetative cells may save the time used for germination and growth. A direct detection method is also independent of the germination efficiency, which may be variable. A direct immunodetection assay for *C. tyrobutyricum* spores after membrane filtration of the milk was described previously (4).

In this paper we describe a direct detection method, based on PCR, for *C. tyrobutyricum* spores in raw milk. A direct PCR detection method for vegetative bacterial cells in raw milk was successfully used for the detection of *Listeria monocytogenes* (8). The use of PCR on DNA from spores of *Bacillus anthracis* disrupted by glass or zirconia beads has been reported by Johns et al. (10) and Reif et al. (14).

In the method presented here, the *C. tyrobutyricum* spores are concentrated from the raw milk after a chemical extraction of the milk components. The vegetative bacterial cells are selectively lysed, and their DNA is digested and removed. The genomic DNA is liberated from the spores by microwave treatment and used as the template for the PCR.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used are described in Table 1. *Clostridium* spp. were anaerobically incubated at 37°C for 2 days in reinforced clostridial medium from Oxoid (Hampshire, United Kingdom). *Listeria* spp., *Bacillus* spp., *Escherichia coli*, and yogurt cultures (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophilus*) were grown for 2 days in brain heart infusion (Oxoid) at 37°C; *Hafnia halvei* was grown in Bacto Peptone (Oxoid) at 37°C, *Brochotrix* spp. were grown in nutrient broth (Oxoid) at 28°C, *Lactococcus lactis* and *Enterococcus faecalis* were grown in De Man, Rogosa, Sharpe (Oxoid) at 37°C, *Agrobacterium tumefaciens* was grown in yeast extract broth (19) at 28°C with aeration, and *Brucella* spp. were swabbed from a tryptone soya broth (Oxoid) agar plate, where they had been grown at 37°C.

DNA preparation. Pure bacterial cultures were centrifuged for 2 min at 13,000 $\times g$ to collect the bacterial cells. Bacterial chromosomal DNA was extracted by the method of Flamm et al. (7), and the concentration was determined spectophotometrically. Ten nanograms of DNA was used as the template in the PCR. Crude bacterial cell lysates were prepared by adding 100 μl of 0.05 M NaOH and 0.125% sodium dodecyl sulfate to the bacterial pellet and subsequently heating for 17 min at 90°C. One microliter of crude cell lysate was added as the template in the PCR.

Spore induction and preparation. Spores were prepared by inoculating 20 ml of a 20-h-grown *C. tyrobutyricum* culture in 100 ml of tryptone-glucose-yeast extract broth in a dialysis sac and culturing this anaerobically in 900 ml of tryptone-glucose-yeast extract broth for at least 4 days at 37°C (5, 6). The vegetative cells were killed by heating for 10 min at 74°C and were digested by incubation with 30 mg of lysozyme (Boehringer GmbH, Mannheim, Germany) and 2 mg of DNase I (Boehringer) in 0.067 M phosphate buffer (pH 7)–15 mM $MgCl_2$ for 1 h at 37°C. Afterwards, 10 mg of trypsin was added and the incubation was continued for 90 min. The suspension was heated for 10 min at 90°C in order to destroy all DNase activity. The spores were concentrated by centrifugation at 12,000 $\times g$ and 4°C for 30 min, washed three times with sterile H_2O, and stored at 4°C in sterile H_2O (6). Microscopically, no intact cells were visible. The spore concentration was determined microscopically (in a Bürker counting cell) to be 3.8×10^8/ml. The spore concentration was also determined by a most-probable-number method, FNZ (16), after an incubation time of 7 days to be 2.3×10^8/ml. For further calculations, an average concentration of 3.0×10^8/ml was assumed.

Concentration and processing of *C. tyrobutyricum* spores. Volumes of 1, 10, and 100 ml of raw milk were artificially contaminated with 10 μl of 10-fold dilutions of the *C. tyrobutyricum* spore preparation, which had an estimated concentration of 3.0×10^8 spores per ml. Milk components were extracted by a two-step chemical extraction procedure essentially as described by Herman et al. (8). Because of the safety risk from the reagents used (NH_3, diethyl ether, and petroleum ether), working in a fume cupboard with protective gloves is recommended. The vegetative bacterial cells were selectively lysed in an ultrasonic bath at 60°C for 15 min in 120 μl of 2 M NaOH. The vegetative DNA was digested by incubation with 5 to 10 U of DNase I (fast protein liquid chromatography-purified DNase I from bovine pancreas; Pharmacia Biotech Inc., Piscataway, N.J.) for 15 min at 37°C. The DNase I was inactivated by heating it at 100°C for

TABLE 1. Bacterial strains and PCR results

Species	Strain	Source[a]	PCR result with[b]:			
			Ct1F-Ct1R		Ct2F-Ct2R	
			DNA	Crude cell lysate	DNA	Crude cell lysate
C. tyrobutyricum	1285^T	LMG	+		+	
	1520	RZS	+		+	
	1715	RZS	+		+	
	MB 102	RZS	+		+	
	MB 103	RZS	+		+	
	510	INRA	+		+	
	596	INRA	+		+	
	566	INRA	+		+	
	573	INRA	+		+	
	608	INRA	+		+	
	500	INRA	+		+	
	526	INRA	+		+	
	562	INRA	+		+	
	607	INRA	+		+	
	613	INRA	+		+	
	620	INRA	+		+	
	515	INRA	+		+	
	523	INRA	+		+	
	587	INRA	+		+	
	609	INRA	+		+	
Clostridium butyricum	1217^T	LMG	–		–	
	1218	LMG	–		–	
	1219	LMG	–		–	
C. acetobutylicum	5710^T	LMG	–		–	
	5711 (t1)	LMG	–		–	
	5711 (t2)	LMG	–		–	
	5712	LMG	–		–	
	5715	LMG	–		–	
Clostridium beijrinckii	528 (t1)	INRA	–		–	
	533 (t1)	INRA	–		–	
	556	INRA	–		–	
	528 (t2)	INRA	–		–	
	533 (t2)	INRA	–		–	
	5716^T	LMG	–		–	
	5717	LMG	–		–	
C. pasteurianum	3285^T	LMG	–		–	
	6655	LMG	–		–	
C. perfringens	11264^T	LMG	–		–	
	10468	LMG	–		–	
	12224	LMG	–		–	
C. sporogenes	8421^T	LMG	–		–	
	126	INRA	–		–	
	692	INRA	–		–	
C. tetanomorphum	4474	DSM	–		–	
	695	INRA	–		–	
Clostridium bifermentans	3029	LMG	–		–	
Clostridium thermosaccharolyticum	2811^T	LMG	–		–	
	6659^T	LMG	–		–	
Clostridium sphenoides	10390	LMG		–		–
Clostridium novyi	12322	LMG		–		–
Clostridium carnis	1293^T	DSM		–		–
A. tumefaciens	$C_{58}C_1$Rif	RUG	–		–	
Rhizobium loti	4284	LMG	–		–	
Phyllobacterium rubiacearum	1 (t1)	LMG	–		–	
Ochrobactrum anthropi	3331	LMG	–		–	
Bradyrhizobium japonicum	4252	LMG	–		–	
Brucella ovis	1051	NCTC	–		–	
Brucella neotomae	10084	NCTC	–		–	
Brucella melitensis	1	NIDO	–		–	
Brucella abortus	TULYA	NIDO	–		–	
L. monocytogenes	MB	RZS	–		–	
Listeria seeligeri	100.000	CIP	–		–	
Listeria ivanovii	78.42	CIP	–		–	
H. halvei	711 (LAC+) E7	INRA	–		–	
E. coli	MC1061	RUG	–		–	

Continued on following page

TABLE 1—*Continued*

Species	Strain	Source[a]	PCR result with[b]:			
			Ct1F-Ct1R		Ct2F-Ct2R	
			DNA	Crude cell lysate	DNA	Crude cell lysate
L. lactis	MRZ 1076-E11	INRA	–		–	
E. faecalis	EF S1-E12	INRA	–		–	
Enterococcus faecium	EFMI-E22	RZS	–		–	
Bacillus cereus	S1	RZS	–		–	
Bacillus megantum	S11	RZS		–		–
Bacillus brevis	S12	RZS	–		–	
Bacillus coagulans	S13	RZS	–		–	
Bacillus pumilus	S19	RZS	–		–	
Bacillus subtilis	S20	RZS	–		–	
Bacillus macerans	S21	RZS	–		–	
RR yogurt starter culture (*S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*)			–		–	
IST yogurt starter culture (*S. salivarius* subsp. *thermophilus* and *L. delbrueckii* subsp. *bulgaricus*)			–		–	

[a] CIP, Collection Nationale de Cultures de Micro-organismes de l'Institut Pasteur, Paris, France; NCTC, National Collection of Type Cultures, London, United Kingdom; RUG, Laboratory of Genetics of the University of Ghent, Ghent, Belgium; NIDO, National Institute of Veterinary Research, Brussels, Belgium.
[b] PCR was performed with each of the two primer pairs (Ct1F-Ct1R or Ct2F-CT2R) on DNA or crude cell lysate.

10 min and washing the spores two times with 2 ml of sterile H_2O. The spores were opened by intensive microwave treatment (700 W), three times for 5 min each. The liberated DNA was precipitated and dissolved in 40 μl of H_2O. Five microliters of this solution was used as the template in the first PCR.

PCR amplification. A two-step PCR amplification procedure with two nested pairs of primers specific for *C. tyrobutyricum* was applied. Specific primers were deduced from the 16S-23S rRNA spacer region of *C. tyrobutyricum* (CTRGA DG; GenBank accession number L08062). In the first PCR the primers Ct1F (5′ AACTGAAACAGCATGACT 3′), located at positions 1987 to 2004, and Ct1R (5′ GCTTGACCTTTATCTACA 3′), located at positions 2207 to 2224, were used as forward and reverse primers, respectively. As nested primers, Ct2F (5′ GTTCGGTTTATTTTACTCTG 3′), located at positions 2017 to 2036, and Ct2R (5′ CTTAGCTGTATCTAGTATAC 3′), located at positions 2175 to 2194, were used.

PCR was performed in a final volume of 50 μl containing 5 μl of 10× PCR buffer (200 mM Tris [pH 8.3], 15 mM $MgCl_2$, 500 mM KCl), 5 μl of deoxynucleoside triphosphate mix (dGTP, dTTP, dATP, and dCTP, each at 2 mM), 0.7 U of Goldstar DNA polymerase (Eurogentec s.a., Seraing, Belgium), and 0.5 μg of each appropriate primer (1 μg/μl). For the nested PCR, 5 μl of product from the first PCR was used as the template. PCR was performed in a PCR 9600 thermal cycler (Perkin-Elmer Corporation, Branchburg, N.J.). The first and nested PCRs were performed as follows: denaturation at 95°C for 1 min for the first cycle and then at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 30 s for 30 cycles, and extension at 72°C for 8 min. Eight microliters of the PCR products was analyzed on a 1.5% Seakem ME agarose gel (FMC BioProducts, Rockland, Maine) by a standard protocol (11).

DNA sequencing. The 16S-23S rRNA spacer regions were manually sequenced after PCR amplification with primers chosen from the conserved regions of the 16S and 23S rRNA sequences. One of these primers was labeled with biotin. Magnetic solid phase DNA sequencing with Dynabeads M-280 streptavidin was used (9). The dideoxy sequencing reactions were performed with a deaza-T7 sequencing kit (Pharmacia Biotech) with ^{35}S-dATP as the labeled nucleotide. The spacer regions of *C. tyrobutyricum* 510 (Institut Nationale de Recherche Laitières [INRA], Paris-Grignon, France), *C. tyrobutyricum* 1285^{T} (Culture Collection of the Laboratory of Microbiology [LMG], Ghent, Belgium), *C. tyrobutyricum* MB 102 (Government Dairy Research Station [RZS], Melle, Belgium), *Clostridium pasteurianum* 3285 (LMG), *Clostridium acetobutylicum* 5715 (LMG), *Clostridium sporogenes* 8421 (LMG), *Clostridium tetanomorphum* 4474 (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSM], Braunschweig, Germany), and *C. tetanomorphum* 695 (INRA) were sequenced. These sequences were manually aligned together with the sequences of *Clostridium difficile* 9689 American Type Culture Collection [ATCC] (3) and *Clostridium perfringens* 13124 (ATCC) (CL016 SRRNB; GenBank accession number M69264).

RESULTS

Specific identification of *C. tyrobutyricum* by PCR. On the basis of the sequence alignment of the 16S-23S rRNA spacer regions of different *Clostridium* spp., two forward primers (Ct1F and Ct2F) and two reverse primers (Ct1R and Ct2R) were chosen. The sequence alignment of these primers is shown in Fig. 1. No differences between the three *C. tyrobutyricum* species were found. The sequences align perfectly with the NIZO 51 strain (GenBank accession number L08062) as well. The primer pairs Ct1F-Ct1R and Ct2F-Ct2R amplify fragments of 233 and 173 bp, respectively, which is in accordance with the sizes predicted from the sequence data for the 16S-23S rRNA spacer region. Because each fragment is the correct size and because in a two-step PCR the second fragment is amplified with the first fragment as a target, it may be assumed that the amplified fragments belong to the spacer region.

The primer pairs Ct1F-Ct1R and Ct2F-Ct2R were tested for specific identification of *C. tyrobutyricum* (Table 1). All 20 *C. tyrobutyricum* strains reacted positively with both primer pairs. The other *Clostridium* spp. and other bacteria which may be found in raw milk reacted negatively.

Detection of *C. tyrobutyricum* spores in raw milk. *C. tyrobutyricum* spores were concentrated by centrifugation from 1, 10, and 100 ml of raw milk after chemical extraction of the milk fats and proteins. The efficiency of concentration was microscopically analyzed by counting the spores in a Bürker counting cell. The efficiency was estimated to be 90%.

Before liberation of the DNA from the spores, the vegetative bacterial cells were lysed by treatment in an ultrasonic bath. The complete lysis of the vegetative cells was microscopically estimated to be 95%.

The DNA liberated from the vegetative cells was digested with DNase I. The DNA from vegetative cells has to be removed because it may interfere with the sensitivity of the method (data not shown). Small amounts of nondigested DNA were removed by two washes with 2 ml of H_2O. The efficiency of the removal of vegetative DNA was tested with six 10-fold dilutions (1 to 10^5) of *C. tyrobutyricum* bacterial cells that were counted on reinforced clostridial medium. After lysis, DNA digestion, and washing, the solution was filtered through a 0.22-μm-pore-size filter (Millipore type GV) in order to remove the spores. As a control the same 10-fold dilutions were

Ct1F

```
C. tyrobutyricum 1     AA CT GAAA CAGCAT   G       ACT
                       || || |||  ||||||   |       |||
C. tyrobutyricum 2     AA CT GAAN CAGCAT   G       ACT
                       || || |||  ||||||   |       |||
C. tyrobutyricum 3     AA CT GAAA CAGCAT   G       ACT
                       || || | ||  |||||   |       |||
C. pasteurianum            T GTAATT GCAT   GCAGA TAACT
                       ||  | | |||| | |    | ||| | ||
C. sporogenes          AA  TAGAAA          GAAGA
                       ||  ||||||          |||||
C. difficile           AA  TAGAAA          GAAGA
                       ||  || |  |||| |     |||| | ||
C. perfringens          A  TACATCTTAGGACAACTAAGA TGACA
                        |  || |  |||| |     | |  |||||
C. tetanomorphum           TAG   TTAGTATC
                       || |||||    || ||    | |  |||||
C. acetobutylicum      AATCTAGAT  CAGCAT   GATGTCTGACA
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Ct1R

```
C. tyrobutyricum 1     TGT AGA             T A          AAGGTCAAGC
                       ||| |||             | |          |||||||||
C. tyrobutyricum 2     TGT AGA             T A          AAGGTCAA
                       ||| |||             | |          |||||||||
C. tyrobutyricum 3     TGT AGA             T A          AAGGTCAAG
                        || |||             | |          |||||||||
C. pasteurianum        A  AAGAG            TNAC         AAGGTCAAGC
                        ||||||             | ||         ||||||||||
C. sporogenes          TGTAAGAA            T AC AATTTT  AAGGTCAAGC
                       ||||||||            | || |||||   ||||||||||
C. difficile           TGTAAGAA            T  C AATTT   AAGGTCAAGC
                       ||||| ||            | || ||| |   ||||||||||
C. perfringens         TGTAA AATGAGAA CT ATAACTAATAT     AGGTCAAGC
                       |||||   || ||| || ||||   ||| |   ||| ||||||
C. tetanomorphum        GTAA    TGCGAAGCTTATAAG AATTTNTAAAGATCAAGC
                        |||                              || ||||
C. acetobutylicum                                         AGGTCAA
                       ||||
C. butyricum           TGTATATCAAAACT
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Ct2F

```
C. tyrobutyricum 1     G  TTC    GGTTTATTTTA        CTCTG
                       |  |||    |||| ||||||        |||||
C. tyrobutyricum 2     G  TTC    GGTTNATTTTA        CTCTG
                       |  |||    |||| ||||||        |||||
C. tyrobutyricum 3     G  TTC    GGTTTATTTTA        CTCTG
                       |  |||    || | |   |||       |||||
C. pasteurianum        GGTTTC             TTACTCTTGTCTCTG
                         ||||    || | |   |||||    ||||||
C. sporogenes          C TTTCTAAAGGCTGAA T         TCTCTG
                       | ||||||||||||||| |         ||||||
C. difficile           C TTTCTAAAGGCTGAA T         TCTCTG
                       | |    ||||| | || |||       ||||||
C. perfringens         C T       GGAT AA  TA       TCTCTG
                                      ||  || ||    ||||||
C. tetanomorphum                          TT CTGAGTTCTCTG
                              |||     ||   |||| |||  ||||
C. acetobutylicum      T  AGACAAATAC AAA  TACTi GT  TCTG

                       i- TGCACAAAGGTTACTTATGTATTT
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Ct2R

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C. tyrobutyricum 1     G TAT ACTAGATACAG CTAA G
                       | ||| ||||||||||| |||| |
C. tyrobutyricum 2     G TAT ACTAGATACAG CTAA G
                       | ||| ||||||||||| |||| |
C. tyrobutyricum 3     G TAT ACTAGATACAG CTAA G
                         ||  || ||||| || |||| |
C. pasteurianum        NTTAAAACAA ATAAAGACTAA G
                         ||||     ||||||||||| |
C. sporogenes            TAAAG    TAAAG CTAAAG
                         |||||    ||||| ||||||
C. difficile             TAAAG    TAAAG CTAAAG
                         |||||    |||||  ||| |
C. perfringens                           TA
                         |||||    |||||  ||
C. tetanomorphum         TAAAG    TAAAG
                                 ||||  || ||| |
C. acetobutylicum              TGTGATAT GA TAATG
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FIG. 1. Multiple sequence alignment at the primer positions. Ct1F, forward primer of the first PCR (positions 1987 to 2004 of the *C. tyrobutyricum* 16S-23S rRNA spacer region; GenBank accession number L08062); Ct1R, reverse primer of the first PCR (positions 2207 to 2224); Ct2F, forward primer of the nested PCR (positions 2017 to 2036); Ct2R, reverse primer of the nested PCR (positions 2175 to 2194). Vertical lines indicate matching sequences, with blank regions left horizontally to fit the alignment. *C. tyrobutyricum* 1, *C. tyrobutyricum* 510 (INRA); *C. tyrobutyricum* 2, *C. tyrobutyricum* MB 102 (RZS); *C. tyrobutyricum* 3, *C. tyrobutyricum* 1285^{T} (LMG); *C. pasteurianum*, *C. pasteurianum* 3285^{T} (LMG); *C. sporogenes*, *C. sporogenes* 8421^{T} (LMG); *C. difficile*, *C. difficile* 9689 (ATCC) (3); *C. perfingens*, *C. perfingens* 13124 (ATCC) (3); *C. tetanomorphum*, *C. tetanomorphum* 4474^{T} (DSM); *C. acetobutylicum*, *C. acetobutylicum* 5715 (LMG).

lysed and filtered without DNase treatment. In the samples in which the DNA was digested and washed away, no amplification was obtained with the two-step PCR, while for all of the control samples, amplification occurred.

Genomic DNA was liberated from the concentrated spores by microwave treatment. The conditions applied caused about 99% lysis of the spores as microscopically estimated by using the Bürker counting cell. The genomic DNA was precipitated and used for a two-step PCR protocol with the primers Ct1F-Ct1R and Ct2F-Ct2R.

The effect of the lysis procedure on the bacterial DNA and the efficiency of precipitation were determined with purified *C. tyrobutyricum* DNA. Either 135, 1.35, 0.135, or 0.0135 ng of DNA was subjected to the lysis and precipitation procedures. These treated DNAs were used together with untreated DNAs at the same concentration as templates in the PCR. Treated and untreated DNAs gave the same results: with 135, 1.35, and 0.135 ng, amplification occurred, and with 0.0135 ng, amplification was hardly seen on the agarose gel.

The detection method was applied with 1-, 10-, and 100-ml raw milk samples artificially contaminated with 10 μl of 10-fold dilutions of the washed and DNase-treated spore suspension, which had a concentration of about 3.0×10^{8} spores per ml. The raw milk was analyzed by the PCR method described above and by the most-probable-number method, FNZ, and was found to be negative for spores of *C. tyrobutyricum*. *C. tyrobutyricum* spores were detected in a 10^{-6} dilution of the raw milk, which corresponds to three spores in 1, 10, and 100 ml (Fig. 2). After the first PCR, only 3.0×10^{5} *C. tyrobutyricum* spores could be detected in 1, 10, and 100 ml of raw milk. The same sensitivity was obtained with milk with background floras of 9.98×10^{2} and 5.0×10^{5} CFU/ml.

To determine the sensitivity of detection for the method, two groups of seven 100-ml samples of raw milk were artificially contaminated with 30 and 3 *C. tyrobutyricum* spores, respectively. For an artificial contamination with 30 *C. tyrobutyricum* spores, seven of seven samples gave a positive result. For the contamination with three *C. tyrobutyricum* spores, only five of seven samples gave a positive result. With $n = 7$, the 90% confidence limits on the positive proportion were 0.53 to 1.00

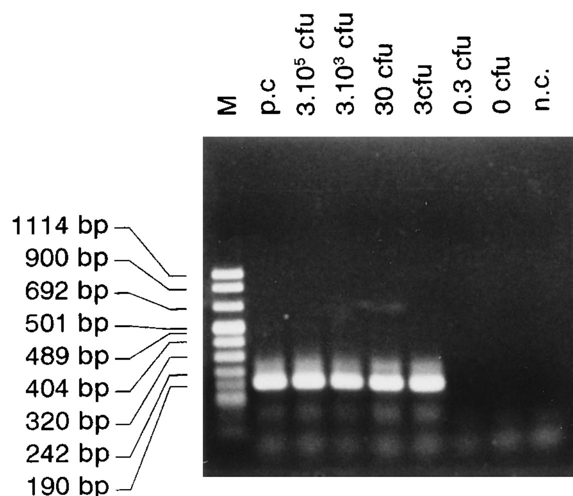

FIG. 2. Detection of *C. tyrobutyricum* spores in 100 ml of raw milk by a two-step PCR with nested primers. One hundred milliliters of raw milk was artificially contaminated with different amounts of *C. tyrobutyricum* spores. p.c., positive control for the two consecutive PCRs; n.c., negative control for the PCR; M, molecular size markers (pUCBM21-*Hpa*I plus pUCBM21-*Dra*I-*Hin*dIII). For a positive control, 100 ng of purified genomic *C. tyrobutyricum* DNA was used.

for the first experiment and 0.25 to 0.96 for the second one. The confidence limits were calculated by using the approximation proposed by Bailey (2). This results in a high probability for detecting *C. tyrobutyricum* if the contamination is 30 spores, while for 3 spores the probability is much lower.

The detection procedure took about 7 h for six samples of 100 ml. When a smaller number of samples or samples of 1 and 10 ml were analyzed, the detection time was reduced to a minimum of about 6 h.

DISCUSSION

The PCR detection method for *C. tyrobutyricum* spores proposed here takes less than 1 day to be performed, which delivers a result before cheese production. For the screening of the cheese milk before production, quantitative data on the presence of *C. tyrobutyricum* spores are necessary. With the positive-negative detection by the PCR method, an estimation of the amount of *C. tyrobutyricum* spores will be possible by testing different samples (three to five) of different 10-fold dilutions. The most probable number of *C. tyrobutyricum* spores may be statistically defined. A comparison with the FNZ method will clarify the practical significance of the PCR detection method for cheese production.

The detection method is relatively inexpensive because the spores are concentrated from the milk by a chemical extraction of the milk components and a centrifugation. No enzymatic digestion or filtration of the milk is done. However, the investment in a PCR laboratory is necessary, which at this time would only be justified for medium-size and large cheese factories. The method of concentration by centrifugation is very efficient and corresponds to what was found for the concentration of vegetative bacterial cells (8).

A direct detection method for *C. tyrobutyricum* spores has the advantages that the time used for germination and growth is saved and that it is independent of the efficiency of spore germination. Direct detection of *C. tyrobutyricum* spores was also achieved by immunodetection of the spores after membrane filtration of the milk (4). A drawback of this immunological approach is the presence of background signals which hamper the interpretation of the results. A PCR detection method, on the other hand, gives not only a very sensitive but also a very reliable result.

Direct detection of mechanically disrupted spores by PCR has also been reported for *B. anthracis* (10, 14). Without mechanical disruption, the release of DNA from the spores during PCR was inefficient and the detection sensitivity was low. This was also established for *C. tyrobutyricum* spores. Without physical disruption of the spores, about 10^4 pure *C. tyrobutyricum* spores have to be present in the PCR mixture in order to be detected after one PCR (data not shown). By using a minibead beater for 10 min at the maximum speed, a detection sensitivity of one *B. anthracis* spore per PCR mixture was achieved for a pure culture. About the same sensitivity was obtained for *C. tyrobutyricum* spores in raw milk.

To obtain this highly sensitive detection in raw milk, a two-step PCR amplification procedure with two nested pairs of primers has to be applied. We could not achieve this high degree of sensitivity with one PCR, even after its optimization. A high degree of sensitivity of the nested PCR has also been reported for the direct detection of vegetative bacterial cells in raw milk. *L. monocytogenes* was detected in 25 ml of raw milk with a detection limit of between 10 and 5 CFU (8). A detection limit of about 10 *Campylobacter jejuni* and *Campylobacter coli* organisms per 40 ml of raw milk, about 10^2 times higher than that with a one-step PCR, was achieved (19).

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