APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1997, p. 3139–3143 Vol. 63, No. 8
0099-2240/97/$04.00+0

Identification and Detection of *Bacillus sporothermodurans* Spores in 1, 10, and 100 Milliliters of Raw Milk by PCR

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Received 11 February 1997/Accepted 9 June 1997

A PCR method was developed to detect spores of *Bacillus sporothermodurans* in 1, 10, and 100 ml of raw milk. Two primers were derived from a unique sequence after subtractive hybridization of *B. sporothermodurans* DNA with DNA of MB 397, a not yet identified spore-forming bacterium isolated from raw milk, closely related to *B. sporothermodurans*. Specific identification was proven on a large collection of *Bacillus* strains and on strains from relevant taxa. The detection of *B. sporothermodurans* in raw milk is based on activation, germination, and outgrowth of the spores, followed by PCR identification. Spores from 10 and 100 ml were concentrated by centrifugation after chemical extraction of the milk components. The total test takes 28 h. The detection limits are 9, 0.4, and 0.22 CFU/ml for 1, 10, and 100 ml, respectively.

Bacillus sporothermodurans is a gram-positive bacterium which produces highly heat-resistant endospores (7, 11, 17). These exceptionally heat-resistant endospores may survive sterilizing and ultrahigh temperature (UHT) treatment.

UHT-treated and sterilized milk products are considered commercially sterile when after incubation for 15 days at 30°C the total count is ≤10 CFU/0.1 ml (1). *B. sporothermodurans* can reach up to 10^5 CFU/ml in sterilized and UHT-treated milk products and therefore causes the problem of nonsterility. The bacterium is considered nonpathogenic (8, 21) and causes in certain circumstances some spoilage effects (12).

B. sporothermodurans is characterized by phenotypic tests, 16S rRNA sequencing, and molecular typing techniques (12, 17). Pettersson et al. (17) showed the occurrence of two different types of *B. sporothermodurans*. These two types differed phenotypically and genotypically. The collection of *B. sporothermodurans* strains, described by Klijn et al. (12), was homogeneous on the basis of 16S rRNA sequences, randomly amplified polymorphic DNA (RAPD) and repetitive extragenic palindromic (REP) fingerprinting patterns.

All phenotypic and genetic characterization methods described till now for *B. sporothermodurans* are quite time-consuming and can be applied only on pure bacterial cultures. Application of PCR offers the opportunity to develop specific identification methods which can be applied for bacterial detection in complex food matrices like raw milk (15). Sensitive detection can be obtained even in the presence of a background flora of 10^5 CFU/ml (9).

In this paper, a PCR identification method for *B. sporothermodurans* is presented. Specific sequences of *B. sporothermodurans* are obtained by subtractive hybridization, which allows the removal of homologous sequences of two different strains so that unique sequences for the target organism can be isolated (3). The unique sequence of *B. sporothermodurans* forms the basis of the specific PCR identification method. The specificity of this method was tested on pure bacterial strains and on raw-milk samples. The analysis of raw-milk samples was necessary because false-positive reactions can be obtained due to the presence of not yet identified *Bacillus* spp., very closely related to *B. sporothermodurans* (12). By artificial inoculation of 1, 10, and 100 ml of raw milk, detection limits were determined.

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MATERIALS AND METHODS

Bacterial strains and culture conditions. *B. sporothermodurans* strains and strains of not yet identified spore-forming bacteria are listed in Table 1. Other bacterial strains used were *B. alcalophilus* 7120^T, *B. badius* 7122^T, *B. circulans* 6926^T, *B. coagulans* 6326^T, *B. lautus* 11157^T, *B. licheniformis* 6933^T, *B. mycoides* 7128^T, *B. pallidus* 11159^T, *B. sphaericus* 7134^T, *B. stearothermophilus* 6939^T, *Clostridium acetobutylicum* 5710^T, *C. beijerinckii* 5716^T, *C. bifermentans* 3029, *C. butyricum* 1217^T, *C. pasteurianum* 3285^T, *C. perfringens* 11264^T, *C. sporogenes* 8421^T, *C. thermosaccharolyticum* 2811^T, *C. tyrobutyricum* 1285^T, *Hafnia alvei* 2791, and *Lactobacillus bulgaricus* 6901, all obtained from the Laboratory of Microbiology of the University of Ghent, Ghent, Belgium; *B. brevis* S12, *B. cereus* S1, *B. macerans* S21, *B. pumilus* S19, and *B. subtilis* 12S-O from the Government Dairy Research Station, Melle, Belgium; *C. tetanomorphum* 695, *Enterococcus faecalis* EF S1, *E. faecium* EF M1, *Lactococcus lactis* MRZ 1076, and *Listeria monocytogenes* Scott A from Institut National de la Recherche Agronomique, Paris, France; *Agrobacterium tumefaciens* $C_{58}C_1$Rif and *Escherichia coli* MC1061 from the Laboratory of Genetics of the University of Ghent, Ghent, Belgium; *Brucella ovis* 1051 from the National Collection of Type Cultures, London, United Kingdom; and *Listeria ivanovii* 78.42 and *Listeria seeligeri* 100.000 from Collection Nationale de Micro-organismes de l' Institut Pasteur, Paris, France. *B. sporothermodurans* strains and other spore-forming bacteria were grown on nutrient broth 2 (Oxoid Ltd., London, England) supplemented with vitamin B_{12} (1 mg/liter) (Sigma, St. Louis, Mo.) and bacteriological agar 1 (15 g/liter) (Oxoid) at 37°C for 24 h. Spores were obtained by growing the strains on nutrient broth 2 supplemented with $MnSO_4 \cdot 2H_2O$ (7 mg/liter), $CaCl_2 \cdot 2H_2O$ (1 g/liter), vitamin B_{12} (1 mg/liter), and bacteriological agar (15 g/liter) (Oxoid), pH 6.8. The 66 strains of *B. sporothermodurans* and the 4 strains of not yet identified spore-forming bacteria were isolated as described by Klijn et al. (12). All other strains were grown as recommended by the University of Ghent Laboratory of Microbiology Culture Collection.

DNA extraction from cultured cells. Pure bacterial cultures were centrifuged for 2 min at 13,000 × *g* to collect the bacterial cells. Bacterial chromosomal DNA was extracted by the method of Flamm et al. (5), and the concentration was determined spectrophotometrically.

Crude bacterial cell lysates were prepared by adding 50 μl of 0.1 M NaOH and 50 μl of 0.25% sodium dodecyl sulfate to the bacterial pellet and subsequently heating for 17 min at 90°C.

Subtractive hybridization. The subtractive hybridization between probe strain DNA and subtracter DNA was basically performed as described by Bjourson et al. (3). *Sau*3A-digested DNA from *B. sporothermodurans* (strain H2-34) was ligated to linker L-P, obtained after hybridization of primers TB7006 (5′ HO-AGCGGATAACAATTTCACACAGGA-OH 3′) and TB7008 (5′ P-GATCTC CTGTGTGAAATTGTTATCCGCT-OH 3′). *Sau*3A-digested DNA from MB 397 was ligated to the biotinylated linker L-S, obtained after hybridization of primers TB7007 (5′ Biotin-CGCCAGGGUUUUCCCAGUCACGAC-OH 3′) and TB7009 (5′ P-GAUCGUCGUGACUGGGAAAACCCUGGCG-OH 3′). The linker sequences were the same as those described by Bjourson et al. (3).

TABLE 1. Bacterial strains and PCR results

Strain (source[a])	PCR result[b]
B. sporothermodurans	
H2-34 (FDRC)	+
M 215 (FDRC)	+
TP 1248 (FDRC)	+
32 (FDRC)	+
NV9/8 (FDRC)	+
66 MB isolates[c] (RZS)	+
Not yet identified spore-forming bacteria	
MB 382 (RZS)	−
MB 385 (RZS)	−
MB 397 (RZS)	−
CAB 1 (NIZO)	−

[a] FDRC, Federal Dairy Research Center, Kiel, Germany; RZS, Government Dairy Research Station, Melle, Belgium; NIZO, Netherlands Institute for Dairy Research, Ede, The Netherlands.

[b] With primers SH2-F1 and SH2-R.

[c] MB 336, MB 338 to MB 357, MB 359, MB 360, MB 375, MB 377 to MB 380, MB 389 to MB 391, MB 404 to MB 410, MB 412, MB 413, MB 456, MB 509, MB 510, MB 512 to MB 524, and MB 565 to MB 574.

The ligated DNAs were purified by phenol-chloroform extraction and concentrated by precipitation (18). Probe strain DNA was prepared by PCR amplification of the ligated DNA with primer TB7006 according to the PCR protocol described below with an annealing temperature of 55°C and 45 amplification cycles. Subtracter DNA was prepared by PCR amplification of the ligated DNA with the biotinylated primer TB7007 according to a PCR protocol basically as described below. Instead of dTTP, 200 μM dUTP was added, an annealing temperature of 55°C was applied, and 45 cycles were performed. After purification by phenol-chloroform extraction, denatured probe strain DNA and subtracter DNA were hybridized in solution with an excess of subtracter DNA. The first hybridization was performed at 64°C; the three subsequent hybridizations were performed at 68°C. Subtracter DNA and hybrids between probe DNA and subtracter DNA were removed by 150-μl Dynabeads M-280 streptavidin (Dynal A.S., Oslo, Norway) according to the supplier's instructions. The supernatants were purified by phenol-chloroform extraction and dissolved in 20 μl of H_2O. Five microliters was applied in the next round of subtraction. One microliter was diluted 10 and 100 times. From each dilution 10 μl was treated with uracil DNA glycosylase (Life Technologies Inc., Paisley, United Kingdom) in order to remove traces of subtracter DNA. Finally, the remaining sequences were amplified by PCR with the TB7006 primer. The PCR program, described below, was used with an annealing temperature of 55°C and 45 cycles.

Cloning of subtracted probe DNA fragments. The *B. sporothermodurans* sequences, generated by three and four rounds of subtractive amplification, were cloned in the pMOS*Blue* vector according to the supplier's instructions (Amersham plc., Buckinghamshire, United Kingdom). The insert DNA was recovered from agarose gel by Geneclean II (Bio 101, La Jolla, Calif.). After blue-white screening of recombinant colonies, white and bull's-eye colonies were confirmed as containing an insert by using a miniprep plasmid preparation method (2) and a *Bam*HI/*Hin*dIII restriction digest.

^{32}P labelling of DNA. The 16S rRNA PCR fragment of *B. sporothermodurans*, the subtracted probe strain DNA, and the recombinant pMOS DNA were radiolabelled by using the Random Primers DNA labelling system according to the supplier's instructions (Life Technologies Inc.). Unincorporated nucleotides were removed after separation on a Sephadex G-50 fine (DNA grade) column (18).

Analysis of DNA dot blots and Southern blots. Total genomic target DNA (1 μg) was denatured and spotted onto a Hybond-N membrane (Amersham) by a dot blot manifold (Bio-Rad, Richmond, Calif.). The DNA was fixed onto the filter by baking for 2 h at 80°C. Transfer of DNA to nylon membranes after separation on agarose gel was performed according to the recommendations of the manufacturer (Amersham).

Filters were hybridized at 68°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M tri-sodium citrate–5,5-hydrate) according to a general protocol (18). Filters were washed at 68°C once with 6× SSC, once with 3× SSC, once with 1.5× SSC, and once with 0.75× SSC, each time for 1 h, and subjected to autoradiography for 1 day.

DNA sequencing. *B. sporothermodurans* DNA fragments, cloned in the pMOS vector, were manually sequenced in both directions with the T7 and the U-19-mer primers (pMOS*Blue* T-vector kit; Amersham). The dideoxy sequencing reactions were performed with a deaza-T7 sequencing kit (Pharmacia Biotech Inc., Piscataway, N.J.) with ^{35}S-dATP as the labelled nucleotide. Sequence data were analyzed with the Genetics Computer Group sequence analysis package (6). Homology between the different cloned *B. sporothermodurans* DNA fragments was searched for with the BESTFIT (20) and GAP (14) programs in both directions. Homology with the bacterial sequences of the EMBL database was searched for with the FASTA program (16).

Specificity testing of PCR on raw-milk samples. Raw-milk samples were collected from milk transport tankers from different dairies. One milliliter of raw milk was heated for 10 min at 80°C and subsequently streaked on nutrient broth 2 (Oxoid) supplemented with vitamin B_{12} (1 mg/liter) and bacteriological agar 1 (15 g/liter) (Oxoid). Plates were incubated at 37°C for 24 h. Bacterial colonies were swabbed from the plate and tested by PCR using the primers SH2-F1 and SH2-R.

Determination of detection limits. Spores and vegetative cells were harvested from slants by swirling with sterile H_2O. Detection limits were determined on raw tanker milk which was first judged to be free from *B. sporothermodurans* spores. Volumes of 1, 10, and 100 ml of raw tanker milk were artificially contaminated with 100 μl of 10-fold dilutions of *B. sporothermodurans* cultures (strain H2-34). Spores were activated by heat treatment for 30 min at 100°C. For 1-ml volumes, the milk was subjected immediately to heat treatment. For 10 and 100 ml of raw milk a chemical extraction method, essentially as described by Herman et al. (9), was used with the exception that ethanol was replaced by *n*-propanol. The pellet was washed twice with sterile H_2O, centrifuged at 770 × *g* for 2 min, suspended in 1 ml of sterile H_2O, and heat treated. After heat treatment, the suspensions were immediately plated on brain heart infusion (Oxoid) supplemented with bacteriological agar 1 (15 g/liter) (Oxoid) and vitamin B_{12} (1 mg/liter) (Sigma), pH 6.8. Plates were incubated at 37°C for 24 h, and colonies were swabbed from the plate and suspended in 1 ml of sterile H_2O. Crude bacterial cell lysates were prepared as described above. For the relation between detection and numbers of bacteria a Pearson chi-square was calculated. Confidence intervals are set on the numbers of positive samples as percentages of the sample frequencies (1a).

PCR amplification. One microliter of crude cell lysate or 10 ng of genomic DNA was added as the template in the PCR. 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), 0.2 mM each deoxynucleoside triphosphate (Pharmacia Biotech Inc.), 0.7 U of Goldstar DNA polymerase (Eurogentec, Seraing, Belgium), and 1 μg of each appropriate primer. The mixture was subjected to 29 cycles of amplification in a PCR 9600 thermal cycler (Perkin-Elmer Corporation, Branchburg, N.J.). The first cycle was preceded by initial denaturation at 95°C for 1 min. Each cycle consisted of denaturation at 95°C for 30 s, annealing at the appropriate temperature (see below), and extension at 72°C for 30 s. The last cycle was followed by a final extension step at 72°C for 4 min.

The following annealing temperatures were applied: SH1-F1/SH1-R, 60°C; SH1-F2/SH1-R, 60°C; SH2-F2/SH2-R, 65°C; SH2-F2/SH2-R, 60°C; SH3-F1/SH3-R, 60°C; SH3-F2/SH3-R, 65°C. The 16S rRNA fragment of *B. sporothermodurans* was amplified by using the universal 16S rRNA primers described by Heyndrickx et al. (10). The annealing temperature was 55°C.

Agarose gel electrophoresis and plasmid analysis. For preparative purposes, the subtracted probe strain DNA was separated on 3% (wt/vol) NuSieve GTG agarose (FMC BioProducts, Rockland, Maine) according to a standard protocol (18). Ten microliters of the PCR product was analyzed on a 1.5% (wt/vol) SeaKem ME agarose gel (FMC BioProducts).

Plasmid DNA was prepared from *B. sporothermodurans* by the Qiagen method (Qiagen Inc., Chatsworth, Calif.) and analyzed on a 1.5% (wt/vol) SeaKem ME agarose gel (FMC BioProducts).

Determination of the percentage of outgrowth of *B. sporothermodurans* spores. Spores and vegetative cells were harvested from slants with sterile H_2O. The vegetative cells were digested by incubation with lysozyme (0.03%) (Boehringer GmbH, Mannheim, Germany) in a 0.067 M phosphate buffer (pH 7) plus 15 mM $MgCl_2$ for 1 h at 37°C. Afterwards, trypsin (0.1%) (BDH Chemicals Ltd., Poole, England) was added, and the incubation was continued for 90 min. The spores were concentrated by centrifugation at 12,000 × *g* at 4°C for 30 min, washed twice with sterile H_2O, and stored at 4°C in sterile H_2O. The spore concentration was determined microscopically in a Bürker counting cell. The corresponding outgrowth of the spores was determined after heat activation (10 min at 80°C and 30 min at 100°C) and plating of the untreated cell suspension on brain heart infusion supplemented with vitamin B_{12} (1 mg/liter) and bacteriological agar (15 g/liter) (Oxoid). For the outgrowth percentages a two-way analysis of variance was applied with heating and duration (10 min at 80°C, 30 min at 100°C) as an applied factor and milk samples as controlling factors to reduce experimental error.

RESULTS

Specific identification of *B. sporothermodurans* by PCR. Specific *B. sporothermodurans* DNA fragments were subtracted from homologous DNA fragments of MB 397, a not yet identified spore-forming bacterium, probably highly related to *B. sporothermodurans* (12). Each subsequent round of subtraction/amplification resulted in an increasing concentration of

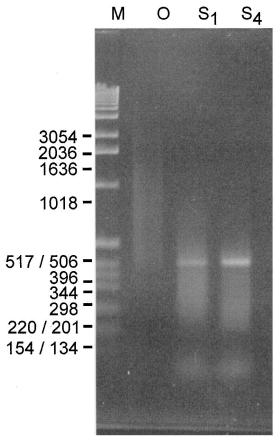

FIG. 1. *B. sporothermodurans* DNA remaining after subtraction/amplification. Lanes 1 to 3: *B. sporothermodurans* DNA fragments generated by zero (0), one (S1), and four (S4) rounds of subtraction/amplification against DNA from MB 397. M, molecular size marker X (Boehringer GmbH).

DNA fragments of about 350 bp (Fig. 1). After the first round of subtraction/amplification, an enrichment for *B. sporothermodurans*-specific DNA fragments was already established (Fig. 2). After three rounds, a higher specificity of the fragments was reached, which was not improved by a fourth round at the same hybridization temperature (Fig. 2).

DNA fragments, generated by three and four rounds of subtraction/amplification, were cloned in the pMOS vector. Four cloned fragments of each round of subtraction/hybridization were sequenced. Three different DNA sequences (SH1, SH2, and SH3) (Fig. 3) were obtained without mutual sequence homology. No homology was found with bacterial sequences of the EMBL database. Each of the three different sequences hybridized with *B. sporothermodurans* DNA and not with related *Bacillus* spp.

One reverse primer and two forward primers, derived from each fragment (Fig. 3), were tested for specific identification of *B. sporothermodurans*. All six primer pairs reacted with all the *B. sporothermodurans* strains tested. No primer pair cross-reacted with any well-described bacterial species tested. Four primer pairs (SH1-F1/SH1-R, SH1-F2/SH1-R, SH2-F2/SH2-R, and SH3-F1/SH3-R) reacted with MB 397, and one pair (SH3-F2/SH3-R) reacted with MB 385 and MB 397. Only one primer pair, SH2-F1/SH2-R, did not react with four strains of not yet identified spore-forming bacteria which were isolated from raw-milk products (Table 1).

The SH2-F1/SH2-R primer pair was tested for specificity on

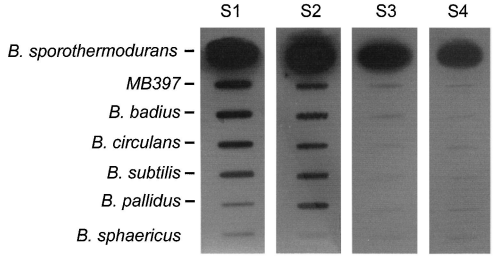

FIG. 2. Specificity of *B. sporothermodurans* DNA sequences generated by subtraction/amplification. *B. sporothermodurans* DNA fragments generated by one (S1), two (S2), three (S3), and four (S4) rounds of subtraction/amplification against DNA from MB 397 were used as probes. Hybridization was performed with 1 μg of total genomic DNA from the bacterial species indicated.

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SH1
                SH1 - F1
   1 ccctaaatgg ggcgtgaaaa agaactcaga aggacaaaat gttttttggt
                SH1 - F2
  51 ttggctacaa aggtcattta gccgttggta catcaagcca atacattcta
 101 caggctcttt tttcatctgg gagtctaaat gatggtaagg cagctatccc
 151 tttactgaaa ggaattcagg aacgccttca ccttccatta cgttatcaaa
 201 caatggatgc gggctatgac tatgaaccca tatacgagca ggtacatcga
                                              SH1 - R
 251 atgggacaac aatccgtcat cgcgtataat aagcgaaatg aaggggaaat
 301 cattggctat gataaacact ttgccccaac ttgtttcaga gagnattctt
 351 atcgttatga tagtttt

SH2
                                  SH2 - F1
   1 aatacatgcc ctggtcaaaa atgattcagg cagaatgtag caaataaatg
                                        SH2 - F2
  51 aaatagccgt aattcgatta aaaaagtcag aatttacggc tatttacgat
 101 gcgtaccgaa aggtacactt atttttata attcgggctt acgttgacca
 151 caaaccataa aaagacttgg aggagtactt cttatgacta ttatacgaca
 201 acctagccta tttggcatac aggaattatt tgacatggaa cctacccaaa
                                              SH2 - R
 251 aatatgaagc cattatttca gcggtagatt tggattcgat gtaccatcaa
 301 gtggcgaaaa aatcacggtt gggtgcaccg gaagaactaa actatgcagc
 351 catgattatc tccacc

SH3
                                                    SH3 - F1
   1 gnttgataat ttgtccgtat ntttcatcga catgcaaaac cgattttgcc
                SH3 - F2
  51 atcatcaact ctttcattcg ttcgtataca ggtaacagcc aatcatgtgc
 101 tacacggata acccaattcg ataggttttt atcattggta ttcagtccat
 151 agcgattcca ttccctttacc tgacggtaaa gaggtaaata ttgtgcaaat
 201 ttatcataga tgactttggc aagtacncta ggacttgcga tgctacnctg
 251 aatcgctggt tgtggtgctt tcccgcgttg attgtgctgg ctgtgatgaa
                                              SH3 - R
 301 tcaactttgc agtcttacat tcatacgcat gttcaatgtg gtgtactttc
 351 atcatttttg caggaataaa ttttgcttct tcacgtacaa gtgtactacc
 401 aatttcaatc atttgccctt ggcaacagtc gcagatt
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FIG. 3. DNA sequences of three different DNA fragments obtained by subtraction/amplification. Two forward (F) primers and one reverse (R) primer are indicated in each fragment.

1 ml of 93 raw-milk samples. All samples reacted negatively without aspecific DNA bands.

The chromosomal or plasmid location of the SH2 DNA sequence was determined. A large circular plasmid (>15 kb) was isolated and purified. The purity of the plasmid DNA was determined by the lack of hybridization with a 16S rRNA probe of *B. sporothermodurans*. On the other hand, a clear hybridization signal confirmed the plasmid location of the SH2 sequence. To test the stability of the PCR method, three *B. sporothermodurans* strains (MB 351, MB 581, and MB 582) were restreaked 28 times on agar plates. No loss in detectability was established.

Detection of *B. sporothermodurans* in raw milk. The detection method of *B. sporothermodurans* spores in raw milk is based on transformation of the spores to vegetative cells, followed by vegetative growth overnight and PCR identification. In order to detect *B. sporothermodurans* in raw milk, the influence of the background flora on the outgrowth efficiency has to be considered. By visual examination, a high reduction of the background flora was established with increasing temperature (80 to 100°C) and heat treatment time (10 to 30 min). Heat treatment for 30 min at 100°C increased the sensitivity of detection of *B. sporothermodurans* in raw milk about 100 times compared to 10 min at 80°C (Table 2). Therefore, heat treatment for 30 min at 100°C was preferred for the detection of *B. sporothermodurans* in raw milk.

The efficiency of spore activation with heat treatment for 30

TABLE 2. Sensitivity of detection of *B. sporothermodurans* in 1 ml of heat-treated raw milk

No. of colonies[a]	PCR detection[b]	
	10 min, 80°C	30 min, 100°C
75	+	+
7.5	−	+
0.75	−	+
0	−	−

[a] Average number of colonies found after incubation for 24 h at 37°C.
[b] +, positive for *B. sporothermodurans*; −, negative for *B. sporothermodurans.*

min at 100°C was studied in comparison with heat treatment for 10 min at 80°C. Two different heat treatments (10 min at 80°C and 30 min at 100°C) were compared for their efficiency of spore activation. The outgrowth percentages were determined six times, and the data were statistically calculated. Heat treatment for 10 min at 80°C yielded an average outgrowth of 53.12%, while with heat treatment for 30 min at 100°C an average outgrowth of only 12.72% was obtained ($F = 0.025$). It was also observed that after heat treatment, colonies could be obtained sooner on brain heart infusion agar than on nutrient agar. An incubation period of 24 h at 37°C was necessary after treatment for 30 min at 100°C in order to obtain sufficient outgrowth. A ±4-h delay in recovery from the heat-induced stress was found relative to treatment for 10 min at 80°C.

Spores from 10 and 100 ml of raw milk were concentrated by centrifugation after chemical extraction of the milk components. The removal of chemical substances from the bacterial pellet by two washes with sterile H_2O was necessary for the spores to survive the heat treatment.

Detection limits of *B. sporothermodurans* for 1, 10, and 100 ml of raw milk were determined. The negative/positive ratios were affected by the numbers of bacteria, for 1 ml of raw milk ($\chi^2 = 0.0014$) as well as for 10 and 100 ml ($\chi^2 < 10^{-5}$). In Table 3 the 95% confidence intervals are given, expressed as percentages of the sample frequencies. We define the detection limit as the lowest number of bacteria for which the lower confidence limit is above or equal to 50%. Based on this definition, the detection limits were 9, 4, and about 22 CFU for 1, 10, and 100 ml, respectively. In CFU per milliliter, the values are 9, 0.4, and 0.22, respectively. A higher detection sensitivity for 10 ml (4 CFU) than for 1 ml (9 CFU) was established, while the detection sensitivity for 100 ml was dependent on the raw-milk sample tested.

DISCUSSION

A reliable and sensitive PCR detection method for *B. sporothermodurans* in raw milk was developed. Till now, identification of *B. sporothermodurans* was possible only by sequencing of the 16S rRNA and by a set of phenotypic tests which are discriminative towards the related described *Bacillus* spp. (17). These methods, however, can be applied only to pure bacterial cultures which are difficult to isolate from food products with a high background flora in raw milk. The PCR identification method forms the indispensable basis of the reliable detection method for *B. sporothermodurans*, described here. Specific sequences were isolated by the combined subtractive-hybridization–PCR method described by Bjourson et al. (3). An aerobic spore-forming bacterium isolated from raw milk was used as the subtraction strain. The partially determined 16S rRNA sequence (12) was not homologous to the 16S rRNA sequence of any *Bacillus* sp. in the EMBL database. Based on the very high homology with the 16S rRNA sequence of *B. sporothermodurans*, this strain was chosen as the subtracter strain. The subtractive hybridization was carried out at a hybridization temperature of 64°C for the first round and 68°C for the subsequent ones. After three or four rounds of subtraction/amplification, specificity for *B. sporothermodurans* was shown by dot blot hybridization. Although this specificity was very obvious in this dot blot hybridization, some primer combinations cross-reacted with some other *Bacillus* spp., including the subtracter strain MB 397, probably as a result of the rather low-stringency hybridization conditions used during subtraction.

The 66 *B. sporothermodurans* strains were isolated from sterilized and UHT-treated products as pure bacterial cultures. The identity of most of them was confirmed by sequence analysis of the V2 region of the 16S rRNA (12). All isolates showed the typical RAPD and REP-PCR fingerprinting profiles (12). The four strains of not yet identified spore-forming bacteria were isolated from raw milk and were differentiated from *B. sporothermodurans* by REP-PCR (data not shown) and by the sequence of the V2 region of the 16S rRNA (12). The 16S rRNA sequences of the V2 regions showed differences from the homologous sequences of all described *Bacillus* species (12). Because not yet identified spore-forming bacteria can cross-react with presumptive specific primer pairs, it can be concluded that differentiation from identified collection strains is not sufficient for proving specific identification of *B. sporothermodurans*. Therefore, it can be assumed that validation of the method will be necessary for each complex food sample before routine application. In this article, the validation of the identification method for 93 raw-milk samples is described.

Computer analyses did not show any special homology with DNA sequences from the EMBL database. Because no homology could theoretically support the stability of the target sequence, possible plasmid location was investigated. A circular plasmid was isolated, and the plasmid localization of the SH2 DNA fragment was determined. The stability of the target sequence was confirmed when, after 28 consecutive cultures of *B. sporothermodurans*, no loss in PCR detectability was observed.

A PCR detection method for *B. sporothermodurans* spores in 1, 10, and 100 ml of raw milk was developed. The detection method is based on activation and germination of the spores, followed by vegetative growth and PCR identification. Heat treatment for 30 min at 100°C resulted in spore activation and substantial reduction of the background flora. Reduction of the competing flora is a crucial factor for the outgrowth efficiency of *B. sporothermodurans* on agar plates. The reduction by heat of the background flora of raw milk, consisting mainly of *B. licheniformis*, *B. subtilis*, and *B. cereus* (4, 13), was also established by Pettersson et al. (17), who suggested heat treat-

TABLE 3. Detection limits for *B. sporothermodurans* spores in 1, 10, and 100 ml of raw milk

Spore CFU[a]		
1 ml	10 ml	100 ml
0.9 (5–83)	1.2 (0–55)	0.22 (0–40)
3.0 (36–99)	4.0 (50–100)	1.20 (0–67)
9.0 (50–100)	12.0 (50–100)	2.20 (50–100)
30.0 (50–100)	40.0 (50–100)	12.00 (14–96)
90.0 (50–100)	120.0 (50–100)	22.00 (50–100)
300.0 (50–100)	400.0 (50–100)	120.00 (38–100)

[a] 95% confidence limits, expressed as percentages of sample frequencies, are given in parentheses.

ment for 40 min at 100°C. To obtain a maximum germination and outgrowth after activation, growth factors are important as well. A better outgrowth was observed on brain heart infusion agar than on nutrient agar, which confirms the results of Pettersson et al. (17). Because activation of bacterial spores due to heat treatment is often a reversible process (19), the time between heat treatment and plating of the spore suspension has to be as short as possible.

Detection limits were determined for 1, 10, and 100 ml of raw milk. The detection sensitivity in 10 ml of raw milk is higher than for 1 ml of raw milk, which can be due to the difference in sample preparation. While 1 ml of raw milk is heat treated and plated as such, the spores present in 10 ml are concentrated after chemical extraction of the milk components and subsequently heat treated. Important is the fact that no negative effects from the chemical treatment of the milk on detection sensitivity were observed, indicating that no substantial fraction of the spores was lost during this procedure. For 100 ml of raw milk, sensitivity was variable, probably due to the influence of the background flora, which increases in larger amounts of raw milk. The detection method for *B. sporothermodurans* spores in raw milk presented here is the first described. This method opens new perspectives for investigation of sources of *B. sporothermodurans* contamination on the farm or during processing of milk.

ACKNOWLEDGMENTS

We thank Ann Vanhee for excellent technical work and N. Klijn (Netherlands Institute for Dairy Research, Ede, The Netherlands) and K. Goethals (University of Ghent, Ghent, Belgium) for interesting discussions.

This work was financed by the Ministry of Small Enterprises, Traders and Agriculture, DG 6, division of research (Belgium).

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