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Development of Real-Time PCR Assays for Detection and Quantification of *Bacillus cereus* Group Species: Differentiation of *B. weihenstephanensis* and Rhizoid *B. pseudomycoides* Isolates from Milk▽†

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Quantitative real-time PCR (qRT-PCR) offers an alternative method for the detection of bacterial contamination in food. This method provides the quantitation and determination of the number of gene copies. In our study, we established an RT-PCR assay using the LightCycler system to detect and quantify the *Bacillus cereus* group species, which includes *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, and *B. pseudomycoides*. A TaqMan assay was designed to detect a 285-bp fragment of the *motB* gene encoding the flagellar motor protein, which was specific for the detection of the *B. cereus* group species, excluding *B. pseudomycoides*, and the detection of a 217-bp gene fragment of a hypothetical protein specific only for *B. pseudomycoides* strains. Based on three hydrolysis probes (MotB-FAM-1, MotB-FAM-2, and Bpm-FAM-1), it was possible to differentiate *B. weihenstephanensis* from the *B. cereus* group species with nonrhizoid growth and *B. pseudomycoides* from the whole *B. cereus* group. The specificity of the assay was confirmed with 119 strains belonging to the *Bacillus cereus* group species and was performed against 27 other *Bacillus* and non-*Bacillus* bacteria. A detection limit was determined for each assay. The assays performed well not only with purified DNA but also with DNA extracted from milk samples artificially contaminated with bacteria that belong to the *B. cereus* group species. This technique represents an alternative approach to traditional culture methods for the differentiation of *B. cereus* group species and differentiates *B. weihenstephanensis* and *B. pseudomycoides* in one reaction.

Members of the *Bacillus cereus* group species include *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis*. Transitional strains, namely, *Bacillus* sp. strain Ba813$^+$, are a new group of *Bacillus* that carry a chromosomal marker, Ba813, that is characteristic only for *B. anthracis* strains (19, 22). However, other features, e.g., hemolysis of blood agar, motility, and penicillin resistance, are not associated with *B. anthracis* but with other members of the *B. cereus* group. *B. cereus* is the most important of the spore-forming microorganisms. In 2010, The European Food Safety Authority (EFSA) Community Summary Report on trends and sources food-borne outbreaks in the European Union (EU) in 2008 showed 124 outbreaks caused by *Bacillus* species from 10 EU member states; 2 nonmember states reported nine *Bacillus* species outbreaks. Forty-five of the *Bacillus* outbreaks were verified (36.3%) for 1,132 cases; 41 patients were hospitalized. The total number of outbreaks in the EU, including 27 member states, caused by *Bacillus* species toxins increased by 18.1% above levels for 2007 (105 outbreaks) (6). *Bacillus* species spores are ubiquitous in raw milk, survive the pasteurization process, and produce different enterotoxins that may cause food poisoning of the diarrhea or emetic types (28). The consumption of food containing 10^5 to 10^6 bacteria (spores)/g or toxins is sufficient to cause infection (5). *B. cereus* can contaminate the milk from soil, air, water, processing equipment, and "milkstone" residue on bulk tanks. *B. cereus* is also of particular concern in the baby formula industry (1, 23). These foods are controlled by ensuring a low initial level of the bacteria in the product. This is achieved by using well-designed equipment with effective cleaning methods to prevent biofilm formation. EU regulation 2073/2005 (4), on the microbiological criteria for foodstuffs, details acceptable microbiological levels in food stuffs and sampling plans required to ensure that the microbiological criteria are met. The EFSA summary report on *B. cereus* in food (5) stated that the lowest numbers in food that resulted in a food poisoning outbreak was 3 to 4 log per g.

PCR was one of the first DNA-based assays for detecting pathogens. PCR is sensitive (12, 20) but can be limited by problems with the sensitivity of polymerase to environmental contaminants, difficulties in quantification, or the contamination of the samples (13, 29). In real-time PCR (RT-PCR), unlike in conventional PCR, the amplification is monitored continuously during the reaction, which permits the user to quantify the target earlier (21). RT-PCR is performed in a closed-tube system and requires no post-PCR manipulation of the sample, preventing PCR mix contamination (8). In the RT-PCR mix, different chemistries allow for the detection of the PCR product via the generation of a fluorescent signal,

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either SYBR green or sequence-specific labeled probes (such as Molecular Beacons, Scorpions, and TaqMan) (15).

The quantitation of the target amount in unknown samples can be estimated by generating a standard curve (7, 26). The establishment of a standard curve using the quantitative RT-PCR (qRT-PCR) process is a key step in determining the copy number of a given target sequence. The standard curve method produces a linear plot of the log of the initial copy number for a set of standards versus the Cp (crossing point) value (16). The Cp is the point in the reaction when the amplicon has been generated to give a fluorescence signal above the baseline (16). A perfect amplification reaction produces a standard curve with an efficiency of 2, because the amount of target DNA should double with each cycle.

In our study, the gene fragment (575 bp) of the *motB* gene, encoding a flagellar motor protein, MotB (681 bp), which is classified as an outer membrane protein (OmpA) (2, 9, 10) in *B. cereus* group species, and a 217-bp DNA fragment, which encodes a hypothetical protein, were used to distinguish the *B. cereus* group species. Two TaqMan probes, MotB-FAM-1 and MotB-FAM-2, targeting the *motB* gene, enabled the detection and identification of *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *Bacillus* sp. strain Ba813$^+$, and some *B. mycoides* strains. The second probe, MotB-FAM-2, allowed for the differentiation of *B. weihenstephanensis* strains from other *B. cereus* group species with nonrhizoid growth. Some *B. mycoides* strains that gave a positive signal with the MotB-FAM-2 probe were easily discriminated from *B. weihenstephanensis* by their rhizoid growth on agar plates. The third TaqMan probe, Bpm-FAM-1, made possible the identification and differentiation of *B. pseudomycoides* strains. The DNA method presented here is an improvement over slow traditional culture methods.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. Strains were stored at −20 and −80°C in nutrient broth-glycerol (15%, vol/vol) (BD Difco, Oxford, United Kingdom) and also in Luria-Bertani (LB) (BD Difco, Oxford, United Kingdom) broth containing 50% glycerol. *Bacillus* strains were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany), the American Type Culture Collection (ATCC; Middlesex, United Kingdom), the Bacillus Genetic Stock Center (BGSC; Ohio State University), and the National Collection of Type Cultures (NCTC; London, United Kingdom). Some of the strains, described in detail in the table in the supplemental material, were kindly provided by Stenfors Arnesen (Norwegian School of Veterinary Medicine, Oslo, Norway), A. H. Bishop (University of Greenwich, United Kingdom), M. Ehling-Schulz (Technische Universität, München, Germany), Noura Raddadi (Milano, Italy), and Kieran Jordan (Moorepark Food Research Centre, Cork, Ireland); all of the *B. mycoides*/*B. pseudomycoides* strains were provided by I. Święcicka (University of Białystok, Poland). Bacteria with rhizoidal growth were classified as *B. mycoides*/*B. pseudomycoides* without further discrimination (27).

Since the handling of *B. anthracis* is restricted to laboratories with a biosafety level of 3 (BSL3), DNA of *B. anthracis* strains and *Bacillus* sp. strain Ba813$^+$ were provided by the Biological Threats Identification and Countermeasure Centre of the Military Institute of Hygiene and Epidemiology, Puławy, Poland. In addition, a selection of *B. cereus* group species that were isolated from soil and food are included. See the table in the supplemental material for details.

Template DNA preparation. All bacteria were grown in 10 ml of nutrient broth (BD Difco, Oxford, United Kingdom) at 33°C for 24 h. Genomic DNA was extracted by the method of Schraft and Griffiths (25), with modifications. The cells were pelleted at 5.0 × *g* for 10 min, resuspended in 200 μl lysis buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 0.75 M sucrose, 10 U lysostaphin, 10 mg/ml lysozyme), and incubated at 37°C for 30 min. Twenty microliters of proteinase K (Fluka, Sigma-Aldrich, Dublin, Ireland) solution (20 mg/ml) and 1% SDS (Fluka, Sigma-Aldrich, Dublin, Ireland) were added to each, followed by digestion at 37°C for 2 h. The lysates were extracted with phenol-chloroform-isoamylalcohol (25:24:1; Fluka, Sigma-Aldrich, Dublin, Ireland) and precipitated with 1/10 volume of 3 M sodium acetate solution and a 0.9 volume of isopropanol (Fluka, Sigma-Aldrich, Dublin, Ireland). Purified DNA was dissolved in 100 μl nuclease-free water or 10 mM Tris-HCl, pH 8.0. All samples were aliquoted and stored at −20°C. Purified chromosomal DNA was used as a template in RT-PCR assays. In addition, the Genomic Mini purification kit (A&A Biotechnology, Gdynia, Poland) was used according to the manufacturer's instructions.

Oligonucleotide primers and hydrolysis probe. Two sets of primers (BCFomp2/BCRomp2 and BpmF/BpmR2) and three TaqMan probes (MotB-FAM-1, MotB-FAM-2, and Bpm-FAM-1) were designed based on the nucleotide sequence of the *motB* gene of the *B. cereus* group species and a hypothetical gene of *B. pseudomycoides* (Table 2) using Primer3 software (version 0.4.0) (24). The expected size of the DNA fragment amplified using BCFomp2/BCRomp2 primers (synthesized by Eurofins MWG Operon, Martinsried, Germany) was 285 bp. Conserved regions for cross-species primers and MotB-FAM-1 and MotB-FAM-2 probe hybridization were identified from multiple sequence alignments for the 575-bp amplified fragment (18) of the *motB* gene from *B. cereus* ATCC 14579 (GenBank accession number NC_004722), *B. cereus* E33L ZK (GenBank accession number NC_006274), *B. thuringiensis* serovar konkukian strain 97-27 (GenBank accession number NC_005957), and *B. anthracis* strain Ames (GenBank accession number NC_007530). The MotB-FAM-2 probe differs from MotB-FAM-1 in three nucleotides, where one was wobbled (Table 2).

The expected size of the DNA fragment amplified using BpmF/BpmR2 primers was 217 bp. Unique DNA regions for primer hybridization were identified after blasting the genome sequence draft of *B. pseudomycoides* DSM 12442 (GenBank accession number NZ_CM000745) against other *B. cereus* group species sequences available at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi. Using those primers, three 217-bp fragments of *B. pseudomycoides* WS 3118, WS 3119, and GRD 1/17 were sequenced and analyzed, and a unique sequence for the hybridization of Bpm-FAM-1 probe was distinguished.

The hydrolysis TaqMan probes (manufactured by TIB Molbiol, Berlin, Germany) with 6-carboxyfluorescein (FAM) label were designed using Primer3 software (version 0.4.0) (24), optimized with TIB Molbiol, and used in RT-PCR detection. Probes were detected in channel F1 of the LightCycler 1.2 (Roche, West Sussex, United Kingdom).

The amplification using BCFomp2/BCRomp2 and BpmF/BpmR2 primers against 31 total sequenced *Bacillus* strains (*B. cereus* ATCC 14579, ATCC 10987, ZK, AH187, B4264, G9842, AH820, Q1, and 03BB102; *B. anthracis* strain Ames, strain Ames 0581, strain Sterne, strain CDC 684, and strain A0248; *B. thuringiensis* 97-27, strain Al Hakam, and BMB171; *B. weihenstephanensis* KBAB4; *B. cereus* subsp. *cytotoxis* NVH 391-98; *B. subtilis* subsp. *subtilis* strain 168; *B. halodurans* C-125; *B. licheniformis* ATCC 14580 and DSM 13;*B. clausii* KSM-K16; *B. amyloliquefaciens* FZB42; *B. pumilus* SAFR-032; *B. pseudofirmus* OF4; *B. megaterium* QM B1551 and DSM 319; *B. tusciae* DSM 2912; and *B. selenitireducens* MLS10) were analyzed using the software program *in silico* PCR available on line at http://in silico.ehu.es/PCR/.

Construction of plasmid. The fragment of the *motB* amplicon (575 bp) of *B. cereus* ATCC 14579 and *B. weihenstephanensis* WSBC 10389 was generated by PCR using the primer set BCFomp1/BCRomp1 (18) and cloned into the pGEM-T Easy vector system (Promega, Heidelberg, Germany) using the manufacturer's instructions. BpmF/BpmR2 primers were used to generate the 217-bp fragment of *B. pseudomycoides* WS 3118 encoding a hypothetical protein. The PCR product was ligated with 50 ng of vector DNA at a 1:3 molar ratio of vector/insert. The ligated product was transformed into *E. coli* JM 109 cells by electroshock (Electroporator Easyject Prima; Equibio, Ashford, United Kingdom). Transformants were selected on LB plates with ampicillin (100 μg/ml) and analyzed by PCR for the target gene. BCFomp1/BCRomp1 and BCFomp2/BCRomp2 primers specific to the cloned fragment *motB* gene and BpmF/BpmR2 primers (Table 2) specific to 217-bp PCR product were used to confirm the correct insert. DNA was extracted by suspending a 3-mm loopful of colony in distilled water and boiled at 95°C for 15 min. After centrifugation, 5 μl of supernatant was used as the DNA template. Only clones positive for the insert were used to purify the recombinant plasmid (pGEM-*motB*, pGEM-*Bpm*) using the Promega plasmid kit (Wizard plus purification system; Promega, Heidelberg, Germany). PCRs with the same set of primers were repeated for confirmation. Two vectors with the insert for each clone were sequenced by Eurofins MWG Operon (Martinsried, Germany). Both T7 (forward) and SP6 (reverse) primers were used for sequencing.

Determination of recombinant plasmid DNA concentration and template copy number. DNA concentration was measured using a UV-Vis NanoDrop 1000 (Fisherbrand, Fisher Scientific). The calculation of the gene copy numbers was based on the assumption that the average weight of a base pair was 650 Da. The

TABLE 1. Bacterial strains used in this study

Species	No. of strains	Bacterial strains	RT-PCR result with BCFomp2/BCRomp2 and:		RT-PCR result with BpmF/ BpmR2 and Bpm-FAM-1	Collection source[a]
			MotB-FAM-1	MotB-FAM-2		
Bacillus cereus	25	NCTC 7464	+	−	−	NCTC, ATCC, DSMZ, BGSC, MIHE, UL laboratory strains
		ATCC 14579	+	−	−	
		ATCC 13472	+	−	−	
		ATCC 19637	+	−	−	
		ATCC 23261	+	−	−	
		UW 85	+	−	−	
		F17289	+	−	−	
		F16959	+	−	−	
		DSM 4312	+	−	−	
		6A1	+	−	−	
		6A2	+	−	−	
		6A3	+	−	−	
		6A6	+	−	−	
		6A15	+	−	−	
		6A48	+	−	−	
		6A51	+	−	−	
		BCSUL1	+	−	−	
		BCSUL2	+	−	−	
		BCFUL1	+	−	−	
		BCFUL2	+	−	−	
		BCFUL4	+	−	−	
		BCFUL6	+	−	−	
		BMeSUL1	+	−	−	
		BCMUL1	+	−	−	
		BCMUL2	+	−	−	
Bacillus thuringiensis	22	DSM 2046	+	−	−	DSMZ, ATCC, MIHE, BGSC, A. Bishop, UL laboratory strains
		DSM 6017	+	−	−	
		DSM 6025	+	−	−	
		DSM 6029	+	−	−	
		DSM 6032	+	−	−	
		DSM 6094	+	−	−	
		DSM 6102	+	−	−	
		DSM 6107	+	−	−	
		BT1	+	−	−	
		ATCC 35646	+	−	−	
		T7-101	+	−	−	
		T07-113	+	−	−	
		T7-055	+	−	−	
		T07-202	+	−	−	
		H36	+	−	−	
		BT3	+	−	−	
		T07-146	+	−	−	
		T07-148	+	−	−	
		33	+	−	−	
		T07-001	+	−	−	
		BTSUL7	+	−	−	
		BTFUL1	+	−	−	
Bacillus anthracis	4	34F2	+	−	−	MIHE
		1583	+	−	−	
		1584	+	−	−	
		211	+	−	−	
Bacillus sp. Ba813^{+}	11	7 (II/3)	+	−	−	MIHE
		11 (9594/3)	+	−	−	
		13 (PC1)	+	−	−	
		15 (11614-2)	+	−	−	
		16 (PJ572)	+	−	−	
		18 (T2 97-76)	+	−	−	
		19 (T5 97-77)	+	−	−	
		22 (BU−1B)	+	−	−	
		25 (97-27)	+	−	−	
		31	+	−	−	
		3403	+	−	−	
Bacillus mycoides	20	BMFUL1	−	+	−	BGSC, DSMZ, N. Raddadi, UL laboratory strains

Continued on following page

TABLE 1—*Continued*

Species	No. of strains	Bacterial strains	RT-PCR result with BCFomp2/BCRomp2 and:		RT-PCR result with BpmF/ BpmR2 and Bpm-FAM-1	Collection source[a]
			MotB-FAM-1	MotB-FAM-2		
		BMSUL1	−	+	−	
		BMSUL2	−	+	−	
		6A11	−	+	−	
		6A12	−	+	−	
		6A13	−	+	−	
		6A14	−	+	−	
		6A19	−	−	+	
		6A20	−	+	−	
		6A47	−	+	−	
		6A49	+	−	−	
		6A68	+	−	−	
		DSM 307	−	−	+	
		DSM 309	−	+	−	
		DSM 384	−	+	−	
		Nov1	−	−	+	
		Nov2	−	−	+	
		A81	−	−	+	
		BiF	−	+	−	
		BmF	−	+	−	
Bacillus pseudomycoides	10	WS 3118	−	−	+	DSMZ, M. Ehling-Schulz, N. Raddadi
		WS 3119	−	−	+	
		WS 3120	−	−	−	
		DSM 12442	−	−	+	
		DSM 12443	−	−	+	
		B346	−	−	+	
		B618	−	−	+	
		TP1	−	−	+	
		DA	−	−	+	
		A82	−	−	+	
Bacillus mycoides/*Bacillus pseudomycoides*	11	PID 1/21	+	−	−	I. Święcicka
		PID 2/43	+	−	−	
		PID3/2	+	−	−	
		GRD 1/17	−	−	+	
		GRD 2/71	−	−	+	
		1/1	+	−	−	
		1/2	−	−	+	
		12/2	+	−	−	
		17/3	−	−	+	
		22/2	−	+	−	
		29/2	−	+	−	
Bacillus weihenstephanensis	16	WSBC 10389	−	+	−	L. P. Stenfors, DSMZ, BGSC, UL laboratory strains
		WSBC 10392	−	+	−	
		WSBC 10416	−	+	−	
		WSBC 10405	−	+	−	
		WSBC 10201	−	+	−	
		WSBC 10202	−	+	−	
		WSBC 10045	−	+	−	
		WSBC 10206	−	+	−	
		DSM 11821	−	+	−	
		BWMUL1	−	+	−	
		BWMUL2	−	+	−	
		BWMUL3	−	+	−	
		BWMUL4	−	+	−	
		BWMUL5	−	+	−	
		BWMUL6	−	+	−	
		BWMUL7	−	+	−	
Bacillus pumilus	1	BPFUL1	−	−	−	UL laboratory strain
Bacillus amyloliquefaciens	1	10A6	−	−	−	BGSC
Bacillus licheniformis	3	BLFUL1 BLMUL1 ATCC 12759	−	−	−	ATCC, UL laboratory strains
Bacillus circulans	1	BCiFUL1	−	−	−	UL laboratory strain
Bacillus subtilis	2	BSFUL1 BSFUL2	−	−	−	UL laboratory strain
Brevibacillus brevis	1	BRFUL1	−	−	−	UL laboratory strain
Lactobacillus acidophilus	2	5378, DPC 6060	−	−	−	K. Jordan
Lactobacillus casei	1	DPC 6059	−	−	−	K. Jordan

Continued on following page

TABLE 1—*Continued*

Species	No. of strains	Bacterial strains	RT-PCR result with BCFomp2/BCRomp2 and:		RT-PCR result with BpmF/BpmR2 and Bpm-FAM-1	Collection source[a]
			MotB-FAM-1	MotB-FAM-2		
Lactococcus lactis	2	HP, 3054	–	–	–	K. Jordan
Ralstonia pickettii	3	NCTC 11149, ULM001, ULM003	–	–	–	NCTC, UL laboratory strain
Staphylococcus aureus	2	ATCC 25923, ATCC 29213	–	–	–	ATCC
Campylobacter jejuni	2	ULCV38 ULCV48	–	–	–	UL laboratory strain
Salmonella enterica serovar Typhimurium	2	Derby 180, NCTC 74	–	–	–	NCTC, RVL
Yersinia enterocolitica	1	YEFUL1	–	–	–	UL laboratory strain
Listeria monocytogenes	1	LMFUL1	–	–	–	UL laboratory strain
Escherichia coli	1	ATCC 25922	–	–	–	ATCC
Proteus mirabilis	1	ATCC 29906	–	–	–	ATCC

[a] UL, University of Limerick; NCTC, National Collection of Type Cultures, London, United Kingdom; BGSC, *Bacillus* Genetic Stock Center; ATCC, American Type Culture Collection; DSMZ, German Collection of Microorganisms and Cell Cultures; MIHE, Military Institute of Hygiene and Epidemiology, Puławy, Poland; and RVL, Regional Veterinary Laboratory, Limerick, Ireland.

amount of the template expressed in molecules (gene copies) of DNA and the conversion of the mass to molecules was carried out using the following formula (3): (mass [in grams] × Avogadro's number)/(average molecular weight of base *x* template length) = molecules (gene copies) of DNA.

Tenfold serial dilutions of the standard were made in sterile water, aliquoted, and stored at −20°C until use. Each aliquot was thawed only once for the reaction.

Real-time PCR assay and product detection. Real-time PCR amplification was performed using the LightCycler and FastStart DNA master hybridization probe kit (Roche Diagnostics, Lewes, Sussex, United Kingdom). A typical 20-μl PCR mixture for the TaqMan-based PCR assay contained 4 μl LightCycler TaqMan reaction mix, 0.8 μM each primer, 0.07 μM specific probe (when probes were used separately), and 5 μl of the DNA. When the two probes MotB-FAM-1 and MotB-FAM-2 were combined in one reaction mix, their concentrations were 0.025 and 0.035 μM, respectively. When the reaction mix included three designed probes, their concentrations were the following: 0.035 μM MotB-FAM-1, 0.035 μM MotB-FAM-2, and 0.035 μM Bpm-FAM-1. The final concentration of each primer (BCFomp2/BCRomp2/BpmF/BpmR2) in one reaction mix was 0.8 μM. No-template controls (NTC) containing 5 μl water instead of DNA were included in each run to detect contamination. Samples initially were incubated at 95°C for 10 min to denature the template DNA and to activate the FastStart *Taq* DNA polymerase. The amplification cycle was the same for both sets of primers and was the following: 35 cycles at 95°C for 10 s, 59°C for 40 s, and 72°C for 01 s. The transition rate of temperature was set as 20°C for denaturation to annealing, 20°C from annealing to extension, and 20°C from extension to denaturation. Two different primer pairs had the identical optimal PCR annealing temperature. The intensity of fluorescence was monitored at the end of each extension step. The LightCycler software (version 4.1) produced the standard curve by measuring the crossing points (Cp) of each standard and plotting them against the logarithmic values of the construction.

Spiking milk samples with *B. cereus* group species with known gene copy numbers. Fat and nonfat milk were purchased from local stores and tested for bacterial contamination, including the *B. cereus* group species, by spread plating the milk onto PEMBA (*B. cereus* selective agar base; Oxoid, Basingstoke, United Kingdom) agar plates and PCA (plate count agar; Difco). After that, milk was autoclaved at 121°C for 15 min, treated with UV light (254 nm) for 30 min and spread plated again on the same plates, and incubated overnight at 35°C. PEMBA plates were left for an additional 24 h at room temperature. No *B. cereus* bacteria were detected. The artificial contamination of milk samples with overnight cultures of *B. cereus* ATCC 14579, *B. thuringiensis* DSM 6017, *B. weihenstephanensis* WSBC 10389, and *B. pseudomycoides* WS 3118 then was carried out. Milk was inoculated with a calculated amount of bacterial cells (CFU) per ml of contaminated milk to achieve 10^5 gene copy numbers per 5 μl for RT-PCR. In the DNA extraction from spiked milk, 100 μl of sample was used in the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) and Genomic Mini AX food kit (A&A Biotechnology, Gdynia, Poland), and 5 μl of DNA was used as a template during the RT-PCR to check the efficiency of the kits.

RESULTS

Results of *in silico* PCR. Two sets of primers (BCFomp2/BCRomp2 and BpmF/BpmR2) and three TaqMan probes (MotB-FAM-1, MotB-FAM-2, and Bpm-FAM-1) were designed based on the nucleotide sequence of the *motB* gene of the *B. cereus* group species and a hypothetical gene from *B. pseudomycoides* (Table 2) using Primer3 software (version 0.4.0) (24). An *in silico* PCR program was used to confirm the newly designed primers. *In silico* PCR integrates complete genome sequencing only and allowed for only two mismatches between primers and template, making the stringency of the PCR high.

The primer set BCFomp2/BCRomp2 gave *in silico* results for 18 total sequenced *B. cereus* group strains available in the National Centre for Biotechnology Information (NCBI) and

TABLE 2. Primers and probes used for RT-PCR amplification

Oligonucleotide	Sequence (5′–3′)	Target gene	Target bp in amplified product	Primer or probe
BCFomp2	CGCCTCGTTGGATGACG	*motB*	1 to 17	Primer
BCRomp2	GATATACATTCACTTGACTAATACCG	*motB*	260 to 285	Primer
BpmF	TAATTTAGGGGGGCATCTTTACTTTTC	*bpm*	1 to 27	Primer
BpmR2	CTATACCCAAAACTTAGATATGCTC	*bpm*	193 to 217	Primer
MotB-FAM-1	FAM-TTCAAGCATCTTTGACAATTTTACTGCAT-BBQ	*motB*	113 to 86	Probe
MotB-FAM-2	FAM-TTCAAGCATCTTYGATAATTTTACTGTAT-BBQ (Y = T/C)	*motB*	113 to 86	Probe
Bpm-FAM-1	FAM-CTGAGAAGGTAGTCATACGCTATACATG-BBQ	*bpm*	161 to 134	Probe

the *in silico* database, while *B. weihenstephanensis* KBAB4 (GenBank accession number NC_010184) gave a positive result with two allowed mismatches; however, none of them were located in the 3′ end. The *B. cereus* subsp. *cytotoxis* NVH 391-98 strain was analyzed, and the nucleotide sequences of the conserved genes varied greatly from the conserved genes of the 18 complete genome sequences and showed negative results for both pairs of primers tested, allowing for mismatches. These results confirmed results published earlier (18) that this strain, although identified as *B. cereus*, is different from other members of the *B. cereus* group. Our results confirm that this strain should be a representative of a novel bacterial species.

When this study was under way, there were no *B. pseudomycoides* strains with complete genome sequences available for *in silico* PCR. *In silico* positive testing of the BpmF/BpmR2 could not be determined and was checked only for negative results against the 31 available strains belonging to the *Bacillus* genus. None of those strains presented a 217-bp amplification product, even with two allowed mismatches. Our results therefore demonstrated that the newly designed primers presently are specific for *B. pseudomycoides*.

Amplification of DNA from *B. cereus* group species and specificity of the assay. Oligonucleotide primers and hydrolysis probes were designed to specifically identify the *B. cereus* group species using Primer3 software. Primers BCFomp1/BCRomp1 were reported previously (18). New primers BCFomp2/BCRomp2 and BpmF/BpmR2 generate 285- and 217-bp products, respectively (Table 2). Designed TaqMan probes MotB-FAM-1, MotB-FAM-2, and Bpm-FAM-1 also are listed in Table 2. Experimental thermocycling conditions were established, including PCR primer and hydrolysis probe concentrations, to enable reliable RT-PCR amplification on the LightCycler. BCFomp2/BCRomp2 primers were specific for the detection of *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, and *Bacillus* sp. strain Ba813^{+}. None of the *B. pseudomycoides* strains gave a positive reaction using these primers.

Two probes, MotB-FAM-1 and MotB-FAM-2, used in one RT-PCR with BCFomp2/BCRomp2 primers generated a fluorescence signal to identify *B. cereus* group species except the *B. pseudomycoides* strains. The two probes used together at final concentrations of 0.025 μM MotB-FAM-1 and 0.035 μM MotB-FAM-2 gave the same sensitivity as that when used separately (see Fig. S1A to C in the supplemental material). Using only the MotB-FAM-2 probe, designed specifically for *B. weihenstephanensis*, in the RT-PCR, it was possible to differentiate 16 tested *B. weihenstephanensis* strains from other strains belonging to the *B. cereus* group with nonrhizoid growth. Some *B. mycoides* strains identified in the reaction using probe MotB-FAM-2 were easily discriminated from *B. weihenstephanensis* by their rhizoid growth on agar plates. Three single-nucleotide polymorphisms in the probe were enough to achieve this result. One nucleotide was wobbled (Y = T/C) (Table 2), because in this position *B. weihenstephanensis* strains presented double profiles.

The evaluation of the specificity of the real-time PCR assay was performed by including DNA from a wide range of bacteria listed in Table 1. One hundred nineteen strains belonging to *B. cereus* group species and 27 other *Bacillus* or non-*Bacillus* species were used as control isolates. All control strains were negative in reactions using BCFomp2/BCRomp2 and BpmF/BpmR2 primers.

The RT-PCR assay with MotB-FAM-1 and MotB-FAM-2 TaqMan probes successfully detected 100 tested *B. cereus* group species strains. Seven from 11 strains of *B. mycoides*/*B. pseudomycoides* that were not previously discriminated showed a positive reaction with either MotB-FAM-1 or MotB-FAM-2 probe. Five strains, Nov 1, Nov 2, 6A19, A81, and DSM 307, earlier classified as *B. mycoides*, did not present the fluorescence signal and PCR product during experimentation. Negative results were obtained for 27 other *Bacillus* and non-*Bacillus* strains.

In the detection and differentiation of *B. pseudomycoides* strains, the reaction with primers and probe based on the gene encoding the hypothetical protein was successful. Newly designed BpmF/BpmR2 primers and Bpm-FAM-1 TaqMan probe were specific for the amplification and detection of the 217-bp product. Strains that showed positive reactions with BCFomp2/BCRomp2 primers and MotB-FAM-1 or MotB-FAM-2 probe did not present the amplicon with BpmF/BpmR2 primers or a signal from Bpm-FAM-1 probe (see Fig. S1D in the supplemental material). Ten of 11 tested *B. pseudomycoides* strains showed positive reactions and the expected products. One strain, WS 3120, was negative with BpmF/BpmR2 and BCFomp2/BCRomp2 primers.

Four of the 11 *B. mycoides*/*B. pseudomycoides* strains that did not present the positive reaction with BCFomp2/BCRomp2 primers showed positive results with Bpm-FAM-1 probe and BpmF/BpmR2 primers. This result demonstrated that the described assay can be used for the differentiation of *B. pseudomycoides* from *B. mycoides* strains, which to date can be distinguished only using fatty acid analysis.

Five strains, Nov 1, Nov 2, 6A19, A81, and DSM 307, earlier classified as *B. mycoides*, were not amplified with BCFomp2/BCRomp2 primers, but positive fluorescence signals were obtained with Bpm-FAM-1 probe in an RT-PCR. The size of the product amplified with BpmF/BpmR2 was as expected (217 bp). These results demonstrate that the five strains possibly are *B. pseudomycoides* and not *B. mycoides*.

When three designed TaqMan probes were used in one reaction mix, it was possible to detect and identify 118 tested *B. cereus* group species. Probes used in the concentrations 0.035 μM MotB-FAM-1, 0.035 μM MotB-FAM-2, and 0.035 μM Bpm-FAM-1 showed the same efficiency as when used separately (see Fig. S1E in the supplemental material). RT-PCR products showed the expected bands on a 1.5% agarose gel, with 217- and 285-bp products (see Fig. S2 in the supplemental material). None of the 27 other *Bacillus* and non-*Bacillus* strains showed a positive reaction.

Sensitivity of the real-time PCR assay. A 575-bp fragment of the *motB* gene of *B. cereus* ATCC 14579 and *B. weihenstephanensis* WSBC 10389 and a 217-bp fragment of *B. pseudomycoides* WS 3118 were cloned into pGEM-T easy vector. The positive transformants were verified by sequencing. Serial dilutions were applied to define the sensitivity of the assay. The detection limit of the RT-PCR assays was determined using these recombinant plasmids. A dilution series (10^{10} to 1 gene copy/20-μl reaction) of recombinant plasmids was tested in triplicate by the real-time assay using the LightCycler system (Roche). The obtained Cp values were plotted against log-

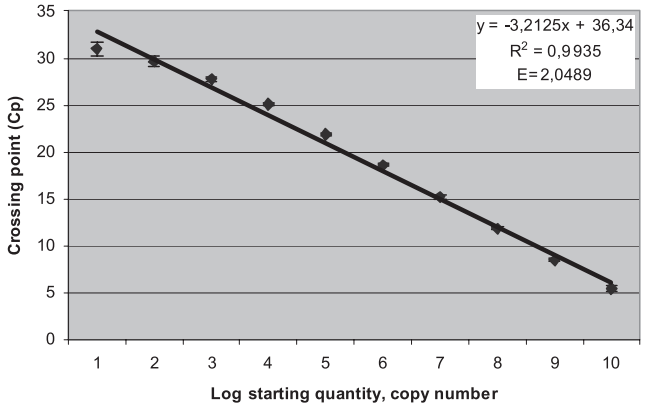

FIG. 1. Standard curve for determination of *motB* gene copy numbers using BCFomp2/BCRomp2 primers and MotB-FAM-1/MotB-FAM-2 probes. Crossing-point values were plotted against the log of the initial template DNA concentration. Plus or minus 1 standard deviation is indicated for each gene copy number. The average efficiency of every real time amplification was 2.0489 ± 0.078.

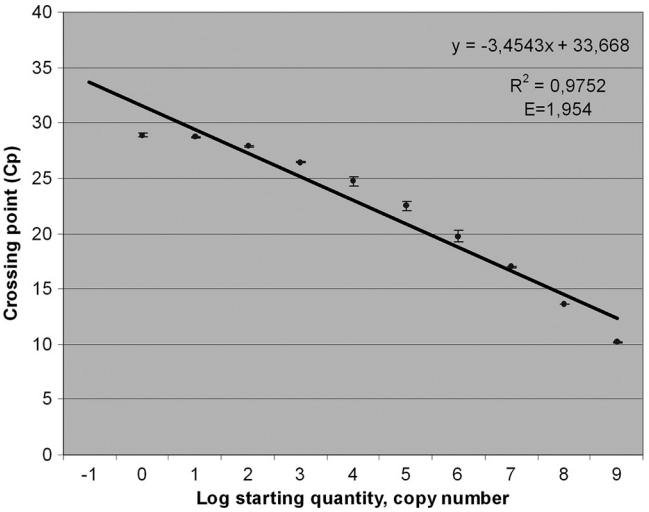

FIG. 2. Standard curve for determination of gene copy numbers of *B. pseudomycoides* based on hypothetical gene using BpmF/BpmR2 primers and Bpm-FAM-1 probe. Crossing point values were plotted against the log of the initial template DNA concentration. Plus or minus 1 standard deviation is indicated for each gene copy number. The average efficiency of every real-time amplification was 1.95 ± 0.007.

transformed concentrations of serial 10-fold dilutions of the target (Fig. 1 and 2).

Significant signals were detected between 10^{10} to 10 gene copy numbers per reaction with BCFomp2/BCRomp2 primers (Fig. 1) and between 10^9 and 1 gene copy numbers per reaction with BpmF/BpmR2 primers (Fig. 2). For the target sequence, in the assay using BCFomp2/BCRomp2 primers, as little as 10 copies per reaction could be detected, whereas during the standard PCR (data not shown) with the same primers the limit of the detection was 10^3 gene copy numbers per reaction (25 μl). Similar results were found with BpmF/BpmR2 in ordinary PCR, where the limit of detection was 10^3 gene copy numbers per reaction (25 μl), and in the real-time assay 1 gene copy number was detected.

Data analysis showed that in all experiments with BCFomp2/BCRomp2 primers and MotB-FAM-1 probe, the mean amplification average efficiency was 2.0489 ± 0.078. This corresponds to 104.8% efficiency, where the average slope was −3.21.

The correlation (R^2) between the Cp value and the $\log_{\text{gene copy number}}$ was 0.99. This linear relationship makes the Cp value a reliable way to estimate the gene copy number.

The efficiency of the MotB-FAM-2 probe was compared to the efficiency of the MotB-FAM-1 probe. Serial dilutions of recombinant plasmid with a fragment of the *motB* gene of *B. weihenstephanensis* WSBC 10389 showed the same averaged Cp values, and the limit of the detection was 10 gene copy numbers per reaction.

The analysis with BpmF/BpmR2 primers and the Bpm-FAM-1 TaqMan probe showed an average slope of −3.45. The efficiency of amplifications was 1.95 ± 0.007, which corresponded to 95%. The correlation (R^2) between Cp value and $\log_{\text{gene copy number}}$ was 0.97.

Real-time analysis of artificially contaminated milk. The detection of the *B. cereus* group species was evaluated in milk. Milk samples, inoculated as described above, were used for DNA extraction directly by using two column-based systems: a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) and a Genomic Mini AX food kit (A&A Biotechnology, Gdynia, Poland). Both kits worked with similar efficiencies. Total DNA extracted from 100 μl of spiked milk (with a known number of CFU) gave similar results during the RT-PCR with both sets of primers. Achieved Cp values for the DNA extracted from the same artificially contaminated milk sample had similar results using both extraction kits. The accuracy of the assays was assessed by comparing the values of the gene copy numbers of inoculated cells to the extrapolated values using earlier generated standard curves.

After inoculation with bacterial culture, the CFU was calculated to achieve 10^5 gene copy number in 5 μl. Kits were able to extract 5.7×10^4 to 7.83×10^4 gene copy numbers from fat milk and 8.91×10^4 to 1×10^5 gene copy numbers from nonfat milk (Table 3). Relative accuracy values ranging from 57.4 to 105% were observed in milk samples using standard curves.

TABLE 3. Accuracy of the real-time PCR assays for the quantification of *B. cereus* group species in milk

Quantity of DNA used for milk contamination (gene copy numbers)	Estimated quantity of DNA after kit extraction (gene copy numbers)	Milk type used for contamination	Relative accuracy (%)
1×10^5	$7.78 \times 10^4 \pm 0.14 \times 10^4$[b]	Fat	77.8
1×10^5	$7.58 \times 10^4 \pm 0.14 \times 10^4$[a]	Fat	75.8
1×10^5	$6.12 \times 10^4 \pm 0.08 \times 10^4$[a]	Fat	61.2
1×10^5	$5.74 \times 10^4 \pm 0.08 \times 10^4$[b]	Fat	57.4
1×10^5	$1.05 \times 10^5 \pm 0.19 \times 10^5$[a]	Nonfat	105.0
1×10^5	$9.84 \times 10^4 \pm 0.09 \times 10^4$[b]	Nonfat	98.4
1×10^5	$9.05 \times 10^4 \pm 0.08 \times 10^4$[b]	Nonfat	90.5
1×10^5	$8.87 \times 10^4 \pm 0.12 \times 10^4$[a]	Nonfat	88.7

[a] Genomic Mini AX food kit (A&A Biotechnology, Gdynia, Poland).
[b] DNeasy blood and tissue kit (Qiagen, Hilden, Germany).

DISCUSSION

The genomes of the *B. cereus* group species are closely related, and attempts to date to design DNA primers allowing differentiation has proven difficult. Lechner et al. (11) designed primers BcF2 and CSPU3 against the *cspA* gene encoding a major cold shock protein. These primers allowed the differentiation of the *B. weihenstephanensis* strains by PCR. However, their results did not demonstrate the quantification and limit of detection. Gas-chromatographic fatty acid methyl ester analysis is the only method to differentiate the *B. pseudomycoides* from *B. mycoides* strains. This method distinguishes *B. mycoides* by differences in 12:0 iso and 13:0 anteiso fatty acid levels (14, 17).

RT-PCR is sensitive and provides a quantitative method for the determination of the copy number of PCR templates (29), plus the earlier observation of PCR amplification. The specific amplification of target sequences is directed by custom-designed primers and probes. This provides an advantage over traditional PCR in food-borne pathogen detection.

The aim of this study was to provide a reliable method to measure the amount of *B. cereus* group species in milk samples. We evaluated real-time PCR with a LightCycler (Roche) for the detection and quantification of the *B. cereus* group species. Two different sets of primers (BCFomp2/BCRomp2 and BpmF/BpmR2) and three TaqMan fluorescence-labeled probes (MotB-FAM-1, MotB-FAM-2, and Bpm-FAM-1) were used to identify this bacterial group. The BCFomp2/BCRomp2 primers and MotB-FAM-1/MotB-FAM-2 identified the *B. cereus* group species except *B. pseudomycoides* strains. The addition of probe MotB-FAM-2 to primers in the reaction mix allowed for the differentiation of *B. weihenstephanensis* strains from the *B. cereus* group species with nonrhizoid growth. *B. mycoides* strains that were identified in reactions using this probe were easily discriminated from *B. weihenstephanensis* by their rhizoid growth on agar plates. The BpmF/BpmR2 primers and Bpm-FAM-1 probe made possible the differentiation of *B. pseudomycoides* strains without fatty acid analysis; however, one strain (WS 3120) classified as *B. pseudomycoides* did not present the expected product size (217 bp). This may be due to a single-nucleotide polymorphism (SNP) that caused the lack of primer annealing during PCR.

For each assay, the standard curve was generated and the limit of the detection estimated. The reactions were reproducible and sensitive, able to detect 10 gene copy numbers in amplification with BCFomp2/BCRomp2 primers and 1 gene copy number with BpmF/BpmR2 primers. Nonspecific products were not observed in these experiments, demonstrating that the designed primers are appropriate for LightCycler quantification. The products with expected sizes (217 and 285 bp) were observed on 1.5% agarose gel after each RT-PCR (see Fig. S2 in the supplemental material).

This is the first description of a PCR technique that is able to distinguish *B. pseudomycoides* from other members of the *B. cereus* group species and the first real-time PCR using the *motB* gene as the diagnostic target. After the various amplification parameters were optimized, the assays proved to be sensitive, reproducible, and quantitative. The assays performed well, not just with purified templates but also with DNA extracted from milk samples artificially contaminated. The testing of environmental samples may give false positives due to residual naked DNA.

In conclusion, a rapid and sensitive quantitative method for the detection of *B. cereus* group species was developed using TaqMan hydrolysis probes. This assay has the potential for the detection of *Bacillus cereus* group species including *B. cereus*, *B. thuringiensis*, *B. anthracis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis*, and *Bacillus* sp. strain Ba813$^+$. In one reaction mix it was possible to differentiate *B. weihenstephanensis* and *B. pseudomycoides* strains.

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