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Real-Time PCR Detection of *Paenibacillus* spp. in Raw Milk To Predict Shelf Life Performance of Pasteurized Fluid Milk Products

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Psychrotolerant sporeformers, specifically *Paenibacillus* spp., are important spoilage bacteria for pasteurized, refrigerated foods such as fluid milk. While *Paenibacillus* spp. have been isolated from farm environments, raw milk, processing plant environments, and pasteurized fluid milk, no information on the number of *Paenibacillus* spp. that need to be present in raw milk to cause pasteurized milk spoilage was available. A real-time PCR assay targeting the 16S rRNA gene was designed to detect *Paenibacillus* spp. in fluid milk and to discriminate between *Paenibacillus* and other closely related spore-forming bacteria. Specificity was confirmed using 16 *Paenibacillus* and 17 *Bacillus* isolates. All 16 *Paenibacillus* isolates were detected with a mean cycle threshold (C_T) of 19.14 ± 0.54. While 14/17 *Bacillus* isolates showed no signal ($C_T > 40$), 3 *Bacillus* isolates showed very weak positive signals ($C_T = 38.66 \pm 0.65$). The assay provided a detection limit of approximately 3.25×10^1 CFU/ml using total genomic DNA extracted from raw milk samples inoculated with *Paenibacillus*. Application of the TaqMan PCR to colony lysates obtained from heat-treated and enriched raw milk provided fast and accurate detection of *Paenibacillus*. Heat-treated milk samples where *Paenibacillus* (≥1 CFU/ml) was detected by this colony TaqMan PCR showed high bacterial counts (>4.30 log CFU/ml) after refrigerated storage (6°C) for 21 days. We thus developed a tool for rapid detection of *Paenibacillus* that has the potential to identify raw milk with microbial spoilage potential as a pasteurized product.

Despite advances in food preservation techniques, bacterial spoilage remains a leading cause of global food loss (14). Nearly one-third of all food produced worldwide is estimated to be lost postharvest, and much of this loss can be attributed to microbial spoilage (16). Dairy products constitute one of the leading sectors impacted by food loss in the United States, as nearly 20% of conventionally pasteurized (high temperature, short time [HTST]) fluid milk is discarded prior to consumption each year (23). In the United States, the shelf life of fluid milk ranges from approximately 1 to 3 weeks. Most consumer complaints result from the growth of psychrotolerant bacteria, typically, either non-spore-forming Gram-negative rods or Gram-positive spore-forming bacteria (12, 18, 19, 28, 34, 39). The presence of psychrotolerant, non-spore-forming bacteria (e.g., *Pseudomonas*) in pasteurized milk indicates either inadequate heating of the milk or, more commonly, postpasteurization contamination (6). Therefore, pasteurized milk contamination with *Pseudomonas* and other non-spore-forming bacteria can be controlled or eliminated by adhering to pasteurization specifications for minimum time and temperature combinations (8) and by adhering to proper sanitation and equipment maintenance protocols, particularly with respect to milk filler sites (33). Conversely, Gram-positive psychrotolerant sporeformers can survive pasteurization as spores, germinate, and then grow during refrigerated storage to numbers capable of causing off flavors or curdling of milk (5, 20, 34, 36).

The predominant Gram-positive spore-forming bacteria isolated from milk are *Bacillus* spp. and *Paenibacillus* spp. Both *Bacillus* spp. and *Paenibacillus* spp. have been isolated from farm environments (e.g., soil, water, and feed), raw milk, dairy-processing plants, and pasteurized milk (5, 19, 38, 42). In HTST pasteurized milk, when postpasteurization contamination is excluded, *Bacillus* spp. represent the predominant bacteria found early in the shelf life (<7 days). However, during refrigerated storage of pasteurized milk, *Paenibacillus* spp. become the predominant spoilage organisms, typically representing over 95% of the bacterial population identified late in shelf life (>10 days) (35). *Paenibacillus* spp. are generally present in very low numbers in raw milk and early in pasteurized milk shelf life, yet they can reproduce to high numbers during cold storage. Numerous microbiological tests have been applied to raw milk with the goal of predicting the shelf life performance of the milk, but none are adequately predictive of HTST pasteurized fluid milk shelf life (25). This, in part, is likely due to the inability of traditional microbiological tests to identify or quantify low levels (<10 spores/ml) of *Paenibacillus* spp. Currently, only limited phenotypic methods are available to differentiate between *Bacillus* spp. and closely related *Paenibacillus* spp., including cold growth, which requires 7 to 10 days of incubation, and lactose utilization, which can be difficult to interpret and is not a consistent indicator of sporeformer genus (21).

The aim of this study was to develop a novel PCR assay targeting the 16S rRNA gene so that specific identification of *Paenibacillus* spp. could be performed rapidly. The objectives of this study were to (i) design primers and a probe for detection of *Paenibacillus* spp. while limiting nonspecific detection of closely related *Bacillus* spp., (ii) validate the primers and probe using a real-time PCR assay on select *Paenibacillus* and *Bacillus* isolates from a collection of over 1,200 isolates from fluid milk and dairy environments, and (iii) develop a systematic approach to aid in identification of *Paenibacillus* spp. from raw milk. The results of this study will provide the food industry with an assay to monitor the quality

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TABLE 1 TaqMan primers and probe designed for detection of *Paenibacillus* spp. 16S rRNA gene

Primer or probe	Sequence (5′–3′)	Denaturation temp (°C)[a]
Primers		
MR-18_16S F	AAA TCA TCA TGC CCC TTA TG	61.1
MR-19_16S R	CGA TTA CTA GCA ATT CCG ACT	59.8
MR-21_16S probe	CGT ACT ACA ATG GCC GGT ACA ACG	69.6

[a] Denaturation temperatures were calculated using the Sigma-Aldrich DNA calculator (Sigma-Aldrich, St. Louis, MO).

of raw milk. This assay may even be adapted to aid in the development of strategies to limit spoilage of other pasteurized, refrigerated foods like vegetable purees (2, 15) and fermented beverages (17). Finally, our assay has potential for use as a screening tool to isolate novel enzyme-producing *Paenibacillus* spp. from other foods (31) and the natural environment (37), as previous identification of *Paenibacillus* strains has led to the discovery of many compounds with promising applications in agriculture and medicine (29).

MATERIALS AND METHODS

TaqMan probe and primer design. *rpoB* and 16S rRNA gene alignments were performed in the MegAlign program (DNAStar, Inc. Madison, WI). *rpoB* sequences (632 bp) from a total of 1,288 isolates representing the *Paenibacillus* genus ($n = 737$), the *Bacillus* genus ($n = 467$), and genera formerly classified as *Bacillus* (e.g., *Viridibacillus*; $n = 84$) collected from farm environments, raw milk, fluid milk-processing plants, and HTST fluid milk products were analyzed to identify unique subtypes (21). *rpoB* sequences lacked sufficient conservation for design of TaqMan primers and probes that could detect all 737 *Paenibacillus* sequences represented in this collection. Therefore, alignments of partial (>600-bp) 16S rRNA gene sequences representing each of the 283 *rpoB* subtypes identified among these *Bacillus* and *Paenibacillus* spp. were used to create consensus sequences for (i) all *Paenibacillus* rRNA gene sequences and (ii) all non-*Paenibacillus* rRNA gene sequences (which includes sequences for *Bacillus*, *Lysinibacillus*, *Oceanobacillus*, *Psychrobacillus*, *Solibacillus*, and *Viridibacillus*). The consensus sequences were exported to Primer Express software (version 2.0.0; Applied Biosystems, Foster City, CA) for primer-probe design. Primers were designed to detect a conserved region within the *Paenibacillus* genus, while excluding *Bacillus* spp. and other closely related genera. The designed amplicon was 158 bp and included a 24-bp probe located 34 bp downstream from the 5′ end of the forward primer (see Table 1 for primers and probe). The probe was labeled on the 5′ end with 6-carboxyfluorescein (FAM) and the 3′ end with tetramethylrhodamine (TAMRA). Detailed information on all isolates used in this study, including 16S rRNA and *rpoB* sequences, can be accessed at www.pathogentracker.net.

TaqMan conditions. Real-time PCR was conducted in a 12.5-μl reaction mixture containing 6.25 μl of 2× TaqMan universal master mix (Applied Biosystems), 900 nM each forward and reverse primer (MR-18_16S F, MR-19_16S R), 250 nM TaqMan probe (MR-21_16S probe), and 1.375 μl water (Table 1). Each reaction mixture also contained 1.25 μl of 10× exogenous internal positive control (IPC) mix and 0.25 μl of 50× exogenous IPC DNA (PE Applied Biosystems). Finally, 1.0 μl of DNA template was added to each reaction mixture.

Real-time PCR was performed as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, and 40 cycles of denaturation at 95°C for 15 s, followed by extension and annealing at 60°C for 1 min. Threshold cycle (C_T) values represent the fractional PCR cycle in which fluorescence first passed a defined threshold for each sample amplification plot.

Bacterial isolate selection and assay validation. To validate the primers and probe, *Paenibacillus* and closely related *Bacillus* strains ($n = 9$ for each genus) were selected to represent the *rpoB* allelic types (ATs; i.e., those isolated ≥10 times) most frequently found among a collection of over 1,200 isolates collected from dairy farms, processing plants, raw milk, and pasteurized fluid milk (21). An additional 8 *Bacillus* strains (or strains of the closely related genera *Lysinibacillus*, *Oceanobacillus*, and *Viridibacillus*) and 7 *Paenibacillus* strains were included to represent genetic diversity (Table 2).

Pure bacterial cultures, stored in 15% glycerol at −80°C, were streaked onto brain heart infusion (BHI) agar (Difco, BD Diagnostics, Franklin Lakes, NJ) and grown for 18 to 24 h at 32°C. A single colony from plates that confirmed a pure culture was inoculated into 5 ml of BHI broth (Difco) and grown for 18 to 24 h at 32°C. Total genomic DNA was extracted from 1 ml of overnight culture according to the QIAamp DNeasy kit instructions (Qiagen Inc., Valencia, CA). Purified DNA concentrations were determined using Hoechst dye assay (Thermo Fisher Scientific, Wilmington, DE) and standardized to 10^5 genomes/μl.

To determine amplification efficiency, genomic DNA from *Paenibacillus odorifer* isolate FSL H7-592, representing the predominant spoilage allelic type (AT15), was serially diluted (10^7 to 10^1 genomes/ml) to produce a standard curve. Amplification efficiency (E) was calculated using the following equation: $[10^{(-1/\text{slope})}] - 1$.

Detection limit and raw milk sample testing. To determine the detection limit for *Paenibacillus* in the presence of other bacteria in a complex matrix, raw milk was obtained from the Cornell Teaching and Research Center (Dryden, NY). An overnight culture of *Paenibacillus odorifer* (FSL H7-592; AT15) was grown in BHI broth (Difco) and then centrifuged at 10,000 × *g* (5417C; Eppendorf, Hamburg, Germany) and resuspended in phosphate-buffered saline solution (Weber Scientific, Hamilton, NJ) before serial dilution into the raw milk; final *Paenibacillus* concentrations of 10^5, 10^4, 10^3, 10^2, and 10^1 CFU/ml of milk were achieved. A negative control containing no added *Paenibacillus* DNA was also included. To test the sensitivity of the PCR assay with a high background flora of mesophilic spore-forming bacteria typically found in milk, 100 ml of raw milk was heated to 80°C and held for 12 min, cooled, and then incubated at 32°C for 18 h before inoculation with *Paenibacillus odorifer* to achieve final *Paenibacillus* DNA concentrations of 10^5, 10^4, 10^3, 10^2, and 10^1 CFU/ml of enriched milk. A Norgen milk bacterial DNA isolation kit (Norgen Biotek Corp., Ontario, Canada) was used according to the manufacturer's instructions to extract DNA from 1 ml of all milk samples, and a final elution volume of 100 μl was obtained.

***Paenibacillus* assay testing of raw milk samples.** Approximately 400 ml of raw milk was collected from 10 different farms across upstate New York from March to May of 2011. Bulk tank raw milk samples ($n = 24$) were shipped on ice to the Cornell University Milk Quality Improvement Laboratory (Ithaca, NY). Upon receipt, raw milk was spore shocked (80°C for 12 min) to eliminate vegetative cells and activate spores (11). Approximately 150 ml of milk was aliquoted to 4 sterile 250-ml screw-cap Pyrex containers for aerobic plate count (APC) determination on the initial day of heat treatment and at days 7, 14, and 21 of storage at 6°C; APCs were performed according to *Standard Methods for the Examination of Dairy Products* (11). An additional 25 ml of spore-shocked milk was aliquoted into a sterile vial; this sample was incubated at 13°C for 48 h to encourage growth of *Paenibacillus* spp. while limiting *Bacillus* sp. growth. Bacterial counts in the 13°C enrichment were monitored immediately following the spore shock, at 24 h after spore shock, and 48 h after spore shock; bacterial counts were determined by plating 1 ml of milk over 5 BHI medium plates (200 μl per plate) supplemented with bromo-chloro-indolyl galactopyranoside (X-Gal; 100 mg/liter; Gold Biotechnology, St. Louis, MO). Plates were incubated at 32°C for 24 h before enumeration. Plating onto BHI agar supplemented with X-Gal allowed simultaneous APC determination and identification of β-galactosidase (β-Gal)-positive spore-forming bacteria, which, in milk, have generally been found to be *Paenibacillus* spp. (21). From APC plates, both X-Gal-positive and -negative colony counts

TABLE 2 Bacterial isolates used to evaluate the specificity of a real-time PCR assay for detection of *Paenibacillus* spp.

Isolate	AT[a]	Group ID[b]	No. of isolates in AT[c]	Mean C_T[d]
FSL R5-510	1	*Bacillus licheniformis* sensu lato 1	134	>40
FSL H7-687	3	*Bacillus weihenstephanensis*	19	>40
FSL R5-450	6	*Bacillus licheniformis* sensu lato 1	35	39.53
FSL R5-213	17	*Viridibacillus* spp.	24	>40
FSL H7-346	20	*Bacillus pumilus*	24	>40
FSL H7-608	59	*Bacillus cereus* sensu lato	26	>40
FSL R5-280	73	*Viridibacillus* spp.	18	>40
FSL H8-103	75	*Bacillus weihenstephanensis*	23	>40
FSL R5-860	158	*Bacillus cereus* sensu lato	137	>40
FSL H3-288	34	*Lysinibacillus* spp.	3	>40
FSL H7-305	55	*Bacillus clausii*	2	>40
FSL H7-431	64	*Bacillus* sp. 2	2	>40
FSL H7-432	65	*Bacillus subtilis* sensu lato 1	6	>40
FSL H7-719	84	*Oceanobacillus chironomi*	1	>40
FSL H7-729	85	*Bacillus flexus*	1	38.65
FSL H8-493	135	*Bacillus aerophilus* sensu lato	9	>40
FSL R5-231	140	*Bacillus safensis*	6	38.22 ± 0.34
FSL F4-077	2	*Paenibacillus odorifer* 1	52	19.06 ± 0.06
FSL F4-126	13	*Paenibacillus odorifer* 1	21	18.31 ± 0.25
FSL H7-592	15	*Paenibacillus odorifer* 1	112	19.09 ± 0.11
FSL F4-190	21	*Paenibacillus odorifer* 3	28	18.62 ± 0.18
FSL H7-689	23	*Paenibacillus amylolyticus* sensu lato	35	18.88 ± 0.04
FSL F4-242	25	*Paenibacillus odorifer* 1	19	18.40 ± 0.08
FSL F4-248	27	*Paenibacillus odorifer* 1	79	19.01 ± 0.10
FSL R5-925	30	*Paenibacillus odorifer* 3	12	20.28 ± 0.22
FSL H3-442	32	*Paenibacillus odorifer* 1	16	19.43 ± 0.10
FSL F4-100	8	*Paenibacillus lautus*	3	18.92 ± 0.21
FSL H3-318	41	*Paenibacillus* sp. 1	3	19.87 ± 0.28
FSL R7-277	45	*Paenibacillus graminis* 1	3	19.02 ± 0.13
FSL H7-331	58	*Paenibacillus* sp. 10	5	19.95 ± 0.06
FSL H8-287	100	*Paenibacillus* cf. *xylanilyticus*	9	18.94 ± 0.04
FSL H8-551	157	*Paenibacillus* cf. *peoriae*	8	19.38 ± 0.23
FSL R5-978	163	*Paenibacillus graminis* 2	9	19.01 ± 0.14

[a] Bacillus AT1, AT6, and AT17 and *Paenibacillus* AT2, AT15, AT23, and AT27 represent ATs commonly isolated from HTST milk produced in plants throughout the United States (34). AT1, AT15, AT21, and AT27 also represent ATs commonly isolated throughout the dairy-processing continuum (i.e., dairy farm environment, tank trucks, plant storage silos, and pasteurized milk) in New York State (19).

[b] Group identifier based on previously described phylogenetic comparison (21); cf. denotes closely related species according to 16S rRNA sequence analysis.

[c] Numbers are based on a total of 737 *Paenibacillus* and 551 non-*Paenibacillus* (i.e., *Bacillus*, *Lysinibacillus*, *Oceanobacillus*, *Viridibacillus*) dairy-associated isolates characterized by *rpoB* sequence-based subtyping (21). ATs isolated >10 times were considered predominant and used to test assay specificity; all other isolates were included to represent unique phylogenetic clades based on partial *rpoB* sequence comparison.

[d] Samples not detected in 40 cycles were assigned a value of >40. Samples without SDs were detected in only one of two replicates.

were recorded. Up to 5 isolates representing colonies with unique morphologies and including colonies with both β-Gal-positive and -negative activity were selected from each plate for *Paenibacillus* TaqMan PCR; crude lysates were prepared by touching a single colony with a sterile toothpick, transferring the cells into 100 μl of sterile water in a 1.5-ml Eppendorf tube (Eppendorf, Hamburg, Germany), vortexing briefly, and then microwaving on high for 4 min. TaqMan PCR results from colony lysates were interpreted as positive for *Paenibacillus* if the C_T value was <36.71; this cutoff value was the mean C_T for the non-*Paenibacillus* isolates (38.66 ± 0.65) that were used to evaluate assay specificity (Table 2) minus 3 standard deviations (SDs; to limit false-positive detection). An isolate representing each colony was also characterized to the genus and species levels by 16S rRNA gene or *rpoB* sequence-based subtyping, as previously described (20).

In addition to direct testing of colonies, total genomic DNA was isolated from milk, after incubation of the spore-shocked milk at 13°C for 48 h, using the Norgen milk bacterial DNA isolation kit. Final elution volumes of 100 μl were collected and used in the TaqMan PCR reported here to test for the presence of *Paenibacillus*.

To test for an association between the detection of *Paenibacillus* colonies in raw milk samples (after heat shock of milk, 48 h of incubation at 13°C, and plating onto BHI agar supplemented with X-Gal) and the final bacterial count in heat-treated milk samples stored for 21 days at 6°C, Fisher's exact tests were performed (JMP, version 8.0; SAS Institute Inc., Cary, NC). *Paenibacillus* assay results were coded as present (≥1 *Paenibacillus* colony confirmed by TaqMan PCR) or absent (no detectable *Paenibacillus* colonies), depending on TaqMan colony PCR results. For statistical analysis, final bacterial counts at day 21 were used to assign milk samples into one of two groups ($\leq 2 \times 10^4$ or $> 2 \times 10^4$ CFU/ml), based on the Pasteurized Milk Ordinance (8) bacterial count limit of 2×10^4 CFU/ml for grade A pasteurized fluid milk. For descriptive analysis, milk samples with day 21 bacterial counts of $> 2 \times 10^4$ CFU/ml were separated into intermediate ($> 2 \times 10^4$ and $\leq 1 \times 10^6$ CFU/ml) and high ($> 1 \times 10^6$ CFU/ml) categories, while day 21 bacterial counts of $\leq 2 \times 10^4$ remained designated low. *P* values of less than 0.05 were considered significant.

RESULTS

TaqMan PCR allows specific detection of *Paenibacillus* spp. The TaqMan primers and probes designed here (Table 1) were first

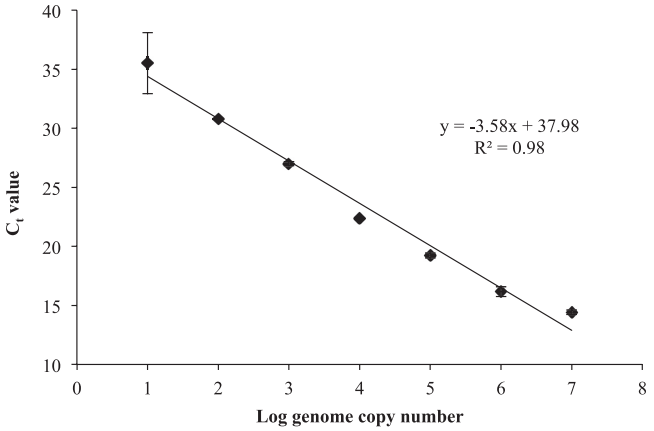

FIG 1 Standard curve for determination of amplification efficiency. Error bars indicate ±1 standard deviation for duplicate tests of each genome copy number. The average efficiency for real-time amplification was 90.11%.

used to generate a standard curve based on mean C_T values from assays performed in duplicate with *Paenibacillus* DNA at levels representing 10^7 to 10^1 log genome copies (Fig. 1). The linear regression line relating log genome copy number to C_T values was $y = -3.58x + 37.98$, and the R^2 value for the linear equation was 0.98. The amplification efficiency for real-time PCR amplification was determined to be 90.11%.

The specificity of the primers and probe for detection of *Paenibacillus* spp. was evaluated using 10^5 copies of genomic DNA isolated from 16 *Paenibacillus* isolates. All 16 *Paenibacillus* isolates were detected with the assay, and the mean C_T value was 19.14 ± 0.54 (Table 2). The 16 isolates tested represented 16 *rpoB* ATs. These *rpoB* ATs represent over 56% (414/737) of *Paenibacillus* isolates previously collected from each of the four fundamental steps in dairy processing (i.e., from dairy farms [feed, bedding materials, manure, soil and milking parlor wash water], tank trucks, plant storage silos, and pasteurized milk). These ATs also represent five of the predominant *rpoB* ATs identified among sporeformer isolates obtained from HTST pasteurized milk processed in different geographical regions throughout the United States (AT2, AT15, AT23, and AT27; representing milk processed in the northeastern, midwestern, western, southern, and southeastern United States) (34).

A total of 17 isolates representing *Bacillus* and other genera closely related to *Bacillus* (i.e., *Viridibacillus*, *Lysinibacillus*, and *Oceanobacillus*) were also tested with the TaqMan PCR. These isolates represented 17 unique *rpoB* ATs, including 9 common ATs (i.e., ATs that represented ≥10 isolates among a total of 551 non-*Paenibacillus* isolates). In total, >85% (470/551) of non-*Paenibacillus* isolates collected and characterized from the fluid milk-processing continuum, including dairy farm environments, tank trucks, plant storage silos, and raw and pasteurized milk, were classified into the 17 ATs tested here. Overall, 14 isolates were negative in the TaqMan PCR ($C_T > 40$), including 8/9 predominant *Bacillus* ATs found in fluid milk- or dairy-processing environments. The remaining three isolates (FSL R5-450, FSL H7-729, and FSL R5-231) yielded weakly positive results in the TaqMan PCR (i.e., C_T values of ≥38.22). Isolate FSL R5-450, which represents a common AT (i.e., AT6; Table 2), was negative in one replicate and weakly positive in the other replicate ($C_T = 39.53$). FSL H7-729 (AT85, an isolate included for genetic diversity; Table 2) was also negative in one replicate and weakly positive in the other replicate ($C_T = 38.65$). *Bacillus* strain FSL R5-231 (AT140, an AT isolated only 6 times) was the only non-*Paenibacillus* strain that yielded a positive result in both TaqMan PCR replicates (C_T = 38.22 ± 0.34).

Detection limit for vegetative *Paenibacillus* cells in raw milk is 3.25×10^1 CFU/ml. The ability of the assay to detect vegetative *Paenibacillus* cells in whole raw milk with and without spore enrichment was tested. Detection of *Paenibacillus* in raw milk (no enrichment) inoculated with *Paenibacillus* isolate FSL H7-592 (AT15) was possible at concentrations ranging from $3.25 \times 10^5 \pm 0.21 \times 10^5$ CFU/ml ($C_T = 26.14 \pm 0.78$) to as few as $3.25 \times 10^1 \pm 0.21 \times 10^1$ *Paenibacillus* CFU/ml (C_T = 39.15; only one of two replicates had a C_T value of <40) (Table 3); background flora in the raw milk was present at $3.85 \times 10^3 \pm 1.91 \times 10^3$ CFU/ml (Table 3). The negative control was not detected in two biological replicates ($C_T > 40$).

The detection of *Paenibacillus* cells inoculated into spore-activated and enriched raw milk ranged from $3.25 \times 10^5 \pm 0.21 \times 10^5$ *Paenibacillus* CFU/ml ($C_T = 26.73 \pm 0.09$) to $3.25 \times 10^2 \pm 0.21 \times 10^5$ *Paenibacillus* CFU/ml (C_T = 39.46; only one of two replicates detected). *Paenibacillus* was not detected ($C_T > 40$) in the enriched milk sample containing $3.25 \times 10^5 \pm 0.21 \times 10^5$ CFU/ml or the negative control. While the C_T values at higher *Paenibacillus* concentrations (3.25×10^4 and 3.25×10^5 CFU/ml) were similar for both raw milk and spore-enriched raw milk, at lower *Paenibacillus* concentrations (3.25×10^1, 3.25×10^2, and 3.25×10^3 CFU/ml), the C_T values were higher for heat-shocked and enriched samples. The sensitivity of detection for *Paenibacillus* was approximately 10-fold lower when *Paenibacillus* was inoculated in the nonenriched raw milk (with a mean background flora of $3.85 \times 10^3 \pm 1.91 \times 10^3$ CFU/ml) than when *Paenibacillus* was inoculated in the enriched milk samples, which showed a background flora of $4.65 \times 10^7 \pm 0.21$ CFU/ml. A high concentration of a mesophilic spore-forming (i.e., *Bacillus*) bacterial 16S rRNA gene may have contributed to the decreased *Paenibacillus* sensitivity observed in the enriched milk samples.

Assay detects low levels of *Paenibacillus* spores capable of germination and outgrowth to spoilage levels in milk. In order to evaluate the utility of the *Paenibacillus* TaqMan colony PCR, we also compared results from *Paenibacillus* detection in raw milk by

TABLE 3 Sensitivity of *Paenibacillus* detection using real-time PCR

Paenibacillus count (no. of CFU/ml)	C_T after:	
	Paenibacillus inoculated into raw milk[a]	*Paenibacillus* inoculated into heat-shocked and enriched raw milk[b]
$3.25 \times 10^5 \pm 0.21 \times 10^5$	26.14 ± 0.78	26.73 ± 0.09
$3.25 \times 10^4 \pm 0.21 \times 10^4$	29.47 ± 0.40	30.80 ± 0.50
$3.25 \times 10^3 \pm 0.21 \times 10^3$	31.76 ± 1.20	38.22 ± 0.06
$3.25 \times 10^2 \pm 0.21 \times 10^2$	35.61 ± 0.95	39.46[c]
$3.25 \times 10^1 \pm 0.21 \times 10^1$	39.15[c]	>40[d]
Negative control	>40	>40

[a] Mean aerobic plate count of raw milk, $3.85 \times 10^3 \pm 1.91 \times 10^3$ CFU/ml; mean aerobic plate count postenrichment, $4.65 \times 10^7 \pm 0.21 \times 10^7$ CFU/ml.
[b] Milk was incubated at 32°C for 18 h to achieve high levels of competitive microflora.
[c] Only one of two sample replicates was detected in 40 cycles.
[d] Samples not detected in 40 cycles were assigned a C_T value of >40.

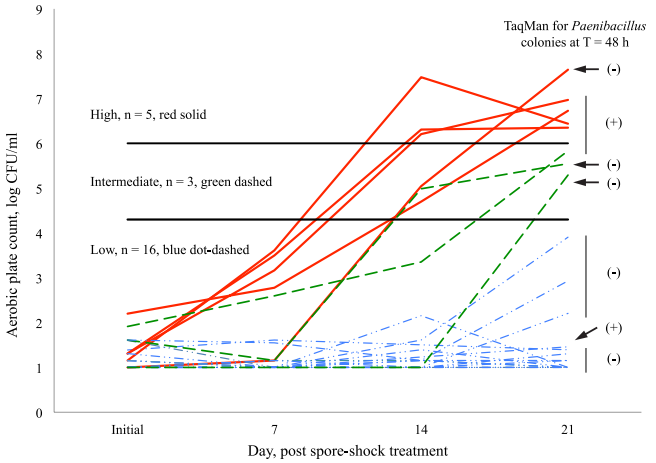

FIG 2 Aerobic plate counts of spore-shocked milk stored at 6°C for 21 days. For each milk sample ($n = 24$), the *Paenibacillus* TaqMan assay was applied to individual colonies following heat treatment (80°C for 12 min), enrichment (13°C for 48 h), and plating of raw milk samples. Assay results indicate the presence (+) or absence (−) of one or more *Paenibacillus* colonies. The horizontal line at 4.3 log CFU/ml indicates the maximum permissible bacterial count in high-temperature, short-time-pasteurized milk in the United States (8). The horizontal line at 6 log CFU/ml indicates the maximum bacterial count typically associated with sensory scores of 8 and above (good flavor) on a 10-point scale (1).

TaqMan colony PCR to bacterial counts of milk stored at 6°C after heat treatment. Briefly, 24 raw milk samples collected from farm bulk tanks were (i) evaluated by the TaqMan colony PCR and (ii) subjected to simulated HTST pasteurization, followed by monitoring of bacterial numbers in the HTST-treated milk over a simulated shelf life of 21 days (i.e., incubation at 6°C) (Fig. 2). While initial-day counts for all 24 milk samples were below 2×10^2 spores/ml and ranged from <1 spore/ml to 117 spores/ml (mean, 11 spores/ml), subsequent bacterial outgrowth varied. At day 21 after spore shock treatment, bacterial numbers in the milk samples ranged from <10 CFU/ml (8 samples) to 4.37×10^7 CFU/ml (Table 4, sample D-3). Bacterial numbers after storage at 6°C for 21 days were categorized: 5 samples had bacterial counts of $>1 \times 10^6$ CFU/ml (high), 16 samples had counts that remained at $<2 \times 10^4$ CFU/ml (low), and 3 had counts of between 2×10^4 and 1×10^6 CFU/ml (intermediate). In 4/5 milk samples that reached bacterial numbers over 1×10^6 CFU/ml by day 21, *Paenibacillus* was detected by applying the *Paenibacillus* TaqMan assay to β-Gal-positive colonies recovered from raw milk after a 48-h enrichment at 13°C (48-h assay result, positive [+]; Fig. 2). For example, for sample D-4, total bacterial counts were 4, 15, and 153 CFU/ml after enrichment at 13°C for 0, 24, and 48 h, respectively. Of these counts, 0 β-Gal-positive CFU/ml were identified at time zero, 5 β-Gal-positive CFU/ml were identified at 24 h, and 114 β-Gal-positive CFU/ml were identified at 48 h. Representative blue colonies selected from 24 h (FSL R7-693) and 48 h (FSL R7-708) were identified as *Paenibacillus* by the TaqMan colony PCR reported here (C_T values = 22.56 and 21.69, respectively). Confirmation of genus and species was performed by *rpoB* or 16S rRNA gene sequence-based characterization, and isolates FSL R7-693 and FSL R7-708 were determined to be *Paenibacillus peoriae* and *Paenibacillus polymyxa*, respectively. By day 21, milk sample D-4 reached a bacterial count of 9.33×10^6 CFU/ml. The predominant spoilage bacteria identified in the heat-treated milk stored at 6°C for 21 days were also determined to be *Paenibacillus*.

Only one sample reached the high bacterial count category ($>1 \times 10^6$ CFU/ml) after storage for 21 days at 6°C and did not contain detectable *Paenibacillus* after enrichment (sample D-3; Table 4). For the raw milk corresponding to this sample, the aerobic plate counts were 2, 2, and <1 CFU/ml following 0, 24, and 48 h of enrichment, respectively. The only colonies obtained at 0 and 24 h were determined to be *Bacillus* (Table 4). After 21 days of storage at 6°C, the bacterial count of sample D-3 reached 4.37×10^7 CFU/ml; the predominant organisms detected at this time were *Paenibacillus*, suggesting that very low levels (<1 spore/ml) of *Paenibacillus* are still capable of reaching high numbers in pasteurized products stored at refrigeration temperatures.

Among the 3 milk samples reaching intermediate bacterial counts by day 21 of cold storage (samples C-5, D-5, and J-5), only sample C-5 contained detectable *Paenibacillus* colonies during the 13°C enrichment and plating on BHI medium supplemented with X-Gal. Aerobic plate counts during enrichment of sample C-5 were 117 (6 β-Gal-positive colonies), 87, and 550 (10 β-Gal weakly positive [partial or light blue] colonies) at the 0-, 24-, and 48-h enrichment times, respectively. Two isolates, FSL R7-726 and FSL R7-727, from the time zero plating were β-Gal positive and were determined to be *Paenibacillus* by the TaqMan colony PCR (C_T values = 21.5 and 23.9, respectively). Characterization by *rpoB* sequence analysis confirmed that both isolates were *Paenibacillus*. Plating at 24 h of enrichment yielded only *Bacillus* colonies ($n = 3$); however, one *Paenibacillus* colony was identified after 48 h of enrichment (FSL R7-739; $C_T = 18.98$). After storage at 6°C for 21 days, the bacterial count for milk sample C-5 reached 6.76×10^5 CFU/ml, and the predominant bacteria identified were *Paenibacillus*. The other two milk samples (D-5 and J-5) in the intermediate count category contained no detectable *Paenibacillus*. Plating at 0, 24, and 48 h during sample enrichments yielded no β-Gal-positive colonies. Analysis of colonies using the *Paenibacillus* TaqMan assay determined colonies to be genera other than *Paenibacillus* ($C_T > 40$). *rpoB* sequence-based characterization identified all 5 isolates collected from enrichment samples to be *Bacillus pumilus* or *Bacillus licheniformis* (Table 4). The predominant spoilage organism identified after storage of milk samples at 6°C for 21 days was determined to be cold-tolerant *Bacillus weihenstephanensis*. Final bacterial counts were 3.55×10^5 and 1.95×10^5 CFU/ml for samples D-5 and J-5, respectively.

A total of 16 raw milk samples had bacterial counts below 2×10^4 (4.30 log) CFU/ml after storage at 6°C for 21 days (see Table S1 in the supplemental material). During enrichment of those samples, 54 isolates were collected and only one sample (H-5) contained detectable *Paenibacillus*. After 48 h of enrichment, plating of sample H-5 resulted in 12 CFU/ml, 5 of which were weakly β-Gal positive. β-Gal weakly positive isolate FSL R7-747 was tested with the assay and determined to be *Paenibacillus* (C_T = 20.37). *rpoB*-based characterization confirmed the identification of FSL H7-747 to be *Paenibacillus*. Following storage of milk sample H-5 for 21 days at 6°C, the bacterial count was 2.88×10^1 CFU/ml.

Results for the 24 milk samples were tested for a statistical association between detection of *Paenibacillus* (see Table S1 in the supplemental material; *Paenibacillus* colonies detected in 6 of 24 samples at 48 h) and the final APC after heat treatment and storage of raw milk samples for 21 days at 6°C. In raw milk samples where

TABLE 4 Summary of assay results for raw milk samples that showed evidence of bacterial spoilage after heat treatment and subsequent incubation at 6°C

Spoilage growth category[b]	Milk sample	No. of CFU/ml (no. of β-Gal-positive CFU/ml) after 13°C milk enrichment time of[a]:			Colony screening results after 13°C milk enrichment time of:										Milk assessment following 21 days at 6°C	
					0 h				24 h				48 h			
		0 h	24 h	48 h	Isolate	β-Gal[c]	TaqMan PCR result (C_T)[d]	*rpoB*-based genus	Isolate	β-Gal	TaqMan PCR result (C_T)	*rpoB*-based genus	Isolate	β-Gal	TaqMan PCR result (C_T)	*rpoB*-based genus	Bacterial count (CFU/ml)	Predominant spoilage bacteria[e]
High	D-3	2	2	<1	FSL R7-644	−	>40	*Bacillus*	FSL R7-645	−	>40	*Bacillus*	NA[f]				4.37×10^7	*Paenibacillus*
	D-4	4	15 (5)	153 (114)	FSL R7-677	−	>40	*Bacillus*	FSL R7-692	−	>40	*Bacillus*	FSL R7-708	+	21.69	*Paenibacillus*	9.33×10^6	*Paenibacillus*
	D-4				FSL R7-678	−	>40	*Bacillus*	FSL R7-693	+	22.56	*Paenibacillus*	FSL R7-709	−	>40	*Bacillus*		
													FSL R7-710	−	>40	*Bacillus*		
	C-4	30	29	313 (13)	FSL R7-674	−	>40	*Bacillus*	FSL R7-690	−	>40	*Bacillus*	FSL R7-704	−	>40	*Bacillus*	5.37×10^6	*Paenibacillus*
					FSL R7-675	−	>40	*Bacillus*	FSL R7-691	−	>40	*Bacillus*	FSL R7-705	−	>40	*Bacillus*		
					FSL R7-676	−	>40	*Bacillus*					FSL R7-706	+	25.18	*Paenibacillus*		
													FSL R7-707	+	20.73	*Paenibacillus*		
	C-3	27	23	197 (40)	FSL R7-646	−	>40	*Bacillus*	FSL R7-650	−	>40	*Bacillus*	FSL R7-652	+	18.9	*Paenibacillus*	2.75×10^6	*Paenibacillus*
									FSL R7-651	−	>40	*Bacillus*	FSL R7-653	−	>40	*Bacillus*		
													FSL R7-654	−	>40	*Bacillus*		
													FSL R7-655	−	>40	*Bacillus*		
													FSL R7-656	+	17.9	*Paenibacillus*		
	G-4	14	42 (1)	530 (190)	FSL R7-679	−	18.37	*Paenibacillus*	FSL R7-695	−	>40	*Bacillus*	FSL R7-711	+	21.8	*Paenibacillus*	2.24×10^6	*Paenibacillus*
					FSL R7-680	−	>40	*Bacillus*	FSL R7-696	−	>40	*Bacillus*	FSL R7-712	+/−	>40	*Bacillus*		
					FSL R7-681	−	>40	*Bacillus*	FSL R7-697	−	>40	*Bacillus*	FSL R7-713	−	>40	*Bacillus*		
					FSL R7-682	−	>40	*Bacillus*					FSL R7-714	−	>40	*Bacillus*		
Intermediate	C-5	117 (6)	87	550 (10)	FSL R7-722	−	>40	*Bacillus*	FSL R7-733	−	>40	*Bacillus*	FSL R7-736	−	>40	*Bacillus*	6.76×10^5	*Paenibacillus*
					FSL R7-723	−	>40	*Bacillus*	FSL R7-734	−	>40	*Bacillus*	FSL R7-737	−	>40	*Bacillus*		
					FSL R7-724	−	>40	*Bacillus*	FSL R7-735	−	>40	*Bacillus*	FSL R7-738	−	>40	*Bacillus*		
					FSL R7-725	−	>40	*Bacillus*					FSL R7-739	+/−	18.98	*Paenibacillus*		
					FSL R7-726	+	21.5	*Paenibacillus*										
					FSL R7-727	+	23.9	*Paenibacillus*										
	D-5	<1	3	<1	NA				FSL R7-740	−	>40	*Bacillus*	NA				3.55×10^5	*B. weihenstephanensis*
									FSL R7-741	−	>40	*Bacillus*						
									FSL R7-742	−	>40	*Bacillus*						
	J-5	<1	12	686	NA				FSL R7-744	−	>40	*Bacillus*	FSL R7-745	−	>40	*Bacillus*	1.95×10^5	*B. weihenstephanensis*

[a] Bacterial counts for the remaining 16 milk samples remained below 2×10^4 CFU/ml after heat shock and storage at 6°C for 21 days. See Table S1 in the supplemental material for complete summary of all 24 samples.
[b] Growth category assigned on the basis of APCs following heat shock and storage of milk at 6°C for 21 days. High, APC $> 1 \times 10^6$ CFU/ml; intermediate, APC $\leq 1 \times 10^6$ CFU/ml and $> 2 \times 10^4$ CFU/ml; low, APC $\leq 2 \times 10^4$ CFU/ml.
[c] +, β-Gal-positive (blue) colonies; −, β-Gal-negative colonies; +/−,β-Gal weakly positive colonies (partial blue or light blue colony).
[d] TaqMan C_T values of <36.71 are interpreted as a positive *Paenibacillus* colony.
[e] *rpoB* or 16S rRNA gene-based identification.
[f] NA, not applicable.

Paenibacillus was detected, there was a significant association with higher bacterial counts at day 21 ($>2 \times 10^4$ CFU/ml; $P = 0.0069$).

Overall, a total of 109 bacterial isolates were collected during screening for *Paenibacillus* colonies by 13°C enrichment for 48 h and plating onto BHI medium supplemented with X-Gal. Of these, 97 isolates were β-Gal negative; 96/97 β-Gal-negative isolates were also negative in the TaqMan colony PCR ($C_T > 40$; see Table S1 in the supplemental material). The only β-Gal-negative colony that yielded a positive signal with the TaqMan colony PCR (FSL R7-679, $C_T = 18.37$) was confirmed to be *Paenibacillus* by *rpoB* sequence-based characterization. *rpoB* sequence-based identification identified the remaining 96 isolates as *Bacillus* ($n = 92$), *Brevibacillus* ($n = 2$), *Oceanobacillus* ($n = 1$), and *Staphylococcus* ($n = 1$). All 9 β-Gal-positive colonies were positive in the TaqMan colony PCR (mean $C_T = 21.57 \pm 2.26$). There were also 3 weakly β-Gal-positive (+/−; see Table S1 in the supplemental material) colonies. On the basis of *rpoB* characterization, 2/3 of these colonies were identified to be *Paenibacillus* and were detected with the TaqMan PCR (FSL R7-739 and FSL R7-747; C_T values = 18.98 and 20.37, respectively). The remaining weakly β-Gal-positive colony (FSL R7-712) was determined to be *Bacillus* and was not detected by the TaqMan colony PCR ($C_T > 40$).

In addition to testing individual colonies, total genomic DNA was collected from each of the 24 raw milk samples after 48 h of incubation at 13°C. Among these samples, only one milk sample was positive for *Paenibacillus* with the TaqMan PCR (sample G-4; 190 β-Gal-positive CFU/ml; $C_T = 34.49 \pm 0.81$). This suggests that *Paenibacillus* contamination in the raw milk is typically at levels below the detection limit of the TaqMan PCR when used on DNA directly extracted from milk (i.e., $<3.25 \times 10^1 \pm 0.21$ spores/ml).

DISCUSSION

Our real-time PCR-based approach represents an improved tool for identifying the predominant psychrotolerant spore-forming spoilage bacteria associated with pasteurized fluid milk stored at refrigerated temperatures. Based on a diverse collection of aerobic spore-forming bacteria, which included over 1,200 isolates collected from different segments of the dairy production continuum (21), we targeted *Paenibacillus* spp., the microbes that present the current biological limit to extension of pasteurized fluid milk shelf life. Our detection method requires heat treating raw milk at 80°C for 12 min to activate spores and eliminate vegetative bacterial cells, followed by a 48-h enrichment at 13°C to enrich for psychrotolerant bacteria. After enrichment, milk samples are plated onto BHI medium supplemented with X-Gal to allow direct screening of colonies, including β-Gal-positive colonies, which, in milk, generally represent *Paenibacillus* spp. Next, crude colony lysates are prepared for immediate testing of individual colonies using our TaqMan PCR, and final testing results (i.e., *Paenibacillus* or non-*Paenibacillus* spp.) can be obtained within a few hours. Overall, this colony-screening strategy combined with a TaqMan PCR presents a novel approach for detecting *Paenibacillus* in raw milk and for predicting psychrotolerant bacterial outgrowth in milk held at 6°C.

A *Paenibacillus* real-time PCR assay has potential applications for detection of psychrotolerant spore-forming bacteria in a variety of foods. Few rapid, molecular-based detection methods targeting spore-forming bacteria responsible for food spoilage have been developed (9, 22, 24), and of these, none have focused on *Paenibacillus*, the psychrotolerant spore-forming genus associated with dairy spoilage. The absence of appropriate tools may reflect, in part, the fact that the bacterial ecology of pasteurized fluid milk has only recently been characterized at the molecular level, which led to identification of *Paenibacillus* as the predominant fluid milk spore-forming spoilage genera (12, 19, 21, 34). Rapid methods to detect spore-forming bacteria have primarily focused on food-borne pathogens, e.g., *Bacillus cereus* (13, 27, 43), that pose a significant health threat. However, the presence of spore-forming bacteria that can resist multiple processing hurdles and affect food product quality represents considerable economic and food security concerns. One commercial assay for the detection of spore-forming bacteria in food has been developed by Pall GeneSystems (32). However, when testing 34 food matrices, the authors reported that the detection system was unable to identify any *Paenibacillus* organisms. Conversely, when applying standard methods to the same 34 food matrices, researchers were able to identify *Paenibacillus* in sliced nuts and chocolate (32), which illustrates the difficulty of reliably identifying low levels of *Paenibacillus* in food. Other than this method, development of assays for *Paenibacillus* spp. to date has focused on *Paenibacillus larvae* (3, 26), an important honeybee pathogen. Thus, an assay targeting psychrotolerant *Paenibacillus* associated with milk spoilage represents a new and important tool for the dairy industry to identify *Paenibacillus* spp. in high-quality raw milk, as well as potential contamination sites at the farm and processing facility level. Sporeformers, including *Paenibacillus*, have the potential to form biofilms (44) and reside within processing facilities (20) and have been isolated from paperboard packaging (30). Thus, it is important to develop sensitive tools for detection of spoilage organisms and to apply them throughout the processing chain to identify entry points to enable development of control strategies to reduce spoilage and improve the quality of our foods. In the future, our assay could be extended to other refrigerated and pasteurized foods, including processed vegetables (2, 7, 15), where psychrotolerant *Paenibacillus* organisms are a potential spoilage concern.

Direct PCR-based detection of *Paenibacillus* in raw milk to predict shelf life is challenging due to the high sensitivity required. Previous studies have demonstrated that low spore levels are typically found in raw milk. For example, sampling of raw milk from 43 processing plant silos in New York State yielded a mean aerobic spore count of 52 spores/ml (25). Additional studies in Europe reported similar findings, as mean counts of 131 mesophilic aerobic spores/ml (41) and <100 spores/ml of raw milk (42) were detected. Of these aerobic spores, only a small percentage are likely to be *Paenibacillus*, as a number of studies have found that *Bacillus* spp. comprise the majority of spores identified in raw and in recently heat-treated milk (4, 12, 19, 36). Consistent with this, only 12/109 (11%) isolates collected during our study represented *Paenibacillus* spp., and 9/12 of those *Paenibacillus* isolates were detected only after enrichment for 24 or 48 h at 13°C. Thus, due to the low levels of spores naturally present in raw milk, particularly spores of psychrotolerant *Paenibacillus* spp., an enrichment or concentration step is needed to improve assay sensitivity.

In addition to low levels of *Paenibacillus* spp., high levels of closely related *Bacillus* spp. further complicate detection, particularly for assays targeting the 16S rRNA gene. The detection limit for our assay increased nearly 10-fold when *Paenibacillus* was inoculated into heat-shocked and enriched (32°C for 18 h) raw milk. This reduction in sensitivity is likely due to high levels of closely

related *Bacillus* spp. competing for primers and probe. Postollec and colleagues (32) encountered cross-reactivity when testing a commercial assay based on 16S rRNA gene primers and probes and reported *Paenibacillus* detection with *Bacillus* primers and vice versa. Many *Bacillus* and *Paenibacillus* spp. share over 99% identity, based on partial (632-bp) 16S rRNA gene analysis (21). Therefore, continued development of new assays, particularly through leveraging of full-genome sequencing technologies and concentration on definition of the characteristics of spore-forming bacteria, such as the differential presence of cold growth genes (10), will be critical to further improve detection capabilities.

PCR-based detection of individual colonies after enrichment and plating allows sensitive and specific detection of *Paenibacillus* spp. Results from TaqMan PCR detection, performed on DNA extracted from milk samples, were predominantly negative due to low levels of *Paenibacillus* and competition from closely related *Bacillus* spp. Our observed detection limit for *Paenibacillus* inoculated into raw milk was 3.25×10^1 CFU/ml, which explains why only 1/24 raw milk samples tested positive for *Paenibacillus*. However, plating the same spore-shocked and enriched milk samples onto BHI medium supplemented with X-Gal allowed detection of *Paenibacillus* in 6/24 raw milk samples. Therefore, use of a TaqMan colony PCR following a short enrichment and plating on BHI medium supplemented with X-Gal greatly improves the reliability of the assay. Direct colony screening allowed us to lower the detection limit for *Paenibacillus* from 3.25×10^1 CFU/ml to 1 CFU/ml when a 1-ml sample was plated. In addition to improved sensitivity, the colony-screening method avoids the time and costs associated with genomic DNA purification steps.

The colony-screening method employs two important phenotypes that aid in distinguishing *Paenibacillus* from other sporeformers: cold growth and β-galactosidase activity. In general, *Paenibacillus* spp. are capable of growth at 6°C, whereas most *Bacillus* spp. are not; the most notable exception is *Bacillus weihenstephanensis* (21). By applying a 48-h incubation step for heat-shocked milk at 13°C, we were able to enrich for psychrotolerant *Paenibacillus* without promoting growth of mesophilic *Bacillus* spp., whose spores typically represent a higher proportion of spores in raw milk. However, two samples (C-5 and J-5) reached counts above the Pasteurized Milk Ordinance (8) limit for pasteurized milk (>20,000 CFU/ml) after storage at 6°C for 21 days and were not detected by our assay. The predominant spoilage organism in the two milk samples was determined to be *B. weihenstephanensis*. This outcome demonstrates the need for a detection system that utilizes genetic targets, such as cold-growth genes, shared by the psychrotolerant spoilage organisms of concern (i.e., *Paenibacillus* spp. and *B. weihenstephanensis*).

In addition to cold growth, β-galactosidase activity proved useful in identification of *Paenibacillus*. Previous work has shown that the majority of dairy-associated *Paenibacillus* subtypes are β-galactosidase positive, whereas the majority of *Bacillus* subtypes are not (5, 21). However, as some dairy-associated *Bacillus* isolates have expressed positive or weakly positive β-Gal activity, this phenotypic test cannot be completely relied upon to distinguish *Paenibacillus* from other sporeformers (21). In fact, we identified two β-Gal weakly positive isolates and one β-Gal-negative isolate as *Paenibacillus* by TaqMan colony PCR and *rpoB* sequence-based characterization. Thus, the combination of β-Gal screening and a 16S rRNA gene TaqMan assay proved necessary for accurate and sensitive detection of *Paenibacillus* spp. Application of this culture-dependent assay to screen for *Paenibacillus* spp. in nondairy environments could facilitate identification of strains with important metabolic capabilities (e.g., production of polymyxin, bioremediation, or nitrogen-fixing ability) of importance to agriculture, food processing, and medicine (29, 37, 40).

Conclusion. We developed a sensitive and specific TaqMan assay that can detect psychrotolerant spore-forming *Paenibacillus* spp. associated with dairy spoilage. While the low levels of spores initially present in raw milk prevented direct detection of *Paenibacillus* in DNA extracted from raw milk or from enriched milk samples, an alternative colony-screening method proved feasible. A 16S rRNA gene-based TaqMan assay on crude colony lysates obtained from heat-shocked milk that had been enriched at 13°C for 48 h and plated on BHI medium supplemented with X-Gal provided fast and accurate identification of *Paenibacillus*. Overall, the assay provides an improved tool for the dairy industry to differentiate raw milk with the potential for lower postpasteurization bacterial outgrowth. Further development of rapid and effective detection methods for psychrotolerant sporeformers within a comprehensive farm-to-fork framework is needed for improved control of these important spoilage organisms in the food supply.

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