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Short communication: Typing and tracking *Bacillaceae* in raw milk and milk powder using pyroprinting

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ABSTRACT

Contamination of fluid and processed milk products with endospore-forming bacteria, such as *Bacillaceae*, affect milk quality and longevity. Contaminants come from a variety of sources, including the dairy farm environment, transportation equipment, or milk processing machinery. Tracking the origin of bacterial contamination to allow specifically targeted remediation efforts depends on a reliable strain-typing method that is reproducible, fast, easy to use, and amenable to computerized analysis. Our objective was to adapt a recently developed genotype-based *Escherichia coli* strain-typing method, called pyroprinting, for use in a microbial source-tracking study to follow endospore-forming bacillus bacteria from raw milk to powdered milk. A collection of endospores was isolated from both raw milk and its finished powder, and, after germination, the vegetative cells were subject to the pyroprinting protocol. Briefly, a ribosomal DNA intergenic transcribed spacer present in multiple copies in *Bacillaceae* genomes was amplified by the PCR. This multicopy locus generated a mixed PCR product that was subsequently subject to pyrosequencing, a quantitative real-time sequencing method. Through a series of enzymatic reactions, each nucleotide incorporation event produces a photon of light that is quantified at each nucleotide dispensation. The pattern of light peaks generated from this mixed template reaction is called a pyroprint. Isolates with pyroprints that match with a Pearson correlation of 0.99 or greater are considered to be in the same group. The pyroprint also contains some sequence data useful for presumptive species-level identification. This method identified groups with isolates from raw milk only, from powdered milk only, or from both sources. This study confirms pyroprinting as a rapid, reproducible, automatically digitized tool that can be used to distinguish bacterial strains into taxonomically relevant groups and, thus, indicate probable origins of bacterial contamination in powdered milk.
Key words: *Bacillaceae*, biotechnology, strain typing, microbial source tracking

Short Communication

The quality of milk and milk products is often evaluated by testing for the presence and quantity of spoilage-causing microbes and their endospores. These bacterial contaminants can produce proteases, lipases, and acid that degrade milk components leading to unpleasant flavor and customer dissatisfaction (Barbano et al., 2006). Raw milk may contain contaminants from the farm environment or dairy equipment, from the host cows, or from farm workers. In addition, microbes present on equipment used to process milk and milk powders can be a source of contamination. Thermophilic and thermoduric species within the family *Bacillaceae* are particularly difficult to control because their ability to form biofilms or endospores allows them to resist common methods of pasteurization and equipment-cleaning procedures (Etoa and Michiels, 1988; Parkar et al., 2003). In fact, temperatures used in the evaporation of milk to generate milk powder (45–75°C) promote the growth or sporulation of many common contaminants (Scott et al., 2007). Identifying the source of such bacterial contamination can focus prevention techniques on specific steps in food processing, thus reducing spoilage.

Microbial source-tracking methods depend on the ability to reliably match bacteria, either with or without isolation, from a contamination site to a site of origin. Strain-typing methods generally use either a

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collection of phenotypic traits or some interrogation of the bacterial genotype to distinguish strains (van Belkum et al., 2007). The collection of traits used, whether phenotypic or genotypic, is often referred to as a fingerprint because it can be used to identify the same strain isolated from multiple samples, thus providing evidence of a connection between the samples. In general, good typing methods use traits that remain stable for the duration of the study period, are applicable to all relevant isolates, discriminate well between isolates already known to be different strains, and have reproducible output. Convenient typing methods are also flexible, rapid, accessible, easy to use, and amenable to computerized analysis (van Belkum et al., 2007; Li et al., 2009). A recently introduced strain-typing method called pyroprinting (Black et al., 2014), based on pyrosequencing, fulfills many of these requirements and is amenable for strain typing of isolates from several bacterial genera.

Pyrosequencing of a homogeneous population of DNA molecules employs a sequencing-by-synthesis method for the real-time detection of the inorganic pyrophosphates produced when nucleotides are incorporated into a growing DNA strand (Ronaghi et al., 1996, 1998). Through a series of enzymatic reactions, the pyrophosphate by-products drive a luciferase-catalyzed reaction that generates a light signal proportional to the number of nucleotides incorporated (Ronaghi et al., 1998). The sequence is determined based on the order in which nucleotides were added to the reaction and the magnitude of the light peaks produced at each nucleotide dispensation.

Pyroprinting uses the pyrosequencing method, but differs in that pyroprinting simultaneously sequences multiple polymorphic loci within a genome (Black et al., 2014). As in the multilocus sequence-typing method (Maiden et al., 1998), this interrogation of many genetic sites increases the power of discrimination between closely related organisms. The light output peak heights generated by pyroprinting produce a pattern that represents the unique combination and ratio of the targeted templates present in the bacterial isolate. The pattern of peak heights is a type of genetic fingerprint that results from a unique combination of loci that are amplified and pyrosequenced and is referred to as a pyroprint. Pyroprints generated from different bacterial isolates are compared with one another using the Pearson's correlation distance metric.

The ribosomal DNA (**rDNA**) operons of bacteria contain 2 intergenic transcribed spacer regions (**ITS**): ITS1 between the 16S and 23S genes and ITS2 between the 23S and 5S genes. Multiple ITS alleles are generally present in a single organism, as bacterial genomes often harbor many copies of the rDNA operon. These intergenic regions are variable due to polymorphic sequences and insertions of one or more transfer RNA genes (Brosius et al., 1981; Jensen et al., 1993). The highly conserved flanking rRNA genes are useful for primer design. These features make the ITS regions good targets for pyroprinting, and underlie the power of pyroprinting to distinguish between bacterial strains. They have been used successfully in the pyroprinting of *Escherichia coli* isolates (Black et al., 2014).

The current paper describes how pyroprinting was used as a rapid way to track the potential sources of endospore-forming *Bacillaceae* found in milk powder samples. The multiple (10 to 16) ITS regions in *Bacillaceae* rDNA operons (Stewart et al., 1982) were simultaneously interrogated in this pyroprinting protocol. Contaminants in a powdered milk product could come from (1) raw milk containing pasteurization-resistant endospores or vegetative cells that sporulated during processing, or (2) processing equipment contaminated with bacterial biofilms or endospores resistant to many cleaning procedures. In the former case, pyroprints from powder-isolated contaminants were expected to match those from endospores found in raw milk; in the latter case, the pyroprints generated from endospores from the 2 sources would not match. In our study, some endospores in the analyzed powder milk samples matched those in raw milk whereas others did not. In addition, the sequence data obtained from pyroprinting was able to provide a presumptive identification of the *Bacillaceae* genera and species contaminating the milk powder.

The variable ITS region that lies between the 23S and 5S rDNA genes (ITS2) was used to generate pyroprints in our study. The primers for PCR amplification and pyrosequencing were designed using sequences from 34 completed genomes of *Bacillus* spp. and *Geobacillus* spp. retrieved from the integrated microbial genomes site (Markowitz et al., 2012). Multiple sequence alignments were generated using CLUSTALW (Thompson et al., 1994) and used to define boundaries of the highly conserved regions appropriate for primer design. Pyromark primer design software (Qiagen, Valencia, CA) confirmed the following primer collection: biotin-labeled forward [Biotin~5] AGCCCCCCTCAAGATGAG and reverse CGTGTTCGGBATGGGAACG primers for PCR, and 5′GGBATGGGAACGGGT for the pyrosequencing step. An in silico pyrosequencing algorithm was developed to model the relative light output at each nucleotide dispensation for all ITS2 sequences in a bacterial genome simultaneously (Brandt et al., 2012). Simulated pyroprints from the 34 genomes were evaluated by pairwise Pearson correlations between pyroprints (Black et al., 2014). The order of nucleotides dispensed during pyrosequencing was optimized using

the simulated data and empirical data to maximize differences between the *Bacillaceae* expected in milk products. To extend the read length, cyclic dispensation was preceded by 2 nucleotides (GT) corresponding to the highly conserved bases near the primer. Replicate dispensations were added at the start and end of the 98-step dispensation sequence to evaluate nucleotide incorporation efficiency, resulting in a final dispensation order of GGT–(GACT) × 24–T.

From a 22-h milk processing run, 88 *Bacillaceae* isolates (48 raw, 40 powder) were collected from 12 raw milk samples, as well as 2 finished powder samples from that raw milk. From a contiguous 28-h run, 71 (34 raw, 37 powder) isolates were collected from 8 raw milk and 2 associated finished powder samples. Ten milliliters of raw milk or reconstituted powder samples (11 g in 99 mL of Butterfield's Phosphate Buffer) were heated to 80°C for 12 min or 100°C for 30 min to reduce background microbiota (i.e., vegetative cells) and then immediately cooled on ice. Bacterial isolates were obtained by plating 1 mL of the heat-treated raw milk or reconstituted powder onto replicate tryptic soy agar plates (instead of standard methods agar) according to procedures outlined in the *Standard Methods for the Examination of Dairy Products* (Wehr and Frank, 1992) and were grown at 55°C for 48 h (Burgess et al., 2010). Isolated colonies were selected and streaked twice to ensure colony purity. Isolates were subsequently resuspended in tryptic soy broth and then frozen at −50°C in 20% glycerol for permanent storage.

For each isolate analyzed, the ITS2 loci were amplified by PCR. Each reaction contained a final concentration of 1× Quickload Taq Master Mix (New England Biolabs, Ipswich, MA), 1 μL of the frozen-thawed bacillus culture in tryptic soy broth, and 0.2 μ*M* forward and reverse primers described above. Thermocycling conditions were 95°C for 2 min followed by 45 cycles of 95°C for 30 s, 56°C for 30 s, and 68°C for 1 min as well as a single 5-min extension at 68°C. The presence of appropriately sized PCR products (240–350 bp) was confirmed by agarose gel electrophoresis. The PCR products were pyrosequenced as described in the Pyromark Q24 pyrosequencing protocol (Qiagen) using the sequencing primer and aforementioned dispensation sequence. Replicates from 4 randomly selected isolates were used to evaluate pyroprint reproducibility and identify an empirical matching threshold between pyroprints. Two technicians pyroprinted isolates in triplicate on 2 different days, resulting in 12 replicate pyroprints for each of the 4 isolates. Pairwise comparison within each group of replicates resulted in Pearson correlations ranging from 0.9866 to 0.9999, which fits well into a β distribution for calculating a 95% confidence limit for pyroprints originating from the same organism. Thus, a correlation of 0.9900 between 2 pyroprints was chosen as the matching threshold indicating pyroprints from the same origin (data not shown).

A total of 159 endospore-derived isolates were obtained from samples of raw milk (82) and milk powder (77) from the 2 contiguous processing runs. All of the isolates produced PCR products and pyroprints and fewer than 15% required repeated effort. On average, data could be obtained from a set of 48 purified isolates in under 8 h. Isolates were classified into 25 groups by calculating Pearson correlations for all pairs and matching them using the 0.9900 threshold (Table 1). Fourteen isolates did not match to any other isolates (8 from raw milk and 6 from powder), and 11 groups (designated A–K) contained from 2 to 60 isolates with matching pyroprints across the 2 runs (Table 1). Group B contained only isolates from raw milk, indicating that these endospore formers did not survive or were not propagated in the pasteurization and drying processes. A total of 26 isolates from powder (33.7% of all powder isolates) were clustered into 5 groups (C, G, H, I, and J) also containing matching isolates in the raw milk from their same run. This indicates that these endospore formers originated in the raw milk and remained or possibly multiplied during the drying process. The 2 most common raw milk isolates, groups G and H, were also among the most abundant powder contaminants. The one powder isolate in group K, isolated from run 2, had a matching raw milk isolate from run 1, which is possible as some milk was common for both runs. A total of 44 isolates (groups A, D, E, and F) were only found in powder samples (57.1% of all powder isolates) and may have been introduced during processing. For example, group E contains a large number of isolates (30) found only in powder and across both runs, which may indicate a source of contamination independent of the raw milk input. Although we cannot rule out the possibility that these strains originated in raw milk and were missed in the original sampling, it is unlikely given the correlation between abundance in raw milk and abundance in powder of groups G and H.

The location of the reverse PCR primer within the 5S rDNA gene allows the sequencing of approximately 25 conserved base pairs before sequencing the adjacent potentially polymorphic ITS region. Thus, despite the mixed PCR template, valid sequence data are produced until sequencing proceeds into the ITS region, where reliable sequence information declines as variability increases. The Pyromark Q24 software assesses sequence quality based on proprietary benchmarks. To determine if the observed pyroprint groups have taxonomic relevance or share a common taxonomic identification, the reliable sequence from each pyroprint (average total length of 39 nucleotides) was matched

to known nucleotide sequences in the National Center for Biotechnology Information GenBank nucleotide sequence database (www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLASTn; Altschul et al., 1990). A group was given a presumed taxonomic identification if all isolates in the group returned at least one BLASTn E-value (a measure of probability for random sequence matching) less than or equal to 1.00×10^{-3} (Table 1). Out of 11 groups containing more than one isolate (groups A–K), 9 were assigned to a presumptive species, as all of the isolates within a group could be assigned to the same species. One powder isolate in group J had only 20 nucleotides of reliable sequence, and could not be assigned to a presumptive taxonomic group. Six of the 14 unmatched isolates were of *Bacillaceae* origin, whereas 8 of the unmatched isolates and isolates from groups C and F matched nothing in the GenBank database. Whereas further tests, such as full-length 16S rRNA sequencing, would need to be performed to confirm presumptive taxonomic identifications, these initial results support the taxonomic relevance of groupings determined by pyroprinting.

Group B sequences matched to DNA from *Thermoactinomyces vulgaris*, a gram-positive, endospore-forming bacterium thought to be a member of the *Actinomycetes* because of the formation of aerial and substrate mycelia. However, analysis has shown that *Thermoactinomyces vulgaris* is related to the *Bacillus* genus based on analysis of 5S and 16S rRNA (Park et al., 1993; Yoon and Park, 2000). *Thermoactinomyces vulgaris* is commonly found in decaying compost, stores of grain and hay, and in animal bedding (Kotimaa et al., 1991); thus, it is likely that this contaminant originated from the dairy farm, consistent with it being found only in raw milk. Powder-specific groups A, D, and E were presumed to belong to thermoduric thermophiles (endospores survive high temperatures; optimum growth temperature above 60°C), whereas groups with isolates from both raw milk and powder (G–K) match to sequences of thermoduric mesophiles (endospores survive high temperatures; optimum growth temperature of 30°C).

The most commonly identified endospore-forming contaminants from dairy farms and fluid milk are psychrotolerant and thermoduric endospore-forming bacteria, species of *Bacillus* and *Paenibacillus* (Huck et al., 2008). This is consistent with our findings that the most commonly found isolates in raw milk, belonging to groups G, H, and I, were identified as *Bacillus licheniformis*. Yet isolates from these groups were also found in milk powder, suggesting that *B. licheniformis* endospores can survive both pasteurization and the evaporation processes leading to the formation of powdered milk. Pasteurization requires temperatures high enough to kill contaminating vegetative cells, but low enough so that the germination of spores is not initiated. Several studies have been performed to identify the combination of time and temperature yielding the lowest level of bacterial contamination. Whereas no pasteurization parameters kill everything, less contamination was detected in milk heated to 72.9°C for 25 s

Table 1. Classification of isolates from raw and powder milk into groups, and presumptive taxonomic match for groups as classified by pyroprint matching of 5S rDNA sequences

	Run 1[2]		Run 2[2]			
Group[1]	Raw	Powder	Raw	Powder	Presumptive taxonomic match[3]	Average E-value[3]
Group A	0	5	0	0	*Geobacillus thermoleovorans*	1.30×10^{-4}
Group B	3	0	0	0	*Thermoactinomyces vulgaris*	3.30×10^{-4}
Group C	0	0	1	1	No match	NA[4]
Group D	0	0	0	7	*Anoxybacillus flavithermus*	1.31×10^{-7}
Group E	0	22	0	8	*Anoxybacillus flavithermus*	1.96×10^{-6}
Group F	0	0	0	2	No match	NA
Group G	6	2	9	8	*Bacillus licheniformis*	3.99×10^{-7}
Group H	32	8	16	4	*Bacillus licheniformis*	4.91×10^{-8}
Group I	1	2	2	0	*Bacillus licheniformis*	2.22×10^{-6}
Group J	1	0	2	1[5]	*Bacillus pumilis*	1.33×10^{-10}
Group K	1	0	0	1	*Bacillus amyloliquefaciens*	2.67×10^{-13}
Unmatched pyroprint	4	1	4	5	6 *Bacillaceae*, 8 no match	—
Total	48	40	34	37		

[1]The set of isolates whose pyroprints matched each other with a Pearson correlation ≥0.99.

[2]The number of isolates of either raw milk or powdered milk origin in a given group from a given run.

[3]Genus and species sequence match that gave the lowest E-value (a measure of probability for random sequence matching) when compared with the isolate pyroprint sequences by BLASTn (Altschul et al., 1990). The E-values from each isolate match within a group were used to determine the average E-value for that group.

[4]Not applicable.

[5]This isolate did not match anything in the NCBI database (www.ncbi.nlm.nih.gov), so it was not included in the average E-value calculation.

than in milk heated to higher temperatures (77–85°C) for that time period (Ranieri et al., 2009; Martin et al., 2012). Finding *B. licheniformis* in both raw and powdered milk samples could thus target prevention efforts toward optimizing pasteurization temperatures in both fluid and powder milk processing.

Groups identified as *Geobacillus* and *Anoxybacillus* were found only in powder samples (A, D, and E). This is consistent with studies reporting low abundances of endospores from these 2 genera in raw milk. However, both vegetative cells and endospores of these species were found in the preheating plate heat exchanger and evaporator components of milk powder-processing equipment (Scott et al., 2007). As milk flows over these components, bacterial biofilms develop protecting vegetative cells and endospores from heat and cleaning processes, and these can later slough off to contaminate the milk powder. Finding these thermoduric species only in powder might thus focus prevention efforts on the frequency and methods of equipment cleaning processes. Whereas the presumptive taxonomic identifications support the pyroprint groupings, this level of identification is not necessary for source tracking. The pyroprinting method can pinpoint the source of contamination as specifically as the testing scheme allows.

Previous microbial source tracking studies of endospores in milk powders used species- or genus-specific 16S rDNA PCR primers to identify members of *Anoxybacillus flavithermus* or *Geobacillus*, respectively (Scott et al., 2007). Multilocus variable number tandem repeat together with high-resolution melt analysis has also been used to identify *Geobacillus* in milk powders (Seale et al., 2012). In other studies, sequence analysis of 632 bases of the *rpoB* gene has been performed to identify different *rpoB* alleles that distinguish *Bacillus* and *Paenibacillus* strains isolated from raw and processed fluid milk (Durak et al., 2006; Huck et al., 2007, 2008). Pyroprinting is a new typing tool that requires bacterial isolation (but no further confirmation before pyroprinting), a PCR step directly from bacterial culture, and then pyrosequencing. The PCR primers used for our study were designed to recognize both *Bacillus* and *Geobacillus* species from endospores isolated from raw milk or milk powder, and pyroprinting incorporates information from at least 10 genomic sites, giving this method both breadth in isolate detection and depth in discriminatory power. Our results show that pyroprinting can (1) fingerprint isolates quickly with a high degree of reproducibility, (2) group isolates at a subspecies level from a wide variety of raw milk and milk powder isolates, and (3) cluster isolates into taxonomically relevant groups. Pyroprinting presumptively identified groups within several *Bacillaceae* genera, and even groups within a species, although further analysis would be required to confirm species-level identifications. Three distinct groups were presumptively identified as *Bacillus licheniformis* (groups G, H, and I) and 2 as *Anoxybacillus flavithermus* (groups D and E). The fact that our primers detected a presumptive *Thermoactinomyces vulgaris* isolates (group B) is further support for the assertion that *Thermoactinomyces* are members of the *Bacillaceae*. Finally, pyroprint data are automatically digitized and instantly amenable to computerized analysis. Thus, pyroprinting is an easy, rapid tool that may also provide presumptive identification of a broad range of contaminants with a specificity that allows source tracking of *Bacillaceae* groups.

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