Molecular Analysis of Spoilage-Related Bacteria in Pasteurized Milk during Refrigeration by PCR and Denaturing Gradient Gel Electrophoresis

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ABSTRACT

Bacterial diversity in fluid milk products has been extensively studied in order to improve milk quality. Here, we illustrate the utility of viable counts and PCR–denaturing gradient gel electrophoresis (DGGE) for monitoring the microbial spoilage of pasteurized milk during shelf life. Five pasteurized milk samples stored at 4°C were examined at 10 and 5 days before expiration and on the expiration day. With bacterial DNA extracted directly from the samples, PCR-DGGE analysis indicated that Pseudomonas became dominant in four samples. Meanwhile, the aerobic plate count of these four samples exceeded the regulatory limit of 20,000 CFU/ml at 5 days before expiration, and the rapid psychrotrophic count markedly surpassed the aerobic plate count on the expiration day. Streptococcus and Buttiauxella spp. were detected in several samples. Sequence analysis of DGGE fragments revealed high diversity among Pseudomonas spp. in the milk samples. P. putida and P. migulae grew to high numbers during refrigerated storage. Further identification of Pseudomonas at the species level was facilitated by PCR and multiplex PCR using species-specific primers; consequently, P. fluorescens and P. fragi were observed. These results highlight an important role of Pseudomonas in the shelf life of pasteurized milk.

The limited shelf life of high-temperature, short-time pasteurized milk is generally due to microbial growth and metabolism. There are two main groups of organisms involved in pasteurized milk spoilage: (i) thermoduric bacteria present in raw milk such as Bacillus, Microbacterium, and Streptococcus, which are able to survive pasteurization, and (ii) psychrotrophic gram-negative bacteria commonly associated with postpasteurization contamination (3, 12). By comparison, psychrotrophic bacteria are by far the most important factor influencing pasteurized milk quality. These ubiquitous microbes are remarkable for their rapid growth and short generation times under low temperatures. The extracellular proteolytic and lipolytic enzymes produced by these bacteria can degrade nutritional components of milk and cause undesirable changes in sensory characteristics. This problem is of particular concern to the dairy industry (7, 12, 24).

It is critical to investigate the microbial diversity and dynamics in pasteurized milk during its shelf life and determine the spoilage-related bacterial species. However, conventional microbiological methods, typically involving culturing the organisms on appropriate agar media and identifying isolates according to their morphological, biochemical, and/or immunological characteristics, are labor-intensive and time-consuming. A culture-independent approach, PCR–denaturing gradient gel electrophoresis (DGGE), has been introduced for rapid profiling of complex microbial populations (18). Based on the analysis of bacterial 16S rRNA genes, PCR-DGGE allows separation of DNA fragments with the same size but different sequences and gives a sample-specific fingerprint of microbial species (5). In recent years, PCR-DGGE has proved to be a powerful tool for characterization of microbial communities in various foods, such as beef (10), pork (17), sausages (5), cod (14), and raw milk (16).

Furthermore, psychrotrophic Pseudomonas strains have been commonly identified as an important contributor to the spoilage of chilled food. But not all members of the genus are equally responsible for the degradation of nutrients in milk. P. fluorescens, P. fragi, and P. putida have been frequently isolated from processed milk samples (6, 26). Ensuring the quality of pasteurized milk requires reliable and rapid identification of these potentially spoilage-related Pseudomonas spp. Nevertheless, previous studies have shown that it is difficult to identify Pseudomonas at the species level due to a high homogeneity in 16S rRNA sequences of different species of Pseudomonas (10, 11). Alternatively, several genes have been explored as targets for PCR-based differentiation of common Pseudomonas spp. causing food spoilage (9, 23).

This study was primarily aimed at illustrating the use of PCR-DGGE to monitor the bacterial diversity in pasteurized milk during refrigerated storage. Moreover, an additional objective was to identify predominant Pseudomonas spp. involved in milk spoilage by PCR and multiplex PCR using species-specific primers.

MATERIALS AND METHODS

Collection and storage of milk samples. Five high-temperature, short-time pasteurized fluid milk samples (A to E) were
TABLE 1. **Species-specific primers used in PCR and multiplex PCR assays for the identification of Pseudomonas spp.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>Target</th>
<th>Target gene</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td><em>P. fluorescens</em></td>
<td>16S rRNA</td>
<td>Pf1 forward: 5'-CGTACGACCCGAAAAAGCC-3'</td>
<td>850</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pf1 reverse: 5'-CGTACGACCCGAAAAAGCC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiplex PCR</td>
<td><em>P. fragi</em></td>
<td>carA</td>
<td>Pfa forward: 5'-ATGCTTTGTGCGCTGCGCC-3'</td>
<td>370</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td><em>P. putida</em></td>
<td>carA</td>
<td>Pput forward: 5'-TGTGCGATTGCGTGCC-3'</td>
<td>230</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>P. lundensis</em></td>
<td>carA</td>
<td>Plun forward: 5'-TGTGCGATTGCGTGCC-3'</td>
<td>530</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pseu reverse: 5'-TGATGRCSSAOGCGATRCC-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Universal reverse primer.

**Microbiological analyses.** The milk samples were serially diluted in 0.1% peptone water and spread on plate count agar (Difco, Becton Dickinson, Sparks, MD). The plates were incubated at 35°C for 48 h to determine aerobic plate count or at 21°C for 48 h to determine rapid psychrotrophic count (RPC) (21).

**DNA extraction and PCR-DGGE.** Total bacterial DNA was extracted from each milk sample with the Milk Bacterial DNA Isolation Kit (Norgen, Thorold, Ontario, Canada) according to the manufacturer’s instructions. The extracted DNA was amplified with universal primers targeting the V3 region of the 16S rRNA gene. A GC clamp (5'-CAGGTTGTCATCTTCTCATGGC-3') (19) was attached to the 5' end of the forward primer 338F (5'-ACTTCACGGGAGGCAGCA-3') (2). The reverse primer HDA2 had the following sequence: 5'-GTATTACCGGCTGTGCGCC-3') (20). A reaction volume of 50 µl of PCR mixture contained 25 µl of PCR MasterMix (400 µM each dNTP, 3 mM MgCl2, and 1.25 U of Taq DNA polymerase; Promega, Madison, WI), 0.5 µM each primer, and 5 µl of template DNA. Sterile distilled water was included as a negative control. PCR was performed in a Personal Mastercycler (Eppendorf, Hamburg, Germany) by using the following program: 2 min at 94°C, 30 cycles of 1 min at 94°C, 30 s at 58°C, and 1 min at 72°C, and 7 min at 72°C. Then, 5 µl of PCR products were electrophoresed on a 2% agarose gel in 0.25X TAE buffer, pH 8.0 (TAE buffer). PCR markers (Promega) were included in the gel to estimate the size of amplicons. The gel was stained with SYBR Green for 20 min at 20 V and an additional 15 h at 70 V, which was followed by staining and photography as described above.

**Sequencing of DGGE fragments.** DNA bands on the denaturing gradient gel were excised with a sterile scalpel and eluted in 40 µl of sterile distilled water overnight at 4°C. Two microliters of the supernatants was reamplified with the primers 338F and HDA2. The PCR products were checked via electrophoresis and subsequently sequenced on an ABI PRISM 377 system (PerkinElmer, Norwalk, CT) at the Molecular Biology Core Facility of University of Hawaii at Manoa. To avoid amplification of artifact products, sequencing was performed from both the 5' and the 3' ends of each PCR product. Two sequences of sense and antisense strands were edited and assembled into a consensus sequence of corresponding amplicon. To determine the closest known relatives of the partial 16S rDNA sequences obtained, searches were performed on the GenBank database with the Basic Local Alignment Search Tool (BLAST) program (1). Ninety-five percent similarity was used as the criterion for species identification.

**Identification of predominant Pseudomonas spp. in milk.** Species-specific PCR was used to detect the following Pseudomonas spp. potentially present in each milk sample: *P. fluorescens*, *P. fragi*, *P. putida*, and *P. lundensis*. The target gene, sequence, amplicon size, and reference of the primers are shown in Table 1. The bacterial DNA extracted from the milk samples was subjected to a PCR assay described by Scarpellini et al. (23) and a multiplex PCR assay described by Ercolini et al. (9). Sterile distilled water was included in each assay to act as a negative control. After amplification, PCR products were electrophoresed on a 2% agarose gel, stained, and photographed as described above.

**RESULTS**

**Microbial growth in milk.** The microbiological quality of five pasteurized milk samples was examined at three time points during their shelf life. At 10 days before expiration, sample D had the highest aerobic plate count at 2.0 log CFU/ml, whereas sample A had the highest RPC at 2.8 log CFU/ml (Fig. 1). After 5 days of storage at 4°C, the RPC of four milk samples (A, C, D, and E) exceeded 20,000 (4.3 log) CFU/ml, the legal bacterial limit for grade “A” pasteurized milk, established by the U.S. Food and Drug Administration (25). On the expiration, the RPC increased to above 7.1 log CFU/ml, and the RPC markedly surpassed the aerobic plate count for the four samples. Only sample B during its shelf life exhibited the bacterial counts lower than the U.S. Food and Drug Administration regulatory limit. An average increase of 5.0 log was observed in the RPC of the other four milk samples during 10 days of refrigeration (Fig. 1), suggesting that psychrotrophic bacteria became dominant and represented a major causative agent for milk spoilage.

**Fingerprinting of bacterial populations in milk.** In order to monitor successive changes of microbial communities in pasteurized milk during storage, total bacterial DNA was extracted from the five milk samples and analyzed by PCR-DGGE. PCR with the primers 338F and HDA2 was used for DGGE analysis. A volume of 50 µl of PCR mixture contained 25 µl of PCR MasterMix (400 µM each dNTP, 3 mM MgCl2, and 1.25 U of Taq DNA polymerase; Promega, Madison, WI), 0.5 µM each primer, and 5 µl of template DNA. Sterile distilled water was included as a negative control. PCR was performed in a Personal Mastercycler (Eppendorf, Hamburg, Germany) by using the following program: 2 min at 94°C, 30 cycles of 1 min at 94°C, 30 s at 58°C, and 1 min at 72°C, and 7 min at 72°C. Then, 5 µl of PCR products were electrophoresed on a 2% agarose gel, stained, and photographed as described above.

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FIGURE 1. The aerobic plate count (APC) and rapid psychrotrophic count (RPC) of pasteurized milk samples (A to E) at 10 and 5 days before expiration (lanes -10 and -5) and on the expiration day (lane 0).

the GC clamp) and HDA2 yielded amplicons of 263 bp from all samples at three time points, but not from sterile distilled water (data not shown). In spite of the same size, the amplicons displayed different mobility features in the denaturing gradient gel. Numerous DGGE bands, derived from distinct 16S rRNA gene sequences, were observed (Fig. 2). Very few bands were observed for the samples at the early stage of storage. Over time, there was little change for the DGGE pattern of sample B, which met the microbiological standard for pasteurized milk. By contrast, several new bands, such as bands 2, 3, 4, 5, 9, 18, and 19, emerged for samples A, C, D, and E during refrigeration (Fig. 2).

Twenty major DGGE bands, as described in Figure 2, were excised and sequenced. Searches of the GenBank database with the BLAST program showed that 

...Pseudomonas spp. were found to become dominant in the microbial communities. For example, P. migulae (band 2) was detected in sample A at 5 days before expiration and on the expiration as well as in sample C on the expiration. A number of DGGE bands, including bands 4, 7, 9, 10, 11, 12, 14, 15, 18, 19, and 20, could be identified to the genus Pseudomonas. Nevertheless, further conclusion to the species level was unsatisfactory, while P. fragi, P. fluorescens, and P. putida appeared frequently in the lists of closest relatives (Table 2).

Identification of predominant Pseudomonas spp. in milk. In the 16S rRNA gene-based species-specific PCR assay (23), an 850-bp amplicon was produced for all the samples except sample B (Fig. 3). Its increased intensity over time proved that P. fluorescens was a predominant bacterial species in pasteurized milk under refrigerated conditions. In the multiplex PCR assay targeting the carA gene (9), P. fragi, evidenced via a 370-bp amplicon, was detected in sample C on the expiration as well as in samples D and E at 5 days before expiration and on the expiration day (Fig. 4). These results suggest that P. fragi grew to high numbers in milk during its shelf life. Further, the multiplex PCR assay did not create a product of 530 bp from

FIGURE 2. DGGE analysis of PCR-amplified 16S rRNA gene fragments from pasteurized milk samples (A to E) at 10 and 5 days before expiration (lanes -10 and -5) and on the expiration day (lane 0). Bands marked were excised and sequenced. The identifications are reported in Table 2.
any samples, indicating that *P. lundensis* is not an important contributor to milk spoilage in this study. Surprisingly, no amplicon corresponding to the *carA* gene of *P. putida* (230 bp in expected size) was yielded while the species was identified in PCR-DGGE profiles above.

**DISCUSSION**

Spoilage of processed milk is primarily due to bacterial activity that results in loss of sensory quality. However, not all microorganisms in milk are equally detrimental during refrigerated storage. Members of certain groups of bacteria only account for a small fraction of the initial microbial community in products; however, they may evolve into predominant populations and contribute to milk spoilage. In the present study, the use of PCR and DGGE provides critical information on dynamics of the bacterial populations in pasteurized milk during shelf life and the sanitary conditions of milk production.

Conventional high-temperature, short-time pasteurization can kill nearly all heat-sensitive psychrotrophic bacteria present in raw milk. Therefore, the typical microflora in fresh pasteurized milk are thermotolerant organisms that can survive pasteurization, and the total bacterial numbers are usually less than 1,000 CFU/ml. This study detected *Streptococcus* in virtually every milk sample tested, probably because that certain species of this genus are heat-resistant (3). Since most thermotolerant bacteria are unable to grow or grow slowly under refrigeration, this type of organisms, under the regulatory limit, seldom causes problems in quality of processed milk.

A close relative of *S. agalactiae* or *S. difficilis* was detected in samples B and D. It is reported that *S. agalactiae* and *S. difficilis* have very high similarity in their 16S rRNA sequences and biochemical characteristics (15). These *Streptococcus* spp. are known as major mastitis agents, which may lead to reduced milk production (27). Since these organisms can be eliminated or substantially reduced by pasteurization, experimental results here suggest that they may be distributed to dairy processing plants and contaminate milk after pasteurization.

Postpasteurization contamination by psychrotrophic bacteria present in environment is currently a detrimental factor for extending the shelf life of high-temperature, short-time pasteurized milk (3). This is supported by our results showing a significant increase in the RPC of samples A, C, D, and E during refrigerated storage. A number of studies indicate that *Pseudomonas* spp. are the most important psychrotrophic organisms implicated in spoilage of pasteurized milk (13, 22). Strains of *Pseudomonas* have been commonly isolated from product filling machines as

![Species-specific PCR for detection of *P. fluorescens* potentially present in pasteurized milk samples (A to E) at 10 and 5 days before expiration (lanes -10 and -5) and on the expiration day (lane 0). *M*, PCR markers; *W*, distilled water (negative control).](image-url)
well as from a variety of dairy processing units (13, 22). In fact, less than 1 CFU/ml of these contaminants may present a great concern in the shelf life of pasteurized milk. For instance, 1 CFU/ml of P. fluorescens cells with a generation time of 9.4 h at 4°C can reach 20,000 CFU/ml, the legal limit, in less than 1 week, under refrigeration (4). Enzymes and other metabolites produced by Pseudomonas can eventually cause milk spoilage.

As a matter of fact, great efforts have been devoted to characterizing Pseudomonas spp. associated with the spoilage of refrigerated milk. Wiedmann et al. (26) determined the predominant strains as P. fluorescens, P. fragi, and P. putida when they evaluated 70 putative Pseudomonas isolates from raw and processed milk. Dogan and Boor (6) observed that P. fluorescens and P. putida made up 90% of 338 Pseudomonas isolates from raw milk, processed milk, and environmental samples. In this study, analyses of the microbial communities in pasteurized milk by PCR-DGGE combined with species-specific PCR assays were in agreement with previous reports. The milk spoilage occurred during the last 5 days before expiration. Most of the causative bacteria belonged to one distinct group Pseudomonas spp. Within the genus, P. putida, P. fluorescens, and P. fragi were identified to play a significant role in milk shelf life, whereas P. migulae was for the first time detected in pasteurized milk. The advantage of PCR-DGGE is that this technique is capable of analyzing bacterial 16S rDNA extracted directly from milk samples during storage and rapidly screening multiple samples simultaneously, hence giving an overall picture of spoilage-related changes of the microbial communities. A well-defined trend in bacterial succession was observed over time in DGGE profiles of the pasteurized milk samples.

Theoretically, each band on the DGGE gel represents a bacterial species, and bands at different positions contain DNA fragments with different sequences. However, the identification of Pseudomonas spp. was unsatisfactory in the present study. It may be because of homogeneity of DGGE bands derived from different species (8). Most resulting sequences of the DGGE bands were homologous to sequences in several different Pseudomonas spp. In fact, previous research indicates that the differentiation of Pseudomonas at the species level based on 16S rRNA sequences is problematic due to insufficient degree of resolution of the gene for intragenic classification (28). In order to overcome this limitation, species-specific PCR assays were employed to detect those Pseudomonas spp. with great potential for food spoilage. Although P. fluorescence and P. fragi were identified, P. putida observed in DGGE profiles gave no signal for all samples in the multiplex PCR assay. This may reflect the fact that the forward primer targeting P. putida was designed based on the carA sequences of biotype A of this species (9). P. putida in tested milk samples may be biotype B.

PCR-DGGE has been widely used for characterization of microbial communities in fermented foods. It was shown here that this technique provides a powerful approach to monitoring the dynamic behavior of bacterial populations in pasteurized milk during refrigerated storage. The bacterial counts, DGGE profiles, and species-specific PCR outcomes consistently reflect the development of milk spoilage over time. Sequence analysis revealed a high diversity of Pseudomonas spp. responsible for the spoilage of pasteurized milk.

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REFERENCES