Direct Detection and Identification of Lactic Acid Bacteria in a Food Processing Plant and in Meat Products Using Denaturing Gradient Gel Electrophoresis

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ABSTRACT

We established a novel system using denaturing gradient gel electrophoresis (DGGE) to quickly identify bacteria known to be responsible for spoilage in meat processing plants and meat products. We extracted bacterial DNA from swabbed samples at various locations in the plant and from meat products and performed PCR amplification, targeting 16S rDNA from the dominant organisms. The amplification products were subjected to DGGE, and the contaminating bacteria in the meat products and the plant were analyzed. This analysis indicated that lactic acid bacteria and spoilage-causing bacteria are widely distributed within the meat processing plant. We developed molecular size markers to identify the dominant organisms obtained from the plant and meat products. The establishment of the present method allows quick and simple identification of bacteria causing the possible deterioration of products and contamination and thus permits constant monitoring of any harmful bacteria within meat processing plants.

Rapid detection of contaminating bacteria is important for preventing the spread of microbial contamination, and molecular methods may be employed to detect bacteria, including LAB. The denaturing gradient gel electrophoresis (DGGE) method (23), which is one of the typing methods, is an attractive method for the analysis of mixed cultures or bacterial communities. DGGE uses a polyacrylamide gel with a urea and formamide gradient to differentiate mixed DNA fragments amplified from multiple species. This method has been widely used in the analysis of bacterial succession in fermented foods such as whiskey (30), fermented sausages (7), and cheese (26). In the present study, DGGE was used for analysis of bacterial contamination in a large food processing plant and in the associated meat products.

MATERIALS AND METHODS

Isolation of LAB strains and their identification. LAB strains were isolated with conventional culture methods from different types of spoiled meat products such as Vienna sausage, ham, and roast pork that exhibited the formation of ropy slime or gas and had viable bacterial counts of more than 10⁸ CFU/g. A 25-g portion of spoiled meat (n = 30) was mixed with 225 ml of 0.85% saline in a stomacher bag, homogenized, and plated on BCP agar (Eiken, Tokyo, Japan). After incubation for 48 h at 30°C, 10 to 20 typical colonies were randomly picked and cultured in enrichment cultures for the extraction of DNA. All LAB strains isolated from meat products were identified by amplifying and sequencing an approximately 450- to 500-bp portion of 16S rDNA (Escherichia coli positions 50 to 450 or 500) (6). Amplification was performed using universal primers 27F and 1492R (21), and

Meat products such as ham and Viennese sausage are either vacuum packed or packed under a modified atmosphere after heat processing, and these products often become contaminated with lactic acid bacteria (LAB) (1, 2, 18, 22). Several main genera of LAB are reported to be responsible for the decomposition of meat products, including Lactobacillus (12, 13), Leuconostoc (20), and Weissella (formerly Lactobacillus) (5). The metabolic activities of LAB produce a sour flavor and odor changes (17).

Because the cooking process inactivates LAB, spoilage of meat products by LAB is mainly due to contamination after cooking. Preventing the growth of LAB on meat products is difficult because these bacteria can grow below 10°C (19) and are microaerophilic. Therefore, it is important to prevent contamination with these bacteria. In addition to LAB, Bacillus (4, 18) and Clostridium (10, 14) are ubiquitous in raw meat. However, these bacteria do not cause problems in many cases because they cannot grow at low temperatures.

At food processing plants, it is essential to quickly detect contaminating bacteria in the manufacturing line. However, to monitor LAB, it is necessary to swab surfaces, culture and isolate the bacteria, and then test all isolates for Gram stain, catalase, and carbohydrate fermentation. These procedures are time consuming and labor intensive.

PCR detection and DNA typing techniques have been widely used in epidemiological studies of pathogenic bacteria and studies on environmental bacterial communities.

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products were purified and sequenced directly using primer 27F. The BLAST 2.0 algorithm was used to compare the derived sequences to 16S rDNA sequences in the DDBJ database (http://www.ddbj.nig.ac.jp; Shizuoka, Japan).

Sampling procedure for meat products and at the processing plant. A total of 60 samples were obtained at a large meat processing plant, including samples of unspoiled meat products such as vacuum-packed sliced hams and Vienna sausages.

Swabs were taken on sterile gauze pads premoistened with sterile distilled water from 100-cm² areas of the processing rooms, a cold-storage room, a heating room, machines and equipment in the slicing and packaging area of the production line, and other areas used in processing at this industrial meat processing plant. The gauze pads were added to 9 ml of 0.85% saline and vigorously shaken. Aliquots of each extraction (1 ml) were transferred and incubated at 30°C for 24 to 48 h until growth became obvious in Gifu anaerobic medium (GAM) broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; 10.0 g of peptone, 3.0 g of soy peptone, 10.0 g of proteose peptone, 13.5 g of digested serum, 5.0 g of yeast extract, 2.2 g of meat extract, 1.2 g of liver extract, 3.0 g of dextrose, 2.5 g of potassium dihydrogenophosphate, 3.0 g of sodium chloride, 5.0 g of soluble starch, 0.3 g of L-cystein hydrochloride, 0.3 g of sodium thioglycollate, and 2% d-glucose dissolved in 1 liter of distilled water; final medium pH of 7.1).

Extraction of bacterial DNA for DGGE analysis. Bacterial DNA from 1 ml of enriched culture from plant swabs or meat product swabs was extracted using phenol-chloroform and ethanol precipitation (27). A 1-ml sample of enriched culture was centrifuged at 10,000 × g for 10 min, and bacterial cells were incubated in 567 μl of Tris-EDTA buffer containing lysozyme (2 mg/ml) for 1 h at 37°C. Cells were lysed by the addition of 30 μl of 10% (wt/vol) sodium dodecyl sulfate and 3 μl of 20-mg/μl proteinase K followed by incubation for 1 h at 37°C. Next, 100 μl of 5 M NaCl was added, and DNA was extracted with chloroform–isooamyl alcohol (24:1) followed by phenol–chloroform–isooamyl alcohol (25:24:1). DNA was then precipitated with isopropanol, washed with 70% ethanol, and dried. Purified DNA was dissolved in Tris-EDTA buffer and used as the DNA template for PCR.

PCR amplification for DGGE analysis. The following primer pair was chosen for the amplification of the V3 region (approximately 220 bp) of the 16S rDNA gene: forward primer with GC clamp (29) GC-339f (5′-CGC CCG CGC CCC CGC GCC CCC GTC CCG CCC CCC CCC CCT ACG GCA GGC AGC AG-3′) and reverse primer V3-53r (5′-GTA TTA CCG CGG CTG CTG CCG-3′). PCR amplification was performed in 100-μl reaction mixtures composed of 10 mM Tris-HCl (pH 8.3) 50 mM KCl, 1.5 mM MgCl₂, 50 pmol each of primer, 0.2 mM each of 4 dNTPs, and 2.5 U of Taq DNA polymerase (Takara Bio, Shiga, Japan) and 50 ng of template DNA. To minimize amplification of nonspecific products, “touchdown” PCR (11) was performed, where the initial annealing temperature was set at 8°C above the expected annealing temperature and decreased by 0.8°C every second cycle until the expected annealing temperature, 60°C, was reached (total 20 cycles), and then 5 additional cycles were carried out. Amplification was carried out using the following protocol: denaturation at 94°C for 1 min, annealing for 1 min, and primer extension at 72°C for 1 min in a GeneAmp 9600 thermal cycler (Applied Biosystems, Foster City, Calif.). Aliquots (5 μl) of the PCR products were analyzed first by electrophoresis in 2% (wt/vol) agarose gels.

DGGE analysis of PCR products. DGGE analysis of PCR amplification products was performed as described previously (23) using the DCode System apparatus (Bio-Rad Laboratories, Hercules, Calif.). Polyacrylamide gels (8% [wt/vol] acrylamide–bisacrylamide [37.5:1]) in 1× Tris-acetate-EDTA buffer with a denaturing gradient ranging from 30 to 60% denaturant (100% denaturation corresponds to 7 M urea and 40% [vol/vol] formamide) were prepared with the model 475 gradient delivery system (Bio-Rad). Polymerization was achieved by adding 0.9% (vol/vol) ammonium persulfate (10% solution) and 0.09% (vol/vol) N,N,N,N-tetramethylethylenediamine. The gels were electrophoresed at a constant 200 V at 60°C for 3 h. The DNA fragments were stained with ethidium bromide and washed with distilled water prior to UV transillumination.

Recovery of bands from DGGE gels and sequence analysis. Main DGGE fragments were selected for nucleotide sequence determination. Each band was excised with a sterile razor. The DNA of each fragment was eluted in 50 μl of Tris-EDTA buffer at 100°C for 10 min. The extracts were reamplified by PCR using the same primers and purified with the Ultrafree-MC 30,000 NMWL filter unit (Millipore, Bedford, Mass.) according to the manufacturer’s instructions. Purified DNA fragments were ligated in pT7blue vectors (Novagen, Darmstadt, Germany) and transformed into E. coli JM109. The transformants were grown on Luria-Bertani agar containing ampicillin and were screened by β-galactosidase assay. Plasmid DNA of selected transformants was isolated using a plasmid miniprep kit (Bio-Rad). The inserted DNA sequence, approximately 200 bp of 16S rDNA (E. coli positions 389 to 530) (6), was determined using an ABI310 genetic analyzer (Applied Biosystems) with the Big Dye terminator v3.0 cycle sequencing kit (Applied Biosystems). To identify the inserted sequences, the BLAST 2.0 algorithm was used to compare the derived sequence to 16S rDNA sequences in the DDBJ database. Species identification was made based on percent similarity to database sequences (98.0 to 100.0% similarity).

RESULTS

Optimization of DGGE conditions for LAB strains. The conditions of DGGE were optimized to efficiently separate the species of LAB that most frequently cause problems at food processing plants. DGGE was performed with eight strains of LAB (Leuconostoc citreum, Leuconostoc argentinum, Leuconostoc mesenteroides, Leuconostoc carnosum, Enterococcus malodoratus, Enterococcus faecalis, Lactobacillus sakei, and Lactobacillus fermentum) that were isolated as dominant species from spoiled meat products, and the following running conditions were determined to be optimal: 8% acrylamide gel with a denaturing gradient ranging from 30% to 60% and electrophoresis at 200 V for 3 h (Fig. 1, lanes 1 to 8). Fragments obtained from the same species had identical mobility, whereas those originating from distinct species had characteristically different mobilities.

DGGE analysis of enrichment cultures swabbed from meat products and the food processing plant and development of molecular markers. To develop molecular markers for DGGE analysis, we carried out swab sampling of meat products and several areas of the processing plant and processed these samples by PCR and DGGE analysis. These bands were recovered from DGGE gels, cloned, and sequenced (Table 1) to identify the bacteria based on band characteristics. In normal or spoiled meat products, only
one or two bands were observed by DGGE analysis. The bands detected from normal ham and sausage products were identified as Bacillus sp. (Bacillus cereus group), those detected in normal Vienna sausage were identified as Clostridium bifermentans, and those detected in spoiled products were identified as Leuconostoc and Weissella. LAB distribution was also monitored in two different production lines of a large meat processing plant. Although LAB can survive on various working surfaces, populations are very low when the production environment is adequately cleaned (data not shown). Therefore, to detect contaminating bacteria in the meat processing plant, swabbing samples were enriched in GAM broth. When all swabbing samples obtained from various working areas of the production line and surfaces of the machines in the meat processing plant were analyzed on the same DGGE gel, bands with the same migration patterns were observed in samples 1, 3, 4, 5, 9, 10, and 11 (Table 1). When these bands were excised from the gel and sequenced, all of them were identified as Lactococcus lactis. In these samples, some bands were also identified as Enterococcus dispar, Streptococcus pasteuri, Enterobacter sp., and Pseudomonas sp. Two bands from samples 3 and 4 associated with S. pasteuri showed the same migration. Two bands detected in sample 8 were both identified as E. dispar by 16S rDNA sequencing.

Based on all the sequenced samples, the PCR products produced species-specific migration patterns. Using these patterns, we developed two molecular size markers for the identification of bacterial species on the DGGE gels. One marker (lane M1, Fig. 2) was constructed with amplification

FIGURE 1. Optimized DGGE analysis using undesirable LAB species obtained from spoiled meat products. Lane 1, L. carnosum; lane 2, E. faecalis; lane 3, L. sakei; lane 4, L. argentinum; lane 5, E. malodoratus; lane 6, L. mesenteroides; lane 7, L. fermentum; lane 8, L. citreum.

### TABLE 1. 16S rDNA sequence identification of DGGE gel bands from enrichment cultures of swabs from meat products and a meat processing plant

<table>
<thead>
<tr>
<th>Sample</th>
<th>Origin</th>
<th>Band</th>
<th>Closest relative</th>
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<tbody>
<tr>
<td>PH1</td>
<td>Unspoiled ham</td>
<td>15-2</td>
<td>Bacillus sp.</td>
</tr>
<tr>
<td>PH2</td>
<td>Unspoiled ham</td>
<td>16-2</td>
<td>Bacillus sp.</td>
</tr>
<tr>
<td>A1</td>
<td>Unspoiled sausage</td>
<td>13a</td>
<td>Bacillus sp.</td>
</tr>
<tr>
<td>A2</td>
<td>Unspoiled sausage</td>
<td>14-2a</td>
<td>Clostridium bifermentans</td>
</tr>
<tr>
<td>D1</td>
<td>Spoiled meat product</td>
<td>a</td>
<td>Leuconostoc carnosum</td>
</tr>
<tr>
<td>D2</td>
<td>Spoiled meat product</td>
<td>b</td>
<td>Weissella hellenica</td>
</tr>
</tbody>
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<thead>
<tr>
<th>Meat processing plant</th>
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<tr>
<td>Sample</td>
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<tr>
<td>1</td>
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*a Bands included in the DGGE marker M2.*
products obtained from undesirable LAB (Leuconostoc citreum, L. mesenteroides, L. argentinum, Enterococcus faecalis, and L. carnosum) isolated from spoiled meat products (Fig. 1), and the other (lane M2, Fig. 2) was constructed with five species (Enterococcus dispar, S. pasteurii, Lactococcus lactis, Bacillus sp., and C. bifermantans) considered to be dominant species in this meat processing plant and the meat products (Table 1). These markers were produced from an equal volume mixture of PCR products amplified from LAB strains or an equal volume mixture of reamplified PCR products of recovered DGGE fragments.

**Identification of DGGE bands by constructed markers.** To evaluate whether identification of a band were possible using the constructed markers (M1 and M2), we collected additional swabs from the processing plant and meat products and performed DGGE. To evaluate the accuracy of the DGGE identification, we sequenced all the bands shown in Figure 2 (Table 2).

Of the eight swab samples, the bands identified as *L. lactis* were present in samples 1, 3, 4, and 6 (Fig. 2), and *E. dispar* was present in sample 7 (Fig. 2). The species identity of all of these bands was confirmed by sequence analysis (Table 2). Similarly, the fragments observed in ham (R1) and in sausage (S1) were identified as *Bacillus* sp. (B. cereus group) and *C. bifermantans* based on band migration (Fig. 2), and identification was confirmed by sequencing (Table 2). However, *Bacillus* sp. detected by sequencing in samples 3 and 4 and *Pseudomonas putida* detected in samples 1 and 2 were not identified with the marker because these sequences had not been included in the marker. The band detected in another position of sample 2 was identified as *P. putida* by 16S rDNA sequence analysis but could not be differentiated from *S. pasteurii* using the marker.

**DISCUSSION**

We selected the universal region of 16S rDNA as the target sequence for PCR primers and developed two DGGE markers, one for spoilage-causing LAB and one for strains obtained from the meat processing plant and unspoiled meat products. The first marker of spoilage LAB was constructed with strains *L. citreum, L. mesenteroides, L. argentinum, E. faecalis,* and *L. carnosum.* The second marker was constructed using swabblings at the meat processing plant and from meat products and represented *L. lactis, S. pasteurii,* and *E. dispers,* which were widely found in the meat processing plant, and *C. bifermantans* and *Bacillus* sp. (B. cereus group), which were widely found in meat products. To verify the markers developed in this study, swabs from the meat processing plant and meat products were analyzed. Almost all the main bands were excised from gels and sequenced for precise identification. We confirmed that most

![FIGURE 2. DGGE analysis of PCR-amplified 16S rDNA fragments obtained from swab samples from the meat processing plant and meat products. Lane M1, bacterial marker derived from amplification PCR products of recovered DGGE fragments. Lane M2, bacterial marker constructed from reamplified PCR products obtained from bands detected in DGGE analysis of the processing plant and meat products. Samples 1 to 7 were enrichment cultures obtained from swab samples from the meat processing plant, and samples R1 and S1 were swabbed from meat products.](http://meridian.allenpress.com/jfp/article-pdf/67/11/2515/1674386/0362-028x-67_11_2515.pdf)
bands with identical migration patterns on the gel represented the same species.

There are limitations associated with DGGE analysis for identification of bacteria. We observed bands in nearly the same positions that belonged to different organisms, such as *Pseudomonas putida*, which was detected in sample 2 but could not be differentiated from *S. pasteurii* in the marker. Because the band position on DGGE gels is affected by the melting point of the 16S rDNA fragments (23), it is likely that species having different sequences but the same melting point will show the same migration patterns (16, 25). Because DGGE detects the differences in the secondary structure of amplified DNA (22), there is no relation between sequence similarity and band position. Also, because we used the very short domain of V3, closely related species may be indistinguishable. This problem was also described by Ogier et al. (25), who insisted that some species including members of the *Lactobacillus casei* group or some subspecies of *L. mesenteroides* could not be distinguished on temporal temperature gradient gels because their sequences were closely related. Aside from the LAB, the *Bacillus cereus* group, which includes *B. cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, and *Bacillus mycoides*, are closely related (3, 15) and are indistinguishable in this type of analysis (8). We observed multiple bands identified as *E. dispar* in different positions on the DGGE gel (Table 1). This intraspecific heterogeneity has often been observed in DGGE banding patterns and may represent 16S rDNA heterogeneity (9, 24, 28).

Although the limitations of DGGE analysis for identification of bacteria should be considered, the DGGE analysis presented here could be helpful for detecting spoilage and other undesirable bacterial flora in meat processing plants. The most important advantage of this method is that it can be used to rapidly monitor the contamination sources in a meat processing plant. For example, when spoilage of a product occurs, it is possible to find the contamination source within 1 day using this method. We analyzed a spoiled ham sample using the DGGE method and detected a single strong band (data not shown). We then compared the band with the bands obtained from swabbing samples from various locations in the meat processing plant. Swabbing samples from two locations produced bands identical to that of the spoiled meat sample, one strong and the other weak (data not shown), and these bands were identified as *Leuconostoc* sp. by DGGE markers. In this way, we successfully and rapidly found the contamination sources within the plant. Although the resolution of this method is not high and it cannot be used to distinguish bacteria to strain, it can still be effective enough as a practical means for monitoring a meat processing plant. In the course of routine monitoring when different microorganisms are detected in swab samples from different sampling times in the same area, it has been assumed that disinfection is being adequately performed. However, when the same organism is consistently detected, it is assumed that the area has not been completely disinfected. At the processing plant in this study, *L. lactis* was detected twice in areas 1, 3, and 4, suggesting that disinfection of these areas was not sufficient. In addition, *L. lactis* and *E. dispar* were isolated from a wide area, indicating the prevalence of these organisms in this plant. *Leuconostoc* species, which are well-known meat spoilage organisms, were not detected in swab samples.

We have succeeded in developing a DGGE screening procedure to quickly assess bacterial contamination at a meat processing plant. This method can be applied to any meat processing plant. Adoption of this method for monitoring bacterial flora in products and at meat processing plants should facilitate more rapid assessment of contamination.

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REFERENCES


