Detection of Meat Spoilage Bacteria by Using the Polymerase Chain Reaction

K. S. VENKITANARAYANAN, M. I. KHAN, C. FAUSTMAN,* and B. W. BERRY

ABSTRACT

The growth of spoilage bacteria results in a shorter shelf life of meat, causing economic losses to the meat industry. Based on 23S rDNA sequence data of Pseudomonas aeruginosa, two primers designated as PF (23 bases) and PR (20 bases) were synthesized for use in the polymerase chain reaction. A unique 207-base-pair DNA product from nine different bacteria typically associated with meat spoilage was amplified by the primers. Dot blot analysis with the internal DNA probe specific for the amplified products confirmed that the amplified DNA sequence is specific for the spoilage bacteria studied.

Key words: Meat spoilage, Pseudomonas, PCR, DNA probe, beef

Meat is contaminated by a variety of microorganisms during slaughter and subsequent processing operations. The bacterial flora in meat can be broadly divided into two groups, namely pathogenic and spoilage bacteria. The pathogenic bacteria are of public health significance, whereas spoilage bacteria are primarily responsible for quality losses to the food industry. The most common bacteria typically associated with spoilage of aerobically stored meat include Pseudomonas fluorescens, P. putida, P. fragi, P. aerofaciens, Acinetobacter calcoaceticus, Enterobacter liquifaciens, Flavobacterium spp., Moraxella spp., and Brochothrix thermosphactum.

Several studies have revealed that ribosomal RNA (rRNA) is the molecule of choice for evaluating phylogenetic relationships among microorganisms (3, 16). Studies on cataloguing and sequence analysis of 16S and 23S rRNAs have shown that these macromolecules contain regions exhibiting a high degree of sequence conservation among bacteria. Genes coding for ribosomal RNA are especially useful for the construction of specific DNA probes for various groups of microorganisms such as the Pseudomonas fluorescens group (6), legionellas (4), members of the genus Proteus (10), and mycoplasmas (9). Regensberger et al. (21) reported that the regions of sequence conservation are longer and more distinct in 23S rRNA than in 16S rRNA. The same researchers developed probes specific for Micrococcus luteus-Micrococcus lysae, for the Arthrobacter-Micrococcus group, for eubacteria and a universal probe using a gene coding for 23S rRNA from M. luteus.

The polymerase chain reaction (PCR) and DNA probes provide powerful and rapid tools for the detection of specific microorganisms (14, 19). Although numerous PCRs and DNA probes have been used for the rapid detection of food-borne pathogens (12, 13, 18), relatively little research has been performed in developing these techniques for detecting spoilage bacteria. In the present study, we developed a novel DNA-based PCR which detects all of the aforementioned meat spoilage bacteria.

MATERIALS AND METHODS

Bacterial species and culture medium

The bacterial species used in this study are listed in Table 1. All bacteria except E. liquifaciens and B. thermosphacta were cultivated aerobically in 50 ml of nutrient broth (Difco Laboratories, Detroit, MI) overnight for 18 h at 30°C on a rotary shaker at 250 rpm (17). E. liquorifaciens and B. thermosphacta were grown at 30°C without shaking.

Isolation of chromosomal DNA from bacteria

Chromosomal DNA of the bacteria was isolated as described by Sambrook et al. (23). After overnight growth in the culture medium as specified above, bacteria were pelleted by centrifugation at 8,000 x g for 30 min, washed twice with 5 ml of phosphate-buffered saline, and resuspended in 2 ml of TE buffer (10 mM Tris and 1 mM EDTA, pH 7.6). Bacteria were lysed with sodium dodecyl sulfate (SDS) at a final concentration of 1% and incubated at 37°C for 1 h. The mixture was then treated with proteinase K (100 µg/ml, final conc.) (Sigma Chemical Co., St. Louis, MO) and incubated at 37°C for 1 h. The solution was extracted twice with an equal volume of phenol: chloroform:isoamyl alcohol (1:1:24). The DNA was precipitated from the aqueous layer with 0.1 volume of 3 M sodium acetate (4°C) and two volumes of absolute ethanol (−20°C) and was incubated overnight at −20°C. The precipitated DNA was pelleted by centrifugation at 8,000 x g for 10 min and resuspended in 1 ml of TE buffer. The concentration of DNA was estimated from absorbance at 260 nm and the DNA was stored in aliquots of 50 µl at −20°C.
TABLE 1. Typical meat spoilage bacteria detected by PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas fluorescens</td>
<td>NRRL B-10(^a)</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>NRRL B-22</td>
</tr>
<tr>
<td>Pseudomonas fragi</td>
<td>NRRL B-727</td>
</tr>
<tr>
<td>Pseudomonas aureofaciens</td>
<td>NRRL B-1576</td>
</tr>
<tr>
<td>Acinetobacter calcoaceticus</td>
<td>ATCC 19976</td>
</tr>
<tr>
<td>Enterobacter liquefaciens</td>
<td>ATCC 27502(^b)</td>
</tr>
<tr>
<td>Flavobacterium breve</td>
<td>ATCC 43319</td>
</tr>
<tr>
<td>Moraxella osloensis</td>
<td>ATCC 9200</td>
</tr>
<tr>
<td>Brochothrix thermosphacta</td>
<td>ATCC 11509</td>
</tr>
</tbody>
</table>

\(^a\) NRRL: National Center for Agricultural Utilization Research, Peoria, IL (Northern Regional Research Laboratory).
\(^b\) ATCC: American Type Culture Collection, Rockville, MD.

Polymerase chain reaction
Primer determination and synthesis. The primers were selected from the 23S rDNA sequence of P. aeruginosa (6). The forward (PF) and reverse (PR) primers (Table 2) were selected on the basis of criteria described by Innis and Gelfand (11). The two sets of primers were synthesized on a model 380B DNA synthesizer (Applied Biosystems Inc., Foster City, CA) with the assistance of the University of Connecticut Biotechnology Center. Before use, the primers were desalted by passage through a Sephadex G-25 column (Pharmacia Biotech, Inc., Piscataway, New Jersey). The concentration of primers was determined by measuring absorbance at 260 nm using a spectrophotometer and the primers were stored at -20°C in 20-μl volumes.

Optimization of PCR. PCR was carried out as described by Saiki et al. (22) using reagents from a GeneAmp PCR Kit (Perkin Elmer Cetus, Norwalk, CT). A reaction volume of 100 μl of PCR mixture contained 100 mM Tris-HCl (pH 8.3); 500 mM KCl; 200 μM each of dATP, dCTP, dGTP, and dTTP; 0.9 μM of each primer; 2.5 units of AmpliTaq\(^R\) (Perkin Elmer Cetus, Norwalk, CT) DNA polymerase; and 2.5 mM MgCl\(_2\). Template DNA (2 μg) from each of the bacteria was used for the amplification. Two micrograms of calf thymus DNA (Clontech labs, Inc., Palo Alto, California) and sterile distilled water were used as the negative controls. The reaction mixture was overlaid with 50 μl of mineral oil. PCR was performed in an automatic DNA thermal cycler (Model 480, Perkin Elmer Cetus, Norwalk, Connecticut). Following preliminary trials with different annealing temperatures and times, the thermal cycler was programmed for optimum PCR conditions. Initially, the reaction mixture was heated at 95°C for 5 min, then the PCR run for 30 cycles at a melting temperature of 92°C for 1 min, annealing temperature of 56°C for 1 min, and extension temperature of 74°C for 1 min. The sample was then heated at 74°C for 7 min.

Meat samples. The samples used in this experiment were from top round (seminembrinosus) beefsteaks purchased from a local supermarket and stored at -18°C until the experiment was started. The experiment was performed on beef steaks stored at 4°C for 0, 1, 3, 6, 9, and 11 days. From each sample, a circular piece of meat (diameter 3 cm, depth 1 cm) was removed using a sterile stainless-steel meat corer and transferred into a sterile stomacher bag (Integrated Biosolutions Inc., Monmouth Junction, New Jersey) containing 50 ml of sterile 0.1% peptone water (1 g of peptone [Difco Laboratories, Detroit, MI] in 1,000 ml of distilled water). The stomacher bag was placed in a stomacher (Model 400, Tekmar, Cincinnati, OH) and the sample homogenized at high speed for 1 min. A volume of 10 μl of homogenate from the stomacher bag was aseptically transferred into a sterile microfuge tube.

We performed PCR directly with bacteria isolated from meat by using Gene Releaser (Bioventures, Murfreesboro, TN) rather than first isolating bacteria and then extracting chromosomal DNA as separate steps. All samples (10 μl of meat homogenate) were treated with 20 μl of Gene Releaser and subjected to a temperature regimen according to the manufacturer’s protocol. Then the PCR reagent mixture was added to the samples and PCR performed as described. Initial experiments performed with and without Gene Releaser yielded similar results and indicated that Gene Releaser is advantageous in eliminating a separate step for extraction of DNA from bacteria.

The aerobic plate counts of the meat samples were determined by using the spread plate technique (26) using plate count agar (Difco). Duplicate 0.1-ml aliquots from appropriate dilutions of the meat homogenate in the stomacher bag were each spread on plate count agar plates and incubated at 25°C for 48 h.

Detection of PCR products. Gel electrophoresis was used to detect the amplified PCR products in each of the samples. A volume of 10 μl of PCR products was electrophoresed on a 1.5% agarose gel; the gel was stained with ethidium bromide and exposed to ultraviolet light to determine the presence and size of the amplified DNA product.

DNA probe and dot blot hybridization. The PCR product (approximately 200 ng as determined from A\(_{260}\)) from each of the nine spoilage bacteria diluted in 100 μl of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was denatured at 95°C for 10 min, rapidly cooled in ice, and then spotted onto a nitrocellulose membrane (Biorad, Hercules, CA). The filter was then dried, baked, prehybridized and hybridized as reported by Nguyen et al. (19). A 50-base oligonucleotide selected from the internal sequence of the PCR-amplified DNA product was synthesized using a DNA synthesizer (Model 380B, Applied Biosystems Inc., Foster City, CA) at the University of Connecticut Biotechnology Center. The oligonucleotide was labelled with [\(^{32}\)P]ATP (Amersham Corporation, Arlington Heights, Illinois) using T4 polynucleotide kinase (Life Technologies, Inc., Grand Island, NY). DNA hybridizations were performed in a buffer containing 1 mM EDTA, 0.5 M NaH\(_2\)PO\(_4\) (pH 7.2), and 7% SDS at 60°C for 18 h. The membrane was washed twice (30 min each time) in a buffer containing 1 mM EDTA, 40 mM NaH\(_2\)PO\(_4\) (pH 7.2), and 5% SDS and again twice in a buffer containing 1 mM EDTA, 40 mM NaH\(_2\)PO\(_4\) (pH 7.2), and 1% SDS. The membrane was allowed to dry and then exposed to X-ray film for 24 hr at -70°C.

RESULTS

When the forward primer (PF) and reverse primer (PR) were used in the PCR, they directed the amplification of a 207-bp sequence in the chromosomal DNA from the pure cultures of the nine spoilage bacteria, but not in calf thymus DNA or distilled water (Figure 1). Similar results were also obtained when the bacteria isolated from meat samples were subjected to the same PCR conditions (Figure 2). The 50-base oligonucleotide probe synthesized from the internal sequence of the PCR product also hybridized with the amplified products from all the samples except calf thymus

---

TABLE 2. Base sequence of oligonucleotide primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF</td>
<td>AAGCTTGCTGGAGGTATCATGAAAGTGCC</td>
</tr>
<tr>
<td>PR</td>
<td>CTCCGGCCTCCATGCGACTG</td>
</tr>
</tbody>
</table>
observed that Gene Releaser could be used successfully to perform the PCR directly with samples, since the results were the same as in the PCR with purified chromosomal DNA.

**DISCUSSION**

In detecting food-borne pathogens, DNA probes and PCR have been developed for the specific identification of each microorganism, as it is essential to confirm the causative agent from a public-health standpoint. However, in the case of spoilage bacteria, as they are mainly of economic significance, there is no necessity for detecting them individually in foods. Therefore, it would be more appropriate to design a single probe or PCR that would detect the majority of spoilage bacteria as a group for a given commodity. A common detection system would be more economical and require less time for results than an assay for each species of spoilage bacteria present in meat. The ultimate application of such an assay would be in the rapid quantitation of the total spoilage bacteria load in meat. Genes coding for rRNA are commonly used for developing probes specific for various groups of bacteria (6, 21). Therefore, we selected primers for the PCR from the published 23S rDNA sequence of *P. aeruginosa*. The primers resulted in the amplification of a common and unique 207-bp fragment in the DNA of all the spoilage bacteria under study. A similar approach was adopted by Tsai and Olson (28) who developed a PCR to amplify two specific 16S ribosomal gene fragments for detecting low numbers of bacterial cells in soil. Specific DNA sequences from native bacterial populations present in soil, sediment, and sand samples were amplified by using primers for either "universal" eubacterial 16S rRNA genes or mercury-resistance (mer) genes (2). The PCR with eubacterial primers amplified a 1.5-kb fragment, whereas the primers for mer genes resulted in the amplification of a 1-kb DNA fragment.

While meat contains a heterogenous bacterial flora at the start of retail display, *Pseudomonas* species usually outcompete and dominate other bacteria at spoilage (1, 27). Therefore PCR was performed on bacteria from beef steaks stored at 4°C for different periods of time to determine if the bands of PCR products became narrower with decreases in storage time and bacterial population of the samples. These results showed that the concentration of the amplified DNA product following controlled amplification of bacterial genomic DNA from meat appeared dependent on bacterial number, thus indicating that the PCR could potentially be developed into a quantitative assay for enumeration of spoilage bacteria in meat. It was also found that the use of Gene Releaser replaced the necessity for separate extraction of bacterial DNA for PCR, thereby making the assay more rapid and simple.

Quantitative PCR is a relatively new technique which has the potential for rapid detection and quantification of
target nucleic acid sequence. Sninsky and Kwok (25) developed a QPCR for rapid quantification of human immunodeficiency virus (HIV) RNA in the plasma or sera of acquired immune deficiency syndrome (AIDS) patients, as a tool for monitoring the efficacy of therapeutic intervention. A similar approach was reported by Ferre et al. (5), who developed a QPCR for the quantitation of HIV-1 DNA in the blood cells of AIDS patients undergoing immunotherapeutic treatment. Currently in our laboratory, experiments are in progress to modify the present assay into a quantitative PCR (QPCR) for rapid estimation of the spoilage bacteria load in meat. Successful application of PCR technology to an assay that determines bacterial load in meat would offer a unique and rapid technology alternative to conventional methods for determining bacterial load and thus the spoilage status of meat. The present work elucidating appropriate PCR primers for detecting spoilage bacteria is a logical first step in achieving this goal.

ACKNOWLEDGEMENT

Support for this research was provided by the United States Department of Agriculture, ARS, Beltsville, MD, and U.S. Army Natick R.D. & E Center, MA. Thanks are expressed to Drs. Gary Shults, Frank DiLeo, Curtis Blodgett, and Richard Worfel for their assistance. We also thank the Storrs Agricultural Experiment Station, College of Agriculture and Natural Resources, University of Connecticut. Scientific contribution no. 1659, Storrs Agricultural Experiment Station.

REFERENCES