

Detection of Beer Spoilage Organisms by Polymerase Chain Reaction Technology

Youichi Tsuchiya, Hirotaka Kaneda, Yukinobu Kano, and Shohei Koshino, *Brewing Research Laboratories, Sapporo Breweries, Ltd. 10, Okatohme, Yaizu-shi, Shizuoka, 425, Japan*

ABSTRACT

A sensitive detection and identification method for beer spoilage organisms was developed. The procedure involves the electrophoretic analysis of DNA fragments amplified by the polymerase chain reaction. When genomic DNA of *Lactobacillus brevis* was amplified, using a set of primers, to give a 117-base pair fragment encompassing the 5S rRNA gene (rDNA) and then electrophoretically analyzed, a single cell of *L. brevis* was detected. When various populations of *L. brevis* were added to 250-ml volumes of pasteurized beers, collected by a membrane filter, and then analyzed by this method, the detection limit was about 30 cells. It was expected that these primers could specifically be used to detect lactic acid bacteria. Another set of primers encompassing the 100-base pair fragment of 5S rDNA of *Saccharomyces cerevisiae* was synthesized, and using the two sets of primers in mixtures, *L. brevis* and *S. cerevisiae* were detected and distinguished. These results suggested that the detection and identification of beer spoilage organisms can be done at the same time using suitable primers.

Keywords: Beer spoilage organisms, Contamination, Detection, Lactic acid bacteria, PCR, Yeast

The microbiological safety of food is a primary concern and implicit obligation of any food manufacturer. Luckily, pathogens die in beer. Therefore, spoilage microbes are the only concern in normal brewing products. The recent increase in the consumption of draft beer as opposed to pasteurized beer in the Japanese beer market has made the biological monitoring of beer spoilage microorganisms even more important.

The plate count method for enumerating microbiological contamination has remained unchanged for over a century, but it requires several days before the microorganisms are detected. Many rapid detection methods for low concentrations of microorganisms recently have been developed (7,11,13,15,18,20). However, organisms cannot be reliably identified to genus with these methods nor are the methods sensitive.

The polymerase chain reaction (PCR) process is a simple and powerful method first described by K. Mullis (12). It allows amplification of DNA segments in vitro through a succession of incubation steps at different temperatures. The basis of the technique is shown in Figure 1. The double-stranded DNA is heat-denatured and the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a cycle. The PCR process is based on the repetition of this cycle, and the process can amplify the target DNA segment specifically by at least 10^5 -fold and potentially as high as 10^9 -fold (16). The PCR technology has been used successfully for biochemical, genetic, medical, and medicolegal studies (3).

In this article, we report that the analysis of PCR-amplified DNA fragments can be applied to the rapid and sensitive detection of beer spoilage organisms.

EXPERIMENTAL

Cultures

Bacterial and yeast strains were obtained from the American Type Culture Collection (ATCC), Maryland; Japan Collection of Microorganisms (JCM), Saitama, Japan; Institute for Fermen-

tation Osaka (IFO), Osaka, Japan; and Institute of Applied Microbiology (IAM), Tokyo, Japan. They included *Lactobacillus brevis* JCM 1059^T, *L. pastorianus* JCM 1113, *L. casei* JCM 1163, *Pediococcus damnosus* JCM 5886^T, *Corynebacterium facians* IAM 1079, *Microbacterium flavum* IAM 1642, *Sarcina lutea* IFO 3232, *Streptococcus lactis* IFO 12007, *Pseudomonas fragi* IAM 1650, *Escherichia coli* JCM 1649^T, *Saccharomyces batatae* ATCC 44970, *S. bisporus* IFO 0723, *S. exiguus* ATCC 44972, and *S. cerevisiae*, our brewery strain. They were inoculated into dextrose-yeast extract medium (19) and incubated at 28°C. The cell population was determined using agar plates of the same medium incubated at 28°C. Each developed colony was assumed to have grown from one viable unit.

Recovery of DNA

Bacterial and/or yeast cells in 100- μ l cultures were transferred into 1.5- μ l Eppendorf tubes containing 150 μ l of the mixture of 1.67M sucrose, 0.67% 2-mercaptoethanol, zymolyase (0.33 mg/

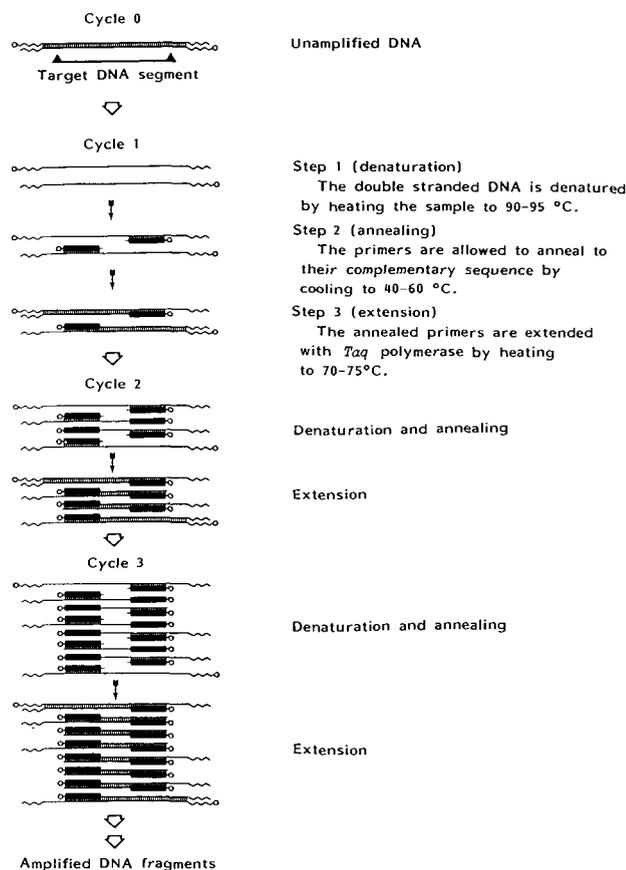


Fig. 1. DNA amplification system by polymerase chain reaction. One set of three steps (denaturation, annealing, and extension) is referred to as a cycle. The number of target DNA copies approximately doubles every cycle. *Thermus aquaticus* DNA polymerase (*Taq* polymerase) is thermostable and is not inactivated by the high temperature; therefore, the reaction components (template, primers, deoxynucleoside triphosphates, *Taq* polymerase, and buffer) can all be assembled at the beginning of the procedure, and the amplification reaction is carried out by simply cycling the temperature within the reaction tube.

ml, Seikagaku Kogyo Co., Tokyo, Japan), lysozyme (0.33 mg/ml, Sigma Chemical Co., St. Louis, MO), and mutanolysin (6.7 μ g/ml, Sigma) in 16.7 mM potassium phosphate buffer (pH 6.8) and incubated at 37°C for 1 hr. Then 150 μ l of the mixture of 13.3 mM MgCl₂, 2.7% Triton X-100, 2.7% diethyl pyrocarbonate, proteinase K (0.27 mg/ml, Merck, Darmstadt, Germany), and 6.7% sodium dodecyl sulfate in 10 mM potassium phosphate buffer (pH 6.8) was added and incubated at 70°C for 10 min. Extracted DNA was further purified by phenol extraction and ethanol precipitation as described by Wallace (21). In the ethanol precipitation, 1 μ g of λ -DNA (Wako Pure Chemical Industries, Osaka, Japan) was used as a coprecipitant for effective DNA recovery.

Recovery of DNA of *L. brevis* in Beer

Pasteurized beer (250 ml) containing various populations of *L. brevis* was filtered through a 47-mm-diameter, 0.22- μ m filter (GVWP04700, Nihon Millipore Kogyo K. K., Yonezawa, Japan). The filter was transferred into a 10-ml polyallomer tube containing 5 ml of ethanol. After the tube was capped and shaken at a speed of 140 strokes per minute for 30 min at room temperature, the filter was removed. The ethanol in the tube was completely dried with a centrifugal concentrator (Tomy Seiko Co., Ltd., Tokyo, Japan). Cells remaining in the tube were resuspended in 100 μ l of double distilled water, and their DNA was extracted as already described.

PCR Amplification

Buffer, deoxynucleotide triphosphates, and *Thermus aquaticus* DNA polymerase (*Taq* polymerase) were obtained from GeneAmp kits (Perkin-Elmer Cetus, Norwalk, CT) and were used according to the manufacturer's protocols. Custom-made and purified oligonucleotide primers were purchased from Takara Shuzo Co., Ltd., Kyoto, Japan. Temperature cycles were carried out in a TR-100 thermoprocessor (Taitec Co., Saitama, Japan), using step-cycle settings of 1 min at 94°C (denaturation), 2 min at 50°C (primer annealing), and 1.5 min at 72°C (DNA extension) for 35 cycles.

Detection of Amplified DNAs

PCR-amplified DNAs were detected with gel electrophoresis. The DNAs were separated using an 8% vertical polyacrylamide gel with a bed size of about 80 \times 80 mm. Ten-microliter samples were applied in each lane. Polyacrylamide gels were run in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM ethylenediaminetetraacetic acid, pH 8.0) at 43 V/cm for 12 min. The

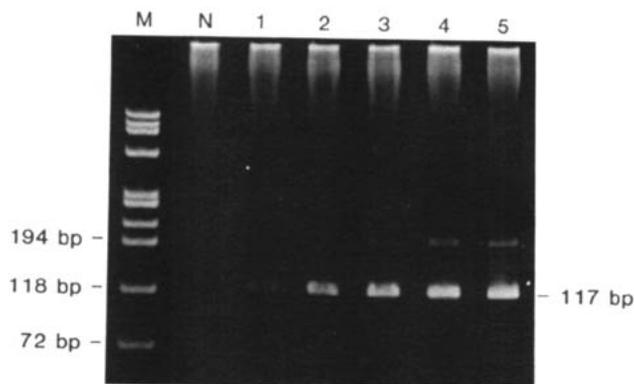


Fig. 2. Polyacrylamide gel (8%) electrophoretic analysis after polymerase chain reaction amplification of 100- μ l samples of various populations of *L. brevis* using primers Lb5S-1 and Lb5S-2. The cell population was determined by the colony count method. Lane M, DNA size marker, ϕ X174/*Hae*III digest (Wako); N, negative control with λ -DNA and no added cells; 1, one cell; 2, eight cells; 3, 50 cells; 4, 10³ cells; and 5, 10⁴ cells. bp = Base pair.

gels were stained in ethidium bromide solution (0.5 μ g/ml), visualized with a UV transilluminator (SL-800F, Funakoshi Co., Ltd., Tokyo, Japan), and photographed.

RESULTS AND DISCUSSION

Sensitivity for the Detection of *L. brevis*

Very low levels of contamination by lactic acid bacteria (*Lactobacillus* and *Pediococcus*) can lead to the spoilage of final packaged products (1). *L. brevis* was therefore selected as the monitor organism for this procedure. Based on the 5S rRNA sequence of *L. brevis* reported by Woese et al (22), the 20-mer primers, Lb5S-1 (5'-TGTGGTGGCGATAGCCTGAA) and Lb5S-2 (5'-GCGTGGCAACGTCCTATCCT), were synthesized. These primers encompass a 117-base pair (bp) region of the 5S rRNA gene (rDNA). We selected 5S rDNA for the target sequence because the 5S rDNA is generally represented by multiple copies in the genome. The size of the target region, 117 bp, was planned as small as possible for faster polymerization and electrophoretic analysis. Using 100- μ l samples of various populations of *L. brevis*, the sensitivity for the detection of *L. brevis* by the PCR then was determined (Fig. 2). A 117-bp product, the amplified DNA, was produced from only one cell. It was suggested that the PCR allows single genomic copy (single cell) detection and that this detection level for *L. brevis* is as sensitive as that reported for coliform bacteria (2) and eukaryotic cells (6,10).

Of course, when we assess the microbiological safety and spoilage of beer in breweries, we must start from a collection of microorganisms from beer. Therefore, various populations of *L. brevis* were added to 250 ml of pasteurized beer, collected by the filter, and analyzed by the PCR method (Fig. 3). The detection limit of *L. brevis* was about 30 cells per 250 ml of beer. The detection limit was, however, about one cell when a 100- μ l sample and a filter were put into the 10-ml tube without the filtration process (data not shown). It seemed that because cells were buried in the filter and could not be washed out sufficiently, the filtration process reduced the detection limit. With a more efficient method of cell collection, the detection limit can be improved. Although 250 ml of beer was filtered in this case, a filter can deal with much more beer; therefore, the detection limit per volume of beer can be increased. This method requires only about 11 hr for the detection without the culturing of *L.*

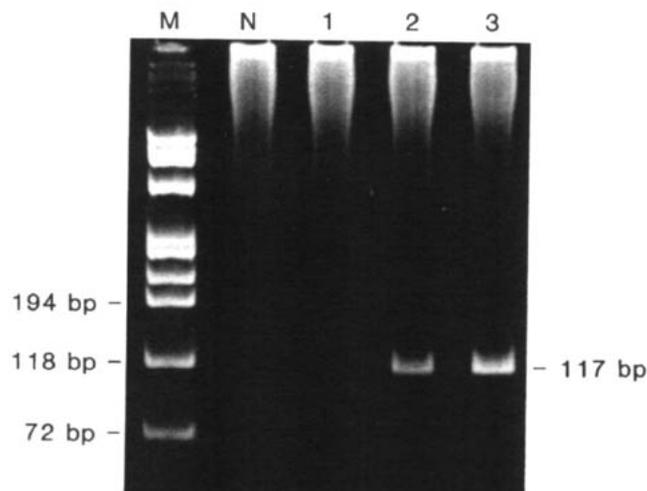


Fig. 3. Polyacrylamide gel (8%) electrophoretic analysis after polymerase chain reaction amplification of various populations of *L. brevis* in beer using primers Lb5S-1 and Lb5S-2. The cell population was determined by the colony count method. Lane M, DNA size marker, ϕ X174/*Hae*III digest (Wako); λ -DNA, negative control with λ -DNA and no added cells; 1, five cells; 2, 30 cells; and 3, 10² cells. bp = Base pair.

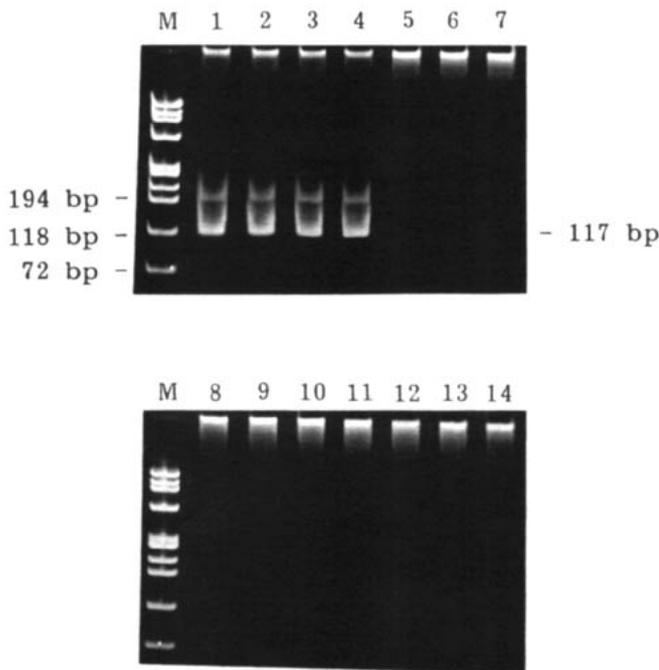


Fig. 4. Polyacrylamide gel (8%) electrophoretic analysis after polymerase chain reaction amplification of various cells, the populations of which were more than 10^4 cells in 100- μ l samples. Lane M, DNA size marker, ϕ X174/*Hae*III digest (Wako); 1, *L. brevis*; 2, *L. pastorianus*; 3, *P. damnosus*; 4, *L. casei*; 5, *E. coli*; 6, *M. flavum*; 7, *S. lutea*; 8, *S. lactis*; 9, *P. fragi*; 10, *C. facians*; 11, *S. cerevisiae*; 12, *S. batatae*; 13, *S. bisporus*; and 14, *S. exiguus*. bp = Base pair.

brevis. This compares favorably with the present plate count methods and many rapid detection methods (7,11,13,15,18,20).

Specificity of primers Lb5S-1 and Lb5S-2

Homology of 5S rDNA among genetically related species is high and has been applied in the construction of phylogenetic trees of prokaryotes and eukaryotes (4,5,8,9,17). So these primers, Lb5S-1 and Lb5S-2, which were designed for *L. brevis*, seem to be applicable to the detection of other lactic acid bacteria. To study the specificity of these primers, PCR amplification was tested for some other bacteria and yeast (Fig. 4). Using extracted DNA of *L. brevis*, *L. pastorianus*, *L. casei*, or *P. damnosus*, the respective amplified DNAs were observed. On the other hand, amplified DNAs were not produced when the extracted DNA of *C. facians*, *M. flavum*, *S. lutea*, *S. lactis*, *P. fragi*, *E. coli*, *S. batatae*, *S. bisporus*, *S. exiguus*, or *S. cerevisiae* were used. It was expected that these primers could specifically detect lactic acid bacteria. Of course, all lactic acid bacteria cannot grow in beer, and *L. casei*, which was detected with these primers, is innocuous. We are now searching for more specific primers for the typical beer spoilage organisms.

Distinction between *L. brevis* and *S. cerevisiae*

The identification of microorganisms is very complex and time-consuming. If the detection and identification of beer spoilage organisms can be done at the same time, it is quite useful for brewing microbiology. So, the distinguishable detection of different types of organisms such as *L. brevis* and *S. cerevisiae* was tested (Fig. 5). We synthesized the 20-mer primers, Sc5S-3 (5'-GAGACATTGTGAGACCCTCC) and Sc5S-4 (5'-CGATGCCGCCACGTGCAAAG), based on the sequence of 5S rDNA of *S. cerevisiae* reported by Piper et al (14), to give a 100-bp fragment. When *L. brevis* and/or *S. cerevisiae* in 100 μ l cultures was tested by the PCR with primers Lb5S-1, Lb5S-2, Sc5S-3, and Sc5S-4,

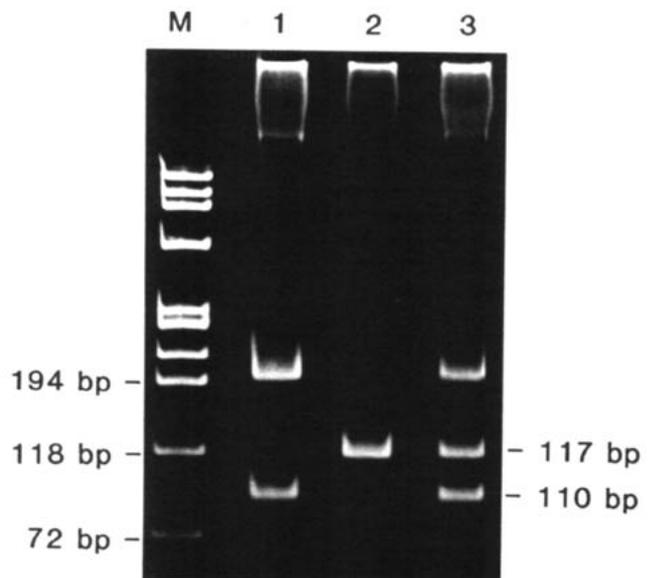


Fig. 5. Polyacrylamide gel (8%) electrophoretic analysis after polymerase chain reaction amplification of *L. brevis* and/or *S. cerevisiae* whose populations were more than 10^4 cells in 100- μ l samples using primers Lb5S-1, Lb5S-2, Sc5S-3, and Sc5S-4 at the same time. Lane M, DNA size marker, ϕ X174/*Hae*III digest (Wako); 1, *S. cerevisiae*; 2, *L. brevis*; and 3, *S. cerevisiae* and *L. brevis*. bp = Base pair.

a 100-bp and some other sized products were produced from *S. cerevisiae*, and a 117-bp product was produced from *L. brevis*. It was, therefore, concluded that *L. brevis* and *S. cerevisiae* can be specifically detected and distinguished by the size of the amplified DNA. Moreover, it is expected that beer spoilage organisms can be detected and identified at the same time by the use of multiple primers that are suitable for plural species and produce different sizes of amplified DNA.

CONCLUSIONS

In this preliminary report, the potential of the PCR technology has been demonstrated as a rapid, sensitive, and specific means of assessing the microbiological contamination of beer. With a more efficient method of cell collection, some simplification of the DNA extraction method, and the development of more appropriate primers for beer spoilage organisms, the PCR can be made to be more useful and convenient and may be applicable to the brewing industry.

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