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Note

Rapid Detection of Acetic Acid Bacteria in the Traditional Pot-Fermented Rice Vinegar *Kurozu*

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Knowledge of the microbial population, particularly that of acetic acid bacteria (AAB), present during the fermentation process is required to produce good quality traditional rice vinegar (*Kurozu*). We focused on the internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes of AAB for easy and rapid detection of AAB from *Kurozu*. Five PCR primer sets were designed to amplify the five specific DNA fragments within the ITS region of AAB. PCR amplification with these primer sets resulted in the detection of specific fragments from AAB chromosomal DNA, but not from other bacteria or yeast. Use of a DNA sample directly isolated from *Kurozu* mash as a template gave the same distinct PCR fragment pattern with these primer sets. This one-step PCR analysis is an easy tool for rapid detection of AAB during the long process of *Kurozu* fermentation and maturation.

Keywords: *Kurozu*, rice vinegar, easy detection, acetic acid bacteria, ITS region, PCR

Introduction

Vinegar is one of the most commonly used cooking flavors with both nutritional and pharmacological value (Hayashi *et al.*, 2007). Many different types of vinegars exist worldwide, made from various sources such as grains, fruits, and roots (Horiuch *et al.*, 1999; Giumanini *et al.*, 2001; Terahara *et al.*, 2003; Ye *et al.*, 2004; Masino *et al.*, 2008). The rice vinegar *Kurozu* is a traditional product of Kagoshima, Japan, which is produced by long manufacturing processes including a ~6-month fermentation step followed by ~3-year maturation step. This long production process is performed entirely in an outdoor field without any temperature control, and observation by skilled workers is continued for successful fermentation. Knowledge of the state of microflora during the long fermentation and maturation periods of *Kurozu* production may aid workers for improving the control of the fermentation conditions.

In addition to the classical culture-dependent method, culture-independent techniques are being used to profile microbial populations in their natural environments (Muyzer, 1999). In the field of food microbiology, a similar approach has been applied to food ecosystems such as cheese, sourdough, and sausage (Giraffa, 2004). In the case of vinegar fermentation, the sequence analysis of the PCR-amplified 16S rRNA gene accompanied by denaturing gradient gel electrophoresis (DGGE) (De Vero *et al.*, 2006; Haruta *et al.*, 2006; De Vero and Giudici, 2008) or restriction fragment length polymorphism (RFLP) (Ilabaca *et al.*, 2008) has been reported.

In this study, we described a rapid and easy one-step PCR amplification method using specific primer sets that does not require additional digestion steps using restriction enzymes. In order to detect and distinguish AAB strains from other bacteria during the *Kurozu* manufacturing process, we focused on the PCR amplification of specific fragments from the acetic acid bacteria (AAB) 16S-23S internal transcribed spacer (ITS) region.

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Materials and Methods

Bacterial strains and growth conditions *Acetobacter aceti* (IAM 1802), *Acetobacter pasteurianus* (IAM 1803), and *Gluconobacter oxydans* (IAM 14436), were obtained from the Institute of Applied Microbiology (IAM) Culture Collection (Tokyo University), and *Gluconacetobacter xylinus* (NBRC13772) was purchased from the National Institute of Technology and Evaluation Biological Resource Center. *A. pasteurianus* strain Sakamoto AN-23 was isolated from fermenting *Kurozu* mash.

AAB were grown in 0.5% yeast extract, 0.5% polypeptone, and 1% glucose medium (pH 6.5). *Bacillus subtilis* Marburg168, *Staphylococcus aureus* Wood46, *E. coli* C600, and *Pseudomonas aeruginosa* PAO1 were cultured in LB broth (1% bacto tryptone, 0.5% bacto yeast extract, and 1% NaCl, pH 7.0). *Chromohalobacter* sp. *#560* was grown in nutrient broth (1% beef extract and 1% polypeptone) supplemented with 2 M NaCl. *Brevibacillus choshinensis* HPD31 was grown in TM medium (1% glucose, 1% polypeptone, 0.5% meat extract, 0.2% yeast extract, 0.001% $FeSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot H_2O$, and 0.0001% $ZnSO_4 \cdot 7H_2O$). *Saccharomyces cerevisiae* 20B12 was grown in YPD medium (1% bacto yeast extract, 2% bacto peptone and 2% glucose, pH 7.0). *Lactobacillus* sp. E523 isolated from *Kurozu* was grown in MRS medium (1% casein peptone tryptic digest, 1% meat extract, 0.5% yeast extract, 2% glucose, 0.1% Tween 80, 0.2% K_2HPO_4, 0.5% sodium acetate, 0.2% ammonium citrate, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.005% $MnSO_4 \cdot H_2O$, pH 6.4). *S. aureus*, *E. coli*, and *Chromohalobacter* sp. *#560* were grown at 37°C and other bacteria were grown at 30°C.

DNA manipulation and DNA sequence analysis DNA manipulation was carried out using standard procedures (Sambrook *et al.* 1989). Chromosomal DNA was isolated by the method of Ausubel *et al.* (1987). The direct extraction of DNA from the *Kurozu* sample was performed according to the method of Haruta *et al.* (2006). Nucleotide sequences were determined by the dideoxy chain termination method using a BigDye terminator cycle sequencing kit (Applied Biosystems, CA, USA).

PCR amplification The PCR conditions for five primer sets were as follows: initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min.

The 40-μL PCR mixture contained 0.5 μL (1 U) Vent polymerase (New England BioLab, MA, USA), 4 μL of 10× Thermpol buffer, 2 μL each of the primer solutions (10 μM), 4 μL dNTP (4 mM for each dNTP), and 40 ng extracted DNA. Reagents used were of the highest grade commercially available.

Results and Discussion

To acquire information about microorganisms in food ecosystems, techniques to isolate and cultivate them are required. There was a detailed report based on culture-dependent analysis of pot vinegar *Kurozu* (Koizumi *et al.*, 1996). However, this approach is time-consuming for rapid detection of ABB. Therefore, several methods for identifying AAB in vinegar involving molecular-based analysis of the coding regions of 16S rRNA and/or 16S-23S rRNA have been developed (Poblet *et al.*, 2000; Trcek, 2005; González *et al.*, 2006; Gullo *et al.*, 2006). However, these methods require additional restriction enzyme treatment after PCR amplification of the target gene. This step complicates these methods for practical use.

As the ITS region between 16S and 23S rRNA genes is diverse among various bacteria, we used this region for detection and possible identification of AAB by analyzing specific PCR-amplified fragments. First, two primers (I and D; Table 1) were designed to amplify the whole ITS region of AAB. The conserved regions were identified by their alignment with the 16S rRNA gene sequences from the 32 AAB strains that have been deposited in DNA Data Bank of Japan (DDBJ), and the forward primer I was made from a region located near the end of the 16S rRNA gene. The sequence corresponding to the 461-441st base of the 23S rRNA gene of *Gluconacetobacter europaeus* DSM6160 (EMBL accession number X89771) was used for reverse primer D (Table 1).

Using chromosomal DNA from four AAB type strains *A. aceti* (IAM 1802), *A. pasteurianus* (IAM 1803), *Gluconobacter oxydans* (IAM 14436), and *Gluconacetobacter xylinus* (NBRC13772), fragments containing the ITS region were amplified by PCR using the specific primer set (I and D), and their nucleotide sequences were determined. These four ITS sequences were deposited in the DDBJ with the accession numbers AB161358, AB161452, AB162115, and AB161453, respectively. The size of the four ITS regions of AAB varied from about 700 to 800 bp. These differences in fragment lengths implied differences between AAB strains. Therefore,

Table 1. Nucleotide sequences of the six designed primers.

Primer name and position	Sequence
16SrRNA gene	
I 1359-1378th	5’-GTTGGTTTGACCTTAAGCCG-3’
ITS region	
II 124-142nd	5’-GCGCCGTCAACATATCCCT-3’
A 142-124th	5’-AGGGATATGTTGACGGCGC-3’
B 324-305th	5’-GCTTGCAAAGCAGGTGCTCT-3’
C 540-521st	5’-CGCACCAACCGATTCACACT-3’*
23SrRNA gene	
D 461-441st	5’-TCACCTTTCCCTCACGGTACT-3’

Primer I; primers II, A, B and C; and primer D are derived from the 16S rRNA gene, ITS region, and 23S rRNA gene, respectively. The position number corresponds to the region of *Gluconacetobacter europaeus* DSM6160. *Primer C has two different nucleotides from the corresponding sequence of *G. europaeus* DSM6160 (EMBL accession no. X85406).

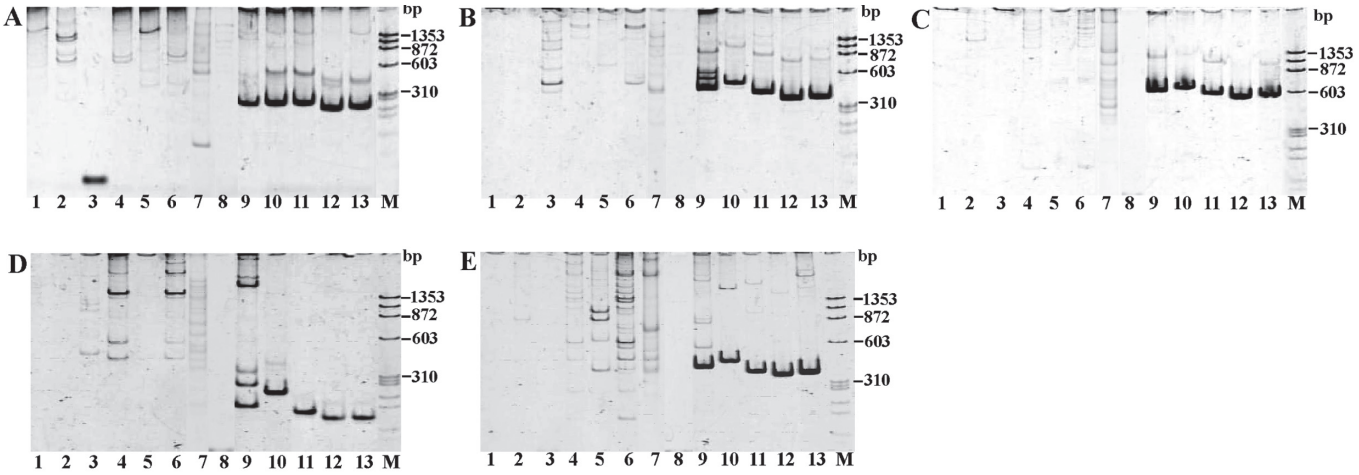

Fig. 1. PCR analysis of chromosomal DNA from various bacterial strains and five type strains of acetic acid bacteria.
The strains in each lane are: lane 1, *Pseudomonas aeruginosa* PAO1; 2, *Escherichia coli*; 3, *Chromohalobacter* sp. 560; 4, *Staphylococcus aureus* Wood46; 5, *Brevibacillus choshinensis*; 6, *Bacillus subtilis* Marburg168; 7, *Saccharomyces cerevisiae*; 8, *Lactobacillus* sp. E523; 9, *Acetobacter aceti* (IAM 1802); 10, *Acetobacter pasteurianus* (IAM1803); 11, *Gluconacetobacter xylinus* (NBRC 13772); 12, *Gluconobacter cerinus* (IAM 1832); and 13, *Gluconobacter oxydans* (IAM 14436). Lane M: *Hae*III-digested ΦX174 fragments as molecular weight marker. The respective primer sets used are: I and A (**A**); I and B (**B**); I and C (**C**); II and B (**D**), and II and C (**E**). Amplified PCR products were detected by electrophoresis on a 5% polyacrylamide gel in Tris-borate buffer (50 mM Tris, 50 mM boric acid, and 2.5 mM EDTA, pH 8.1).

we examined which parts of the sequence were conserved between different strains by aligning 22 ITS sequences from *Acetobacter* spp., *Gluconobacter* spp. and *Gluconacetobacter* spp. from the database (Sievers *et al.*, 1996; De Vero *et al.*, 2006) with the four sequences determined in this study. The conserved sequences, sequence II for forward direction, and sequences A, B, and C for the reverse direction were determined (Table 1). In addition to the four AAB type strains, chromosomal DNA was extracted from *Gluconobacter cerinus* (IAM 1832), several other bacterial strains described in the legend for Fig. 1, baker's yeast, and *A. pasteurianus* Sakamoto AN23 isolated from *Kurozu*. To obtain specific patterns of the amplified fragments for discrimination of AAB from other bacteria and possible identification, five primer sets (I and A, I and B, I and C, II and B, and II and C) were used for PCR amplification.

As shown in Fig. 1, specific fragments were observed in the AAB strains (~250 bp, ~400 bp, ~600 bp, ~200 bp, and ~400 bp in lanes 9-13, respectively), which were not observed in the other bacteria or yeast (lanes 1-8). *Lactobacillus* sp. E523, isolated from *Kurozu*, showed no detectable amplified fragments (lane 8). Thus, these primer sets can be used to specifically detect AAB by one-step PCR amplification. In the case of *A. aceti*, larger bands in addition to 443-bp and 194-bp fragments were seen (Fig. 1B and 1D, lane 9). The length of the 16S-23S rRNA ITS region in ABB, with the exception of the highly conserved $tRNA^{ILE}$ and $tRNA^{Ala}$ genes, shows diversity as described by Trcek (2005). But identification at the species level of AAB is generally difficult. Even with the PCR-RFLP technique, distinction of species such as *Gluconacetobacter liquefaciens*, *Gluconacetobacter xylinus*, and *Gluconacetobacter europaeus* is difficult (Ruiz *et al.*, 2000). The analyses of ITS sequences acquired from the GenomeNet database (i) showed that *Gluconobacter* spp. have shorter ITS regions than *Acetobacter* spp. and *Gluconacetobacter* spp. So the distinction of ABB genera by analyzing the ITS region might be feasible. In Fig. 1D, *Acetobacter* spp. (lanes 9 and 10), *Gluconacetobacter* sp. (lane 11), and *Gluconobacter* spp. (lanes 12 and 13) may be distinguished by comparing the fragment lengths in each gel pattern .

To examine the specificity of the five designed primer sets for direct detection of AAB from *Kurozu* mash, DNA was prepared directly from fermenting *Kurozu* mash (at 60 days) without isolation of microorganisms and subjected to PCR amplification. As shown in Fig. 2A, the five primer sets successfully amplified the specific fragments, giving the same pattern as that of each primer set (each lane 10, Fig. 1A-E). Thus, AAB cells were present in the *Kurozu* sample after 60 days of fermentation and most likely comprised *A. pasteurianus* based on its similar pattern (lane 10) compared with other strains (lanes 9, 11-13). No amplified DNA fragment was obtained from 7-day fermented *Kurozu* (data not shown), suggesting that the growth of AAB was insufficient for detection in the early period of fermentation. No detection of AAB by DGGE was reported by Haruta *et al.* during this early fermentation period. In stages with a very low microbial population, the amount of extracted DNA is likely to be lower than the detection limit for PCR.

The PCR-amplified fragments obtained using the chromosomal DNA extracted from *A. pasteurianus* strain Sakamoto AN23 as a template (Fig. 2B) exhibited the same pattern as that shown in Fig. 2A, confirming that the AAB strain in *Kurozu* is *A.*

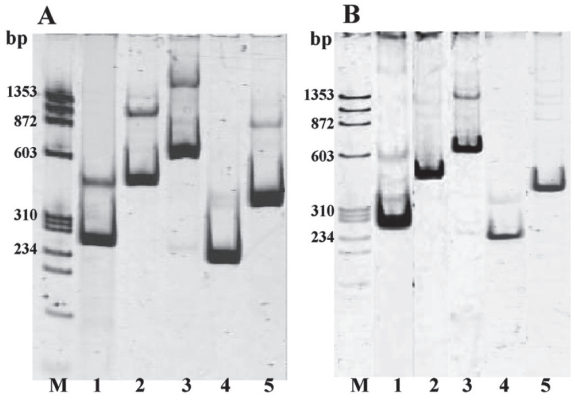

Fig. 2. PCR profile of the five primer sets.
A, DNA extracted directly from *Kurozu* mash (60 days fermentation) was used as the PCR template. B, Chromosomal DNA from *Acetobacter pasteurianus* Sakamoto AN23 isolated from *Kurozu* was used as the PCR template. The primer sets are: lane 1, I and A; 2, I and B; 3, I and C; 4, II and B; and 5, II and C. Lane M, *Hae*III-digested ΦX174 fragments. The electrophoresis conditions are the same as those described in Fig. 1.

pasteurianus.

A major limitation of this molecular method is the inability to distinguish between living and dead microorganisms. Furthermore, biases may occur during DNA extraction and PCR amplification. However, this culture-independent approach is a rapid and sensitive detection method for microbial communities in food like *Kurozu*. This powerful tool should be used while keeping its limitations in mind.

In conclusion, as a first stage detection and possible identification of AAB, this one-step PCR amplification method is applicable to *Kurozu* mash and does not require time-consuming isolation of bacteria.

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