JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 104, No. 6, 481–489. 2007
DOI: 10.1263/jbb.104.481

© 2007, The Society for Biotechnology, Japan

Molecular Monitoring of Bacterial Community Structure in Long-Aged Nukadoko: Pickling Bed of Fermented Rice Bran Dominated by Slow-Growing Lactobacilli

Jiro Nakayama,[1]* Hiroyuki Hoshiko,[1] Mizuki Fukuda,[1] Hidetoshi Tanaka,[1] Naoshige Sakamoto,[1] Shigemitsu Tanaka,[1] Kazutoshi Ohue,[2] Kenji Sakai,[2§] and Kenji Sonomoto[1,3]

Department of Bioscience and Biotechnology, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan,[1] Department of Applied Chemistry, Faculty of Engineering, Oita University, 700 Dannoharu, Oita 870-1192, Japan,[2] and Department of Functional Metabolic Design, Bio-Architecture Center, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan[3]

Received 28 May 2007/Accepted 18 September 2007

Nukadoko is the fermented rice bran bed traditionally used for pickling vegetables in Japan. Here, we investigate the bacterial community structure of nukadoko using several culture-independent methods. Denaturing gradient gel electrophoresis (DGGE) and sequence analysis of V2-V3 16S rRNA gene (16S rDNA) fragments amplified from a long-aged nukadoko bacterial community indicated seven predominant operational taxonomic units (OTUs) closely related to known *Lactobacillus* species. Phylogenetic analysis of these OTUs indicated a major cluster consisting of six OTUs including a dominant OTU closely related to *Lactobacillus acidifarinae* and one distinct OTU corresponding to *Lactobacillus acetotolerans*. *L. acetotolerans* was commonly detected as a dominant species in samples from different seasons. The succession of microbial community structure in the fermentation and ripening processes was investigated using a laboratory model nukadoko. The *L. acidifarinae*-like bacteria grew rapidly with a pH decrease in the first few days after inoculation, whereas *L. acetotolerans* grew slowly and became dominant after one week. Real-time quantitative polymerase chain reaction (Q-PCR) showed that the doubling time of *L. acetotolerans* was 12 h, while that of total bacteria was 4 h. Real-time quantitative reverse transcription polymerase chain reaction (Q-RT-PCR) targeting 16S rRNA showed a low metabolic activity of *L. acetotolerans* throughout the fermentation and ripening processes. Fluorescence *in situ* hybridization (FISH) showed that *L. acetotolerans* was a dominant bacterium in the ripening period and had a low metabolic activity. These results indicate that the slow-growing *L. acetotolerans* stably dominated nukadoko microbiota after the *L. acidifarinae*-like bacteria mainly contributed to the lactic acid fermentation of the rice bran.

[**Key words:** nukadoko, fermented rice bran, microbiota, lactic acid fermentation, denaturing gradient gel electrophoresis, quantitative real-time polymerase chain reaction, quantitative reverse transcription real-time polymerase chain reaction, fluorescence *in situ* hybridization, lactobacilli, *Lactobacillus acetotolerans*]

Nukadoko is the fermented rice bran bed traditionally used for pickling vegetables at home in Japan. Nukadoko is prepared by natural fermentation from salt-water-pasted rice bran with various spices and vegetables for a period lasting from a few days to a few weeks and then can be kept for several weeks or months while being used for the daily pickling of vegetables. It is also possible to maintain a batch of nukadoko for a longer period, sometimes a number of years, by adding a small volume of fresh or roasted rice bran from time to time. As in the case of the long-aged nukadoko used in this study, nukadoko can sometimes be aged for a number of years without spoilage despite its repetitive natural fermentation with nonsterilized rice bran and vegetables. This aging process also tends to enhance its quality, *e.g.*,

* Corresponding author. e-mail: nakayama@agr.kyushu-u.ac.jp
phone: +81-(0)92-642-3020 fax: +81-(0)92-642-3021
§ Present address: Department of Plant Resources, Faculty of Agriculture, Graduate School, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan.

Abbreviations: DGGE, denaturing gradient gel electrophoresis; FISH, fluorescence *in situ* hybridization; OTU, operational taxonomic unit; Q-PCR, real-time quantitative polymerase chain reaction; Q-RT-PCR, real-time quantitative reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate.

favorable flavor and well-balanced microbiota (1, 2).

The microbiota of nukadoko mainly consists of lactic acid bacteria and yeast (1). The balance of lactic acid bacteria and yeast, which is maintained by stirring the nukadoko bed daily, is important for providing moderate sourness and a favorable flavor to the pickled vegetables. Imai *et al.* (2) analyzed the microbial composition of nukadoko using a culture-based method and found not only yeast and lactic acid bacteria (*e.g.*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, *Enterococcus faecalis*, *Tetragenococcus halophila*, and *Lactobacillus brevis* as predominant species) but also other gram-positive and gram-negative bacteria. Such a microbial consortium could play important roles in providing a complex flavor to and stabilizing the quality of aged nukadoko. However, to our knowledge, their paper is the only published scientific report characterizing the microbial composition of nukadoko in detail and our current knowledge is not sufficient to understand microbial ecosystem in nukadoko.

Recently developed culture-independent methods based on 16S rRNA diversity have allowed us to gain an overview of microbial community structure. In particular, PCR-denaturing gradient gel electrophoresis (DGGE) fingerprinting is very efficient for comparing the microbial community structure among a number of samples and monitoring the dynamics in a microbial ecosystem in a certain environment (3, 4). Owing to those advantages, DGGE has been used to study the microbial community structure of fermented foods such as cheese (5), rice vinegar (6), malt whisky (7), and sourdough (8, 9). In this study, we examined the bacterial community structure of a long-aged nukadoko using DGGE. The dynamics and activity of the bacterial community in the fermentation and ripening processes of nukadoko were also monitored in a laboratory model nukadoko by quantitative real-time polymerase chain reaction (Q-PCR), quantitative real-time reverse-transcription polymerase chain reaction (Q-RT-PCR), and whole-cell fluorescence *in situ* hybridization (FISH) analysis in addition to DGGE analysis.

MATERIALS AND METHODS

Nukadoko sampling The long-aged (at least 150 years) nukadoko samples used in this study were obtained from a nukadoko manufacturer in Fukuoka city. During its manufacture, the batch (approximately 50 *l*) of long-aged nukadoko was maintained by adding small volume (about 5% to 10%) of fresh rice bran about every 10 d. The batch was stirred by hand once a day. The batch has been continuously used for pickling vegetables using common procedures. The storage and operations were carried out at room temperature during the manufacture.

To obtain a laboratory model nukadoko to monitor changes in bacterial community structure during fermentation and ripening processes, 25 g of the long-aged nukadoko was mixed with fresh rice bran (500 g) including salts (25 g), and a small amount of other ingredients, including sliced red chili pepper, Japanese pepper, stock from tangle weed, and citron skin, and distilled water (600 ml) was added. The mixture was incubated in a plastic container with a lid at 22°C and stirred well once a day. One gram of the nukadoko sample was collected every day. The sample was vortexed with 9 ml of distilled water and then used for pH measurement and the DNA extraction described below.

DNA isolation from nukadoko DNA isolation from the nukadoko was performed on the basis of the bead beating method. One gram of nukadoko was suspended in 2.5 ml of 10 mM phosphate buffer (pH 8.0) and was then mixed with 1 ml of reagent D (0.1 M NaCl, 0.5 M Tris–HCl, 10% SDS, pH 8.0). After vortexing, the suspension was incubated at 65°C for 1 h. Then, the suspension was transferred into a 2-ml screw-capped tube containing 0.3 g of zirconium beads (diameter, 0.1 mm), and the cells were lysed by 10 cycles of bead beating (3000 rpm, 30 s, multi beads shocker MB-200; Yasui Kikai, Osaka) with a 30 s interval on ice. The beads and cell debris were removed by centrifugation at 20,400×*g* for 5 min. The crude DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. The DNA was further purified using a Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) and finally dissolved in 50 µl of a DNA rehydration solution supplied in the kit and stored at 4°C.

For the model nukadoko experiment, a short-step protocol was employed instead of the above-mentioned method. We have confirmed a similar result to those obtained using the above-mentioned method. Nukadoko (0.5 g) was weighed in a 2.0-ml screw-capped tube containing 10 mM phosphate buffer (pH 8.0) and 0.3 g of zirconium beads. The tube was shaken 3 times at 3000 rpm for 1 min using the multi beads shocker MB-200 with a 1 min interval on ice between shaking. Then 300 µl of phenol-chloroform was added to the lysed suspension and the mixture was shaken twice at 2000 rpm for 1 min. The upper layer was applied onto a QIAamp spin column (Qiagen, Hilden, Germany) and eluted with 100 µl of distilled water after washing the column according to the manufacturer's instructions.

PCR-DGGE and phylogenetic analysis The V2-V3 region of the 16S rRNA gene (rDNA) was amplified by PCR using HDA1-GC and HDA2 primers (Table 1) (10). PCR was performed in a 50-µl solution containing 25 µl of the Premix Ex *Taq* polymerase (Takara Bio, Shiga), 2 µl of the isolated DNA solution (20 ng/µl), and 5 pmol of each primer. The PCR conditions were as follows: 95°C for 3 min, 30 cycles consisting of 95°C for 30 s, 56°C for 30 s and 72°C for 1 min, and finally 72°C for 1 min. The specific amplification of 16S rDNA was confirmed by agarose gel electrophoresis analysis. The PCR products were purified with a QIAquick PCR purification kit (Qiagen) according to the protocol provided by the manufacturer. The purified products were quantified using GENEQUANT *pro* (Amersham Pharmacia Biotech, Uppsala, Sweden), and then stored at −20°C until DGGE analysis.

DGGE analysis was performed as described previously (11, 12) using a Dcode System apparatus (Bio-Rad, Hercules, CA, USA). The amplicons were resolved using an 8% polyacrylamide gel with a denaturing gradient of 30% to 65%, where 100% corresponds to 7 M urea and 40% (v/v) formamide in Tris-acetate buffer (40 mM, pH 8.0) containing 1 mM EDTA. As reference markers, the inserts of some clones in the 16S rDNA clone libraries prepared as described below were amplified using the same set of primers and was then applied onto the denaturant gradient gel. Approximately 650 ng of the PCR amplicons were loaded into each lane and run at 100 V for 6.5 h at 60°C. The resulting gel was stained with 1×SYBR Gold (Molecular Probes, Eugene, OR, USA), visualized under UV light and then photographed. The image of the gel photograph was inverted and is shown in Figs. 1–3.

In order to obtain reference clones to identify the predominant bands in the DGGE gel, the V2-V3 region of 16S rDNA was amplified as described above except that we used the HDA-1 primer instead of the HDA1-GC primer. The amplicons were ligated to the pGEM-T vector (Promega, Madison, WI, USA) and was then transformed into *Escherichia coli* JM109. From the resultant 16S rDNA clone libraries, some colonies were randomly chosen, and the cloned 16S rDNA fragments were amplified by PCR using the HDA1-GC and HDA2 primers as described above and used for DGGE analysis. Clones giving a fragment that migrated to the

Download English Version:

https://daneshyari.com/en/article/22126

Download Persian Version:

https://daneshyari.com/article/22126

Daneshyari.com