Diversity of the Bacterial Community Found in Samma-narezushi (Saury Narezushi) Revealed by the 16S rRNA Gene Clone Library

HIROKI MATSUI*, RIE TSUCHIYA2, YUKA ISOBE2, HIROTO MAEDA1, AND MIYO NARITA2

1Graduate School of Bioresources and 2Faculty of Education, Mie University, Tsu 514-8507, Japan

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Narezushi is one of Japanese traditional foods and is made by fermenting salted fish meat and cooked rice together. In this study, the microbial diversity of samma-narezushi (narezushi using saury, Cobolabis saira) was analyzed by the 16S ribosomal RNA gene (rDNA) clone library. Randomly selected 89 clones were sequenced and phylogenetically analyzed. The sequences were classified into 12 operational taxonomy units (OTUs) at the 97% identity threshold. Most of the clones (89%) were lactic acid bacteria (LAB) and included Lactobacillus sakei, Leuconostoc gelidum, Lactococcus lactis subsp. lactis and L. pisium. The most predominant clone was L. sakei at 72% of the clones. The second most abundant clone was L. gelidum. These results suggest that L. sakei mainly contributes to the lactic acid fermentation process, taste and flavor of samma-narezushi. The present study showed that the culture independent method is useful for the microbial diversity analysis of narezushi.

Key words: Narezushi/Fermented food/Lactic acid bacteria/Lactobacillus sakei/16S rDNA clone library.

INTRODUCTION

Narezushi is a fermented fish food widely found in South-East and North-East Asia and is made by fermenting salted fish meat together with cooked rice for a certain period (Ishige, 1993). It is processed as a preserved food mainly by lactic fermentation, and the long preservation period of the perishable proteinaceous food is achieved by lowering the pH (Cooke et al., 1993). Prototypically, narezushi was fermented for a long period. This type of narezushi in Japan is now known as funazushi and is fermented over 6 months (Isobe et al., 2002). At the present day, narezushi that is fermented for 5 d to a couple of months is commonly found in Japan. This kind of narezushi is known as nama-narezushi and is found in various regions in Japan. In Mie prefecture, Japan, nama-narezushi is made using various kinds of fish, such as samma (saury, Cobolabis saira), ayu (sweetfish, Plecoglossus altivelis altivelis), konoshiro (spotted sardine, Konosirus punctatus), and saba (mackerel, Scomber japonicus), and produced as ritual or celebratory food. Since narezushi is empirically known as an intestinal regulator, narezushi is recognized as a functional food. Therefore, narezushi is considered as an important food for health as well as a culturally important food in the region where narezushi is consumed.

The nucleotide sequence of the 16S ribosomal RNA gene (rDNA) provides essential information for the current identification of bacteria and a considerable number of sequences has been accumulated in the public nucleotide database. The PCR-derived clone library method using the 16S rDNA sequence is the first step for the diversity analysis of an unknown microbial ecosystem. This approach allowed us to perform an encompassing analysis of the microbial community in the various environments. Recently, the bacterial community structure in Kimchi, a Korean...
fermented vegetable food, has been analyzed by the 16S rDNA clone library (Kim et al., 2005). Only a limited number of studies has done on the microbial diversity involved in fermentation process of narezushi (Fujii et al., 1992; Isobe et al., 2002). Furthermore, microbial diversity analysis was carried out by the culture dependent technique in these previous studies. Thus exhaustive analysis of the microbial diversity of narezushi has yet to be done. The purpose of this study is to analyze the diversity of bacteria involved in the fermentation of sammanarezushi (narezushi using saury), produced in Mie Prefecture, by 16S rDNA clone library analysis.

MATERIALS AND METHODS

Narezushi sample
Samma-narezushi was obtained from a local manufacturer in Kiho town, Mie Prefecture, Japan in 2004. Samma-narezushi was produced as follows. After removal of the head and internal organs, the saury was salted for 10-14 days and then the salted saury together with cooked rice was pickled in a tub for about three weeks in winter.

Analysis of chemical composition
Fish meat and rice were separately homogenized with a food processor. The proximate composition of the samples was measured by the general method of food analysis. The moisture content was determined by dryness at 105°C. Protein content was determined by the Kjeldahl methods using the conversion factor 6.24. Lipid content was determined by the Soxhlet methods. Ash content was determined by incineration at 450°C. Carbohydrate content was calculated by subtracting the moisture, ash, protein, and lipid content from the total weight. Lactic acid content was determined by HPLC analysis with post-column detection.

DNA extraction, cloning and sequencing
Each sample was homogenized with a sterilized food processor. The sample was then immediately suspended in acetone at a final concentration of 70% to avoid the degradation of DNA as much as possible (Fukatsu, 1999) and stored at -80°C until analyzed. The stored samples were washed twice with sterilized physiological saline by centrifugation before DNA extraction. DNA was extracted by the bead beating method with the FastPrep instrument (Bio 101, Vista, CA) as described by Godon et al. (1997). The crude DNA was purified with Genomic-tip 100/G (QIAGEN, Hilden, Germany) and dissolved in TE buffer. The DNA concentration was adjusted to 10 ng μl⁻¹.

RESULTS

Chemical composition
The chemical composition of samma-narezushi is shown in Table 1. In fish meat, the moisture value was...
TABLE 1. Chemical composition of samma-narezushi (saury narezushi).

<table>
<thead>
<tr>
<th></th>
<th>Moisture (g/100g)</th>
<th>Protein (g/100g)</th>
<th>Lipid (g/100g)</th>
<th>Ash (g/100g)</th>
<th>Carbohydrate (g/100g)</th>
<th>Lactic acid (g/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish meat</td>
<td>70.88±6.43</td>
<td>19.69±1.35</td>
<td>3.88±0.12</td>
<td>1.97±0.03</td>
<td>3.58</td>
<td>0.63±0.17</td>
</tr>
<tr>
<td>Rice</td>
<td>77.00±0.11</td>
<td>2.53±0.46</td>
<td>0.07±0.02</td>
<td>2.31±0.06</td>
<td>18.09</td>
<td>0.99±0.01</td>
</tr>
</tbody>
</table>

TABLE 2. Abundance and similarity of OTUs to known species of bacteria of samma-narezushi (saury narezushi) according to the 16S rDNA clone library.

<table>
<thead>
<tr>
<th>OTU (number of clone)</th>
<th>Nearest known species</th>
<th>Similarity (%)</th>
<th>Accession Number</th>
<th>Number of clones</th>
<th>Composition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactic acid bacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU-01 (60), OTU-02 (1), OTU-03 (1), OTU-04 (1), OTU-05 (1)</td>
<td>Lactobacillus sakei</td>
<td>98-99</td>
<td>DQ989236, CR936503</td>
<td>64</td>
<td>71.9</td>
</tr>
<tr>
<td>OTU-06 (12)</td>
<td>Leuconostoc gelidum</td>
<td>99</td>
<td>AF175402</td>
<td>12</td>
<td>13.5</td>
</tr>
<tr>
<td>OTU-07 (2)</td>
<td>Lactococcus lactis subsp. lactis</td>
<td>99</td>
<td>AB285124</td>
<td>2</td>
<td>2.2</td>
</tr>
<tr>
<td>OTU-08 (1)</td>
<td>Lactococcus piscium</td>
<td>100</td>
<td>DQ343754</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td></td>
<td></td>
<td>79</td>
<td>88.7</td>
</tr>
<tr>
<td>γ-proteobacteria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTU-09 (1)</td>
<td>Acinetobacter junii</td>
<td>99</td>
<td>DQ859900</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>OTU-10 (1)</td>
<td>Acinetobacter johnsonii</td>
<td>98</td>
<td>X95303</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>OTU-11 (1)</td>
<td>Pseudomonas putida</td>
<td>99</td>
<td>AM411059</td>
<td>1</td>
<td>1.1</td>
</tr>
<tr>
<td>OTU-12 (1)</td>
<td>Rahnella aquatilis</td>
<td>99</td>
<td>DQ440548</td>
<td>7</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>Subtotal</td>
<td></td>
<td></td>
<td>10</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td></td>
<td>89</td>
<td>99.9</td>
</tr>
</tbody>
</table>

Diversity analysis using the 16S ribosomal RNA clone library

A total of 89 clones were randomly selected from the 16S rDNA clone library of samma-narezushi and DNA sequences of cloned fragments were analyzed. Homology search, phylogenetic analysis and estimation of species richness and diversity were carried out. To our best knowledge, this is the first report on the microbial diversity analysis of samma-narezushi by the PCR-derived clone library method.

No chimeric sequence was found using the CHECK_CHIMERA program. Cloned sequences were classified into 12 OTUs at a 97% identity threshold and are summarized in Table 2. All sequences in the library were eubacterial 16S rDNA sequences and no archaeal or eukaryotic sequence was recovered. Expected numbers of OTU by Chao1 estimates and Shannon-Wiener index of samma-narezushi were 44 and 1.2247, respectively. Judging from phylogenetic placement and homology results, 89% of clones were assigned as lactic acid bacteria (LAB) (Figure 1 and Table 2). These clones were classified into 8 OTUs. The results of the homology search of representative sequences showed that these OTUs were classified into Lactobacillus sakei, Leuconostoc gelidum, Lactococcus lactis subsp. lactis, and L. piscium. Among these OTUs, 5 OTUs were classified into Lactobacillus sakei (Table 2). Clones affiliated to L. sakei were the most abundant and made up 72% of the library. Clones affiliated to L. gelidum placed second in abundance in the library. Sequences similar to L. mesenteroides, L. lactis subsp. lactis, and L. piscium were also found in the library; however, these sequences were minor components. Sequence similarities of clones affiliated to LAB ranged from 98-100% to 16S rDNA sequences of known LAB deposited in the database.

Eleven percent of the clones were classified into 4 OTUs and assigned as γ-proteobacteria (Table 2).
The OTUs showed similarity to *Rahnella aquatilis* (7 clones), *Acinetobacter johnsonii* (1), *A. junii* (1), and *Pseudomonas putida* (1). These were minor components in the library. The sequence similarities were ranged between 98-99%.

**DISCUSSION**

Itou et al. (2006) reported time course changes in chemical components in the fish meat and rice of mackerel *narezushi*, which is a prototypical *narezushi*, during the fermentative process. The amount of lactic acid in the mackerel *narezushi* was about 2 g/100 g in both the fish meat and rice portion at 20 d. These values are higher than that of *samma-narezushi* examined in this study (0.63 g/100 g in the fish meat and 0.99 g/100 g in the rice) with almost the same fermentation period. The difference would be due to the mackerel *narezushi* being pickled under high temperatures in summer (from May to September). The amount of lactic acid in the mackerel *narezushi* finally reached 5 g/100 g (fish meat) and 3.3 g/100 g (rice) at 120 d fermentation. Fermentation under high temperatures and over a long term increased the lactic acid content in the mackerel *narezushi*. Difference in the lactic acid content causes the difference in the taste of *narezushi*.
showed that *L. sakei* was the most abundant and *L. gelidum* was second abundant bacterial species in the clone library derived from bacterial community of *samma-narezushi* (Table 2 and Fig. 1). A previous study showed that *L. plantarum*, *L. alimentarius*, *L. coryniformis*, and *Streptococcus lactis* were isolated from *saba-narezushi*, another kind of *nama-narezushi* (Fujii et al., 1992). In the present study, these LABs were not detected. Differences in the composition of LAB species between two kinds of *narezushi* may be due to the difference in the kind of fish meat or difference in the manufacturer. In a recent study of *funazushi*, a prototypical *narezushi*, *L. buchneri* was predominant species of LAB (Isobe et al., 2002). *Funazushi* is an original type of *narezushi* that is fermented for longer than 6 months. Also, the fermentation process is started from summer. Therefore, fermentation is further progressed than in *nama-narezushi*. The difference in the predominant LAB species between *funazushi* and *samma-narezushi* may be due to the fermentation period, kind of fish meat, and season. Our results together with previous results suggest that bacteria that belong to the genus *Lactobacillus* play important roles in the fermentation of *narezushi*.

*L. sakei* is a lactic acid bacterium widely represented in the natural flora of fresh meat and is considered as an important microorganism in the food industry (Chaillou et al., 2005). *L. sakei* is able to grow under low temperatures or in the presence of NaCl (Marceau et al., 2004). The fermentation process of *samma-narezushi* is carried out during the winter season. Also the fish meat is salted before fermentation. These factors would support growth of *L. sakei* and the species became the predominant species in the microbial community of *samma-narezushi*. Clones affiliated to *L. gelidum* were second most abundant in the present study (Table 2 and Fig. 1). *L. gelidum* was the predominant species in kimchi prepared at low temperatures (Kim et al., 2000). Like in the case of *L. sakei*, low temperature fermentation of *samma-narezushi* would support the growth of *L. gelidum*.

Proteolytic activities of LAB play a major role in the development of flavor and texture in fermented dairy foods and sausages (Champomier-Vergès et al., 2002). Proteases and aminopeptidase activities of some *L. sakei* strains in pork muscle sarcoplasm and myofibrillar proteins were demonstrated, and crude cell extract inoculation in meat extracts resulted in the accumulation of glutamic acid and alanine (Champomier-Vergès et al., 2002). *L. sakei* may contribute to the flavor and texture of fish meat in *samma-narezushi*.

*L. sakei* is known as one of the most important microorganisms for meat preservation and fermentation (Champomier-Vergès et al., 2002). Glucose fermentation by *L. sakei* is homolactic. The decrease in pH is of major importance for hygienic safety and determines the quality of the fermented products. In addition to this, 3 bacteriocins: sakacin A, sakacin P and lactocin S are produced by *L. sakei* isolates. Since some strains of *L. sakei* showed antimicrobial activities against meat spoilage microorganisms, these *L. sakei* strains are expected to be used as a protective culture in the production of cooked meat (Vermeiren et al., 2004). *L. gelidum* UAL187 is known to produce a bacteriocin, leucocin A (van Belkum and Stiles, 1995). Our results showed the predominance of *L. sakei* and *L. gelidum* in *samma-narezushi* (Table 2). These bacteria may contribute to the suppression of undesirable bacteria. Bacteriocins of LABs also show antimicrobial activities against LABs (Champomier-Vergès et al., 2002; van Belkum and Stiles, 1995). Therefore, competition between LAB and LAB, and between LAB and other bacteria is possible.

Four OTUs showed homology to γ-proteobacteria (Table 2). One of the OTUs that showed high similarity to *R. aquatilis* was found in the library. *R. aquatilis* has rarely been associated with infection in humans. Therefore, the bacterium is not considered as harmful. OTUs that belong to *Acinetobacter junii* and *A. johnsonii* were also found in the library. Bacteria that belong to the genus *Acinetobacter* are isolated from soil, water, sewage, human skin and a large variety of foodstuffs. *P. putida* is isolated from soil and water. Because proportions of the clones of these OTUs were small, there would be little contribution of these bacteria to the fermentation of *narezushi*.

In summary, it is shown that the culture independent technique can be successfully applied to diversity analysis of bacteria in *samma-narezushi* and it is highly possible that *L. sakei* plays an important role in the fermentation and preservation of *samma-narezushi*. Further study is needed to clarify the role of *L. sakei* in the fermentation process, preservation, and the development of flavor and texture.

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REFERENCES


