J. Gen. Appl. Microbiol., **58**, 273–281 (2012)

Full Paper

Bacterial communities in fish sauce mash using culture-dependent and -independent methods

Youhei Fukui,[1] Mitsuhiro Yoshida,[2] Kei-ichi Shozen,[3] Yasuhiro Funatsu,[4] Takashi Takano,[5] Hiroshi Oikawa,[6] Yutaka Yano,[7] and Masataka Satomi[1,*]

[1] *National Research Institute of Fisheries Science, Fisheries Research Agency, Yokohama 236–8648, Japan*
[2] *Institute of Biogeosciences, Japan Agency for Marine-Earth Science & Technology, Yokosuka 237–0061, Japan*
[3] *Food Research Institute, Toyama Prefectural Agricultural, Forestry & Fisheries Research Center, Toyama 939–8153, Japan*
[4] *Department of Food Science, Faculty of Dairy Science, Rakuno Gakuen University, Hokkaido 069–8501, Japan*
[5] *Umekama Co., Ltd., Toyama 939–3552, Japan*
[6] *National Research Institute of Fisheries and Environment of Inland Sea, Fisheries Research Agency, Hiroshima 739–0452, Japan*
[7] *National Salmon Resources Center, Fisheries Research Agency, Sapporo 062–0922, Japan*

(Received December 7, 2011; Accepted April 19, 2012)

In fish sauce production, microorganisms are associated with the fermentation process; however, the sequential changes in the bacterial communities have never been examined throughout the period of fermentation. In this study, we determined the bacterial floras in a fish sauce mash over 8 months, using three different culture media and 16S rRNA gene clone library analysis. During the first 4 weeks, viable counts of non-halophilic and halophilic bacteria decreased and were dominated by *Staphylococcus* species. Between 4 and 6 weeks, halophilic and highly halophilic bacterial counts markedly increased from 10^7 to 10^8 cfu/g, and the predominant species changed to *Tetragenococcus halophilus*. The occurrence of *T. halophilus* was associated with an increase of lactic acid and a reduction of pH values. In contrast, non-halophilic bacterial counts decreased to 10^6 cfu/g by 6 weeks with *Bacillus subtilis* as the dominant isolate. Clone library analysis revealed that the dominant bacterial group also changed from *Staphylococcus* spp. to *T. halophilus*, and the changes were consistent with those of the floras of halophilic and highly halophilic isolates. This is the first report describing a combination approach of culture and clone library methods for the analysis of bacterial communities in fish sauce mash.

Key Words——bacterial community; clone library; culture-dependent method; fish sauce; *Staphylococcus* spp.; *Tetragenococcus halophilus*

Introduction

Fish sauce is generally a fermented food made by mixing fish and a large amount of salt with a fermentation period in excess of 6 months or more. Such fermentation processes are common in Southeast and East Asia and sauces are known as ‘nampla’ in Thailand, ‘nucmam’ in Vietnam, ‘patis’ in the Philippines, ‘shottsuru’ in Japan, and ‘yeesui’ in China. Nowadays, the characteristic taste and smell have become popular worldwide.

The fish proteins are hydrolyzed by both endogenous and exogenous proteases of fish and microbes, respectively (Fukami et al., 2004a). Despite the large

* Address reprint requests to: Dr. Masataka Satomi, National Research Institute of Fisheries Science, Fisheries Research Agency, Yokohama 236–8648, Japan.
Tel & Fax: +81–45–788–7669
E-mail: msatomi@affrc.co.jp

amounts of salt used (15 to 20%) various bacteria have been isolated from fish sauce and are considered to play important roles in the maturation process (Fujii et al., 1980; Sato et al., 1995). In previous studies, *Achromobacter*, *Bacillus*, *Micrococcus*, *Staphylococcus*, *Streptococcus*, *Tetragenococcus*, and *Vibrio* were isolated from fish sauces (Crisan and Sands, 1975; Fujii and Sakai, 1984; Fukami et al., 2004a; Ijong and Ohta, 1996; Itoh et al., 1985a, b; Ok et al., 1982; Saisithi et al., 1966; Sato et al., 1995; Satomi et al. 1997, 2008; Taira et al., 2007; Tanasupawat and Daengsubha, 1983; To et al., 1997). In most reports, the isolation of bacterial cultures from various fish sauces was limited to a part of the fermentation process; hence, a systematic analysis on bacterial counts and diversity has not been conducted over the consecutive stages of the fermentation process. Additionally, the identification of isolates in many studies has been based on phenotypic characterizations that have led to potential misidentification due to the ambiguity of variable characteristics (Becker et al., 2004; Fukami et al., 2004a, b).

Due to the inherent biases of cultivation-based methods (Amann et al., 1995), culture-independent techniques are regarded as more reliable for the investigation of microbial flora in environmental samples (Cottrell and Kirchman, 2000; Sekiguchi et al., 2002), although the analysis of microbial diversity in fish sauce has yet to be investigated using a culture-independent approach. Recently, the culture-independent method polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was utilized to investigate bacterial diversity in fermented seafoods (Roh et al., 2010; Yoshikawa et al., 2010). Among these studies, some bacterial sequences detected by the culture-independent method had never previously been obtained using culture-based techniques (Roh et al., 2010). In this study, we used molecular cloning to reveal the bacterial diversity in a fish sauce mash throughout the fermentation process. This methodology can be used in conjunction with cultivation studies to provide a better understanding of the fish sauce microbial community.

We sequentially investigated the fluctuations of bacterial viable counts and the bacterial communities through fermentation for 8 months using three different culture media. Additionally, molecular cloning and DNA sequencing were used to investigate fish sauce bacterial communities by targeting the universally conserved 16S rRNA gene.

Materials and Methods

Preparation of fish sauce. Deep sea smelt *Glossanodon semifasciatus* was purchased at fish markets in Toyama and Niigata, Japan. The fish were stored at −20°C until use. After thawing and washing, the fish were minced using a meat grinder, and the heads, skin, and bones removed. 260 kg of prepared deep sea smelt was mixed with 86 L of water, 66 kg of salt to a 15% (w/w) final concentration, and 40 kg of soy sauce *koji* using *Aspergillus oryzae* (Ichimurasaki, Bio'c, Toyohashi, Japan). The mixture was stirred well and put in a covered 500 L polypropylene tank. The fish sauce mash was fermented at room temperature for 8 months from April to November. About 100 ml of the mash was regularly sampled at 0, 2, 4, 6, 8, 12, and 32 weeks of fermentation.

Chemical parameters. Chemical parameters including temperature, pH, total nitrogen, and lactic acid were measured at each fermentation stage. Temperature was measured using a thermometer every 2 weeks during 16 weeks, 22 and 32 weeks of fermentation. pH was measured with a glass electrode (HM-5S, DKK-TOA, Tokyo, Japan). Total nitrogen contents were determined by the Kjeldahl method. Lactic acid content was determined using HPLC (Funatsu, 2001).

Preparation and cultivation of sample. The fish sauce mash was homogenized in sterile 10% sodium chloride solution with a stomacher blender (Basic, IUL, Barcelona, Spain) for 1 min. Samples for cultivation were decimally diluted and 0.1 ml of the serial dilutions was spread on each of three defined media: plate count agar without NaCl (PCA, Eiken Chemical, Tokyo, Japan; 0% NaCl), marine agar (MA, Difco, Franklin Lakes, USA; 2.5% NaCl), and salt marine agar (SMA; 12.5% NaCl) defined as MA with 10% sodium chloride and 1% D-glucose added. Non-halophilic bacteria were cultured on PCA at 37°C for 2 days; halophilic bacteria and highly halophilic bacteria were cultured on MA and SMA, respectively, at 20°C for 2 weeks. After incubation, viable bacterial counts on each medium were enumerated.

Identification of bacterial strains. Twenty colonies were randomly selected from each of the three media at each fermentation stage. Bacterial DNA was extracted by boiling in TE buffer containing Triton X-100 of 1% (v/v), and the protein was removed with chloroform-isoamyl alcohol (24:1). Initially, an identification for *T. halophilus* was performed by PCR detection using a *T.*

halophilus-specific primer set of ThF (5′-CGAACGCTG CTTAAGAAGA-3′) and ThR (5′-GCATTTCCTCTTTCT CCTG-3′) for the 16S rRNA gene. The PCR cycles consisted of 1 min at 94°C followed by 25 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C and for 7 min at 72°C. For non-*T. halophilus* strains, PCR amplification was performed with a bacterial universal primer set of 27F and 1492R for the 16S rRNA gene sequence (Weisburg et al., 1991). To determine the nucleotide sequence of the 16S rRNA gene, sequencing was performed on PCR products with 27F primer using DNA sequencer Model 3100 (Applied Biosystems, Foster City, CA, USA). Approximately 700 bp of the 5' end of the 16S rDNA sequence was used for phylogenetic analysis. To examine the phylogeny of the isolates, representative sequences were searched for sequence similarities using the BLAST algorithm (Altschul et al., 1997) and Ribosomal DNA database project II (RDP-II) (Maidak et al., 2001) in Genbank, EMBL, and DDBJ databases. Isolates having a >95% similarity to the type strain were assumed to be the same genus (Ludwig et al., 1998).

Construction of 16S rDNA clone libraries. Genomic DNA from the microbial community was extracted using an isofecal DNA Kit (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. Amplification of 16S rDNA was conducted with the universal primer set of 27F and 1492R using conditions specified previously. The amplicons were inserted into TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) and transformed into competent *Escherichia coli* strain TOP10, according to the manufacturer's protocol. Twenty colonies at each fermentation stage were randomly selected and sequenced using M13 and M13 RV primers. The presence of chimera was evaluated by sequence similarity using BLAST and RDP-II, and putative chimeric sequences were omitted. Approximately 700 bp of the 5′-16S rDNA sequences was subsequently used for phylogenetic analysis. Sequences with a similarity of >98% were assigned to the same phylogenic clone type (phylotype) (Rosselló-Mora and Amann, 2001; Stackebrandt and Ebers, 2006). The type strain in the database showing the highest similarity to the representative clone is described in the results. Clones having a >95% similarity to the type strain are shown at the level of the genus (Ludwig et al, 1998). The representative sequences were deposited in the GenBank database and assigned the accession numbers AB649148–AB649167. To evaluate bacterial diversity, coverage (*C*) was calculated as $C=1-(n_1/N)$, where n_1 represents the number of phylotype that contained single clone and *N* represents the total number of clones analyzed (Good, 1953).

Results

Chemical components

The temperature increased from 14.7°C to 31.0°C during 22 weeks of fermentation, and then decreased to 16.7°C by 32 weeks (Fig. 1A). The pH value decreased from 5.9 on the starting day to 5.5 after 6 weeks of fermentation, but the pH increased to 6.4 by 32 weeks (Fig. 1B). Total nitrogen markedly increased during the first 4 weeks of fermentation, and then gradually increased until 32 weeks (Fig. 1C). Lactic acid dramatically increased between 4 and 8 weeks of fermentation (Fig. 1D). The highest concentration of lactic acid (1,740 mg/100 ml) was reached at 12 weeks of fermentation, where it remained constant throughout the rest of the fermentation period.

Changes in bacterial counts during fermentation

At the initial stage of fermentation viable counts of both non-halophilic and highly halophilic bacteria, and halophilic bacteria were 10^7 and 10^8 cfu/g, respectively (Fig. 1E, 1F, and 1G). Through week 4, the number of non-halophilic and halophilic bacteria decreased to 10^6 and 10^7 cfu/g, respectively (Fig. 1E and 1F). Between 4 and 6 weeks of fermentation, halophilic and highly halophilic bacterial counts increased up to 10^8 cfu/g (Fig. 1F and 1G), while non-halophilic bacterial counts decreased at 10^6 cfu/g (Fig. 1E). After 6 weeks, the number of halophilic and highly halophilic bacteria continued to decrease, with final counts of 10^7 and 10^6 cfu/g at week 32, respectively (Fig. 1F and 1G). Non-halophilic bacterial counts remained at 10^6 cfu/g between 6 and 32 weeks (Fig. 1E).

Bacterial flora during fermentation by culture method

Until the first 2 to 4 weeks of the fermentation, *Staphylococcus* spp. were the dominant bacteria on all media (Fig. 2A, 2B, and 2C). After 6 weeks, *Bacillus* sp. was the dominant isolates on non-halophilic medium and was similar to *B. subtilis* (AJ276351; 100%) (Fig. 2A). At 4 weeks of fermentation, *T. halophilus* was observed on halophilic and highly halophilic media, and thereafter was the predominant isolate (Fig. 2B and

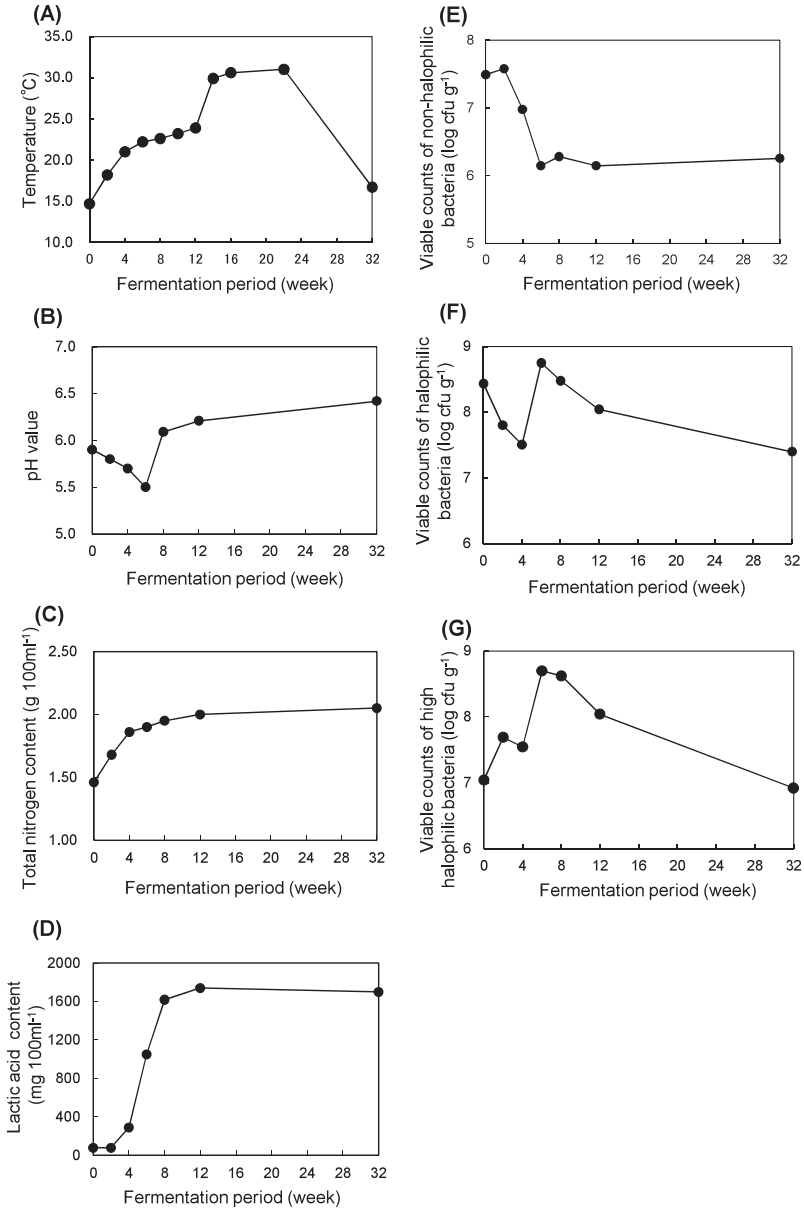

Fig. 1. Changes in chemical components and viable bacterial counts in fish sauce mash during fermentation.

(A) temperature, (B) pH, (C) total nitrogen, (D) lactic acid, (E) non-halophilic bacteria, (F) halophilic bacteria, and (G) highly halophilic bacteria.

2C). *T. halophilus* was never isolated on PCA medium throughout the fermentation period (Fig. 2A). Other species isolated to a lesser extent included *Kocuria* sp. related to *K. koreensis* (FJ607312; 99.9%) isolated from MA medium (Fig. 2B) and *Enterococcus* sp. closely related to *E. casseliflavus* (AF039903; 100%) from PCA medium (Fig. 2A).

Bacterial community by culture-independent methods

Until week 4, the majority of clones were identified as *Staphylococcus* spp. most similar to *S. cohnii* subsp. *urealyticus* (AB009936; 98.9–99.0%), *S. equorum* subsp. *equorum* (AB009939; 100%), *S. equorum* subsp. *linens* (AF527483; 100%), *S. gallinarum* (D83366; 99.6–99.7%), *S. kloosii* (AB009940; 99.1%), *S. lentus* (D83370; 96.4–99.9%), *S. sciuri* subsp. *sciuri* (AJ421446; 98.0–99.6%), and *S. succinus* subsp. *succinus* (AF004220; 99.7%) (Table 1). Clones most related to *T. halophilus* were initially observed at 4 weeks, and thereafter represented the predominant sequences (D88668;

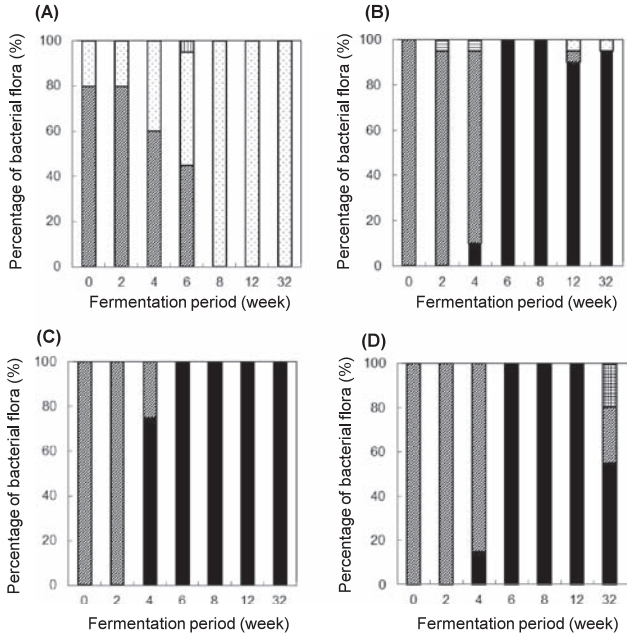

Fig. 2. Phylogenetic classifications at the genus level of bacterial cultures and clones on the basis of 16S rRNA gene sequence.

(A) non-halophilic bacteria, (B) halophilic bacteria, (C) highly halophilic bacteria, and (D) clone library. *Bacillus* sp., *Enterococcus* sp., *Kocuria* sp., *Lentibacillus*-like, *Staphylococcus* spp., *Tetragenococcus halophilus*.

98.9−99.5%). At 32 weeks of fermentation, clones closely related to *S. cohnii* subsp. *urealyticus* (AB009936; 98.0%), *S. saprophyticus* subsp. *saprophyticus* (AP008934; 99.6%), and *Lentibacillus salinarum* (EF601571; 93.1%) were detected. The coverage of the clone library at 0, 2, 4, 6, 8, 12, 32 weeks, was 95%, 90%, 95%, 100%, 100%, 100%, 95%, respectively.

Discussion

We examined the changes in bacterial floras in the fish sauce mash throughout a fermentation period of 8 months, using both culture-dependent and -independent methods. Additionally, we measured specific chemical parameters (e.g., temperature, pH, total nitrogen, and lactic acid) at each fermentation stage to examine associations between bacterial communities and chemical parameters. Changes in these chemical parameters were similar to those observed in previous studies (Ijong and Ohta, 1996; Taira et al., 2007) indicating the fish sauce underwent a normal fermentation process. Culture-dependent methods utilized three different media, PCA, MA, and SMA for isolation of non-halophilic, halophilic, and highly halophilic bacteria, respectively. Each clone library analyzed in this study covered more than 90% of bacterial populations in the fish sauce mash. During the fermentation process, the changes of bacterial genera isolated on each medium and clone libraries differed. While all three media and clone sequences were initially dominated by *Staphylococcus* spp., the non-halophilic bacterial flora became exclusively *Bacillus* spp. (Fig. 2A) whereas both halophilic and highly halophilic bacterial flora and clone sequences became dominated by *Tetragenococcus* spp. (Fig. 2B, 2C, and 2D).

Between the initial day and 2 to 4 weeks of fermentation, *Staphylococcus* spp. were detected as the most dominant bacteria using both culture and clone library methods (Fig. 2 and Table 1). In this study, all of the clone sequences belonged to two groups represented by *S. saprophyticus* and *S. sciuri* (Kloos et al., 1992; Spergser et al., 2003; Takahashi et al., 1999). However, as *Staphylococcus* spp. often have a high degree of mean similarities (97.4−98%) in 16S rRNA gene sequence, it was difficult to discriminate to an accurate species level of *Staphylococcus* spp. (Becker et al., 2004; Fukami et al., 2004b; Poyart et al., 2001; Shah et al., 2007). Therefore, the use of other genes with higher discrimination than 16S rRNA gene for classification of *Staphylococcus* species, such as *dnaJ* (Shah et al., 2007), *rpoB* (Drancourt and Raoult, 2002; Fukami et al., 2004b), and *sodA* (Poyart et al., 2001) would be required to accurately identify these strains. Interestingly, among the *S. saprophyticus* group, *S. saprophyticus* and *S. nepalensis* have been isolated from several fish sauces (Fukami et al., 2004a, b; Itoh et al., 1985b). In particular, the addition of the *S. nepalensis* strain to a fish sauce was shown to change the sauce's volatile composition and improve its odor (Fukami et al., 2004b). Between the initial day and 4 weeks of fermentation, viable counts of non-halophilic and halophilic bacteria decreased (Fig. 1E and 1F). These decreases were related to declines in the detection rate of *Staphylococcus* spp. that were dominant until 4 weeks (Fig. 2A and 2B).

Between 4 and 6 weeks, the dominant bacterial species on MA and SMA plates and clone libraries changed from *Staphylococcus* spp. to *T. halophilus* (Fig. 2B, 2C, and 2D). *T. halophilus* is known as halophilic and lactic acid bacteria, and the isolates have been cultured from various fish sauces (Itoh et al., 1985b; Sato et al., 1995; Satomi et al., 2008; Taira et al., 2007; Tanasupawat

Table 1. Identification of the bacterial 16S rRNA gene sequences of clone library obtained from fish sauce samples during the fermentation period.

Fermentation period (week)	Phylotype	Clone number	Closest sequence	Identity (%)
0	f0-30 (AB649149)	11	*Staphylococcus succinus* subsp. *succinus* (AF004220)	99.7
	f0-3 (AB649148)	5	*Staphylococcus cohnii* subsp. *urealyticus* (AB009936)	99.0
	f0-32 (AB649150)	3	*Staphylococcus sciuri* subsp. *sciuri* (AJ421446)	99.6
	f0-45 (AB649151)	1	*Staphylococcus sciuri* subsp. *sciuri* (AJ421446)	98.0
2	f2-1 (AB649152)	16	*Staphylococcus gallinarum* (D83366)	99.7
	f2-17 (AB649153)	2	*Staphylococcus cohnii* subsp. *urealyticus* (AB009936)	98.9
	f2-22 (AB649154)	1	*Staphylococcus lentus* (D83370)	96.4
	f2-31 (AB649155)	1	*Staphylococcus lentus* (D83370)	97.5
4	f4-1 (AB649156)	9	*Staphylococcus gallinarum* (D83366)	99.6
	f4-3 (AB649157)	5	*Staphylococcus equorum* subsp. *linens* (AF527483)	100
			Staphylococcus equorum subsp. *equorum* (AB009939)	100
	f4-4 (AB649158)	3	*Tetragenococcus halophilus* (D88668)	99.0
	f4-13 (AB649159)	2	*Staphylococcus kloosii* (AB009940)	99.1
	f4-7 (AB649160)	1	*Staphylococcus lentus* (D83370)	99.9
6	f6-2 (AB649161)	20	*Tetragenococcus halophilus* (D88668)	99.5
8	f8-1 (AB649162)	20	*Tetragenococcus halophilus* (D88668)	99.3
12	f12-1 (AB649163)	20	*Tetragenococcus halophilus* (D88668)	99.5
32	f32-35 (AB649164)	11	*Tetragenococcus halophilus* (D88668)	98.9
	f32-45 (AB649165)	4	*Staphylococcus cohnii* subsp. *urealyticus* (AB009936)	98.0
	f32-38 (AB649166)	4	*Lentibacillus salinarum* (EF601571)	93.1
	f32-37 (AB649167)	1	*Staphylococcus saprophyticus* subsp. *saprophyticus* (AP008934)	99.6

and Daengsubha, 1983). As some strains of *T. halophilus* were reported to grow well in media with 30°C (Itoh et al., 1985b), 3−15% NaCl (Kobayashi et al., 2004), and pH 5.5−8.0 (Itoh et al., 1985a), the fermentative condition in this study provided an environment suitable for this species growth. The reduction in pH value between the initial day and 6 weeks resulted from increases in *T. halophilus* and lactic acid concentrations (Fig. 1B and 1D) as reported elsewhere (Ijong and Ohta, 1996; Itoh et al., 1985b; Taira et al., 2007). Recently, *T. halophilus* strains have been shown to have proteinase-producing abilities and to produce major volatile compounds such as 1-propanol, 2-methylpropanol, and benzaldehyde (Udomsil et al., 2010), which were previously identified in fish sauce with *T. halophilus* and *A. oryzae* (Funatsu et al., 2002). In view of the metabolism of *T. halophilus*, it was considered that *T. halophilus* played an important role in the maturity process of the fish sauce mash.

After 6 weeks of fermentation, most isolates of non-halophilic bacteria were closely related to *B. subtilis* (Fig. 2A). *B. subtilis* has been isolated from a fish sauce in Vietnam and a few strains showed collagenase-producing activity (To et al., 1997). However, the total nitrogen level did not sharply increase after 6 weeks when *B. subtilis* was predominant in this study. Instead, the level of total nitrogen markedly increased during the first 4 weeks of fermentation (Fig. 1C), and was attributed mainly to protein-decomposition by *A. oryzae,* which was detected at the level of 10^5−10^6 cfu/g on MY20 medium by 8 weeks of fermentation (data not shown). As growth of *B. subtilis* strains of marine origin were inhibited at 15% of NaCl concentration (Ivanova et al., 1999), *B. subtilis* present in the mash might have formed spores and suspended physiological activity. Furthermore, the gene sequence of *B. subtilis* was not detected in any of the clone libraries. This result may be explained by the observation that halophilic and highly halophilic viable counts were notably higher than the non-halophilic counts dominated by *B. subtilis* strains.

Other infrequently isolated strains of non-halophilic and halophilic bacteria included *E. casseliflavus* and *K. koreensis*, respectively (Fig. 2A and 2B). *K. koreen-*

sis has previously been isolated from fermented seafood in Korea (Park et al., 2010). Additionally, it was reported that an *E. casseliflavus* strain was used as a starter culture for a vegetable product, enhancing consumption of carbohydrates and causing a decrease in pH (Castro et al., 2002). In clone libraries, the clone sequences closely to *L. salinarum,* which was initially isolated from a solar saltern and a moderately halophilic bacterium, were detected (Lee et al., 2008).

When the bacterial communities determined by culture-dependent and -independent approaches were compared, the changes in the detection rates of clones identified as *Staphylococcus* spp. and *T. halophilus* were more similar to those of isolates on halophilic and highly halophilic bacterial media than non-halophilic medium (Fig. 2). This agreement suggested that the microbial flora detected through molecular techniques was more reflective of the results obtained from MA and SMA media which showed higher bacterial numbers than that of PCA medium. On the other hand, at 32 weeks of fermentation, the bacterial floras obtained by clone libraries differed from those of cultural methods using MA and SMA. The difference might be explained by the decreased level of halophilic and highly halophilic counts represented by *T. halophilus* and dead state of *Staphylococcus* spp. and *Lentibacillus*-like bacteria.

In conclusion, we examined the dynamics of bacterial communities in a fish sauce mash throughout the fermentation period using both culture-dependent and -independent methods. Our results indicated the predominant bacteria in the fish sauce were *Staphylococcus* spp. and *T. halophilus*. Interestingly, some species could only be detected either by cultivation or by the clone library method. Accordingly, the approach of a combination of culture and clone library methods should be used for analysis of the whole bacterial community in the mash. An understanding of the bacterial communities detected in this study could help in the use and selection of starter cultures although further study is needed to investigate the roles of naturally occurring *Staphylococcus* spp. and *T. halophilus* in the fish sauce mash with respect to the fermentation process as a whole.

Acknowledgments

We would like to thank Dr. J. Bruckner (Dersert Research Institute) for discussion of this study. We also thank Hatano, N. and Sakai, N. for technical assistance. This research was partly supported by the fund from Society for Research on *Umami* taste.

References

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res*., **25**, 3389–3402.

Amann, R. I., Ludwig, W., and Schleifer, K.-H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol*. *Rev*., **59**, 143–169.

Becker, K., Harmsen, D., Mellmann, A., Meier, C., Schumann, P., Peters, G., and von Eiff, C. (2004) Development and evaluation of a quality-controlled ribosomal sequence database for 16S ribosomal DNA-based identification of *Staphylococcus* species. *J. Clin*. *Microbiol*., **42**, 4988–4995.

Castro, A. de, Montaño, A., Casado, F.-J., Sánchez, A.-H., and Rejano, L. (2002) Utilization of *Enterococcus casseliflavus* and *Lactobacillus pentosus* as starter cultures for Spanish-style green olive fermentation. *Food Microbiol*., **19**, 637–644.

Cottrell, M. T. and Kirchman, D. L. (2000) Community composition of marine bacterioplankton determined by 16S rRNA gene clone libraries and fluorescence in situ hybridization. *Appl*. *Environ*. *Microbiol*., **66**, 5116–5122.

Crisan, E. V. and Sands, A. (1975) Microflora of four fermented fish sauces. *Appl*. *Environ*. *Microbiol*., **29**, 106–108.

Drancourt, M. and Raoult, D. (2002) *rpoB* gene sequence-based identification of *Staphylococcus* species. *J*. *Clin*. *Microbiol*., **40**, 1333–1338.

Fujii, T., Basuki, S. B., and Tozawa, H. (1980) Chemical composition and microflora of fish sauce “Patis”. *Bull*. *Jpn. Soc*. *Sci*. *Fish*., **46**, 1235–1240 (in Japanese).

Fujii, T. and Sakai, H. (1984) Chemical composition and microflora of fish sauce “Shottsuru”. *Bull*. *Jpn. Soc*. *Sci*. *Fish*., **50**, 1061–1066 (in Japanese).

Fukami, K., Funatsu, Y., Kawasaki, K., and Watabe, S. (2004a) Improvement of fish-sauce odor by treatment with bacteria isolated from the fish-sauce mush (Moromi) made from frigate mackerel. *J*. *Food Sci*., **69**, 45–49.

Fukami, K., Satomi, M., Funatsu, Y., Kawasaki, K., and Watabe, S. (2004b) Characterization and distribution of *Staphylococcus* sp. implicated for improvement of fish sauce odor. *Fish Sci*., **70**, 916–923.

Funatsu, Y. (2001) Study on extractive components of fish sauces from the minced meat of frigate mackerel and the waste of frigate mackerel surimi processing. *Bull*. *Toyama Pref*. *Food Res*. *Inst*., **7**, 47–53.

Funatsu, Y., Kawasaki, K., Yuan, C., Uchida, M., Satomi, M., and Fukuda, Y. (2002) A comparison of volatile compounds in fish sauces prepared from silver carp by use of soy sauce *koji* and lactic acid bacteria with those in Chinese commer-

cial fish sauces. *Nippon Shokuhin Kagaku Kogaku Kaishi*, **49**, 106–118 (in Japanese).

Good, I. J. (1953) The population frequencies of species and the estimation of population parameters. *Biometrika*, **40**, 237–264.

Ijong, F. G. and Ohta, Y. (1996) Physicochemical and microbiological changes associated with Bakasang processing—A traditional Indonesian fermented fish sauce. *J. Sci. Food Agric.*, **71**, 69–74.

Itoh, H., Hadioetomo, R. S., Nkkuni, S., and Okada, N. (1985a) Studies on lactic acid bacteria in fish sauces (Part 1) Chemical composition and microflora of fish sauces. *Rept. Natl. Food Res. Inst.*, **47**, 23–30.

Itoh, H., Hadioetomo, R. S., Nkkuni, S., and Okada, N. (1985b) Studies on lactic acid bacteria in fish sauces (part 2) Identification of salt-tolerance and acid-producing bacteria from fish sauces. *Rept. Natl. Food Res. Inst.*, **47**, 31–40.

Ivanova, E. P., Vysotskii, M. V., Svetashev, V. I., Nedashkovskaya, O. I., Gorshkova, N. M., Mikhailov, V. V., Yumoto, N., Shigeri, Y., Taguchi, T., and Yoshikawa, S. (1999) Characterization of *Bacillus* strains of marine origin. *Int. Microbiol.*, **2**, 267–271.

Kloos, W. E., Schleifer, K.-H., and Götz, F. (1992) The genus *Staphylococcus*. *In* The Prokaryotes, ed. by Balows, A. B., Trüper, H. G., Dworkin, M., Harder, W., and Schleifer, K.-H., Springer-Verlag, New York, pp. 1369–1420.

Kobayashi, T., Kajiwara, M., Wahyuni, M., Hamada-Sato, N., Imada, C., and Watanabe, E. (2004) Effect of culture conditions on lactic acid production of *Tetragenococcus* species. *J. Appl. Microbiol.*, **96**, 1215–1221.

Lee, S.-Y., Choi, W.-Y., Oh, T.-K., and Yoon, J.-H. (2008) *Lentibacillus salinarum* sp. nov., isolated from a marine solar saltern in Korea. *Int. J. Syst. Evol. Microbiol.*, **58**, 45–49.

Ludwig, W., Strunk, O., Klugbauer, S., Klugbauer, N., Weizenegger, M., Neumaier, J., Bachleitner, M., and Schleifer K. H. (1998) Bacterial phylogeny based on comparative sequence analysis (review). *Electrophoresis*, **19**, 554–568.

Maidak, B. L., Cole, J. R., Lilburn, T. G., Parker, C. T., Jr., Saxman, P. R., Farris, R. J., Garrity, G. M., Olsen, G. J., Schmidt, T. M., and Tiedje, J. M. (2001) The RDP-II (Ribosomal Database Project). *Nucleic Acids Res.*, **29**, 173–174.

Ok, T., Matsukura, T., Ooshiro, Z., Hayashi, S., and Itakura, T. (1982) Study on the use of halophilic bacteria in production of fish sauce. *Nippon Shokuhin Kogyo Gakkaishi*, **29**, 623–627.

Park, E.-J., Roh, S. W., Kim, M.-S., Jung, M.-J., Shin, K.-S., and Bae, J.-W. (2010) *Kocuria koreensis* sp. nov., isolated from fermented seafood. *Int. J. Syst. Evol. Microbiol.*, **60**, 140–143.

Poyart, C., Quesne, G., Boumaila, C., and Trieu-Cuot, P. (2001) Rapid and accurate species-level identification of coagulase-negative staphylococci by using the *sodA* gene as a target. *J. Clin. Microbiol.*, **39**, 4296–4301.

Roh, S. W., Kim, K.-H., Nam, Y.-D., Chang, H.-W., Park, E.-J., and Bae, J.-W. (2010) Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. *ISME J.*, **4**, 1–16.

Rosselló-Mora, R. and Amann, R. (2001) The species concept for prokaryotes. *FEMS Microbiol. Rev.*, **25**, 39–67.

Saisithi, P., Kasemsarn, R.-O., Liston, J., and Dollar, A. M. (1966) Microbiology and chemistry of fermented fish. *J. Food Sci.*, **31**, 105–110.

Sato, T., Kimura, B., and Fujii, T. (1995) Histamine contents and histamine-metabolizing bacteria flora of fish sauce during fermentation. *Food Hyg. Saf. Sci.*, **36**, 763–768 (in Japanese).

Satomi, M., Furushita, M., Oikawa, H., Yoshikawa-Takahashi, M., and Yano, Y. (2008) Analysis of a 30 kbp plasmid encoding histidine decarboxylase gene in *Tetragenococcus halophilus* isolated from fish sauce. *Int. J. Food Microbiol.*, **126**, 202–209.

Satomi, M., Kimura, B., Mizoi, M., Sato, T., and Fujii, T. (1997) *Tetragenococcus muriaticus* sp. nov., a new moderately halophilic lactic acid bacterium isolated from fermented squid liver sauce. *Int. J. Syst. Bacteriol.*, **47**, 832–836.

Sekiguchi, H., Watanabe, M., Nakahara, T., Xu, B., and Uchiyama, H. (2002) Succession of bacterial community structure along the Changjiang River determined by denaturing gradient gel electrophoresis and clone library analysis. *Appl. Environ. Microbiol.*, **68**, 5142–5150.

Shah, M. M., Iihara, H., Noda, M., Song, S. X., Nhung, P. H., Ohkusu, K., Kawamura, Y., and Ezaki, T. (2007) *dnaJ* gene sequence-based assay for species identification and phylogenetic grouping in the genus *Staphylococcus*. *Int. J. Syst. Evol. Microbiol.*, **57**, 25–30.

Spergser, J., Wieser, M., Täubel, M., Rosselló-Mora, R. A., Rosengarten, R., and Busse, H.-J. (2003) *Staphylococcus nepalensis* sp. nov., isolated from goats of the Himalayan region. *Int. J. Syst. Evol. Microbiol.*, **53**, 2007–2011.

Stackebrandt, E. and Ebers, J. (2006) Taxonomic parameters revisited: Tarnished gold standards. *Microbiol. Today*, **33**, 152–155.

Taira, W., Funatsu, Y., Satomi, M., Takano, T., and Abe, H. (2007) Changes in extractive components and microbial proliferation during fermentation of fish sauce from underutilized fish species and quality of final products. *Fish Sci.*, **73**, 913–923.

Takahashi, T., Satoh, I., and Kikuchi, N. (1999) Phylogenetic relationships of 38 taxa of the genus *Staphylococcus* based on 16S rRNA gene sequence analysis. *Int. J. Syst. Bacteriol.*, **49**, 725–728.

Tanasupawat, S. and Daengsubha, W. (1983) *Pediococcus* species and related bacteria found in fermented foods and related materials in Thailand. *J. Gen. Appl. Microbiol.*, **29**, 487–506.

To, K. A., Tanaka, T., and Nagano, H. (1997) Isolation of a collagenase-producing bacterium from traditional fermented food and its enzyme production. *J. Home Econ. Jpn.*, **48**,

1083–1087.

Udomsil, N., Rodtong, S., Tanasupawat, S., and Yongsawatdigul, J. (2010) Proteinase-producing halophilic lactic acid bacteria isolated from fish sauce fermentation and their ability to produce volatile compounds. *Int*. *J*. *Food Microbiol*., **141**, 186–194.

Weisburg, W. G., Barns, S. M., Pelletier D. A., and Lane, D. J. (1991) 16S ribosomal DNA amplification for phylogenetic study. *J*. *Bacteriol*., **173**, 697–703.

Yoshikawa, S., Yasokawa, D., Nagashima, K., Yamazaki, K., Kurihara, H., Ohta, T., and Kawai, Y. (2010) Microbiota during fermentation of chum salmon (*Oncorhynchus keta*) sauce mash inoculated with halotolerant microbial starters: Analyses using the plate count method and PCR-denaturing gradient gel electrophoresis (DGGE). *Food Microbiol*., **27**, 509–514.