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RESEARCH NOTE

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Bacterial Diversity in a Korean Traditional Soybean Fermented Foods (*Doenjang* and *Ganjang*) by 16S rRNA Gene Sequence Analysis

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Abstract The bacterial diversity in Korean soybean-fermented foods was investigated using a PCR-based approach. 16S rRNA sequences were amplified and cloned from two different soybean-fermented foods such as *doenjang* (soybean paste), and *ganjang* (soybean sauce). *Staphylococcus equorum* (60.6%), *Tetragenococcus halophila* (21.2%), *Leuconostoc mesenteroides* (9.1%), *Lactobacillus sakei* (6.1%), and *Bacillus subtilis* (3.0%) were detected among clones isolated from soybean paste samples and *Halanaerobium* sp. (37.5%), *Halanaerobium fermentans* (37.5%), *T. halophila* (12.5%), *Staphylococcus* sp. (6.3%), *S. equorum* (3.1%), and *B. subtilis* (3.1%) were detected among clones isolated from soybean sauce. Our approach revealed different bacterial distributions and diversity from those previously obtained using culture-dependent methods.

Keywords: soybean fermented food, bacterial diversity, 16S rRNA gene, culture-independent

Introduction

Fermented soybean foods such as *doenjang* (soybean paste, SP), *ganjang* (soybean sauce, SS), and *cheonggukjang* (soybean cook, SC) have served as side dishes which have provided a major source of protein in Korea for thousands of years (1, 2). These foods have been prepared by mixing *meju* (a fermented cooked-soybean), salt and water or with cooked soybeans in a container such as a porcelain pot. Various kinds of microorganisms participated in fermentation of these foods and their unique flavor and taste originate in decomposed products of soybean protein by microbial action during fermentation (3, 4). In recent years fermented soybean foods have attracted considerable interest due to their excellent nutritional value (5-7) and their beneficial impact on human health as an anticarcinogenic agent (8, 9) and as an antioxidant (10-12), as well as playing a potential role in the prevention of heart attacks (13, 14), and in the prevention of blood coagulation (15-17).

Recently many technological advances have facilitated improved community profiling and culture-independent approaches to studying environmental microorganisms, such as soil (18, 19), rumen (20), compost (21), cheese (22), and *kimchi* (23) etc. Several molecular biological methods such as denaturing gradient gel electrophoresis (DDGE) (24, 25) terminal restriction fragment length polymorphism (T-RFLP) (26, 27), ribosomal intergenic spacer (RISA) (28), randomly amplified polymorphic DNA (29), and ribosomal DNA (rDNA) sequences (16S rDNA) have been used to identify, quantify, and visualize microorganisms populations (18, 20, 23, 30).

There is a general agreement that the classification and identification of microorganisms should be performed by using a phylogenetic analysis based on rRNA (31, 32). The rRNA molecules and their genes consist of highly conserved domains interspersed with variable regions. Thus, the comparative analysis of the sequences is a powerful means to infer phylogenetic relatedness among organisms. Methods that have arisen from this work include cloning and sequence analysis of bacterial 16S rRNA genes (to yield clone 'libraries'). A phylogenetic analysis of 16S rRNA sequences together with chemotaxonomic and genomic analyses is one of the most powerful methods for inferring the relationships between genera or between species belonging to a genus (19).

This study attempts to investigate the microbial diversity of the bacterial community in Korean traditional soybean-based fermented foods (*doenjang* and *ganjang*) using a culture-independent approach utilizing DNA extraction and ribosomal RNA (16S rRNA) gene amplification and sequencing.

Materials and Methods

Sampling and DNA extraction Soybean paste (SP, *doenjang*) and soybean sauce (SS, *ganjang*) samples were obtained from a regional market place in Jinju, Korea. Both commercial products (6 samples of *doenjang* and 4 samples of *ganjang*) and homemade products (5 samples of *doenjang* and 4 samples of *ganjang*) were used in this study. Each sample (ca. 1 g or 1 mL) was mixed with 40 mL of phosphate buffered saline (pH 7.2), and vortexed for 30 min. The samples for DNA extraction were collected through 4 layers of cheesecloth, and centrifuged at 14,000×g for 5 min at 4°C. The pellet (approximately 0.2 mL) was then subjected to DNA extraction using the Soil DNA extraction kit (Mo Bio, Solana Beach, CA, USA). The extracted DNA was then used as a template for PCR to amplify 16S rRNA.

PCR amplification of 16S rRNA gene The 16S rRNA

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gene was amplified by PCR with a thermal cycler (model MJ Mini™; Gradient Thermal Cycler, Foster City, CA, USA). The PCR primers used to amplify 16S rDNA fragments were the bacterial-specific primers, 5'-CGGAG AGTTTGATCCTGG-3' (1BF, forward) and 5'-TACGGC TACCTTGTTACGAC-3' (2BR, reverse) (33). Subsequently, rRNAs were amplified by PCR using the metagenomic DNA and Super-Therm DNA polymerase (JMR, Side Cup, Kent, UK). Based on the manufacturer's instructions, the PCR reaction mixture (50 μL) contained 1 μL of *Taq* polymerase (2.5 unit), three all each of primer sets, 1BF-2BR (10 pmol), 5 μL of reaction buffer, 15 mM $MgCl_2$, 5 μL of 2 mM dNTP, 5 μL of template DNA, and 28 μL of sterilized water. Thirty cycles (denaturizing at 94°C for 30 sec, annealing at 50°C for 30 sec, and extension at 72°C for 90 sec) were followed by a final incubation at 72°C for 10 min. The anticipated product of approximately 1.5 kb was isolated by agarose gel electrophoresis (Fig. 1). The bacterial 16S rRNA gene amplicons were purified with a PCR purification kit (Intron Biotechnology, Suwon, Korea).

16S rRNA library Amplified Bacterial 16S rRNA genes were cloned in *Escherichia coli* DH5α with the pGEM-T easy vector (Promega, Madison, WI, USA) according to the manufacturer's instructions. Recombinant clones were randomly picked and recombinant plasmids were extracted using the Plasmid purification kit (Intron Biotechnology). Purified plasmids were checked for correct insert size via standard vector-targeted PCR and gel electrophoresis.

DNA sequencing and sequence analysis Samples for nucleotide sequencing were prepared by the dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CN, USA). The samples were analyzed with an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequencing was done on full-length cloned PCR products. All reference sequences were obtained from the GenBank and Ribosomal Database Project (RDP) (34) databases. Sequences were analyzed using the CHECK_CHIMERA program (34) to identify and exclude sequences arising from chimeric rRNA clones. Similarity searches against database entries were carried out by online BLAST search (35). Sequences were aligned using the multiple sequence alignment program, CLUSTAL W (36). Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using neighbor-joining methods (37). Bootstrap analysis was performed using data resampled 1,000 times using the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada).

Nucelotide sequence accession numbers and nomenclature Nucleotide sequences have been deposited in the GenBank database under a accession numbers DQ922955 – DQ923019. Clones names in the SP bacteria library begin with the letters SPB (e.g., SPB01), and a SSB prefix represents (e.g., SSB01) the SS bacteria library.

Results and Discussion

The diversity of bacterial populations in Korean SP and SS were studied by analysis of PCR-amplified bacterial 16S rRNA molecules. Although there are no exact 16S rRNA similarity limits for defining specific taxa such as genus and species, species definition in general requires sequences similarities greater than 98% (38). Thus, if a sequence has greater than 98% similarity to a 16S rRNA of a known bacterium, it is considered to be a member of that species. Sixty-two sequences in our libraries have greater than 98% similarity to a 16S rRNA of a known bacterium (Table 1). This culture-independent approach offers the possibility of characterizing microbial ecosystems independent of isolation, maintenance, and propagation of bacteria under laboratory conditions. However, PCR-based methodologies are subject to certain limitations such as the PCR amplification error and formation of chimera (39, 40).

At the SP library and SS library, sequencing of the clones were classified into five and six operational taxonomic units (OTUs) following of the 16S rRNA sequence, respectively (Table 1). The predominant species at the two different soybean fermented foods were: *Staphylococcus equorum* (60.6%) in the SP library and *Haloanaerobium* sp. (37.5%) and *Haloanaerobium fermentans* (37.5%) in the SS library. *Tetragenococcus halophila* (21.2%), *Leuconostoc mesenteroides* (9.1%), *Lactobacillus sakei* (6.1%), and *Bacillus subtilis* (3.0%) were also detected in the SP library and *T. halophila* (12.5%), *Staphylococcus* sp. (6.3%), *S. equorum* (3.1%), and *B. subtilis* (3.1%) were detected in the SS library. *S.*

Table 1. Bacterial distribution and diversity of the soybean paste and soybean sauce

Species	Similarity[1] (%)	Soybean fermented food	
		Soybean paste (n=33)[2]	Soybean sauce (n=32)
Bacillus subtilis	99	3.0[3]	3.1
Lactobacillus sakei	99	6.1	
Leuconostoc mesenteroides	99	9.1	
Halanaerobium fermentum	98-100		37.5
Halanaerobium sp.	99		37.5
Staphylococcus equorum	98-100	60.6	6.3
Staphylococcus sp.	99		6.3
Tetragenococcus halophila	99-100	21.2	9.3

[1]Range of 16S rRNA genes sequence is similarity values between soybean fermented food clones and type strain.
[2]n=the number of clones examined.
[3]% compositions.

equorum, *T. halophila*, and *B. subtilis* were found in both libraries.

However, *L. mesenteroides* and *L. sakei* were detected in the SP library only. *Halanaerobium* sp., *H. fermentans*, and *Staphylococcus* sp. were only detected in the SS library (Table 1). Generally, the quality of SP and SS products are determined by the different sources (soybean, wheat, and salt etc.), fermentation conditions, and fermentation periods as well as the different types of bacteria participated during the soybean paste and soybean sauce processing (5). In our culture-independent clone libraries, we did not find certain bacilli such as *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Bacillus pumilis*, and *Bacillus atrophaeus* which had been reported as major bacterial compositions in soybean fermented foods by culture-dependent studies (5, 6, 16, 41). *Doenjang* and *ganjang* are typically fermented at saline concentrations of 12 to 18%. This discrepancy was probably due to presence of thick spore wall of bacillus group which make hard to isolate genomic DNA. In addition, bacillus group are easy to culture than bacteria like *S. equorum*, *Haloanaerobium* sp. and *H. fermentans*. Moreover, the SS fermentation includes halophilic bacteria because of high content of salt (42).

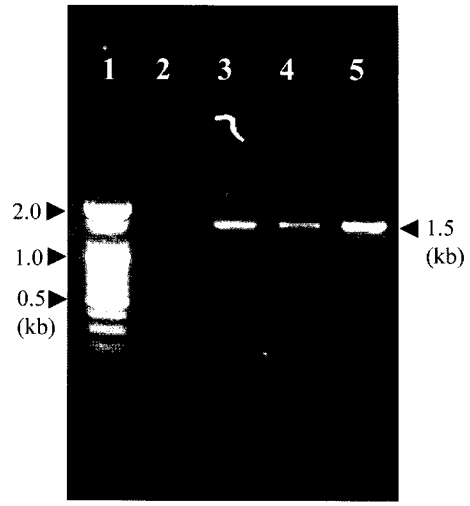

Fig. 1. Electrophoresis of PCR products when the primers 1BF and 2BR were used for the identification and detection of identified 16S rDNA. Lane 1, 100 bop-ladder; lane 2, sterile water for negative control; lane 3, soybean paste; lane 4, soybean sauce; lane 5, *E. coli* KCTC 1682 for positive control.

A phylogenetic analysis of the SP library is shown in Fig. 2A. More than half of the sequences were placed within the *S. equorum*. One clone (SPB28) was related to the typical soybean fermented bacteria, *B. subtilis*. Seven clones (SPB04, SPB07, SPB14, SPB20, SPB23, SPB31, and SPB33) clustered with the type strain of *T. halophila*. Two sequences (SPB08 and SPB15) were related to the typical *kimchi* isolate, *L. mesenteroides*. The sequence of

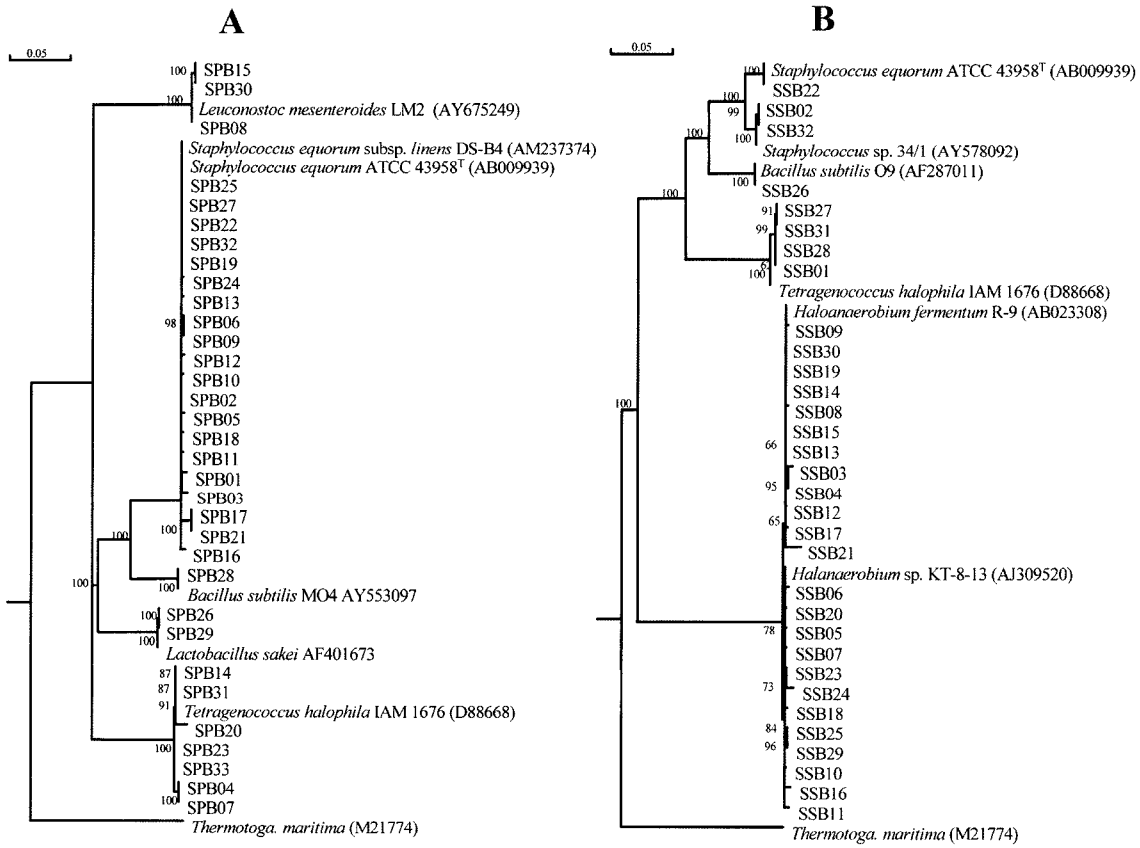

Fig. 2. Phylogenetic placement of 16S rDNA sequences from the soybean paste (A) and soybean sauce (B). Numbers above each node are confidence levels (%) generated from 1,000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position. Only values of 60% or above are shown. *Thermotoga maritima* was used as an out-group.

clones SPB24 and SPB29 was associated with the type strain of *L. sakei*.

The phylogenetic analysis of the SS library is shown in Fig. 2B. The majority of sequences were associated with the *Haloanaerobium* sp. and *H. fermentans* cluster. One clone (SSB26) was related to the typical soybean fermented bacteria, *B. subtilis*. Four clones (SSB01, SSB27, SSB28, and SSB31) clustered with the type strain of *T. halophila*. Two clones (SSB02 and SSB32) were associated with the *Staphylococcus* sp. cluster. One clone (SSB22) was placed in the *S. equorum* cluster.

S. equorum, recently isolated from a surface ripened Swiss mountain cheese, same was the predominant in SP. This strain suggested that as starter culture component of surface ripened cheeses without any detectable antibiotic production (43). *S. equorum* that exhibit antilisterial activity was also described (44). Moreover, salt tolerant and hemophilic staphylococci produce extracellular proteolytic and lipolytic enzymes without excessive modification of the product texture and are mainly involved in flavour development (45-47). *H. fermentans*, a novel halophilic anaerobe species isolated from salted puffer fish ovary, was another same predominant species in SS. This strain was related to manufacturing process of Japanese seafood, puffer fish ovaries fermented with rice bran (*fugunoko nukaduke)* (48). Members of the genus *Haloanaerobium* are widely distributed in surface saline ecosystems such as hyper saline lakes and in subsurface ecosystems such as oilfields (48, 49). *T. halophila* (formerly *Pediococcus halophilus*; 42) was described as halophilic lactic acid bacterium which requires high sodium chloride for growth. Uchida (50) showed that the *T. halophila* was a predominant of Japanese-type fermented vegetables. This strain was also detected in Indonesian soy mash (*kecap*) by random amplified polymorphic DNA (RAPD) analyses methods (51). *Lactobacillus, Lactococcus, Streptococcus,* and *Tetragenococcus* species are also known to be beneficial for flavour development, food preservation, and intestinal disorders action in various fermented foods (50-52).

In previous studies using culture-dependent methods, *B. subtilis* has the predominant which its plays important roles in Korean traditional soybean fermented foods (5, 16, 53). However, we detected only a single clone in the SP library and SS library, respectively. Our approach revealed different distribution and diversity from those reported in previous studies using culture-dependent methods. These results suggest that such different microbial populations may play specific roles in different soybean fermentation conditions such as *doenjang* and *ganjang*.

We have shown that culture-independent methods can be successfully applied to a fermented food environment such as soybean paste and soybean sauce. The data presented in this study may provide a useful frame of reference for further studies of a population dynamics of soybean fermentation, flavor development, control of the fermentation process.

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