African Journal of Microbiology Research Vol. 6(42), pp. 7008-7012, 6 November, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.1341
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Diversity analysis of bacteria in the latter ripening of Pixian soybean paste fermentation based on 16S rDNA analysis

Zou Yan-Ling[1,2], Xiang Wen-Liang[1,2], Rao Yu[1], Xing Ya-Ge[1], Zhang Liang[1] and Li Ming-Yuan[1]*

[1]Key Laboratory of Food Biotechnology of Sichuan, College of Bioengineering, Xihua University, Chengdu 610039, Sichuan, China.
[2]Institute of Traditional Brewing Biotechnology, Xihua University, Chengdu 610039, Sichuan, China.

Accepted 3 September, 2012

The biodiversity of bacteria inhabited in the latter ripe fermentation of Chinese traditional Pixian soybean paste was investigated based on bacterial 16S rDNA culture independent method. A total of 102 clones were recovered and belonged into *Pseudomonadaceae* family (3%), *Enterobacteriaceae* family (14%), *Lactobacillaceae* family (3%), *Bacillaceae* family (44%), *Staphyloccaceae* family (23%), *Brucellaceae* family (3%), *Chloroplast* family (3%) and not determined to family but determined to *Actinomycetales* order clones (6%), respectively. These results suggested that *Bacillaceae* family, *Staphyloccaceae* family and *Enterobacteriaceae* family are suspected to be the domain microorganisms responsible for the latter ripe fermentation and maybe contribute to the production of the characteristic flavor and tastes factors of Pixian soybean paste. To our knowledge, it is the first report about the bacterial diversity in the latter ripening of Chinese traditional Pixian soybean paste.

Key words: Pixian soybean paste, 16S rDNA, bacterial diversity.

INTRODUCTION

Pixian soybean paste is a traditional important ingredient in Sichuan cuisines and is known as the soul of Sichuan cuisines, where it is used in cooking and as a condiment in China, like *doenjang* in Korea and *miso* in Japan. Recently, Pixian soybean paste has gained attention not only because of its aroma value, but also because of its nutritional and health protection function. Pixian soybean paste originated in Pixian county of Sichuan province in China 300 years ago, and to date, it has been one of the most famous Chinese traditional condiments. In 2008, its traditional process was listed in China Intangible Cultural Heritages List.

Traditional Pixian soybean paste fermentation usually has two stages: initial stage fermentation and latter ripe fermentation. The raw materials including fresh capsicum chilli, fermented broad bean, salt, flour and no microbial inoculums, are mixed and fermented as initial stage fermentation under the natural environment for 90 days or more. During the latter ripe fermentation process, the coexistence of diverse microorganisms could provide numerous enzymes to accomplish the biochemical reaction of its unique flavor and tastes. Therefore, these microorganisms were thought to play important roles in latter ripe fermentation process. However, there is report about their microbial diversity.

Traditional cultivation techniques and molecular biological methods were widely used to analyze the microbial diversity. In the 1980s and 1990s, many investigators had used the method of isolation in laboratory to analyze the diversity of microorganism inhabited in Pixian soybean paste. Most of the microorganisms in traditional natural fermenting could escape traditional cultivation techniques because of the selective enrichment cultivation based on the thoroughly described microbial diversity (Xiang et al., 2012). As we

*Corresponding author. E-mail: yanling0205@163.com. Tel: +86-28-8772-0552.

know, the traditional isolate systems have many deviations which could alter the community structure from the original environmental parameter during cultivation (Dunbar et al., 1997). In most cases, these methods have many limiting factors to obtain a complete view of the microbial diversity. In recent years, 16S rDNA analysis has become a standard approach for the investigation of microbial diversity (Xiang et al., 2008). And, it is widely utilized for fermented food to analyze microbial diversity, such as Chinese rice vinegar (Haruta et al., 2006), Korean *Doenjang* (soybean paste) (Kim et al., 2009) and *shochu* (Endo and Okada, 2005).

It is well known that the quality and characteristics of the fermented products were affected by the microbial action. In the current study, we investigated the microbial diversity in the latter ripe fermenting of Pixian soybean paste by 16S rDNA gene libraries. The information gathered may be useful to improve our understanding of the composition and the microbial populations arising of unique flavor and tastes of Pixian soybean paste, and to design the effective management of the latter ripe fermentation.

MATERIALS AND METHODS

Sampling and DNA extraction

The experiment was carried out on an industrial scale traditional fermenting Pixian soybean paste at the Gaofuji Food Co. Ltd (Pixian County of Chengdu City, Sichuan, China). The sample was collected aseptically in sterile tubes from the latter ripening stages and frozen in liquid nitrogen until analysis. Microbial cells were collected using the same method by Xiang et al. (2012). The microbial cell pellets obtained was directly used for extracting DNA using the Bacterial DNA Kit (Omega Inc. USA). Then, total DNA was subsequently estimated by 0.7% (w/v) agarose gels and stored at -20°C.

Construction of clone library

PCR amplification of bacterial 16S rDNA was carried out by using the universal primers EU27F (5'-GAGAGTTTGATCCTGGCTCAG-3') and 1490R (5'-GGTTACCTTGTTACGACTT-3'). The 50 µl PCR mixture contained 0.5 µl *Taq* DNA polymerase (Promega, USA), 5 µl 10 × PCR buffer, 4 µl dNTP mixture, 1 µl of each primer, 1 µl of Pixian soybean paste DNA, and 37.5 µl ddH_2O. The thermal cycling conditions for EU27F and 1490R primers were initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were assessed by electrophoresis on a 1.0% (w/v). The 16S rDNA recombinant plasmids were produced using the pGEM®-T Vector Easy Systems I (Promega, USA) according to the manufacturer's protocol, and then transformed into the *Escherichia coli* DH5α competent cells. The transformants were identified on the solid LB culture medium containing 20 µl Amp (25 mg/ml), 40 µl IPTG (25 mg/ml), and 20 µl X-Gal (20 mg/ml). The white clones were used to construct 16S rDNA clone library of Pixian soybean paste.

Sequencing and phylogenetic analysis of 16S rDNA

Recombined plasmids with 16S rDNA segments were prepared from individual recombined colonies and used as templates for sequencing. Sequencing was performed by a sequencer (Applied Biosystems, Foster City, CA, USA) with a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Bioscience, Piscataway, NJ, USA). The 16S rDNA sequences obtained without chimeras were compared with those in GenBank Database by using the BLAST program and their percent similarities were then determined. Multiple alignments of 16S rDNA obtained in this study and from reference strains in GeneBank were run by using the Clustal X program (Thompson et al., 1994). And, the phylogenetic trees were constructed with the MEGA program version 5.0 by using the neighbor-joining method with the Kimura two-parameter model (Tamura et al., 2011).

Statistical analyses

The comparative sequences in this study were executed by BioEdit Software and sequences with 97% or higher identity score were grouped together and defined an operational taxonomic unit (OTU) (Godon et al., 1997; Schloss and Handelsman, 2005), while the others were grouped alone. Maximal OTU richness was estimated using models of Michaelis-Menten (SMM), Chao1 (SChao1), and ACE (SACE) (Hughes, 2001; Raaijmakers, 1987). Rarefaction analysis, displaying the number of OTUs detected versus the number of clones analyzed, was performed using the Analytic Rarefaction 1.3 calculator and displayed using SigmaPlot 8.02 (SYSTAT Software) (Raaijmaker, 1987). All 16S rDNA represent sequences from the OUT were respectively classified by program RDP Seqmatch and GeneBank Database. When 97% or higher identity of nucleotide was obtained, the OTU was identified to a same species level represented by the strain from the database.

Nucleotide sequence accession numbers

The 16S rDNA gene sequences have been deposited in GenBank with accession numbers JQ966216 to JQ966229.

RESULTS

Estimation of the gene library

For Pixian soybean paste, library of 16S rDNA clones were produced. A total of 102 clones were recovered from the current sample. All clones were selected for sequencing and then obtained sequences without chimeras were aligned and sorted into OTUs. Classification of OTUs at 97% identity of nucleotide level revealed that there is a S*obs* of 12 different OTUs in the gene libraries, representing 35.3% of the clone samples. The rarefaction curve was obtained by plotting the number of OTUs observed against the number of clones. The decrease in the rate of OTUs detection shown on the curve indicated that the major part of the diversity in 16S rDNA libraries had been detected. This conclusion was further supported by 34.9% coverage of gene library. Maximum OTU richness was estimated with two different models S_{Chao1} and S_{ACE}, and revealed S_{Chao1} of 40 and S_{ACE} of 28.6. Based on these estimations, 34.9% of the bacterial diversities were covered by the applied sampling survey.

Table 1. 16S rDNA analysis of the bacteria from the latter ripening of Pixian soybean paste.

Sample ID[a]	Closest species (GenBank accession)	Identity (%)[b]
DB-E14	*Bacillus subtilis* DSM10 ^{T} (AJ276351)	99 (23.6)
DB-A10	*B. thermoamylovorans* CNCM I-1378 ^{T} (L27478)	99 (15.6)
DB-F15	*B. smithii* DSM 4216 ^{T} (Z26935)	98 (2.9)
DB-G3	*B. licheniformis* ATCC 14580 ^{T} (CP000002)	99 (2.9)
DB-A11	*Staphylococcus sciuri* DSM 20345^T (AJ421446)	99 (20.6)
DB-G2	*S. xylosus* ATCC 29971 ^{T} (D83374)	99 (2.9)
DB-G1	*Enterobacter cloacae* ATCC13047 ^{T} (AJ251469)	98 (11.8)
DB-A4	*E. cloacae* LMG 2683 ^{T} (Z96079)	99 (2.9)
DB-F12	*Arthrobacter creatinolyticus* gifu12498 ^{T} (D88211)	99 (2.9)
DB-A3	*A. mysorens*DSM 12798^T (AJ617482)	97 (2.9)
DB-F8	*Pediococcus pentosaceus* DSM 20336 ^{T} (AJ305321)	99 (2.9)
DB-A5	*Pseudomonas plecoglossicida* FPC951 ^{T} (AB009457)	99 (2.9)
DB-D9	*Ochrobactrum pseudogrignonense* LMG 3331 ^{T} (AM114398)	98 (2.9)
DB-D11	*Uncultured organism clone*(HQ781886)	99 (2.9)

a: Sample ID are the representative clone from one OUT; b: Identity represents the % identity shared with the sequences in the GenBank databases.

Taxonomic analyses

The complete 16S rDNA retrieved sequences without chimeras were rapidly and accurately classified by program RDP MultiClassifier. Most of the 102 sequences obtained in this study respectively fell into the class of *Pseudomonadaceae* family, *Enterobacteriaceae* family, *Lactobacillaceae* family, *Bacillaceae* family, *Staphyloccaceae* family, *Brucellaceae* family, *Chloroplast* family and not determined to family but determined to *Actinomycetales* order clones. While, 21 sequences belonged to *Proteobacteria* phylum, 72 sequences sorted into *Firmicutes* phylum were not further classified to unambiguous genus by the RDP MultiClassifier. The family *Bacillaceae* (45/102), *Staphyloccaceae* (24/102), and *Enterobacteriaceae* (15/102) were prevalent bacteria in the sample.

Phylogenetic affiliation

Comparative sequence analysis of 16S rDNA is currently the most widely used approach for the reconstruction of microbial phylogeny (Xiang et al., 2011). All represent 16S rDNA sequences from the every OTU group were submitted to the identity of nucleotide searches in GeneBank to infer a possible phylogenetic classification. The BLAST searches revealed that most represent sequences shared more than 99% identity of nucleotide with the strains available, but the bacteria represented by clone DB- F15, DB-G1, DB-A3, and DB-D9 showed lower than 99% or less to strains in public database at the16S rDNA level (Table 1). To disclose the taxonomic position and relationships, the phylogenetic tree based on the complete 16S rDNA gene of the representative sequences from OTUs by using neighbor-joining method was respectively constructed and shown in Figure 1. The trees revealed the phylogenetic affiliation of microorganism in the latter ripening stage of Pixian soybean paste.

DISCUSSION

The aim of this study was to use 16S rDNA analysis to characterize the diversity of microbes in Pixian soybean paste, which could influence the arising of unique flavor and tastes. The molecular biology method based on PCR technology can objectively evaluate the diversity of the sample. It is well known that the quality of Pixian soybean paste is mostly affected by the microflora involved fermentation process. Various kinds of microorganisms are thought to participate in fermentation of soybean paste and its unique flavor imparts by the decomposed products of soybean protein is mainly dependent on the microbial action of ripening fermentation. In the current study, some microbes which belonged to *Bacillus*, *Staphylococcus*, *Enterobacter*, *Pediococcus*, *Pseudomonas* and *Arthrobacter* genera, were involved in the ripening process of Pixian soybean paste. It has been reported that *doenjang* is dominated by *Bacillus* species, lactic acid bacteria, and *staphylococcus* (Yoo et al., 1999; Kim et al., 2009). In contrast, *Bacillus*, *Staphylococcus* and *Enterobacter* were the dominant species in Pixian soybean paste sample.

Many researchers have reported that *Bacillus subtilis* and *Bacillus licheniformis* are the dominant organisms in soybean paste and play an important role during

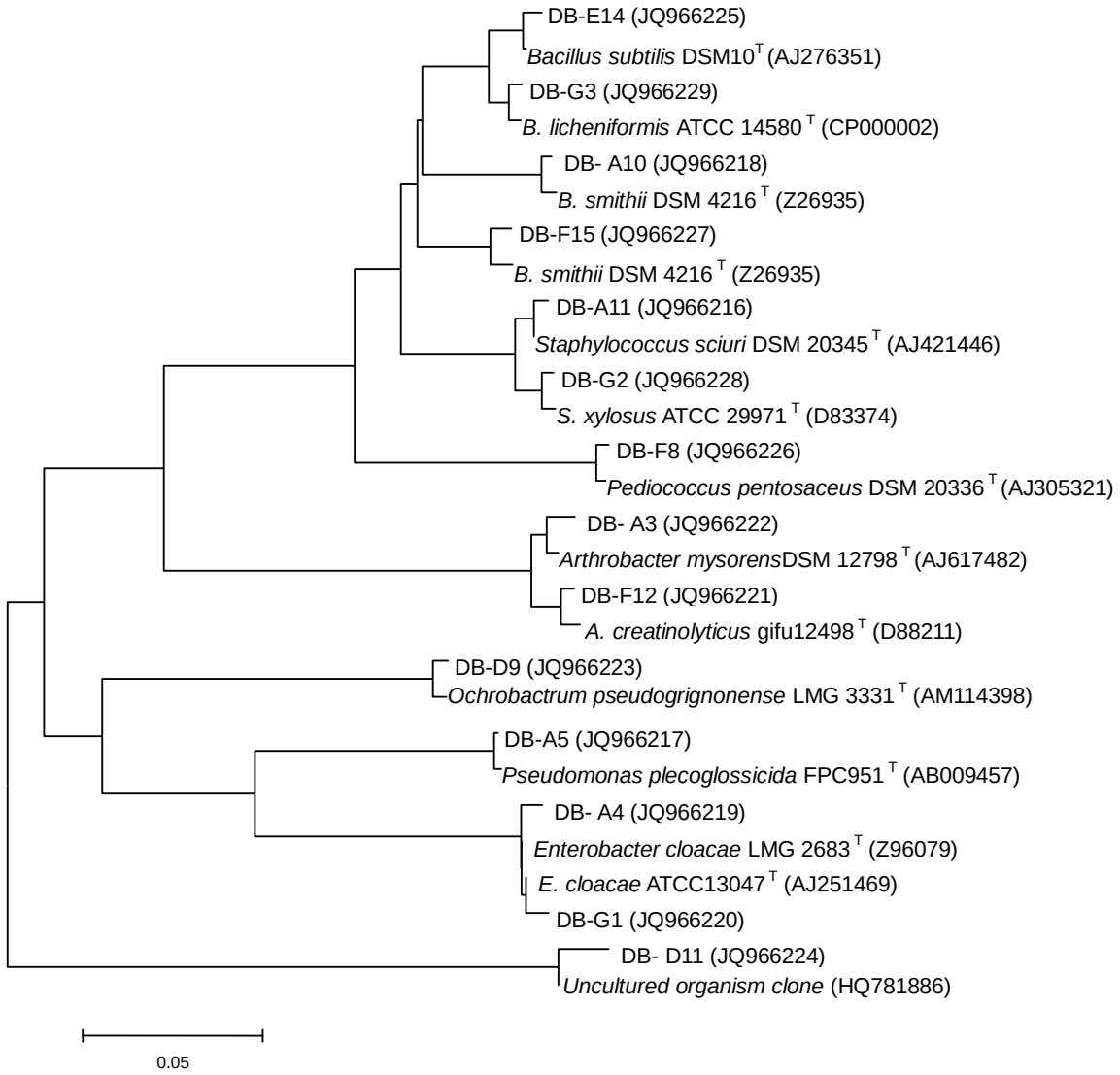

Figure 1. The phylogenetic tree based on the complete 16S rDNA sequences of representative clones from prokaryotic OTUs by using the neighbor-joining method and the representative clone distributions the latter ripening of Pixian soybean paste. The scale bar corresponds to 0.05-estimated nucleotide substitution per sequence position.

fermentation (Yoo et al., 1999). In our sample, *B. subtilis* and *B. licheniformis* were also detected by 16S rDNA technology, while *B. subtilis* were prime prevalent strains with 23.6%, but not *B. licheniformis*, only with 2.9%. They are both likely to grow soybean paste ingredients, bringing into generate the preferable flavors and masking the offensive flavors during the ripening of soybean paste. *Bacillus thermoamylovorans* was also found to be main strains in our study and it can produce lactate, acetate, ethanol, and formate by glucose fermentation. In the genus *Staphylococcus*, the strains assigned to *Staphylococcus sciuri* an opportunistic pathogen of controversial clinical significance. In Korea soybean paste, it was found but not dominant. Interestingly, in Pixian soybean paste, it was also prevalent with 20.6% (Table 1). However, the fermentation function of *S. sciuri* in Korea soybean paste and Pixian soybean paste is not reported till now. *Enterobacter cloacae* can produce β-galactosidase with both hydrolytic and transglycosylation reaction (Lu et al., 2009). In ripening stage of Pixian soybean paste, it also generates some odors. But, the strains represented by DB-G1 were only closest to the *E. cloacae* with 98% identity of nucleotide and they were needed to fourthly identification.

The fermentation process of Pixian soybean paste is different from that of the other fermented soybean paste such as Korea *doenjang*. In addition, due to the regional climate and soil, raw material and proportion and pits

form different size, the microbes grown in it are also different from those in the other soybean paste. This was confirmed by our results based 16S rDNA analysis of ripening stage. And this result was effective in understanding of the composition and the microbial populations arising of unique flavor and tastes of Pixian soybean paste, and designing the management of the latter ripe fermentation.

ACKNOWLEDGEMENTS

This work was financially supported by the Key Laboratory of Food Biotechnology of Sichuan, Xihua University, Sichuan, China (SZJJ2009-014, SZJJ2009-013), the Key Project and High Technology Research, Xihua University, Sichuan, China (Z0910501) and the Sichuan Key Disciplinary of Food Science, Xihua University, Sichuan, China.

REFERENCES

Dunbar J, White S, Forney LJ (1997). Genetic diversity through the looking glass: effect of enrichment bias. Appl. Environ. Microbiol. 63(4):1326-1331.

Endo A, Okada S (2005). Monitoring the lactic acid bacterial diversity during *shochu* fermentation by PCR-denaturing gradient gel electrophoresis. J. Biosci. Bioeng. 99(3):216-221.

Godon JJ, Zumstein E, Dabert P, Habouzit F, Moletta R (1997). Molecular microbial diversity of an aerobic digester as determined by small-subunit rDNA sequence analysis. Appl. Environ. Microbiol. 63(7):2802-2813.

Haruta S, Ueno S, Egawa I, Hashiguchi K, Fujii A, Nagano M, Ishii M, Igarashi Y (2006). Succession of bacterial and fungal communities during a tradition pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. Int. J. Food Microbiol. 109(1-2):79-87.

Kim TW, Lee JH, Kim SE, Park MH, Chang HC, Kim HY (2009). Analysis of microbial communities in *doenjang*, a Korean fermented soybean paste, using nested PCR-denaturing gradient gel electrophoresis. Int. J. Food Microbiol. 131(2-3):265-271.

Lu LL, Xiao M, Li ZY, Li YM, Wang FS (2009). A novel transglycosylating β-galactosidase from *Enterobacter cloacae* B5. Process Biochem. 44(2):232-236.

Raaijmakers JG (1987). Statistical analysis of the Michaelis-Menten equation. Biometrics 43(4):793-803.

Schloss PD, Handelsman J (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. 71(3):1501-1506.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28(10):2731-2739.

Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22(22):4673-4680.

Xiang WL, Guo JH, Feng W, Huang M, Chen H, Zhao J, Zhang J, Yang ZR, Sun Q (2008). Community of extremely halophilic bacteria in historic Dagong Brine Well in southwestern China. World J. Microbiol. Biotechnol. 24(10):2297-2305.

Xiang WL, Liang HZ, Liu S, Luo F, Tang J, Li MY, Che ZM (2011). Isolation and performance evaluation of halotolerant phosphate solubilizing bacteria from the rhizospheric soils of historic Dagong Brine Well in China. World J. Microbiol. Biotechnol. 27(11):2629-2637.

Xiang WL, Liang HZ, Luo F, Liu S, Xing YG, Li MY, Ma L, Che ZM (2012). A novel NhaD type Na^+/H^+ antiporter gene from a metagenomic library of halophiles colonizing in the Dagong Ancient Brine Well in China. Afr. J. Microbiol. Res. 6(3):543-551.

Yoo SK, Cho WH, Kang SM, Lee SH (1999). Isolation and identification of microorganisms in Korean traditional soybean paste and soybean sauce. Korean J. Appl. Microbiol. Biotechnol. 27(2):113-117.