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## Full Paper

# Taxonomic homogeneity of a salt-tolerant lactic acid bacteria isolated from shoyu mash

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**Forty-seven salt-tolerant lactic acid bacteria, which had been isolated from different places and grown in 15% NaCl, were examined to assess their taxonomic heterogeneity. Among the isolates, 42 were isolated from shoyu mash during the acid fermentation phase, 2 were from miso and 3 were from anchovy pickles. All isolates were identified as *Tetragenococcus halophilus* on the basis of DNA relatedness values. We further examined 102 phenotypic characteristics of them. The isolates exhibited differences in only 16, supporting the conclusion obtained from the DNA relatedness analysis.**

**Key Words**——DNA relatedness; lactic acid bacteria; phylogenetic analysis; physiological homogeneity; shoyu; *Tetragenococcus*

## Introduction

During the early stage of shoyu fermentation, salt-tolerant lactic acid bacteria become the dominant species. Those bacteria require sodium chloride (NaCl) for growth and have a tolerance of very high concentrations of it (higher than 18%). Their active growth, preceding the fermentation by yeast, constitutes a prerequisite step to suppress the growth of unwanted microbes. Thus, lactic acid bacteria play an essential role in making shoyu of high quality. The identity of the bacteria growing in the shoyu mash, however, remains ambiguous so far, though some physiological studies have shown that there is heterogeneity in the group. The *Tetragenococcus halophilus* population from shoyu mash is heterogeneous regarding substrate utilization (Uchida, 1982), ecological properties (Roling et al., 1996) and genetic variation with random amplified polymorphic DNA analyses (Gurter et al., 1998).

Currently, the method based on the measurement of the DNA relatedness value is the well-established, standard method for taxonomic study of bacteria. So we carried out the study of the bacteria isolated from anchovy pickles and miso as well as shoyu using the DNA-DNA hybridization technique. In this report, we determined the homogeneity of *T. halophilus* strains from those sources not only on the basis of DNA relatedness but through numerical analysis of phenotypic characteristics as well.

## Materials and Methods

*Bacterial strains and media.* Bacterial strains used in this study are listed in Table 1. These salt-tolerant lactic acid bacteria were isolated from many sources of soy sauce mash during various phases of lactic acid fermentation. GYP media supplemented with 15% NaCl and 0.6% $CaCO_3$ was used for the isolation. Colonies were selected, after being incubated at 30°C for 7 days, by checking haloes of clearing in $CaCO_3$.

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Table 1. Bacterial strain list.

| Strain[a] | Source[b] | History[c] |
|---|---|---|
| Unidentified strains | | |
| HSCC 554 | | |
| \| | | Isolated from shoyu mash. |
| HSCC 593 | | |
| HSCC 1032 | RIFY 5448 | From MK-1, isolated from miso. |
| HSCC 1033 | RIFY 5449 | From MR08, isolated from miso. |
| HSCC 1075 | | Isolated from shoyu mash. |
| HSCC 1076 | | Isolated from shoyu mash. |
| HSCC 1077 | RIFY 5039 | From NRIC 1643, isolated from anchovy pickles. |
| HSCC 1078 | RIFY 5044 | From IAM 1676, isolated from anchovy pickles. |
| *Tetragenococcus halophilus* | | |
| HSCC 1122$^T$ | JCM 5888$^T$ | From ATCC 33315 from NCDO 1635 from H. Hoffman from P. H. Mees, Technol. Hochschule, *Tetracoccus* No. 1 Orlajensen, isolated from anchovy pickles. |
| *Tetragenococcus muriaticus* | | |
| HSCC 1396$^T$ | JCM 10006$^T$ | From T. Fujii, isolated from squid liver sauce. |
| *Pediococcus damnosus* | | |
| HSCC 1314$^T$ | JCM 5886$^T$ | From ATCC 29358 from NCDO 1832 from Q. E. C. Univ. London from D. H. Williamson, isolated from Lager Beer Yeast. |

[a] HSCC, Culture Collection, the R&D Department, Higeta Shoyu Co., Ltd., Chiba, Japan. T indicated type strain.

[b] RIFY, Research Institute of Fermentation, Yamanashi University, Kofu, Japan; JCM, Japan Collection of Microorganisms, Saitama, Japan.

[c] NRIC, NODAI Research Institute Culture Collection Center, Tokyo University of Agriculture, Tokyo, Japan; IAM, Institute of Applied Microbiology, the University of Tokyo, Tokyo, Japan; ATCC, American Type Culture Collection, Rockville, Maryland, USA; NCDO, National Collection of Food Bacteria, AFRC Institute of Food Research, Reading, UK.

*DNA base composition and DNA relatedness.* Chromosomal DNA was prepared as described by Saito and Miura (1963). DNA was isolated from cells grown in GYP broth supplemented with 5% NaCl and 0.5% glycine for 5 days. The DNA base composition (G+C content) was determined as described by Tamaoka and Komagata (1984). DNA relatedness values were determined as described by Ezaki et al. (1989).

*Sequencing of 16S rRNA gene and phylogenetic analysis.* PCR amplification of the 16S rRNA gene from chromosomal DNA was carried out by the method of Fox et al (1992). Origonucleotides 5′-CTGGGATCCATTTACTCGAGAGTTTGATCCTG-GCTCAG-3′ (primer 27FC; 5′ end of the 16S rRNA gene) and 5′-GGTTCCCCTAAGCTTACCTTGTTAC-GACTTC-3′ (primer 1490RC; 3′ end of the 16S rRNA gene) were used as primers for PCR as described by Wisotzkey et al. (1992). Sequencing was carried out as described by Sanger et al. (1977), by using a Dye terminator cycle sequencing FS Ready Reaction kit (Perkin-Elmer Co., Boston, Mass., USA) and a model ABI 373A automatic DNA sequencer (Perkin-Elmer Co.). The nucleotide sequences of 16S rRNA genes were obtained from EMBL/GenBank/DDBJ databases. Multiple alignment of sequences, calculation of evolutionary distance ($K_{nuc}$) values (Kimura, 1980), generation of a neighbor-joining phylogenetic tree (Saito and Nei, 1987), and bootstrap analysis with 1,000 replicates for evaluation of phylogenetic tree topology (Felsenstain, 1985) were carried out with the CLUSTAL W program version 1.5 (Thompson et al., 1994). Alignment gaps and unidentified base positions were not taken into account.

*Phenotypic characterization and numerical analysis.* All phenotypic tests were carried out with GYP media supplemented with 5% NaCl at 30°C. The cell form, cell size, cell arrangement, and colonial appearance of the cells grown for 3 days were examined. Hucker-Coun modification was used for gram stain. Motility was examined by observing how colonies diffused in GYP semisolid agar. Tyramine production was determined by thin layer chromatography (TLC) using Kieselgel 60 (Merck, Darmstedt) with development sol-

vent (buthanol, acetic acid, water=20:3:7). Aspartic acid dehydrolysis was detected by monitoring the change in alanine level using TLC Kieselgel 60 (Merck) with development solvent (1-propanol, 28% ammonia solution=67:33). Lactic acid isomers were analyzed by the method by Otsuka et al. (1994). All other phenotypic tests were performed in accordance with standard protocols (Kozaki et al., 1992). The phenotypical basis of relationships were determined by performing numerical analysis as described previously (Takagi et al., 1993).

## Results

### *DNA base composition and DNA relatedness*

The G+C content of 47 strains from shoyu mash, miso and anchovy pickles (including type strain of *T. halophilus*, which was isolated from anchovy pickles) were examined, and found to range from 36.2 to 38.2 mol% (Table 2). The DNA relatedness value between HSCC 554 and all the remaining isolates was higher than 69%, as shown in Table 2. The DNA relatedness values of the type strain between *T. halophilus* HSCC 1122$^T$ and 15 strains randomly selected were higher than 79% (Table 2). The result means that all the strains examined, including those from miso and anchovy pickles, form one species.

### *Phylogenetic relationships*

The 16S rRNA gene from strains HSCC 554, HSCC 562, HSCC 579, HSCC 1075, HSCC 1076, HSCC 1077, HSCC 1032, HSCC 1033, HSCC 5039 and *T. halophilus* HSCC 1122$^T$ were sequenced. The sequence homology values for all of these strains were greater than 99.2% and the other species were less than 96.2%. They were placed in a monophyletic cluster which included *T. halophilus* (Fig. 1).

### *Phenotypic characterization and numerical analysis*

All of the isolates were gram-positive, nonmotile cocci, and coccal forms appeared in tetrads. Colonies grown on GYP agar plates formed into lenz shapes, and were white in color. They showed negative reactions for catalase, nitrate reduction, gelatin hydrolysis, and slime formation from sucrose. In litmus milk, they showed negative reactions for acidification, coagulation, liquefaction, and reduction. They were homofermentative, and produced L(+)-lactic acid with only trace amounts of D(+)-lactic acid. They were able to

Table 2. DNA base compositions and levels of DNA relatedness among salt-tolerant lactic acid bacteria.

| Strain | G+C content (mol%) | Reassociation with DNA from[a] | |
|---|---|---|---|
| | | *T. halophilus* | |
| | | HSCC 554 | HSCC 1122$^T$ |
| HSCC 554 | 36.5 | 100 | 100 |
| HSCC 555 | 36.7 | 102 | NT |
| HSCC 556 | 36.9 | 76 | NT |
| HSCC 557 | 36.8 | 90 | NT |
| HSCC 558 | 36.8 | 100 | NT |
| HSCC 559 | 36.6 | 99 | NT |
| HSCC 560 | 37.2 | 100 | NT |
| HSCC 561 | 36.6 | 69 | NT |
| HSCC 562 | 38.2 | 101 | NT |
| HSCC 563 | 36.7 | 83 | NT |
| HSCC 564 | 36.2 | 98 | NT |
| HSCC 565 | 36.3 | 79 | 86 |
| HSCC 566 | 36.7 | 87 | NT |
| HSCC 567 | 36.8 | 84 | NT |
| HSCC 568 | 36.8 | 100 | NT |
| HSCC 569 | 36.6 | 87 | NT |
| HSCC 570 | 36.6 | 100 | 98 |
| HSCC 571 | 36.5 | 101 | NT |
| HSCC 572 | 36.4 | 102 | NT |
| HSCC 573 | 36.6 | 92 | NT |
| HSCC 574 | 36.5 | 75 | NT |
| HSCC 575 | 36.8 | 102 | NT |
| HSCC 576 | 36.4 | 93 | NT |
| HSCC 577 | 36.6 | 86 | NT |
| HSCC 578 | 36.8 | 79 | NT |
| HSCC 579 | 36.6 | 89 | 85 |
| HSCC 580 | 36.4 | 90 | 86 |
| HSCC 581 | 36.6 | 82 | NT |
| HSCC 582 | 36.7 | 90 | NT |
| HSCC 583 | 36.7 | 75 | NT |
| HSCC 584 | 36.4 | 100 | NT |
| HSCC 585 | 36.7 | 86 | 79 |
| HSCC 586 | 36.6 | 97 | NT |
| HSCC 587 | 36.5 | 99 | 90 |
| HSCC 588 | 37.0 | 103 | NT |
| HSCC 589 | 36.4 | 100 | NT |
| HSCC 590 | 36.5 | 101 | NT |
| HSCC 591 | 36.7 | 82 | NT |
| HSCC 592 | 36.8 | 99 | NT |
| HSCC 593 | 36.4 | 78 | NT |
| HSCC 1075 | 36.6 | 100 | 92 |
| HSCC 1076 | 36.6 | 100 | 85 |
| HSCC 1077 | 36.6 | 76 | 101 |
| HSCC 1078 | 36.5 | 100 | 103 |
| HSCC 1032 | 36.8 | 102 | 90 |
| HSCC 1033 | 36.8 | 101 | 100 |
| *T. halophilus* HSCC 1122$^T$ | 36.8 | 100 | 80 |

[a] Reassociation values are averages of two determinations.
NT, not tested.

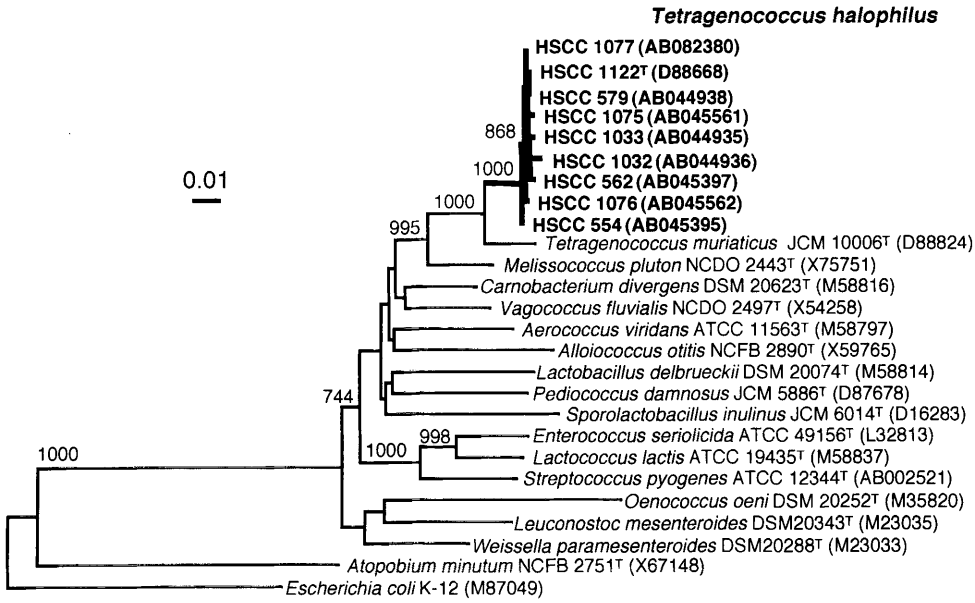



Fig. 1. Phylogenetic relationships of lactic acid bacteria based on 16S rRNA gene sequences.

The branching pattern was generated by the neighbor-joining method. The numbers indicate bootstrap values greater than 700. The boldface lines and boldface type indicate the cluster consisting of *Tetragenococcus halophilus* and the strains sequenced in this study, respectively. Bar=0.01 nucleotide substitution per site.

grow at 37°C but not at 5 or 43°C. Optimal growth occurred at temperatures between 23 and 30°C. Growth occurred at NaCl concentrations of 0 to 15%. The optimal NaCl concentration was about 2.5 to 5.0%. All the isolates produced acid from glucose, fructose, galactose, mannose and inulin, but not from lactose, sorbose, rhamnose, xylose, raffinose, adonitol, glycerol, inositol, starch or xylan. Some produced acid from mellibiose, sorbitol, mannitol, arabinose, ribose, sucrose, cellobiose, trehalose and maltose. No difference was found in the vitamin, amino acid or base requirements. Some strains produced ammonia from arginine, produced alanine from aspartic acid, produced tyramine from tyrosine and exhibited pseudocatalase activity. Some strains indicated sensitivity against bacteriophages ϕ606, ϕ676 and ϕI5, which were isolated from shoyu mash. Differential phenotypic characteristics are shown in Table 3. We analyzed distant relationships on the basis of 22 phenotypes which indicated differences among 49 strains including *T. muriaticus* HSCC 1396$^T$ and *Pediococcus damnosus* HSCC 1314$^T$. Forty-six strains and the type strain of *T. halophilus* were recovered in one cluster at a similarity level of more than 75% (Fig. 2). However the type strain of *T. muriaticus* and *P. damnosus* were not. They exhibited 73% and 55% similarity, respectively (Fig. 2).

Table 3. Differential phenotypic characterization of lactic acid bacteria from shoyu mash.

| Characteristics | Number of positive strains (% of positive strains) |
|---|---|
| Acid formation from: | |
| Mellibiose | 25 (53) |
| Sorbitol | 19 (40) |
| Mannitol | 26 (55) |
| Arabinose | 17 (36) |
| Ribose | 10 (21) |
| Cellobiose | 34 (72) |
| Trehalose | 31 (66) |
| Maltose | 43 (91) |
| Sucrose | 12 (26) |
| Pseudo-catalase activity | 3 (6) |
| Ammonia formation from arginine | 1 (2) |
| Tyramine formation from tyrosine | 9 (19) |
| Alanine formation from aspartic acid | 2 (4) |
| Phage sensitivity against: | |
| ϕ606 | 1 (2) |
| ϕ676 | 4 (9) |
| ϕ15 | 1 (2) |

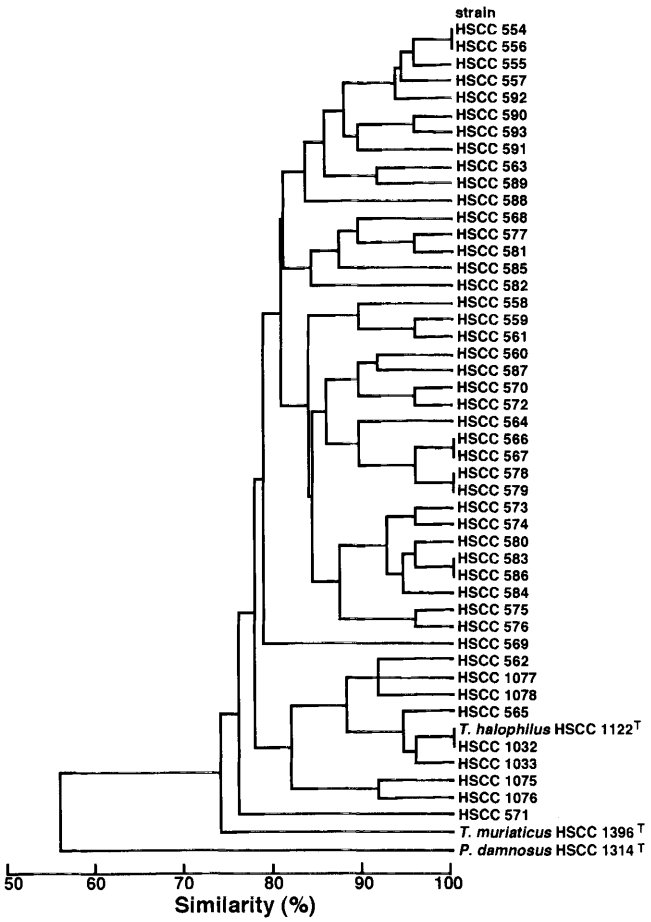



Fig. 2. Phenotypic relationships among strains isolated from soy sauce mash during lactic acid fermentation, *T. muriaticus* and *P. damnosus.*

The dendrogram was produced by using the simple matching coefficient and the unweighted pair arithmetic average algorithm.

## Discussion

Uchida (1982) proposed that *T. halophilus* strains isolated from shoyu mash were heterogeneous, based on the analysis of acid formation from sugars. We obtained a similar result showing their heterogeneity regarding sugar utilization. Based on the analysis of DNA, however, it was concluded that they were highly homogeneous. First, G+C content of all isolates ranged from 36.3 to 38.2 mol%, a very narrow variation range. Second, DNA relatedness values between isolates were higher than 69%. These results indicate that all the strains examined comprised one species despite differences of isolation place and fermentation phase. This conclusion was further supported by the numerical analysis data.

In addition to shoyu mash, salt-tolerant lactic acid bacteria are widely distributed in fermented foods which have a high salt concentration. To discover the taxonomical heterogeneity among them, we took up the isolates from miso and anchovy pickles. We subjected them to the same analysis as that of the isolates from shoyu mash and obtained results showing they belonged to the same species, *T. halophilus.* Furthermore, we determined the nucleotide sequences of 16S rRNA gene and compared them to each other. The similarity level ranged from 99.2 to 100% ($K_{nuc}$ value=0–0.008). Thus, it is concluded that there is no phylogenetic distance among the isolates from shoyu mash, and from at least two additional sources. The fact that bacteria living in different sources with an environment of especially high salt concentration have a same identity suggests a very limited number of species which can adapt to the environment.

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