APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1996, p. 1203–1207 Vol. 62, No. 4
0099-2240/96/$04.00+0

Characterization of *Tetragenococcus halophila* Populations in Indonesian Soy Mash (Kecap) Fermentation

WILFRED F. M. RÖLING AND HENK W. VAN VERSEVELD*

Department of Microbiology, Biological Laboratory, Vrije Universiteit, 1081 HV Amsterdam, The Netherlands

Received 16 October 1995/Accepted 27 January 1996

Chinese- and Japanese-type fermented soy sauces are made of different plant materials. The lactic acid bacterium *Tetragenococcus halophila* is present and grows in both types. On the basis of the difference in sugar composition and content in the plant materials, differences in the populations of *T. halophila* bacteria were expected. However, obvious differences were found only regarding the utilization of L-arabinose. In the Chinese type, almost all isolates utilized L-arabinose, while in the Japanese type only 40% of the isolates did. Also, the population in the Japanese type was more heterogeneous regarding substrate utilization. Random amplified polymorphic DNA analysis revealed that the heterogeneous population at the Japanese-type industrial manufacturer was derived from only three strains at maximum. Genetic relatedness among isolates from different soy sauce manufacturers was low, but protein fingerprinting indicated that the isolates still belonged to one species.

Soy sauce is a well-known food condiment in Southeast Asia, China, and Japan. Its production involves a fungal solid-state fermentation of plant materials, followed by a brine fermentation. Two types of soy sauce can be distinguished: a Chinese type, made of soybeans only, and a Japanese type, made of equal amounts of wheat and soybeans (25). The lactic acid bacterium *Tetragenococcus halophila* (until recently known as *Pediococcus halophilus* [2]) grows in both types during the brine fermentation (7, 18, 19). In the Japanese type, an obvious yeast fermentation occurs after acidification of the brine by *T. halophila*, while such a yeast fermentation is lacking in the Chinese type (17).

The difference relates to the sugar content and composition of the plant materials used (17, 18). Soybeans contain less than 20% (wt/wt) sugars, including a large variety of carbohydrates such as melibiose, sucrose, raffinose, stachyose, and cell wall polysaccharides (13, 25). Starch is the main carbohydrate in wheat and contributes to 60 to 70% of its dry weight (25). During the solid-state fermentation and treatments prior to the solid-state fermentation, the oligo- and polysaccharides in the plant materials are broken down to monosaccharides like fructose, glucose, galactose, L-arabinose, and xylose (8, 13, 18). This results in high glucose concentrations (>100 mM) at the start of Japanese-type brine fermentation. After growth of *T. halophila* cells, a large amount of glucose remains, and this can be used for yeast growth. In Chinese-type brine fermentation, the initial glucose concentration is low (<10 mM), and no fermentable sugars are left for yeast growth after growth of *T. halophila* cells (17, 18).

Because of the low sugar content of Chinese soy mash, its fermentation might differ from Japanese-type brine fermentation not only regarding yeast fermentation but also regarding the properties of its *T. halophila* population. The brine is not inoculated. Growth presumably results from cells that have remained from previous use of the fermentation tanks. Utilization of carbohydrates is under glucose- and fructose-mediated catabolite control (1). Because of its high glucose content, only glucose metabolism seems required for *T. halophila* isolates in Japanese-type brine fermentation, while in the energy-poor Chinese type, one would expect selection for strains able to utilize other carbohydrates and possibly amino acids, since amino acid decarboxylation by lactic acid bacteria can generate energy (12). Amino acid-decarboxylating *T. halophila* strains have been isolated from Japanese mash (22).

Uchida (21) showed that the *T. halophila* population of a single batch of Japanese-type brine fermentation is very heterogeneous regarding substrate utilization. *T. halophila* populations in Chinese-type brine fermentation have not been reported before. Traditional Indonesian kecap is a Chinese type of soy sauce. In order to establish whether *T. halophila* populations in Chinese-type brine fermentation differ from the population in Japanese-type brine fermentation, isolates obtained from three kecap manufacturers were characterized for substrate utilization and general growth characteristics such as maximum growth and acidification characteristics. These characteristics were compared with the characteristics of isolates from an Indonesian manufacturer producing Japanese-type soy sauce. Heterogeneity of the *T. halophila* populations was determined by random amplified polymorphic DNA (RAPD) analyses (6, 11). Information about the composition and abilities of *T. halophila* populations is required for a good understanding of soy sauce fermentation processes and could, for example, be used for developing procedures for inoculation and strain selection. At present, soy sauce fermentation in Indonesia yields a product with a rather inconsistent composition (18).

MATERIALS AND METHODS

Strains. The isolates designated LIB (10 isolates from an 18-day-old batch), IL (6 isolates from a 4-week-old sample), and PUR (10 isolates) were previously isolated from three traditional manufacturers in Middle Java, Indonesia, and identified as *T. halophila* (18). The 43 JV isolates were isolated from a 20-day-old batch at an industrial kecap manufacturer on Java. All strains are in the strain collection of the Department of Microbiology, Vrije Universiteit, Amsterdam, The Netherlands. *T. halophila* reference strains DSM20337, DSM20338, and

* Corresponding author. Mailing address: Department of Microbiology, Biological Laboratory, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands. Phone: 31 20 4447193. Fax: 31 20 4447123. Electronic mail address: verse@bio.vu.nl.

DSM20339 were purchased from the Deutsche Sammlung von Mikroorganismen Zellkulturen GmbH, Braunschweig, Germany.

Physiological characterization. Utilization of 95 different carbon sources was tested in the MicroStation system (Biolog Inc., Hayward, Calif.). Inoculum preparation for the microplate tests was modified from the instructions of the manufacturers, since only slight growth occurred on agar plates without salt. Isolates were grown in standing batches with tryptone soy broth containing 5% NaCl (TSB-5%). Exponentially growing cells were harvested by centrifuging at low speed (5 min, 3,000 × *g*). Cells were washed once with Biolog Lactic Acid bacterial suspension broth (Biolog Inc.) containing 5% NaCl (BLA-5%) and resuspended in BLA-5% to an optical density at 590 nm of 0.3 to 0.45. The suspension was inoculated into a microtiter plate for gram-positive bacteria (150 μl per cupule). The plates were incubated at 30°C for 3 to 4 days. Growth in TSB-5% enabled comparison with the method of Uchida (21). Substrate utilization was comparable in both systems, with the exception that α-D-glucose in the microplates surprisingly seldom gave a positive reaction in the Biolog system, while D-fructose and D-mannose showed day-to-day variation. Tested according to the method of Uchida (21), these three substrates showed positive reactions.

Media and tests for arginine catabolism and amino acid decarboxylation were modified from the method of Skerman (20). Arginine catabolism was determined by growing isolates statically in 0.5% Bacto Peptone–0.5% yeast extract–0.05% glucose–0.3% L-arginine–0.2% K_2HPO_4–5% NaCl (pH 7.0) for 5 days at 30°C. Ammonia formation from arginine was detected by adding one drop of Nessler reagent (Merck) to 3 drops of spent medium. Color formation was compared with that of a control of the isolate grown in medium without arginine. Decarboxylation of lysine, aspartic acid, phenylalanine, and tyrosine was tested by growing isolates for 7 days at 30°C in two media: 0.3% yeast extract–0.5% tryptone–0.1% glucose–2% amino acids–0.004% bromocresol purple–5% NaCl (pH 6.8) and 0.5% Bacto Peptone–0.5% meat extract–0.05% glucose–1% amino acids–0.004% bromocresol purple–5% NaCl (pH 6.2). The color of the medium changed from purple to yellow as the result of acidification due to glucose fermentation. A positive reaction for decarboxylation was noted when the color changed back from yellow to purple. Histamine production from histidine decarboxylation was measured according to the method of Rice et al. (16), after growing isolates for 5 days at 30°C in 1% tryptone–1% yeast extract–1% glucose–0.1% histidine–5% NaCl.

Maximum growth rate was determined in spectronic tubes, completely filled with 1% yeast extract, 2% Bacto Peptone, 1% glucose, 0.016% Tween 80, 0.3% yeast nitrogen base, 0.85% KH_2PO_4, and 5% NaCl. Changes in optical density at 660 nm were measured in the range of 0.1 to 0.8. Measurements were done every half hour after mixing the contents of the tubes by vortexing. Final pH as well as production of L-(+)-lactate, D-(−)-lactate, ethanol, and formate was determined in cultures in stationary phase. Quantities of lactate and ethanol were measured enzymatically (4), and the amount of formate was determined colorimetrically (10).

Salt tolerance was tested in media consisting of 1% yeast extract, 2% Bacto Peptone, 1% glucose, 0.016% Tween 80, 0.85% KH_2PO_4, and salt concentrations up from 15% NaCl (pH 6.5). When growth occurred, a 1% inoculum was added to fresh medium with a 1 to 1.5% higher salt concentration. pH and acid tolerances were tested by adding a 1% inoculum to media composed of 0.5% yeast extract, 1% Bacto Peptone, 0.5% glucose, 0.1% KH_2PO_4, 0.1% K_2HPO_4, and 15% NaCl, with different pHs and/or concentrations of sodium lactate, sodium acetate, and/or ethanol. Ability to grow was judged from final pH and visual examination of opacity.

RAPD analysis. Isolates were cultured statically in TSB-5%. Cells (10 ml) in the stationary phase were centrifuged and incubated for 30 min at 37°C in 570 μl of 4 mM magnesium acetate, containing 150 μg of lysozyme. Next, genomic DNA was isolated according to the method of Ausubel et al. (3). DNA content was determined by measuring the A_{260}. Purity of DNA was checked on 0.8% agarose gels.

Amplification reactions were performed in a DNA thermal cycler (Perkin-Elmer), programmed as follows: initial denaturation, 5 min at 94°C; 40 cycles of 1 min at 94°C, 2 min at 36°C, and 2 min at 72°C; and 5 min at 72°C to provide a final extension. The 50-μl reaction mixture contained Super *Taq* buffer (HT Biotechnology Ltd., Cambridge, United Kingdom) supplemented with 4 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 1 μM primer, 5 ng of DNA, and 2 units of Super *Taq* DNA polymerase. Four decamer primers were used: TCACGATGCA (PHR1), AGGTCACTGA (PHR2), GTATAGCAAC (PHR3), and TAGCATGATC (PHR4). Primers PHR1 and PHR2 have previously been used successfully for RAPD analyses (24), while PHR3 and PHR4 were selected on the basis of the resemblance of their GC content (40%) to that of *T. halophila*.

Twenty microliters of the reaction mixture was electrophoretically separated on a 1.5% agarose gel. A 100-bp DNA ladder (Gibco BRL) was used as a reference. Ethidium-stained nucleic acid bands were visualized by UV light and recorded with a video imager (Appligene Inc.). Gels were normalized with the GELCOMPAR software package (version 3.0; Applied Maths, Kortrijk, Belgium), and matching bands were scored. A similarity matrix was calculated by using the Jaccard coefficient (S_j) and was used in unweighted pair group method using arithmetic averages (UPGMA) cluster analysis (GELCOMPAR), as described previously by Pot et al. (14): $S_j = n_{AB}/(n_A + n_B - n_{AB})$, in which n_{AB} is the number of bands common for tracks A and B, n_A is the total number of bands in track A, and n_B is the total number of bands in track B.

Protein pattern analysis. Cells were cultured statically in TSB-5%. Fifteen to thirty milliliters of exponentially growing cells (optical density at 660 nm, 0.4 to 0.8) was harvested, resuspended in 3.5 ml of 4 mM magnesium acetate, and boiled for 10 min. After the suspension was cooled to room temperature, 50 μl of a 5-mg ml^{-1} solution of lysozyme was added, the suspension was incubated for 30 min at 37°C, and then 1 ml of sample buffer (60 mM Tris HCl [pH 6.6], 50% glycerol, 2% sodium dodecyl sulfate [SDS], 5% β-mercaptoethanol, 0.1% bromophenol blue) was added. Subsequently, the sample was heated for 30 min at 95°C. After cooling, the sample was stored at −20°C. Electrophoresis was performed as previously described by Laemmli (9) on 12% (wt/vol) SDS–polyacrylamide gel slabs. An extract of *Psychrobacter immobilis* LMG1125 (Laboratorium voor Mikrobiologie, Ghent, Belgium) was used as a reference. Coomassie blue-stained gels were scanned with a Hewlett-Packard Scanjet IIcx. Registration of data, normalization of densitometric traces, grouping of isolates by the Pearson product moment correlation coefficient (*r*), and UPGMA cluster analysis were performed with the GELCOMPAR software package. The lysozyme band was not included in the calculations.

RESULTS AND DISCUSSION

Growth and substrate utilization. *T. halophila* populations from three Chinese-type soy sauce manufacturers on Java, named Libra (isolates LIB1 to LIB10), Ikan Lele (isolates IL1 to IL6), and Purwokerto (isolates PUR1 to PUR10), and one Japanese-type soy sauce industrial producer on Java (isolates JV1 to JV43) were characterized. Isolates were obtained from a sample taken at the indicated manufacturer when *T. halophila* growth was finished.

The three Chinese-type soy sauce manufacturers used different salt percentages for the brine fermentation, ranging from 12 to 26% (18). Despite the fact that the isolates came from different types of soy sauce fermentation and from different manufacturers, all isolates and the Deutsche Sammlung von Mikroorganismen Zellkulturen reference strains showed similar growth characteristics. They were homofermentative, since about two lactates per glucose were produced instead of one ethanol, one lactate, and one carbon dioxide per glucose, as would be expected in the case of heterofermentative lactic acid bacteria. All strains were capable of heterolactic fermentation (formation of one acetate, one ethanol, and two formates per glucose). Lactate was mainly (>95%) in the L-(+) configuration. Final pH was 4.8 ± 0.1 in 15% NaCl broth, but the minimum pH for growth was 5.2 to 5.3. Maximum specific growth rates (0.25 ± 0.02 h^{-1}) were comparable. The maximum percentage of salt at which the medium still was acidified to a pH below 5.0 was 22.5% NaCl. Tolerance to acids and ethanol was high, since growth occurred in medium with 150 mM lactate, 50 mM acetate, 4% ethanol, and 15% NaCl (pH 6.0).

However, large differences regarding utilization of substrates were found: in total, 13 distinct substrate utilization patterns were observed (Table 1). Since the oligo- and polysaccharides in the plant materials are converted to monosaccharides during the stages prior to the brine fermentation (1, 18), only the utilization of monosaccharides seems to be important for growth of *T. halophila* cells, and for Japanese-type brine fermentation only the consumption of glucose seems to be important for *T. halophila* growth. While galactose and fructose are utilized by isolates from both Japanese-type and Chinese-type brine fermentations, xylose and rhamnose are not utilized at all, although xylose-fermenting strains have been isolated from Japanese soy mash at low frequency (1, 21). The only obvious difference is found for L-arabinose: it is used by almost all isolates (25 of 26) in Chinese-type brine fermentation and by only about 40% of the isolates from the Japanese type (this study; 21). Arginine catabolism and decarboxylation of other amino acids can yield extra energy for growth and maintenance. Strains from both Japanese- and Chinese-type

TABLE 1. Differential substrate utilization (SU) patterns of *T. halophila* isolates[a]

Substrate(s)	Result[b] for SU pattern:												
	1	2	3	4	5	6	7	8	9	10	11	12	13
L-Arabinose	+	+	+	+	+	+	−	+	−	−	−	−	−
D-Melibiose	+	+	−	−	−	+	+	+	+	+	−	−	−
D-Raffinose	+	+	−	−	−	+	+	w	w	−	−	−	−
D-Trehalose	+	+	+	+	−	−	−	+	w	+	+	+	w
α-D-Lactose, lactulose, α-CH_3 β-galactoside, stachyose	+	−	−	−	−	−	−	−	−	−	−	−	−
D-Sorbitol	+	−	−	−	−	−	−	−	−	−	−	−	+
Sucrose	+	w	+	w	−	−	−	w	w	w	w	−	w
Maltotriose	+	+	+	−	−	+	+	+	+	+	+	w	+
Palatinose, turanose	+	+	+	+	−	+	+	+	+	+	+	−	+
D-Arabitol	−	+	+	+	−	+	+	+	+	+	+	+	−
D-Tagatose	+	+	+	+	+	+	+	+	+	+	+	+	−
N-Acetyl-D-mannosamine	−	+	−	−	−	−	−	−	−	−	−	−	−
Citrate	+	+	+	+	+	−	−	+	+	+	+	+	+
α-CH_3 D-mannoside	−	−	−	−	−	−	−	−	−	−	−	w	−

[a] All strains utilized α-D-glucose, D-fructose, D-mannose, *N*-acetylglucosamine, arbutin, cellobiose, D-galactose, gentiobiose, maltose, D-mannitol, β-methyl D-glucoside, D-ribose, salicin, methyl pyruvate, pyruvic acid, and glycerol. None of the isolates utilized α-cyclodextrin, β-cyclodextrin, glycogen, inulin, mannan, L-fucose, D-galacturonic acid, D-gluconic acid, *m*-inositol, D-melezitose, β-methyl D-galactoside, 3-methyl glucose, D-psicose, L-rhamnose, sedoheptulosan, xylitol, D-xylose, or L-malic acid, as well as the amino acids and other organic acids in the Biolog titer plates.

[b] +, positive reaction; −, negative reaction; w, weak reaction.

brine fermentations catabolized arginine but did not decarboxylate amino acids, although amino acid decarboxylation has been observed for strains in Japanese-type brine fermentation (22).

Heterogeneity of populations. As can be seen in Table 2, substrate utilization patterns varied for isolates obtained from different producers. The isolates from Libra all showed the same substrate utilization pattern, while among the isolates from Purwokerto only isolate PUR10 differed from the others in its inability to utilize arabinose. Within the batch from Ikan Lele, two very different substrate utilization patterns were found. The most heterogeneous population was found at the Japanese-type industrial producer, with eight different patterns. Uchida (21) also observed heterogeneous populations in Japanese-type brine fermentation. Fifty isolates from two batches showed, respectively, 18 and 11 different fermentation patterns. Consequently, populations seem to be more heterogeneous in Japanese-type brine fermentation than in Chinese-type brine fermentation.

RAPD analysis allows intraspecies characterization and a characterization of populations based on genetic relatedness (6, 11). This technique was applied to our isolates, using four primers. DNA fragment patterns with sizes ranging from 100 to 1,500 bp were found to be reproducible when the same amplification conditions were used in different runs and for different DNA preparations from the same isolates. Relative intensities of the fragments within a pattern were found to vary in different runs, an observation also made by others working with RAPD analysis (6).

The four primers generated 77 distinct fragments, with primers PHR1, -2, -3, and -4 amplifying, respectively, 28, 19, 21, and 9 different fragments. At least 29 fragments were generated for each isolate, with a minimum of four fragments per primer.

TABLE 2. Distribution of *T. halophila* isolates according to substrate utilization (SU) pattern

SU pattern	*n*[a]	Isolate(s)[b]
1	4	DSM20337; IL3, -5, -6
2	1	DSM20338
3	15	DSM20339; JV4, -5, -7, -9, -11, -18, -21, -26, -27, -34, -35, -36, -40, -41
4	10	LIB1, -2, -3, -4, -5, -6, -7, -8, -9, -10
5	3	IL1, -2, -4
6	9	PUR1, -2, -3, -4, -5, -6, -7, -8, -9
7	1	PUR10
8	3	JV2, -8, -42
9	10	JV13, -17, -19, -24, -29, -30, -31, -37, -39, -43
10	4	JV1, -16, -28, -38
11	9	JV3, -6, -12, -14, -15, -20, -23, -25, -32
12	2	JV10, -33
13	1	JV22

[a] *n*, number of isolates with a unique SU pattern.

[b] DSM, Deutsche Sammlung von Mikroorganismen Zellkulturen; LIB, isolate from the traditional manufacturer Libra; IL, isolate from the traditional manufacturer Ikan Lele; PUR, isolate from the traditional manufacturer Purwokerto; JV, isolate from an industrial manufacturer.

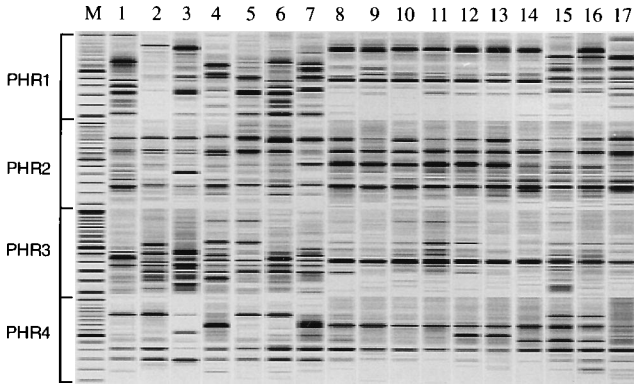

FIG. 1. Normalized RAPD patterns for *T. halophila* isolates, produced with primers PHR1, PHR2, PHR3, and PHR4 (see Materials and Methods) as shown from top to bottom (the four gels are linked head to tail). Of the 20 different combinations of RAPD patterns, 17 are shown. Lanes: 1, DSM20337; 2, DSM20338; 3, DSM20339; 4, LIB; 5, IL(A); 6, IL(B); 7, PUR; 8, JV(A); 9, JV(B); 10, JV(C); 11, JV(D); 12, JV(E); 13, JV(F); 14, JV(G); 15, JV(J); 16, JV(K); and 17, JV(L). M, 100-bp DNA ladder (marker bands from 1,500 to 100 bp are shown).

TABLE 3. Combined RAPD patterns and SU patterns for 69 *T. halophila* isolates obtained from four Indonesian soy sauce manufacturers[a]

Strain(s)	Pattern		%[b]
	RAPD	SU	
LIB1, -2, -3, -4, -5, -6, -7, -8, -9, -10	LIB	SU4	100
IL1, -2, -4	IL(A)	SU5	50
IL3, -6	IL(B)	SU1	33
IL5	IL(C)	SU1	17
PUR1, -2, -3, -4, -5, -6, -7, -8, -9	PUR	SU6	90
PUR10		SU7	10
JV34, -35, -36, -40, -41	JV(A)	SU3	12
JV1		SU10	2
JV6, -25, -32		SU11	7
JV39	JV(B)	SU9	2
JV5	JV(C)	SU3	2
JV3, -20		SU11	5
JV11, -18	JV(D)	SU3	5
JV12, -23		SU11	5
JV26	JV(E)	SU3	2
JV7	JV(F)	SU3	2
JV10, -33	JV(G)	SU12	5
JV14, -15	JV(H)	SU11	5
JV9, -21, -27	JV(I)	SU3	7
JV42	JV(J)	SU8	2
JV29, -30, -31, -37, -43		SU9	12
JV2, -8	JV(K)	SU8	5
JV13, -17, -19, -24		SU9	10
JV16, -28, -38		SU10	7
JV22	JV(L)	SU13	2

[a] For abbreviations, see Table 2.
[b] %, contribution of isolates with a certain combination of RAPD and SU patterns to the population at the indicated kecap manufacturer.

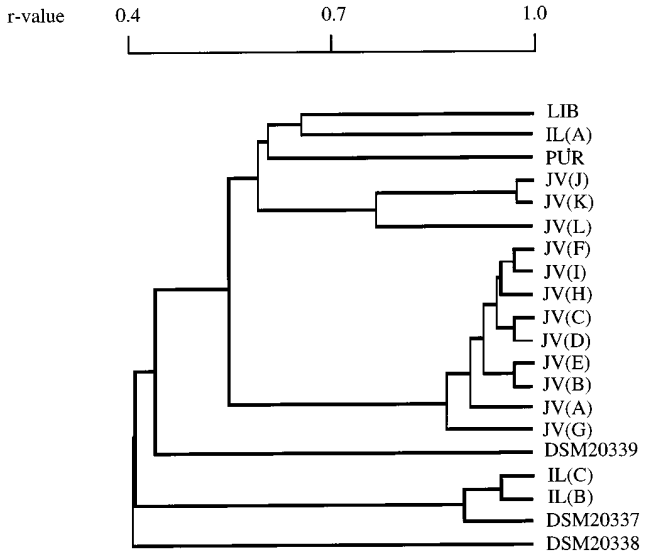

FIG. 2. Dendrogram showing the relationship between *T. halophila* isolates with different RAPD patterns, based on UPGMA clustering of pairwise Jaccard correlation coefficients (*r* values) of densitograms.

Amplification of genomic DNA with the four primers produced, respectively, 13, 11, 9, and 6 distinct patterns. When the patterns were combined for each isolate, 20 different RAPD combinations were observed (Fig. 1). A genetic relationship between isolates was established by hierarchical cluster analysis with the Jaccard coefficient (see Materials and Methods).

Isolates from different producers showed distinct RAPD combinations, agreeing well with the patterns of substrate utilization (Fig. 1, Table 3). Although isolate PUR10 did not metabolize L-arabinose, in contrast to the other isolates from manufacturer Purwokerto, all PUR isolates showed an identical RAPD combination. Three different RAPD combinations were observed for the isolates from the traditional producer Ikan Lele. Isolates with combinations IL(B) and IL(C) were closely related but quite distinct from isolates with RAPD combination IL(A) (see Fig. 2), as was already concluded from the differences in substrate utilization (Table 3). Interestingly, the DSM20337 strain clustered together with the three isolates with RAPD combinations IL(B) and IL(C) at a mean correlation level of $r > 0.85$. These four isolates had identical substrate utilization patterns. As observed for substrate utilization, the *T. halophila* population from the Japanese-type industrial producer also was genetically diverse, with the 43 isolates distributed over 13 RAPD combinations. At a mean correlation level of $r > 0.85$, three clusters were observed (Fig. 2). One group comprised RAPD combinations JV(A) to JV(I), the second contained RAPD combinations JV(J) and JV(K), and isolate JV22 formed a separate branch. Isolates from the cluster JV(J and K) were in general able to utilize melibiose (100% of isolates) and raffinose (80%) but not arabinose (20% positive), while isolates in the cluster JV(A to I) in general did not utilize melibiose (8% positive) and raffinose (4%) but utilized arabinose (54%). Since differences in RAPD patterns within the two large clusters were only slight (Fig. 2), the cluster analysis indicates that the *T. halophila* population from the Japanese-type fermentation is derived from only three strains at maximum.

Thus, compared with the populations from the Chinese-type soy sauce producers, the more heterogeneous population from the Japanese-type soy sauce industrial manufacturer could have evolved from these three strains by a larger number of mutations at this manufacturing site and selection pressure for these mutations. We calculated that at the industrial production site, 10^{18} tetragenococci propagate annually, while at the Chinese-type production sites only 10^{15} tetragenococci do. Therefore, assuming equal mutation rates, more spontaneous mutations can occur at the Japanese-type production site. Furthermore, unfavorable environments do cause stress, and stress is known to induce genetic alternations (15). Such an unfavorable environment could be yeast-fermented brine (with high ethanol concentrations) or the brine in the later stage of lactic acid fermentation, when pH is around 4.5 and unable to support the growth of *T. halophila* cells (19). Also, we observed that after the occurrence of lactic acid fermentation in the industrial soy mash fermentation, viable counts decrease rapidly after the drop in pH, while in Chinese-type brine fermentation the viable counts remain quite stable (18). Thus, stress may be more profound in Japanese-type soy sauce production. Soy mash is never inoculated, and *T. halophila* growth presumably results from cells that have remained from previous use of the fermentation tanks. Mutated cells may therefore remain and grow in new prepared mashes. Mutations which allow better adaptation to certain conditions in the continuously changing brine environment may aid the survival of the cells possessing these mutations and may give rise to a substantial contribution of these cells to the *T. halophila* population. Although no significant differences in growth rate, final pH, and tolerance for pH, acids, and ethanol were observed, even slight

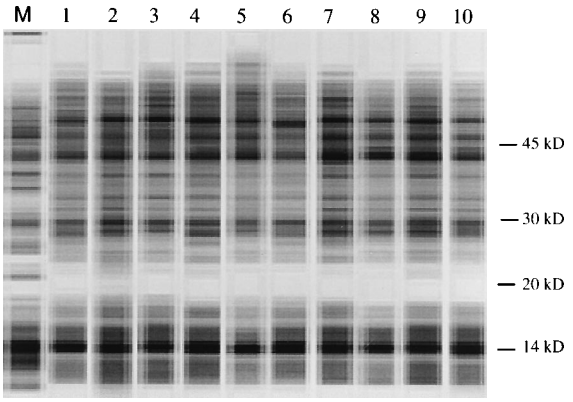

FIG. 3. Normalized electrophoretic protein profiles of *T. halophila* isolates obtained from several manufacturers. Lanes: M, *Psychrobacter immobilis* LMG1125; 1, DSM20337; 2, DSM20338; 3, DSM20339; 4, isolate LIB10; 5, isolate IL1; 6, isolate IL5; 7, isolate PUR5; 8, isolate JV1; 9, isolate JV2; 10, isolate JV10.

differences in these characteristics may support the survival of mutated cells.

Since only the utilization of monosaccharides is essential, the ability to utilize oligosaccharides can be lost without consequences, in this way causing a heterogeneous population. In Japanese-type soy sauce fermentation also, the ability to utilize nonglucose monosaccharides can be lost without consequences.

Protein fingerprinting. The slight genetic relationship between isolates of different manufacturers (Fig. 2) raised some doubts about whether the isolates constitute one species. Lactic acid bacteria can easily be identified to the species level and even to the subspecies level by protein fingerprinting (5, 23). We applied this technique to our isolates. Total cell extracts were electrophoresed, and the Pearson correlation coefficients of the densitograms were calculated and clustered. All the isolates grouped into one cluster at a mean correlation level of $r > 0.90$ (data not shown), indicating strongly that all isolates belong to one species of *Tetragenococcus*, *T. halophila*. Isolates from the same producer did not group together, although they showed a highly identical protein profile. Slight differences with respect to the presence or absence of bands and band intensity between isolates from different kecap producers could be observed visually (Fig. 3).

Final remarks. Despite the differences in plant materials used, differences in the ability to utilize substrates are small. This may indicate either that growth of *T. halophila* cells is not limited by energy availability or that the introduction of new strains with better substrate utilization abilities from outside the soy sauce manufacturing site into the brine seldom occurs. The fact that the heterogeneous population at the Japanese-type soy sauce production site seems to be derived from, at most, three strains through mutation and selection is an argument for the second option.

ACKNOWLEDGMENT

This investigation was supported by The Netherlands Foundation for the Advancement of Tropical Research (WOTRO), with financial aid from the Netherlands Organization for Scientific Research (NWO).

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