研究報告

Characterization and Identification of *Tetragenococcus halophilus* and *Tetragenococcus muriaticus* Strains from Fish Sauce (Nam-pla)

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Eighty strains of homofermentative tetrad-forming lactic acid bacteria were isolated from fish sauce fermentation in Thailand. These bacteria were divided into three groups based on the phenotypic and chemotaxonomic characteristics including DNA relatedness. Forty strains (Group I) showed high degrees (70.0% to 109.1%) DNA-DNA homology with *Tetragenococcus halophilus* ATCC 33315[™]. They were identified as *T. halophilus*. Thirty-eight strains (Group II) showed high degrees (70.9% to 105.6%) of DNA-DNA homology with *Tetragenococcus muriaticus* JCM 10006[™]. They were identified as *T. muriaticus*. Two strains (Group III) showed low degrees (3.7% to 16.3%) of DNA-DNA homology with *T. halophilus* ATCC 33315, *T. muriaticus* JCM 10006, and *Aerococcus viridans* TISTR 393[™]. Then they were left unidentified. Group I and Group II strains are halophilic which grew in 25% NaCl. All the strains tested produced L-lactic acid and contained cellular fatty acids with C18:1 as the major component. Some of the selected strains produced histamine ranging from 0.04 to 52.29mg/100 ml. The differentiation of *Tetragenococcus* strains is discussed.

Key Words : Fish sauce; halophilic; lactic acid bacteria; *Tetragenococcus halophilus*; *Tetragenococcus muriaticus*

The moderately halophilic lactic acid bacteria, *T. halophilus*, formerly known as "*Pediococcus halophilus*" has been reported and described¹⁻⁹⁾. This bacterium was reclassified in the genus *Tetragenococcus* based on 16S rRNA studies¹⁰⁾. Recently, the existence of a second species, *T. muriaticus*, has been proposed¹¹⁾, and the differentiation of these *Tetragenococcus* strains was described by Kobayashi *et al.*¹²⁾. Fish sauce is a clear brown liquid traditionally produced and widely used in Southeast Asia and to some extent in other parts of the world^{13,14)}. This product contains high concentrations of NaCl and it

was in this that "*Pediococcus halophilus*" was found^{6,15)}. *T. halophilus* and *T. muriaticus* strains have been found in Japanese squid liver sauce and puffer fish ovaries^{11,12)}, but their isolation from fish sauce (nam-pla) in Thailand has not been reported. This work deals with the identification of tetrad-forming cocci from fish sauce fermentation in Thailand based on phenotypic and chemotaxonomic characteristics including the results of photobiotin labeling DNA-DNA hybridization studies.

Materials and Methods

Isolation method. The fish sauce samples were obtained from three factories in Samutprakarn, Samutsongkhram and Chonburi Provinces, and lactic acid bacteria were isolated by a pour plate technique

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using MRS agar¹⁰⁾ with 5% NaCl incubating at 30°C for 3-5 days.

Bacterial cultures. Eighty isolates and each of the strains of *Tetragenococcus halophilus* ATCC 33315[†] (American Type Culture Collection, Manassas, VA, USA), *Tetragenococcus muriaticus* JCM 10006[†] (Japan Collection of Microorganisms, Saitama, Japan) and *Aerococcus viridans* TISTR 393[†] (Thailand Institute of Scientific and Technological Research, Bangkok, Thailand) were used in this study. All tests were carried out by incubating the cultures at 30°C, except for the study on the effects of temperature. The strain number PM, PW, PB, PS or P indicated the strain from Samutprakarn; K from Samutsongkhram and C from Chonburi while the number 0, 1, 2, 3, ...18 (month) and the number 30 and 60 (day) of strain number indicated the period of fermentation of batches from which they were isolated.

Morphological and cultural characteristics. Cell form, cell size, cell arrangement, and colonial appearance were examined on the cell grown on MRS agar with 5% NaCl incubated for 5 days. Hucker-Conn modification¹⁷⁾ was used for gram stain. Spore formation was examined in gram-stained specimens. Motility was detected by the appearance of stab cultures in soft agar¹⁸⁾.

Biochemical and physiological characteristics. The following tests were carried out: activities of catalase with hematin in the medium and of oxidase, nitrate reduction, hydrogen sulfide formation, and the hydrolysis of arginine, casein, starch, gelatin and tributyrin. In addition, the oxidation-fermentation test and the Methyl red-Voges-Proskauer reaction were performed, as previously reported¹⁹⁻²¹⁾. The effect of temperature (40, 50°C) and different starting pHs (4.2, 5.0, 6.5, 8.0, and 9.0) were determined in MRS with 5% NaCl while the different concentrations of NaCl (0, 10, 15, 20, and 25 %) were tested by using MRS broth as basal medium. Acid formation from carbohydrates was determined in medium supplemented with 5% NaCl as reported previously²¹⁾. The tested strains were cultivated in histidine containing medium with 10% NaCl and the supernatant was analyzed for histamine by the AOAC method²²⁾.

Isomer of lactic acid. The tested strains were cultivated in GYPB broth with 5% NaCl for 5 days²¹⁾. Lactic acid was extracted from the supernatant of the

fermentation broth with ether and was analyzed by the enzymatic method reported by Okada *et al.*²³⁾.

Cellular fatty acid composition. Methyl esters of fatty acids were prepared using a 5% methanolic hydrochloric acid solution from dried cells (40mg), which were cultivated in MRS broth with 5% NaCl as described by Ikemoto *et al.*²⁴⁾. The samples were analysed by gas-liquid chromatography.

DNA-DNA hybridization. DNAs were isolated from cells grown in MRS broth with 5-10% NaCl after incubating for 1-2 days and were purified by the method of Saito and Miura²⁵⁾. For strains with difficult in isolation of DNA, the medium was supplemented with 0.5% glycine²⁶⁾. Photobiotin labeling DNA-DNA homologies were carried out in 2xSSC (saline trisodium citrate) and 50% formamide solution at 40°C for 12h and detected by the colorimetric method^{27,28)}.

Results and Discussion

A total of 80 strains of tetrad-forming lactic acid bacteria isolated from multiple batches of fish sauce fermentation at 3 factories in Thailand were divided into three groups based on phenotypic and chemotaxonomic characteristics including DNA relatedness. Group I and Group II strains are halophilic and were belong to the genus *Tetragenococcus* while Group III strains are similar to the genus *Pediococcus* and *Aerococcus* as shown in Tables 1 to 4^{10,29-32)}. All the strains were gram-positive cocci (0.6 to 1.0 μ m in diameter) and they appeared singly, in pairs, and in tetrads. Cells were nonmotile and nonsporing. Colonies on MRS agar plate were circular, low convex with entire margin, and nonpigmented. They were homofermentative and microaerophilic. All strains showed negative reactions to oxidase Voges-Proskauer reaction hydrolysis of starch, gelatin and tributyrin hydrogen sulfide formation and nitrate reduction. They produced catalase in the medium containing hematin and showed Methyl red reaction. Most of the strains in Group I and II hydrolyzed arginine and Group II strains hydrolysed casein. All strains tested grew at pH levels of 6.5 and 9.0, but not at pH 4.2, and at 40°C, but not at 50°C. Most of the Group II strains did not grow in 0% NaCl. All produced acid from D-glucose and D-fructose and variable reactions were shown in Table 1. The tested strains produced histamine ranging from 0.04 to 52.29mg/100ml (Table 2).

All tested strains in Group I produced low concentration of histamine while the most of the strains in Group II produced high concentrations. Histamine production was found to be variable in the strains of Group II (Table 2).

The strains in Group I (PM0-8, PM0-10, P7-23, P7-27, K4-2, K2-32, K5-51, K6-35, C1-5, and C1-14); Group II (PW0-8, P7-37, K1-31, K3-26, P7-18, and C2-1; and Group III (C0-1) produced L + DL or L-lactic acid as the major product from glucose as reported for *T. halophilus* ATCC 33315¹¹ and *T. muriaticus* JCM 10006^{11,12}. The tested strains contained cellular fatty acid of C18:1 as the major component. The straight chain acids of C14:0, C14:1, C16:0, C16:1, C18:0, C20:1, and Cy 19 cyclopropane acids were also found as is shown in Table 3. The strains in Group I (PM0-8, P7-23, K2-44, K4-16) and Group II (P7-18, K1-31, K3-26, K5-37, K18-1, C2-1) showed the same fatty acid profiles. Because fatty acids were esterified by refluxing with HCl/Methanol, the amount of Cy 19 cyclopropane acids was not as high as reported for up. "*P. halophilus*" by Ushida and Mogi³⁰. However this result is consistent with the fatty acid profiles described by Satomi et al.¹⁰. Although the C18:1 content of Group I and Group II strains was slightly different, this characteristic was not significant enough for their differentiation. Group III strains (C0-1, C0-2) had no Cy 19 cyclopropane acids and contained the same fatty acid profiles as reported for *Aerococcus viridans*³⁰.

The DNA relatedness as shown in Table 4 indicated that forty strains in Group I (PM0-8, PM0-10, PW0-7, PW0-13, PB30-1, PB30-3, PS60-2, PS60-6, PB60-2, P3-1, P3-2, P3-3, P3-4, P3-5, P7-2, P7-7, P7-23, P7-26, P7-27, K0-2, K2-32, K2-34, K2-44, K4-2, K4-16, K4-40, K4-47, K5-51, K6-32, K6-35, C1-1, C1-2, C1-5, C1-6, C1-8, C1-9, C1-12, C1-14, C1-17, C1-19) showed high degrees (70.0% to 109.1 %) of DNA-DNA homology with *T. halophilus* ATCC 33315¹¹. They grew in MRS medium with no NaCl and fermented a variety of carbohydrates. All grew in 25% NaCl (Table 1). They were identified as *T. halophilus*^{11,30}.

Thirty-eight strains in Group II (PW0-8, PW0-15, P4-3, P4-5, P4-6, P5-1, P5-2, P5-3, P6-1, P6-3, P6-4, P7-18, P7-37, K1-10, K1-31, K1-35, K1-46, K2-9, K2-17, K2-29, K3-6, K3-26, K3-29, K3-40, K3-46, K5-37, K5-55, K6-29, K6-50, K8-1, K8-5, K8-13, K9-1, K9-2, K18-1,

K18-6, K18-12, C2-1) showed high degrees (70.9% to 105.6%) of DNA-DNA homology with *T. muriaticus* JCM 10006¹¹ (Table 4). Few of them grew in MRS with no NaCl, but all did in 25% NaCl. Most of them did not ferment as many carbohydrates as Group I strains (Table 1). They were identified as *T. muriaticus*^{11,30}.

Two strains in Group III (C0-1, C0-2) showed low degrees (3.7% to 16.3%) of DNA-DNA homology with *T. halophilus* ATCC 33315¹¹, *T. muriaticus* JCM 10006¹¹, and *A. viridans* TISTR 393¹¹ (Table 4). They did not grow in 20% NaCl. Their characteristics are similar to *Pediococcus* and *Aerococcus*^{30,32}. Further studies on their 16S r RNA sequences would have required proposing a new species, and were therefore left unidentified.

T. halophilus strains have previously been reported⁹. A total of 11 strains of *T. muriaticus* were isolated from fermented squid liver sauce and proposed as a new species¹⁰. Recently, Kobayashi et al.¹² described the differentiation of *T. halophilus* and *T. muriaticus* strains from fermented puffer fish ovaries based on their ability to grow in medium not supplemented with NaCl and the fermentation of L-arabinose, sucrose and D-mannitol including genotypical typing of the 16S rRNA gene. These two species have been classified based on strains from soy sauce, fermented squid liver sauce and fermented puffer fish ovaries^{5,11,12}. In this study, we found many isolates from fish sauce (narn-plai). These isolates displayed characteristics which varied widely from one another, and also varied from characteristics of the type strains shown in Table 1. Their growth in MRS broth with no NaCl as mentioned by Satomi et al.¹⁰ and Kobayashi et al.¹², appear useful for separating *T. halophilus* and *T. muriaticus* strains. The hydrolysis of casein and acid from sucrose supported their differentiation. Arginine hydrolysis, acids from L-arabinose and D-mannitol, and histamine production are not sufficient to differentiate these two species according to Satomi et al.¹⁰ and Kobayashi et al.¹² Photobiotin labeling DNA-DNA hybridization is simple and useful for the identification of these two species.

T. halophilus and *T. muriaticus* strains are widely distributed in many batches of fish sauce fermentation in Thailand. They fermented many carbohy-

Table 1. Characteristics *Tetragenococcus* strains

Characteristics	<i>T. halophilus</i> ATCC 33315 ^r	<i>T. muriaticus</i> JCM 10006 ^r	Group I (40)	Group II (38)	Group III (2)
Cell shape			Cocci		
Hydrolysis of arginine	+	—	+ (—1)	+ (—8)	+
Hydrolysis of casein	—	+	— (+3)	+ (—7)	+
Growth at 40°C	—	+	+	+	+
Growth at pH 5.0	+	+	+ (—17)	+ (—17)	+
6.5	+	+	+	+	+
9.0	+	+	+	+	+
Growth in 0% NaCl	+	—	+	+ (+2)	+
15% NaCl	+	+	+	+	+
20% NaCl	+	+	+	+	—
25% NaCl	+	+	+	+	—
Acid from:					
Amygdalin	+	+	+ (—1)	+ (—9)	+
L-Arabinose	—	+	+ (—8)	+ (—12)	+
D-Cellobiose	+	—	+ (—1)	+ (—15)	+
Dextrin	—	+	+ (—17)	— (+15)	+
Esculin	+	+	+ (—4)	+ (—7)	+
D-Galactose	+	+	+ (—4)	+ (—11)	+
Glycerol	+	—	+ (—16)	— (+16)	—
Lactose	—	—	— (+17)	— (+3)	+
Maltose	+	+	+ (—3)	+ (—19)	+
D-Mannitol	—	+	+ (—15)	+ (—7)	+
D-Mannose	+	+	+ (—1)	+ (—3)	+
D-Melibiose	—	+	+ (—15)	— (+13)	—
D-Melezitose	+	—	+ (—14)	— (+3)	—
α-Methyl-D-glucoside	+	+	+ (—8)	— (+8)	+ (—1)
Raffinose	—	—	— (+12)	— (+2)	—
Rhamnose	—	+	+ (—20)	— (+3)	—
D-Ribose	+	+	+	+ (—10)	+
Salicin	+	+	+ (—5)	+ (—9)	+
D-Sorbitol	—	+	+ (—10)	— (+18)	—
L-Sorbose	+	+	+ (—9)	— (+18)	+ (—1)
Sucrose	+	—	+ (—9)	— (+5)	+
D-Trehalose	+	+	+ (—8)	+ (—17)	+
D-Xylose	+	+	+	+ (—2)	+

+, positive reaction; —, negative reaction; Numbers in parentheses indicate the number of strains showing the reaction.

drates and showed various characteristics. In Thailand, there are many kinds of fermented fish with high concentrations of NaCl such as pla-ra, pla-chom, kung-chom, shrimp paste, tai-pla, etc.¹⁵⁾. For further investigation, would be interesting to compare their characteristics with the tetracoccal isolates from other fermented products.

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Table 2. Histamine production of *Tetragenococcus* strains

Strain	Histamine (mg/100ml)	Strain	Histamine (mg/100ml)
Group I		Group II	
PM-8	0.42	K3-6	52.29
PW-7	2.32	K3-40	23.99
PM-10	0.05	K3-46	30.52
PM-13	0.15	K5-37	0.04
P7-23	0.70	K5-55	26.65
P7-27	0.45	K6-29	43.39
K2-34	0.19	K8-1	17.79
K2-44	1.89	K8-5	24.87
K4-2	3.46	K8-13	8.37
K4-16	4.46	K9-1	32.39
K4-40	0.10	K9-2	50.24
K6-32	3.27	K18-1	17.79
C1-6	0.12	K18-6	39.87
Group II		K18-12	15.29
PW-8	44.22	Group III	
PW-15	13.11	C0-1	42.81
P7-18	44.00	C0-2	22.87
P7-37	0.20	<i>T. halophilus</i> ATCC 33315 ^T	0.05
K1-10	0.10	<i>T. muriaticus</i> JCM 10006 ^T	44.03
K1-31	29.27		
K1-46	6.82		
K1-35	1.14		
K2-9	0.12		
K2-29	29.16		
K3-26	38.84		

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Table 3. Cellular fatty acid composition (%) of *Tetragenococcus* strains

Group	Number of strain	C14:1	C14:0	C16:1	C16:0	C18:1	C18:0	△19	C20:1	%Unknown
I	4	3.7	7.4	6.4	13.2	27.8	6.2	7.3	11.6	16.3
II	6	3.8	7.8	8.2	14.0	41.7	3.7	3.5	5.7	11.6
ATCC 33315 ^T		2.5	5.1	2.5	12.9	10.9	10.6	12.6	20.5	22.3
JCM 10006 ^T		5.1	6.0	3.1	13.4	11.0	10.2	11.1	19.2	20.7
III	2	4.0	17.5	5.6	14.9	38.9	1.4	-	2.5	15.4
<i>A. viridans</i> *		5.0	8.0	31.0	20.0	22.0	2.0	-	7.0	4.0

*Data from O, Leary and Wikinson (1988); △, Cyclopropane acid

Table 4. DNA relatedness of *Tetragenococcus* strains

Group	Number of Strain	% Homology with labeled strains			
		ATCC 33315 ^T	JCM10006 ^T	K3-26	TISTR 393 ^T
I	40	70.0-109.1	4.7-64.8	5.9-51.8	ND
II	38	8.8-63.6	70.9-105.6	71.5-107.5	ND
	K3-26	24.9	89.5	100.0	ND
III	2	23.7-3.9	3.8-4.1	4.0-4.9	12.3-16.3
<i>T. halophilus</i>	ATCC 33315 ^T	100.0	16.7	24.1	ND
<i>T. muriaticus</i>	JCM 10006 ^T	26.0	100.0	106.6	ND
<i>A. viridans</i>	TISTR 393 ^T	ND	ND	ND	100.0

ND, not determined

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