การพิสูจน์เอกลักษณ์ของแบคทีเรียกรดแลคติคชอบเค็มจากกะปิ

Identification of Halophilic Lactic Acid Bacteria from Shrimp Paste (*Ka-pi*)

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บทคัดย่อ

กะปิเป็นอาหารหมักพื้นบ้านมีลักษณะสีชมพูม่วงกลิ่นแรงที่ทำจากเคยซึ่งเติมเกลือปริมาณมาก ใช้ประโยชน์ในการปรุงอาหารไทยหลายชนิด การวิจัยครั้งนี้ได้คัดแยกแบคทีเรียกรดแลคติคชอบเค็มจากตัวอย่างกะปิที่เก็บจากตลาดในจังหวัดทางภาคใต้ของไทยได้แก่ พังงา ปัตตานี ตรัง สงขลา และนครศรีธรรมราช พบแบคทีเรียกรดแลคติคชอบเค็มปริมาณ 3.3 x10^3 to 3.0x10^6 CFU/กรัม โดยนับจากการเลี้ยงบนอาหารวุ้น MRS ที่เติมเกลือ 5% แบคทีเรียที่แยกได้ 17 สายพันธุ์ จัดอยู่ในสกุล *Tetragenococcus* โดยอาศัยผลจากการศึกษาลักษณะทางสัณฐานวิทยา การเจริญ สรีรวิทยา และชีวเคมี และจากผลการศึกษาความคล้ายคลึงของดีเอ็นเอของแบคทีเรียตัวแทน 5 สายพันธุ์ พบว่าแบ่งได้เป็นสองกลุ่ม กลุ่มที่หนึ่งมีค่าความคล้ายคลึงของดีเอ็นเอมากกว่า 83.6% เมื่อเทียบกับ *T. halophilus* ATCC 33315^T และกลุ่มที่สอง มีค่าความคล้ายคลึงของดีเอ็นเอมากกว่า 94.7% กับ *T. muriaticus* JCM 10006^T ดังนั้นจึงพิสูจน์เอกลักษณ์แบคทีเรียกรดแลคติคชอบเค็มกลุ่มที่หนึ่งได้เป็น *T. halophilus* และกลุ่มที่สอง ได้เป็น *T. muriaticus* จากงานวิจัยนี้ได้อภิปรายผลของลักษณะทางฟีโนไทป์ ความคล้ายคลึงของดีเอ็นเอและการเจริญของ *T. halophilus*

และ *T. muriaticus* ที่เลี้ยงในอาหารเหลว MRS เติมเกลือ 5 และ 10% ซึ่งเป็นประโยชน์ในการแยกความแตกต่างเพื่อพิสูจน์เอกลักษณ์แบคทีเรีย *Tetragenococcus* ระดับสปีชีส์

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Abstract

Traditional fermented food, ***ka-pi,*** a pinkish purple color, strong smelling paste is produced from shrimp containing high salt. It is used in various spicy soups or for flavoring dished in Thailand. Halophilic lactic acid bacteria were isolated from shrimp paste samples collected at the market in Phungnga, Pattani, Trang, Songkhla, and Nakhonsithammarat Provinces in the southern part of Thailand. Lactic acid bacterial cells count on MRS medium with 5% NaCl. are 3.3×10^3 to 3.0×10^6 CFU/g. Seventeen strains of these isolated bacteria were belong to the genus *Tetragenococcus* based on the morphological, cultural, physiological, and biochemical characteristics. On the basis of DNA-DNA hybridization studies, they were divided into 2 groups, the representative strains in Group I showed high degree of similarity over 83.6% with *Tetragenococcus halophilus* ATCC 33315^T and were identified as *T. halophilus.* The representative strains in Group II showed high degree of similarity over 94.7% with *Tetragenococcus muriaticus* JCM 10006^T and were identified as *T. muriaticus.* Their phenotypic characteristics, DNA-DNA similarity and the growth of *T. halophilus* and *T. muriaticus* strains in MRS broth with 5 and 10% NaCl that were useful for the differentiation of species have been discussed.

Introduction

Fermented shrimp paste (***ka-pi***), a dark-coloured strong smelling paste that varied depending on the shrimp used (*Acetes erythraeus, Koei-dtaa-daeng* or *Acetes* sp., *Koei-malet-khao-saan-som-oh).* It contained high concentration of NaCl (14-40.1%). Ingredients, shrimp and salt are mixed and left for 1-2 days. After the contents are drained of liquid, pound the mixture or mince it to paste until it becomes sticky, pack it tightly in a wide-mouthed earthenware and left fermenting for 4-6 months. This product allowed to occur various halophilic

microorganisms including lactic acid bacteria. [1, 2] Moderately halophilic lactic acid bacteria, *Tetragenococcus halophilus*, formerly known as "*Pediococcus halophilus*", were isolated from various fermented fish in Thailand.[3-5] This bacterium was reclassified in the genus *Tetragenococcus* bases on 16S rRNA studies. [6] In 1997, the existence of second species, *Tetragenococcus muriaticus* , has been proposed. [7] Recently, isolation and characterization of halophilic lactic acid bacteria isolated from "terasi" shrimp paste: a traditional fermented seafood product in Indonesia had been reported by Kobayashi et al [8]. This work deals with the identification of terad-forming cocci isolated from shrimp paste in Thailand based on the phenotypic characteristics and photobiotin labelling DNA-DNA hybridization. Growth in MRS with 5 and 10% NaCl of the selected strains were also determined.

Materials and Methods

Isolation method. The fermented fish (ka-pi) samples were obtained from the markets in Trang, Nakhonsithammarat, Songkhla, Ranong, Krabi, Pattani and Phungnga Provinces (Table 1), and lactic acid bacteria were isolated and counted (CFU/g) by a poure plate technique using MRS [9] agar with 5% NaCl incubating at 30 °C for 3-5 days.

Bacterial cultures. Seventeen isolates and each of the strains of *Tetragenococcus halophilus* ATCC 33315^T and *Tetragenococcus muriaticus* JCM 10006 ^{T} were used in this study. All tests were carried out by incubating the cultures at 30 °C, except for the investigation of effects of temperature.

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 modification [10] was used for Gram stain. Spore formation was examined in Gram-stained specimens. Motility was detected by the appearance of stab cultures in soft agar. [11]

Biochemical and physiological characteristics. Catalase (with hematin in the medium); oxidase; nitrate reduction; hydrolysis of arginine, casein , starch, gelatin tributyrin; oxidation-fermentation test; hydrogen sulfide formation; and Methyl red -Voges-Proskauer reaction were tested as reported [2, 12-13]. The effect of temperature (40, 50°C), and different concentrations of NaCl (0, 10,

15, 20, and 25 %) were tested by using MRS broth as a basal medium. Acid formation from carbohydrates was determined as reported previously [2].

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 Miura [14]. For strains with difficult in isolation of DNA, the medium was supplemented with 0.5% glycine [15]. Photobiotin labelling DNA-DNA similarity was carried out in 2xSSC (saline trisodium citrate) and 50% formamide solution at 40°C for 15 h and detected by colorimetric method [16, 17].

Effect of NaCl on Growth. The growth of selected *Tetragenococcus* strains and the type strains of each group in MRS broth (200 ml) with 5 and 10% NaCl were determined by spectrophotometer at 600 nm when incubating at 30°C for 72 h.

Results and Discussion

Morphological and cultural characteristics. All the strains were Gram-positive cocci 0.6-1.0 μm in size, 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 (Table 2).

Biochemical and physiological characteristics. All the strains were homofermentative and microaerophilic. They showed negative reactions to oxidase; Voges-Proskauer reaction; hydrolysis of starch, gelatin, tributyrin; hydrogen sulfide formation and nitrate reduction. All strains produced catalase in the medium containing hematin and showed methyl red reaction. Few strains hydrolysed arginine. All strains grew at pH 5.0 to 9.0, in 10 to 25% NaCl , and at 40°C but not at 50°C. Variable reactions were shown in Table 2. All produced acid from D-glucose, D-fructose, glycerol, D-maltose, D-mannose, D-ribose, salicin and D-sorbitol, and variable reactions were shown in Table 3.

DNA –DNA hybridization.

The selected isolates were divided into 2 groups on the basis of DNA-DNA similarity as shown in Table 4.

Four strains in Group I (SP2-1, SP7-1, SP28-1, and SP36-1) showed high DNA-DNA similarity (83.6-89.1 %) with *Tetragenococcus halophilus* ATCC 33315^T.

Two strains in Group II (SP10-2 and SP11-1) showed high DNA-DNA similarity (94.7-117.4 %) with *Tetragenococcus muriaticus* JCM 10006^T.

Seventeen isolates were included in the genus *Tetragenococcus* [18-21] on the basis of phenotypic characteristics and DNA-DNA similarity as shown in Tables 2 to 4.

Group I contained 4 isolates, as shown in Table 4 were similar to *Tetragenococcus halophilus* ATCC 33315^T. They were identified as *T. halophilus* [7].

Group II contained 2 isolates, as shown in Table 4 were similar to *Tetragenococcus muriaticus* JCM 10006^T. They were identified as *T. muriaticus* [7].

Tetragenococcus halophilus strains had been reported so far by Nakagawa and Kitahara, Coster and White, Whittenbury, Sakaguchi, and Mori, Uchida, Tanasupawat and Daengsubha, and Villar et al.[22-27]. Total of eleven strains of *T. muriaticus* were isolated from fermented squid liver sauce and proposed as the new species [7]. This species was described limited on only the strains from one source. In this study, we found some isolates from shrimp paste and they showed the different characteristics with the type strains as shown in Tables 2 and 3.

The differential characteristics of *T. halophilus* and *T. muriaticus* are the growth in MRS broth with no NaCl and the hydrolysis of casein. The hydrolysis of arginine, acid production from L-arabinose and mannitol are not significant characteristics to separate these two species as discussed by Satomi et al.[7]. As we mention the phenotypic characteristics of 17 isolates in Tables 2 and 3, this result was not significant to separate them at the species level. Photobiotin labelling DNA-DNA similarity is useful to differentiate the two *Tetragenococcus* species.

Effect of NaCl on Growth

Growth in MRS broth with 5 and 10% NaCl of *T. halophilus* SP36-1 and *T. muriaticus* SP11-1 including the type strains were shown in Figs 1 to 2. *T. halophilus* strains grew more faster and better than *T. muriaticus* strains in 5% NaCl broth. *T. halophilus* ATCC 33315^T grew

better than *T. halophilus* SP36-1 while *T. muriaticus* strains preferred growth in 10% NaCl broth. This characteristics also be useful for differentiation of *T. halophilus* and *T. muriaticus.*

As mentioned above, shrimp paste (***ka-pi***) contained over 14% of NaCl [1], the significant amounts of salt that are present in the product may be the cause of the predominant of *Tetragenococcus* species, *T. halophilus* and *T. muriaticus* the same result as reported by Kobayashi et al., 2003. The both species are halophilic bacteria that could grew in 25% NaCl therefore we found them distributed from 3.3×10^3 to 3.0×10^6 CFU/ g of shrimp paste.

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Table 1. Source of samples , lactic acid bacterial cells count and isolate number.

Province(number of sample)	Bacterial count (CFU/ g)	Isolate no.
Trang (4 samples)	1.8×10^6	SP2-1
	3.0×10^6	SP12-1
	3.0×10^6	SP28-1
	1.1×10^6	SP29-1
Nakhonsithammarat (6 samples)	6.0×10^4	SP4-1
	2.4×10^6	SP5-2
	2.5×10^6	SP7-1
	3.0×10^6	SP15-1
	6.2×10^4	SP20-1
	1.5×10^6	SP25-1
Songkhla (3 samples)	2.5×10^4	SP10-2
	1.5×10^4	SP11-1
	2.0×10^6	SP11-2
Ranong (1 samples)	2.9×10^6	SP23-1
Krabi (1 samples)	2.7×10^6	SP24-1
Pattani (1 samples)	3.3×10^3	SP31-2
Phangnga (1 samples)	3.0×10^6	SP36-1

Table 2. Characteristics of *Tetragenococcus* isolates.

Characteristic	*T. halophilus* ATCC 33315^T	*T. muriaticus* JCM 10006^T	Isolates (17)
Cell shape	.....Cocci.....		
Cell size (μm)	..... 0.6-1.0		
Cell arrangment	..... Occurring singly , in pairs and in tetrads		
Colony form	.....White, raised, circular and entire		
Oxidase	-	-	-
Catalase with hematin	+	+	+
Hydrolysis of arginine	+	-	-(+3)
Hydrolysis of casein	-	+	-
Nitrate reduction	-	-	-
MR	+	+	+
VP	-	-	-
Oxidative-Fermentative	F	F	F
H_2S production	-	-	-
Growth at 40°C	-	+	+
50°C	-	-	-
Growth at pH 4.2	-	-	-
5.0	+	+	+
9.0	+	+	+
Growth in 0 % NaCl	+	-	+
10 % NaCl	+	+	+
25% NaCl	+	+	+

+ , positive reaction; -, negative reaction. Numbers in parentheses indicate the number of isolates or numbers of isolates showing the reaction. ATCC, American Type Culture Collection, Manassas, VA, USA; JCM, Japan Collection of Microorganisms, RIKEN BioResource Center, Saitama, Japan.

Table 3. Acid from carbohydrates of *Tetragenococcus* isolates.

Carbohydrate	*T. halophilus* ATCC 33315^T	*T. muriaticus* JCM 10006^T	Isolates (17)
Amygdalin	+	+	+(-4)
L-Arabinose	-	+	+(-4)
D-Cellobiose	+	-	+(-2)
Dextrin	-	+	+(-2)
D-Fructose	+	+	+
D-Galactose	+	+	+(-3)
Glycerol	+	-	+
Lactose	-	-	+(-7)
Maltose	+	+	+
D-Mannitol	-	+	+(-2)
D-Mannose	+	+	+
D-Melibiose	-	+	+(-6)
D-Melezitose	+	-	+(-4)
α-Methyl-D-glucoside	+	+	+(-1)
L-Raffinose	-	-	+(-6)
L-Rhamnose	-	+	+(-7)
D-Ribose	+	+	+
Salicin	+	+	+
D-Sorbitol	-	+	+
Sucrose	+	-	+(-1)
D-Trehalose	+	+	+(-5)
D-Xylose	+	+	+(-5)

+, positive reaction; -, negative reaction. Numbers in parentheses indicate the number of isolates or numbers of isolates showing the reaction.

Table 4. DNA–DNA similarity of *Tetragenococcus* strains.

	Strain	% Similarity with labelled strains	
		ATCC 33315 ^{T}	JCM 10006 ^{T}
Group I	SP2-1	84.4	49.7
	SP7-1	83.6	41.9
	SP28-1	84.5	49.6
	SP36-1	89.1	3.2
Group II	SP10-2	59.3	117.4
	SP11-1	61.5	94.7
T. halophilus	**ATCC 33315^{T}**	100	19.5
T. muriaticus	**JCM 10006^{T}**	30.7	100

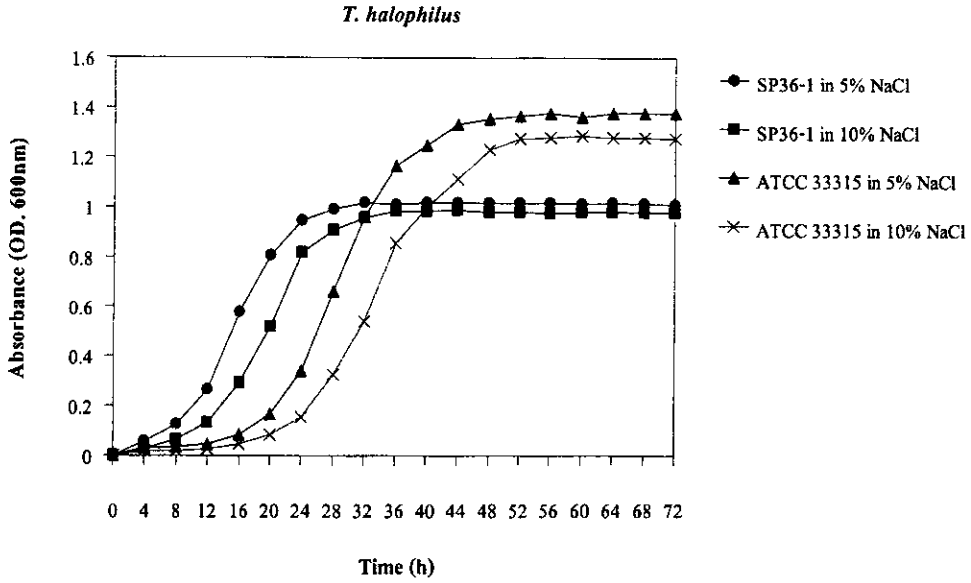

Fig. 1 Growth of *T. halophilus* SP36-1and ATCC 33315^T in MRS broth with 5 and 10 % NaCl.

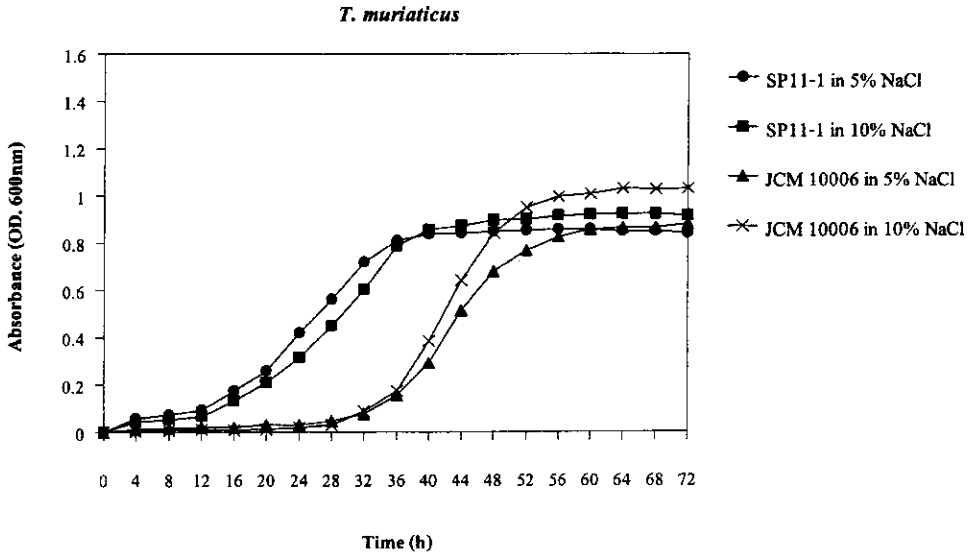

Fig. 2 Growth of *T. muriaticus* SP11-1and JCM 10006^T in MRS broth with 5 and 10 % NaCl.

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