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Identification of moderately halophilic bacteria from Thai fermented fish (*pla-ra*) and proposal of *Virgibacillus siamensis* sp. nov.

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Forty-one isolates of moderately halophilic bacteria were isolated from fermented fish (*pla-ra*) in Thailand. On the basis of their phenotypic and chemotaxonomic characteristics, DNA–DNA relatedness and 16S rRNA gene sequences analyses, they were divided into six groups. The isolates in Group I to V were Gram-positive rod-shaped bacteria. They contained *meso*-diaminopimelic acid in the cell-wall peptidoglycan and menaquinone with seven isoprene units (MK-7). An isolate in Group VI was a Gram-negative rod-shaped bacterium. The DNA G+C contents of tested strains ranged from 36.5–63 mol%. Ten strains (Group I) were identified as *Virgibacillus dokdonensis*, 13 isolates (Group II) as *V. halodenitrificans*, 14 isolates (Group III) as *V. marismortui*, 1 isolate (Group IV) as *Virgibacillus* sp., 2 isolates (Group V) as *Bacillus vietnamensis*, and 1 isolate (Group VI) as *Chromohalobacter salexigens*. Isolate MS3-4 in Group IV was closely related to *V. carmonensis* KCTC 3819^T (95.9%). This strain contained anteiso-C_{15:0} (55.8%) and anteiso-C_{17:0} (17.7%) as major cellular fatty acids and had phosphatidylglycerol, diphosphatidylglycerol and an unidentified glycolipid as polar lipids. The DNA G+C content of MS3-4 was 38.0 mol%. The strain from Group IV is proposed as *Virgibacillus siamensis* sp. nov. and MS3-4^T is the type strain (JCM 15395^T = PCU 312^T = TISTR 1957^T).

Key Words—*Bacillus*; *Chromohalobacter*; fermented fish; halophile; *Virgibacillus*

Introduction

Moderately halophilic bacteria are widely distributed in many kinds of fermented foods: for example, strains of *Jeotgalibacillus alimentarius* were found in jeotgal in Korea (Yoon et al., 2001), *Lentibacillus juripiscarius* and *Lentibacillus halophilus*, *Tetragenococcus halophilus* and *T. muriaticus* in Thai fish sauce (Namwong

et al., 2005; Tanasupawat et al., 2006; Thongsanit et al., 2002), *Bacillus vietnamensis* in Vietnamese fish sauce (Noguchi, 2004), *Salinicoccus siamensis*, *Lentibacillus kapialis*, and *Oceanobacillus kapialis* in Thai fermented shrimp paste (Namwong et al., 2009; Pakdeeto et al., 2007a, b), and *Piscibacillus salipiscarius*, *Salinivibrio siamensis* and *Gracilibacillus thailandensis* in salt-fermented fish (*pla-ra*) in Thailand (Chamroensaksri et al., 2009, 2010; Tanasupawat et al., 2007). In the course of our study on the diversity of halophilic bacteria in fermented fish in Thailand, we newly isolated 41 isolates of moderate halophiles from *pla-ra*. The subsequent taxonomic characterization revealed that they were composed of six groups, distributed

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across three genera *Virgibacillus*, *Bacillus* and *Chromohalobacter*. One *Virgibacillus* isolate, designated MS3-4^T, represented a novel species in this genus, and we propose the name *Virgibacillus siamensis* sp. nov. to accommodate it.

Materials and Methods

Bacterial isolation. Halophilic bacteria were isolated from 38 samples of fermented fish (*pla-ra*) collected from the markets in Thailand by using the spread-plate technique on JCM no. 377 agar plates and incubated at 30°C for 3–7 days.

Identification methods. Cell shape, cell size, and cell arrangement were examined on JCM no. 377 agar at 30°C after 3 days. The Hucker-Conn modification was used for Gram staining (Hucker-Conn, 1923). Cell form and spore formation were observed under the light microscopy. Flagella were examined as described by Forbes (1981). Effects of growth at various NaCl concentrations were investigated in JCM no. 377 broth omitting the MgSO₄·7H₂O with different concentrations of NaCl (0, 0.5 and 1–25%, w/v with an interval of 1). Optimum growth at different pH (5 to 10, with an interval of 1) and temperatures (15, 25, 30, 37, 40, 45, 50 and 55°C) was investigated in JCM no. 377 broth (Namwong et al., 2005). Catalase and oxidase activity, H₂S production, nitrate reduction, and citrate utilization were determined as described by Barrow and Feltham (1993). Hydrolysis of esculin, casein, gelatin, starch, Tween 80, urea, and L-tyrosine was determined as described by Namwong et al. (2005). Arginine hydrolysis was performed by using the medium reported by Thornley (1960). Acid production from carbohydrate was determined in the medium described by Leifson (1963). All tests were carried out in medium supplemented with 10% NaCl, except for the investigation of effects of growth at various NaCl concentrations. Growth under anaerobic conditions on JCM no. 377 agar plate with KNO₃ or without (1%, w/v when present) was performed in the Gaspak (BBL) anaerobic jar.

The cell biomass was produced in JCM medium no. 377 at 30°C for 3 days for chemotaxonomic characterization. Diaminopimelic acid in the cell wall and menaquinone were determined as described by Komagata and Suzuki (1987). Polar lipids were extracted as described by Minnikin et al. (1984) and identified by two-dimensional TLC followed by spray-

ing with appropriate detection reagents (Komagata and Suzuki, 1987). For quantitative analysis of cellular fatty acid compositions, cell mass was harvested and fatty acid methyl esters (FAMES) were prepared and identified according to the instructions of the Microbial Identification System (MIDI) (Kämpfer and Kroppenstedt, 1996; Sasser, 1990).

Chromosomal DNA was isolated and purified from cells grown in JCM medium no. 377 according to the method of Saito and Miura (1963). The DNA G+C content was determined by reversed-phase HPLC (Tamaoka and Komagata, 1984). The DNA-DNA hybridization was conducted in multidilution well plates as reported by Ezaki et al. (1989). The 16S rRNA genes of the isolates were amplified, purified, and sequenced as described by Namwong et al. (2005). The sequence determined was aligned with selected sequences obtained from the GenBank/ EMBL/ DDBJ database employing CLUSTAL_X version 1.83 (Thompson et al., 1997). The alignment was manually edited, and positions with gaps and ambiguous bases were eliminated prior to construction of a phylogenetic tree. The phylogenetic trees based on the neighbor-joining method (Saitou and Nei, 1987) and the maximum-likelihood method were constructed by the NJ plot program (Perrière and Gouy, 1996) in MEGA 4 software version 4 (Tamura et al., 2007). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings.

Results and Discussion

Identification of isolates

Forty-one halophilic bacteria were isolated from 38 samples of *pla-ra* collected from markets in Thailand (Table 1) and were divided into six groups on the basis of their phenotypic properties including the chemotaxonomic traits. The 16S rRNA gene sequence analysis was conducted for representative strains of the six groups in order to allocate their phylogentic positions, and the DNA-DNA hybridization study was also examined to access the integrity and species identity of the respective groups. Most of the isolates (40 strains) were Gram-positive, rod-shaped bacteria distributed across five groups, Groups I to V. They contained *meso*-diaminopimelic acid (*meso*-DAP) in the cell-wall peptidoglycan, and menaquinone with seven isoprene units (MK-7). Four groups were allocated to the genus

Table 1. Sources, isolate number, and identification.

Location of samples	Isolate no.	Group	Identification in this study
Bangkhunsri, Bangkok	BKS1-1	III	<i>V. marismortui</i>
Thaewast, Bangkok	BT3-1	III	<i>V. marismortui</i>
Chainat	CHN1-1, CHN2-2	I	<i>V. dokdonensis</i>
Chiang Mai	CHM1-3, CHM1-4	II	<i>V. halodenitrificans</i>
	CHM2-2	I	<i>V. dokdonensis</i>
Ladkrabang, Bangkok	BKL1-1, BKL2-2, BKL6-1	II	<i>V. halodenitrificans</i>
	BKL4-2, BKL 5-3	I	<i>V. dokdonensis</i>
Lop Buri	LB3-2	I	<i>V. dokdonensis</i>
Mahasarakham	MSK1-1, MSK2-1, MSK3-1	I	<i>V. dokdonensis</i>
Mahasarakram	MS3-4 ^T	IV	<i>V. siamensis</i> sp. nov.
Nakhon Nayok	N20-1	V	<i>B. vietnamensis</i>
	ND2-1	III	<i>V. marismortui</i>
Nakhon Ratchasima	RM1-1, RM2-1	III	<i>V. marismortui</i>
Nonthaburi	TPA4-7	V	<i>B. vietnamensis</i>
	TPA1-3, TPA3-3	III	<i>V. marismortui</i>
	TPA2-1	I	<i>V. dokdonensis</i>
	TPA2-6, TPA3-1, TPA4-3	II	<i>V. halodenitrificans</i>
Prachinburi	PR2-1, PR2-2	III	<i>V. marismortui</i>
Ayutthaya	AY1-2	II	<i>V. halodenitrificans</i>
Roi Et	R5-7	VI	<i>C. salexigens</i>
Samrong, Samut	BSR4-1, TSR5-2	III	<i>V. marismortui</i>
Prakarn	BSR3-1, TSR2-1	II	<i>V. halodenitrificans</i>
Samyan, Bangkok	TSY2-3	II	<i>V. halodenitrificans</i>
Sing Buri	SB2-2	III	<i>V. marismortui</i>
Sukho Thai	BY2-4	III	<i>V. marismortui</i>
Talaadthai, Bangkok	TM4-3	III	<i>V. marismortui</i>
	PT5-1	II	<i>V. halodenitrificans</i>
Total number of isolates	41		

Virgibacillus and one to the genus *Bacillus*. The remaining group, Group VI, was represented by a Gram-negative rod-shaped bacterium. It contained ubiquinone with nine isoprene units (Q-9) as the major component, and was assigned to the genus *Chromohalobacter*. The detailed phenotypic properties and the identity of the species are described below.

Group I contained ten isolates, BKL4-2, BKL5-3, CHM2-2, CHN1-1, CHN2-2, LB3-2, MSK1-1, MSK2-1, MSK3-1 and TP2-1. Cells were $0.6\text{--}0.8 \times 2.5\text{--}5 \mu\text{m}$ and were motile. Colonies were irregular, flat, and milky white in color (0.5–3.5 mm diameter). Terminal and subterminal endospores were observed. Catalase and oxidase activity was positive but L-arginine hydrolysis, urease, nitrate reduction and H₂S formation was negative. They hydrolyzed esculin, casein, gelatin, starch and Tween 80. They grew under aerobic and anaerobic conditions, in 0–20% (w/v) NaCl, at pH 5.0–8.0, optimally at 30–37°C but did not grow at

15 or 55°C. They produced acid from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, glycerol, inulin, *myo*-inositol, lactose, D-maltose, mannitol, D-mannose, melezitose, ribose, salicin, sucrose, and D-xylose, but did not produce acid from melibiose, raffinose, rhamnose or trehalose. The DNA G-C content of MSK2-1 was 36.5 mol% (Table 2). On the basis of 16S rRNA gene sequence analyses, the representative isolate, MSK2-1 in Group I (1,491 nt) was placed in the genus *Virgibacillus* (Fig. 1) and was closely related to *V. dokdonensis* (99.8%). Isolate MSK2-1 and related isolates showed high DNA–DNA relatedness (89.1–100%) to *V. dokdonensis* KCTC 3933^T. Therefore, they were identified as *V. dokdonensis* (Wayne et al., 1987; Yoon et al., 2005).

Group II contained 13 isolates, AY1-2, BKL1-1, BKL2-2, BKL6-1, CHM1-3, CHM1-4, TPA3-1, TP4-3, TPA2-6, TSY2-3, BSR3-1, PT5-1 and TSR2-1. Cells were $0.6\text{--}0.8 \times 2.0\text{--}4.0 \mu\text{m}$ and were motile. Colonies were circular

Table 2. Differential characteristics of the isolates in Group I, II, III and IV and related *Virgibacillus* species.

Characteristics	1	2	3	4	5	6	7	8	9 ^c
Colony color	Milky white	Milky white	Creamy brown	Creamy brown	Creamy white	Creamy white	Red	Pink	Cream
Spore shape	E, S	E, S	S, E	E	E	E	E	E, S	E
Spore position	T, ST	T, ST	T, ST	T, ST	T, ST	T, ST	ST	ST	T, ST
Anaerobic growth	+	+	+	+	–	–	+	–	–
Growth at/in:									
at 50°C	+(–3)	+	–	–	+	+	–	–	–
in 0% NaCl	+	+	–	–	–	–	–	–	w
in 20% NaCl	w	w	w	+	+(–2)	+	+	–	ND
Hydrolysis of:									
Esculin	+	+	–	–	+	+	–	w	–
Casein	+	+	+	+	+	+	w	+	+
Gelatin	+	+	+	+	+	+	+	–	vw
Nitrate reduction	–	–	+	+	+	+	–	+	+
Acid from:									
L-Arabinose	+	+	–	–	–	–	–	–	ND
D-Cellobiose	+	+	–	–	+	+	+	–	ND
D-Fructose	+	+	+	+	+	+	–	–	vw
D-Galactose	+	+	+	+	–	–	–	–	–
D-Glucose	+	+	+	+	+	+	+	–	vw
Glycerol	+	+	–	–	+(–1)	+	–	–	vw
myo-Inositol	+	+	–	–	–	–	–	–	–
Lactose	+	+	+	+	–	–	–	–	ND
D-Mannose	+	+	+	+	+	+	–	–	vw
D-Xylose	+	+	–(+2)	–	–	–	+	–	ND
DNA G+C (mol%)	36.5 ^a	36.7	38.0 ^a	38.0	38.8 ^a	40.7 ^b	38.0	40	37.3

Abbreviations: 1, Group I (10 isolates); 2, *V. dokdonensis* KCTC 3933^T; 3, Group II (13 isolates); 4, *V. halodenitrificans* JCM 12304^T; 5, Group III (14 isolates); 6, *V. marismortui* KCTC 3867^T; 7, Group IV (1 isolate, MS3-4^T); 8, *V. carmonensis* KCTC 3819^T; 9, *V. necropolis* DSM 14866^T.

E, ellipsoidal; S, spherical; T, terminal; S, subterminal. +, positive; w, weakly positive; vw, very weakly positive; –, negative; ND, no data. Number in parentheses indicates the isolates showing the reaction.

^aData for the tested isolates MSK2-1 (Group I), CHM1-4 (Group II) and TPA3-3 (Group III), respectively. ^bData from Arahal et al. (1999). ^cData from Heyrman et al. (2003).

to slightly irregular, raised, cream brown in color and 1–2 mm in diameter. Terminal and subterminal endospores were observed. Catalase, oxidase activity and nitrate reduction were positive but L-arginine hydrolysis, urease activity and H₂S production were negative. They hydrolyzed casein and gelatin but did not hydrolyze esculin, starch, Tween 80 or L-tyrosine. They grew under aerobic and anaerobic conditions, in NaCl up to 20% (w/v), at pH range 5.0–9.0 and at 10–45°C, but did not grow in 0.5% w/v or at 50°C. All produced acids from D-fructose, D-galactose, D-glucose, lactose, D-maltose, mannitol, D-mannose, ribose, and sucrose, but did not from L-arabinose, D-cellobiose, glycerol, inulin, myo-inositol, melibiose, melezitose, raffinose, rhamnose, or salicin. Variable acid production was

found in D-xylose and trehalose. The DNA G-C content of CHM 1-4 was 38.0 mol% (Table 2). On the basis of 16S rRNA gene sequence analyses, a representative isolate, CHM1-4 in Group II (1,457 nt) was placed in the genus *Virgibacillus* (Fig. 1) and it was closely related to *V. halodenitrificans* JCM 12304^T (99.6%). They showed high DNA–DNA relatedness (74.9–100%) with related isolates and *V. halodenitrificans* JCM 12304^T. Therefore, they were identified as *V. halodenitrificans* (Wayne et al., 1987; Yoon et al., 2004).

Group III contained 13 isolates, BT3-1, BKS1-1, BSR4-1, BY2-4, SB2-2, TM4-3, TPA3-3, ND2-1, PR2-1, PR2-2, RM1-1, TP1-3 and TSR5-2. Cells were 0.5–0.7 × 2–4 µm and were motile. Colonies were circular to slightly irregular, raised, and cream white in color

Fig. 1. Phylogenetic tree showing the relationships between strain MSK2-1, CHM1-4, TPA3-3 and MS3-4^T, *Virgibacillus* species and related taxa based on 16S rRNA gene sequences.

The branching pattern was generated by the neighbor-joining method. Bootstrap percentages above 50%, based on 1,000 replications, are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

(2–6 mm diameter). Terminal and subterminal endospores were observed. Catalase, oxidase, urease activity and nitrate reduction were positive but starch, Tween 80, L-tyrosine and H₂S production were negative. They grew under aerobic but not anaerobic conditions. They grew at 15–50°C and at pH range 6.0–8.0. Most of them grew between 1 and 25% NaCl (w/v). No growth occurred in the absence of salt. All produced acid from D-cellobiose, D-fructose, D-glucose, D-mannose and salicin but not from L-arabinose, D-galactose, myo-inositol, lactose, mannitol, melibiose, melezitose, raffinose, rhamnose, trehalose or D-xylose. Variable acid production was found in glycerol, D-maltose, ribose and sucrose. The DNA G+C content of TPA3-3 was 38.8 mol% (Table 2). On the basis of 16S rRNA gene sequence analyses, the representative isolate, TPA3-3 (1,545 nt) was placed in the genus *Virgibacillus* (Fig. 1) and it was closely related to *V. marismortui* (99.6%). Isolates MSK2-1, *V. marismortui* KCTC 3867^T and related isolates showed high DNA–DNA relatedness (87.9–100%). Therefore, they were identified as *V.*

marismortui (Arahal et al., 1999; Wayne et al., 1987).

Group IV contained one isolate, MS3-4. This strain grew under aerobic and anaerobic conditions, in NaCl 1–20% (w/v), at 15–40°C and at pH range 7.0–8 [optimally in NaCl 5% (w/v), at pH 7.0, and at 37°C]. No growth occurred in the absence of salts or in 25% w/v. The strain was differentiated from the isolates in Group I, II, III and type strains *V. dokdonensis* KCTC 3933^T, *V. halodenitrificans* JCM 12304^T and *V. marismortui* KCTC 3867^T as shown in Table 2.

On the basis of 16S rRNA gene sequence analyses, strain MS3-4 (1,524 nt) was closely related to *Virgibacillus* species (93.6–95.9%) and *Lentibacillus* species (93.3–94.9%) (Figs. 1, 2 and 3). The strain was closely related to *V. carmonensis* KCTC 3819^T (95.9%) and *V. necropolis* DSM 14866^T (95.6%). This strain contained 38.0 mol% of G+C content of DNA with in the range (36–43 mol%) of the genus *Virgibacillus* (Heyrman et al., 2003; Lee et al., 2006); that differed from the genus *Lentibacillus* which contained 41.6–49.0 mol% (Lee et al., 2008a, b; Tanasupawat et al., 2006). Therefore it

Fig. 2. Phylogenetic tree showing the relationships between strain MS3-4^T, *Virgibacillus* species, *Lentibacillus* species, and related taxa based on 16S rRNA gene sequences.

The branching pattern was generated by the neighbor-joining method. Bootstrap percentages above 50%, based on 1,000 replications, are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

was included in the genus *Virgibacillus*. It contained anteiso-C_{15:0} (55.8%), anteiso-C_{17:0} (17.7%), iso-C_{15:0} (11.3%), iso-C_{16:0} (6.6%), iso-C_{14:0} (3.9%), C_{16:0} (1.5%) and iso-C_{17:0} (1.5%) as cellular fatty acid profiles (Table 3) which corresponded to *Virgibacillus* species (Lee et al., 2006). This strain showed low DNA-DNA relatedness to *V. carmonensis* KCTC 3819^T (12.7%), *V. halodenitrificans* JCM 12304^T (4.4%) and *V. marismortui* KCTC 3867^T (3.0%). In addition, strain MS3-4 was differentiated from *V. carmonensis* KCTC 3819^T by growth in 20% NaCl, hydrolysis of esculin, gelatin, and

L-tyrosine; nitrate reduction; acid from D-cellobiose, D-glucose and D-xylose (Table 2). In addition, *V. carmonensis* KCTC 3819^T could not grow on medium containing KNO₃ under anaerobic condition or in NaCl concentrations of 0.5 or 15% (w/v). As mentioned above and based on its phenotypic characteristics, the phylogenetic analysis using 16S rRNA gene sequences, and DNA G+C content, including DNA-DNA relatedness, strain MS3-4 represented a novel species of the genus *Virgibacillus* for which the name *Virgibacillus siamensis* sp. nov. is proposed.

Fig. 3. Phylogenetic tree showing the relationships between strain MS3-4^T, *Virgibacillus* species, *Lentibacillus* species, and related taxa based on 16S rRNA gene sequences.

The branching pattern was generated by the maximum-likelihood method. Bootstrap percentages above 50%, based on 1,000 replications, are shown at the nodes. Bar, 0.005 substitutions per nucleotide position.

Table 3. Fatty acid compositions of strain MS3-4^T and *Virgibacillus* species.

Fatty acid	Percentage of total ^a		
	1	2	3 ^b
Saturated straight-chain			
C _{14:0}	0.2		
C _{15:0}	0.8		0.8
C _{16:0}	1.5	1.0	1.6
Saturated branched-chain			
anteiso-C _{13:0}	0.2		
iso-C _{14:0}	3.9	3.3	2.2
anteiso-C _{15:0}	55.8	58.3	67.9
iso-C _{15:0}	11.3	5.7	3.8
iso-C _{16:0}	6.6	6.4	4.2
anteiso-C _{17:0}	17.7	11.1	12.2
iso-C _{17:0}	1.5	0.9	0.9
Unsaturated straight-chain			
C _{16:1} ω7c alcohol		6.5	2.4
C _{16:1} ω11c		0.6	1.0
SF3 ^c		5.0	2.1

Abbreviations: 1, MS3-4^T; 2, *V. carmonensis* KCTC 3819^T; 3, *V. necropolis* DSM 14866^T.

^aValues are percentage of total cellular fatty acids. ^bData from Heyrman et al. (2003). ^cSummed feature 3 contains C_{16:1} ω7c and/or iso-C_{15:0} 2OH.

Group V comprised two isolates, N20-1 and TPA4-7. Colonies were irregular, raised, and cream orange yellow in color (0.5–3.5 mm diameter). Cells were 0.6–1.0 × 2.0–3.5 μm and were motile. Central and subterminal endospores were observed under microscopy. They grew under aerobic and anaerobic conditions, in NaCl concentrations between 0 and 15% (w/v) NaCl (optimum 0–1%), in pH range 5.0–8.0, and at the temperature range 20–45°C. Catalase and oxidase activity were positive but H₂S formation was negative. They hydrolyzed casein, starch, DNA, esculin, and gelatin. Acid was produced from D-fructose, D-glucose, raffinose, salicin, and sorbitol but no acid was produced from D-cellobiose, D-galactose, myo-inositol, melibiose, sucrose, or trehalose.

On the basis of 16S rRNA gene sequence and phylogenetic analyses, the isolate, N20-1 in Group V (1,482 nt) was a member of the genus *Bacillus* (Fig. 4) and it was closely related to *B. vietnamensis* JCM 11124^T (99.6%), *B. aquimaris* (99.3%) and *B. marisflavi* (98.4%). Isolate N20-1 and TPA4-7 showed high DNA–DNA relatedness to each other (100–102.1%) and *B. vietnamensis* JCM 11124^T (85.6–103.9%) but

showed low DNA–DNA relatedness with *B. marisflavi* KCTC 3906^T (23.6%) and *B. aquimaris* KCTC 3903^T (20.7%). The Group V isolates showed almost the same phenotypic characteristics as *B. vietnamensis* JCM 11124^T but were different in the acid produced from myo-inositol and trehalose. Therefore, they were identified as *B. vietnamensis* (Noguchi et al., 2004; Wayne et al., 1987).

Group VI contained isolate R5-7. Cells were 0.6–0.8 × 2.5–5 μm. Colonies were cream in color (0.3–3 mm diameter). Isolate R5-7 grew over a wide range of salinity between 3 and 25%, w/v (optimal in 10%), at pH 6–9, and at 45°C. It was positive for catalase, oxidase, nitrate reduction, citrate utilization, starch hydrolysis, but negative for H₂S production, urease, hydrolysis of casein, esculin, gelatin, and L-tyrosine. The predominant respiratory ubiquinone (Q) was Q-9. The DNA G+C content was 64 mol%. On the basis of 16S rRNA gene sequence analyses, isolate R5-7 (1,499 nt) was placed in the family *Halomonadaceae* and in the genus *Chromohalobacter* (Fig. 5) and it was closely related to *C. salexigens* KTCC 12941^T (98.8% similarity) and *C. israelensis* ATCC 43985^T (98.8% similarity). Isolate R5-7 showed high DNA–DNA relatedness with *C. salexigens* KCTC 12941^T (98.7%). It contained saturated straight chains of C_{10:0} (3.7%), C_{12:0} (5.9%), C_{16:0} (25.3%), unsaturated straight chains of C_{18:1} ω7c (13.5%), C_{17:0} cyclo (7.0%), C_{19:0} cyclo ω8c (29.7%), and hydroxylated fatty acid of C_{12:0} 3OH (8.0%), and summed feature 3 contained C_{16:1} ω7c and/or iso-C_{15:0} 2OH (3.2%). Based on its phenotypic characteristics, fatty acid profiles, and the 16S rRNA gene sequence analyses as indicated, isolate R5-7 was identified as *C. salexigens* (Arahal et al., 2001; Wayne et al., 1987).

The current study revealed that most isolated strains from the salted fermented fish, *pla-ra*, were identified as species of the genus *Virgibacillus*, indicating they may play important roles in the fermentation process of fish. The genus *Virgibacillus* housed 19 species at the present date, and species of this genus are rather broadly distributed according to the 16S rRNA gene-based phylogenetic analysis. On the other hand, genera in the vicinity of the genus *Virgibacillus*, such as *Lentibacillus*, *Oceanobacillus*, *Halobacillus* and *Gracilbacillus* are allocated coherently. More comprehensive studies will be required to re-evaluate the current taxonomic status of the genus *Virgibacillus*.

Fig. 4. Phylogenetic tree showing the relationships between strain N20-1, *Bacillus* species and related taxa based on 16S rRNA gene sequences.

The branching pattern was generated by the neighbor-joining method. Bootstrap percentages above 50%, based on 1,000 replications, are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

Description of *Virgibacillus siamensis* sp. nov.

Virgibacillus siamensis (si.am.en'sis. N.L. masc. adj. *siamensis* pertaining to Siam, the old name of Thailand, from where the first strain was isolated).

Cells are Gram-positive rods ($0.5\text{--}0.7 \times 2\text{--}5 \mu\text{m}$). Colonies are convex, circular with red color and 2–5 mm in diameter. Motile. Terminal and subterminal endospores are observed. Catalase, oxidase, hydrolysis of gelatin, starch and L-tyrosine are positive but L-arginine hydrolysis, H₂S production, nitrate reduction, citrate utilization, hydrolysis of esculin and Tween 80 are negative. It hydrolyzes casein weakly. Growth in NaCl 1–20% w/v, at 15–40°C and at pH range 7.0–8 [optimally in NaCl 5% (w/v), at pH 7.0, and at 37°C]. Growth under aerobic and anaerobic conditions. No growth occurs in the absence of salts or in 25% w/v.

Produces acid from D-cellobiose, D-glucose, D-maltose, D-ribose, L-sorbose, and D-xylose, but does not produce acid from L-arabinose, D-galactose, glycerol, D-mannose, melezitose, melibiose, salicin, sucrose, trehalose or *myo*-inositol. It presents phosphatidylglycerol (PG), diphosphatidylglycerol (DPG) and an unidentified glycolipid. Anteiso-C_{15:0} and anteiso-C_{17:0} are major cellular fatty acids. The DNA G+C content is 38.0 mol%. The type strain is MS3-4 (JCM 15395^T = PCU 312^T = TISTR 1957^T).

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Fig. 5. Phylogenetic tree showing the relationships between strain R5-7, *Chromohalobacter* species and related taxa based on 16S rRNA gene sequences.

The branching pattern was generated by the neighbor-joining method. Bootstrap percentages above 50%, based on 1,000 replications, are shown at the nodes. Bar, 0.01 substitutions per nucleotide position.

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