Allobacillus salarius sp. nov., and *Allobacillus saliphilus* sp. nov., Isolated from Shrimp Paste (*ka-pi*) in Thailand

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Research Article

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Abstract

The moderately halophilic, Gram-stain-positive, spore-forming rods, designated SKP4-8^T and SKP8-2^T isolated from a traditional fermented shrimp paste (*Ka-pi*), were taxonomic studied based on polyphasic approach. Strain SKP4-8^T grew at pH 6.0-9.0 (optimum 7.0), at 25-45°C (optimum 37°C) and in 1-16 % (w/v) NaCl (optimum 5-10). Strain SKP8-2^T grew at pH 6.0-9.0 (optimum 8.0), at 25-45°C (optimum 37°C) and in 0-20 % (w/v) NaCl (optimum 3-10 %). The strains contained *meso*-diaminopimelic acid in cell-wall peptidoglycan and the major menaquinone was MK-7. Strain SKP4-8^T contained iso-C_{15:0}, antesio-C_{15:0} and iso-C_{17:0}; and strain SKP8-2^T contained antesio-C_{15:0}, iso-C_{15:0}, iso-C_{16:0} and antesio-C_{17:0} as major cellular fatty acids. Phosphatidylglycerol, diphosphatidylglycerol, unknown phospholipids and an unknown glycolipid were detected as major polar lipids. On the basis of 16S rRNA gene sequence analysis, strains SKP4-8^T and SKP8-2^T belonged to the genus *Allobacillus* and was closely related to *Allobacillus halotolerans* LMG 24826^T with 98.8 % and 99.3 % similarity, respectively. The comparative genome analysis based on average nucleotide identity (ANI) and digital DNA-DNA hybridization revealed that both strains showed the values below 95 and 70 %, from each other and the *Allobacillus halotolerans* LMG 24826^T, respectively. Based on data from this polyphasic study, strains SKP4-8^T and SKP8-2^T represent the novel species of the genus *Allobacillus* and the name *Allobacillus salarius* sp. nov. was proposed for SKP4-8^T (=KCTC 33905^T =LMG 30016^T =TISTR 2499^T); and *Allobacillus saliphilus* sp. nov. for SKP8-2^T (=KCTC 33906^T =LMG 29682^T =TISTR 2558^T).

Introduction

Shrimp paste, a dark brown, gray or pink brown traditionally called *Ka-pi* is a strong-smelling commonly used as a cooking ingredient. It is produced by fermentation of shrimp with salt for 1–3 months and has rich in various nutrients, particularly amino acids and peptides, and contains a high concentration of NaCl (Phithakpol et al. 1995; Tanasupawat and Komagata 2001). Moderately halophilic rod-shaped, have been isolated from shrimp paste and was proposed as the new genus *Allobacillus* (Sheu et al. 2011). To date, the numerous reports on moderately halophilic bacteria have revealed novel isolates with distinct properties from various salty environments in the member of genera including *Alkalibacillus* (Jeon et al. 2005), *Bacillus* (Ventosa et al. 1989), *Gracilibacillus* (Wainø et al. 1999), *Halobacillus* (Spring et al. 1996), *Lentibacillus* (Yoon et al. 2002), *Marinococcus* (Hao et al. 1984), *Piscibacillus* (Tanasupawat et al. 2007), *Thalassobacillus* (García et al. 2005) and *Virgibacillus* (Heyndrickx et al. 1998). Many species were isolated from fermented foods such as *Gracilibacillus thailandensis* (Chamroensaksri et al. 2010), *Lentibacillus kapialis* (Pakdeeto et al. 2007a), *Lentibacillus salicampi* and *Lentibacillus juripiscarius* (Namwong et al. 2005), *Oceanobacillus kapialis* (Namwong et al. 2009), *Piscibacillus salipiscarius* (Tanasupawat et al. 2007), *Salinicoccus siamensis* (Pakdeeto et al. 2007b), *Virgibacillus kapii* (Daroontpant et al. 2016) and *Virgibacillus siamensis* (Tanasupawat et al. 2010). This study, a strain SKP8-2^T was isolated from shrimp paste (*ka-pi*) in Thailand and found to represent the novel species of the genus *Allobacillus* and was subsequently evaluated by the means of a polyphasic taxonomy.

Materials And Methods

Bacterial isolation

Strains SKP4-8^T and SKP8-2^T were isolated from shrimp paste collected from the market in Samut Sakhon province, Thailand by using spread-plate technique duplicate on modified JCM medium no.377 agar plates, contained (g/L): 5 g casamino acids, 5 g yeast extract, 1 g sodium glutamate, 3 g trisodium citrate, 20 g MgSO₄·7H₂O, 2 g KCl, 50 g NaCl, 0.036 g FeCl₂·4H₂O, 0.0036 g MnCl₂·4H₂O, 20 g agar (pH adjusted to 7.0-7.2) and incubated at 37°C for 3-7 days.

Phenotypic characteristics

The colonies with different cell size, cell shape, and color were examined on the cell grown on modified JCM medium no. 377 agar at 37°C after 3 days. The Hucker-Conn modification was used for Gram staining (Hucker and Conn 1923). Cell form and spore formation were observed under the light microscope (CH-2, Olympus) and electron microscope (JSM-5410LV, Japan). Flagella were examined as described by Forbes (1981). Anaerobic growth was tested by incubating the cultures on modified JCM medium no. 377 plates in an anaerobic jar with AnaeroPack-Anaero (Mitsubishi Gas Chemical, *MGC*). Catalase and oxidase activities and nitrate reduction were determined as described by Barrow and Feltham (Barrow and Feltham 1993). Hydrolysis of aesculin, gelatin, starch, skim milk and Tween 20, Tween 40, Tween 60 and Tween 80 were determined as described by Namwong et al. (2005). Arginine hydrolysis was performed by using the medium reported by Thornley (Thornley 1960). Acid production from carbohydrate was determined in the use of basal medium composed (g/L): of 5 g carbohydrate, 1 g casamino acids, 0.1 g yeast extract, 1 g sodium glutamate, 3 g trisodium citrate, 20 g MgSO₄·7H₂O, 2 g KCl, 50 g NaCl, 0.036 g FeCl₂·4H₂O, 0.0036 g MnCl₂·4H₂O and was adjusted to pH 7.0 and incubated at 37°C for 7 days. Phenol red was used as an indicator. Effects of growth at various NaCl concentrations were investigated in modified JCM medium no. 377 broth omitting the MgSO₄·7H₂O with different concentrations of NaCl (0 and 1-20%, w/v with an interval of 1). Growth at different pH (4 to 10) was investigated in buffer medium (Sorokin 2005) and growth at different temperatures (10-50°C) was investigated on modified JCM medium no. 377 agar plates. Antibiotic susceptibility was examined by disc diffusion assay (Acar and Goldstein 1991) on Mueller-Hinton agar (Difco) supplemented with 5 % (w/v) NaCl. The enzyme activities were determined using API ZYM system (bioMérieux), according to the manufacturer's instructions. All tests were carried out in media supplemented with 5 % NaCl, except for the investigation of effects of growth at various NaCl concentrations.

Chemotaxonomy

The cell biomass for chemotaxonomic characterization was produced in modified JCM medium no. 377 at 30°C for 3 days. Diaminopimelic acid in the cell wall and menaquinone component were determined as described by Komagata and Suzuki (1987). Polar lipids were extracted and analyzed by using the procedure of as described by Minnikin et al. 1984 and identified by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata and Suzuki 1987). For quantitative analysis of

cellular fatty acid compositions, cells were cultivated on modified JCM medium no. 377 agar plates at 30°C for 48 h. Fatty acid methyl esters (FAMES) were prepared and identified according to the instructions of the Microbial Identification System (MIDI) (Sasser 1990).

Genotypic and phylogenetic analysis

Chromosomal DNA was isolated and purified from cells grown in modified JCM medium no. 377 according to the method of Saito and Miura (Saito and Miura 1963). The 16S rRNA gene was 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 (Felsenstein 1981) were constructed in MEGA version 6 (Tamura et al. 2013). The confidence values of branches of the phylogenetic tree were determined using bootstrap analyses (Felsenstein 1985) based on 1,000 resamplings.

Whole genome sequence of strain SKP4-8^T, SKP8-2^T and *Allobacillus halotolerans* LMG 24826^T were performed using an Illumina Miseq platform (Illumina, Inc., San Diego, US-CA) by the World Data Center for Microorganisms (WDCM) under the Globa Catalogue of Microorganisms (GCM) 2.0 project. Assembling the reads to contigs were accomplished by using SPAdes 3.12 (Bankevich et al. 2012). The assembled genome of strain SKP4-8^T, SKP8-2^T and *Allobacillus halotolerans* LMG 24826^T are available public on the GenBank (accession numbers: VMHE000000000, JAGSIE000000000 and J AHLZF000000000). The genome was annotated by using the DFAST sever (Tanizawa et al. 2018) followed the NCBI Prokaryotic Genome Annotation Pipeline (PGAP). Average nucleotide identity (ANI) values were calculated with pairwise genome alignment of the draft genome sequences of *Allobacillus halotolerans* LMG 24826^T (J AHLZF000000000) by using the ANI-BLAST (ANiB) and ANI-MUMmer (ANIm) algorithms (Meier-Kolthoff and Göker 2019) implemented within the JspeciesWS web service (Richter and Rosselló-Móra 2009). Calculation of the digital DNA-DNA hybridization (dDDH) values was achieved by using the Genome-to-Genome Distance Calculator (GGDC 2.1) using the BLAST+ method (Richter et al. 2016). Results were based on the recommended formula 2 (identities/HSP length), which is useful when dealing with incomplete draft genomes.

Results And Discussion

Strains SKP4-8^T and SKP8-2^T were Gram-stain-positive, strictly aerobic and spore-forming rods. Endospores are produced at terminal position in swollen sporangia (Supplementary Fig. S1). Colonies were smooth, circular, low convex and cream in the color (1–2 mm in diameter) after grew on modified JCM medium no. 377 agar at 37°C for 2 days. Motile by peritrichous flagella. No growth in anaerobic condition. Strain SKP4-8^T grew in 1–16 % (w/v) NaCl (optimum 5–10 %), at 25–45°C (optimum 37°C) and at pH 6.0–9.0 (optimum 7.0). Strain SKP8-2^T grew in 0–20 % (w/v) NaCl (optimum 3–10 %), at 25–45°C (optimum

37°C) and at pH 6.0–9.0 (optimum 8.0). The results of phenotypic characteristics are given in the species description and listed in Table 1. The strains SKP4-8^T and SKP8-2^T were sensitive to chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), novobiocin (5 µg), penicillin G (20 U), rifampicin (5 µg) and tetracycline (30 µg) but were resistant to ampicillin (10 µg) and streptomycin (10 µg).

Table 1

Differential characteristics of strains SKP4-8^T, SKP8-2 and *Allobacillus halotolerans* LMG 24826^T. All data are from this study. +, positive reaction; -, negative reaction.

Characteristics	SKP4-8 ^T	SKP8-2 ^T	LMG 24826 ^T
Temperature range for growth (optimum) (°C)	25–45 (37)	25–45 (37)	20–45 (37)
NaCl range for growth (optimum) % (w/v)	1–16 (5–10)	0–20 (3–10)	1–20 (5–10)
Nitrate reduction	+	+	-
Hydrolysis of Tween 80	+	-	-
API ZYM tests			
C14 Lipase	+	-	-
Trypsin	+	-	-
Acid phosphatase	+	+	-
Acid production from:			
D-Fructose	-	+	-
D-Galactose		+	
D-Mannose	-	+	-
D-Ribose	-	-	+

Strain SKP4-8^T and SKP8-2^T contained *meso*-diaminopimelic acid in the cell wall peptidoglycan and the major menaquinone was MK-7. The major fatty acids found in strain SKP4-8^T were iso-C_{15:0} (54.4 %), antesisio-C_{15:0} (11.9 %), and iso-C_{17:0} (11.6 %). In addition, the other fatty acids, C_{16:0} (0.8 %), iso-C_{14:0} (1.8 %), iso-C_{16:0} (3.2 %), anteiso-C_{17:0} (4.5 %), C_{16:1}ω11c (0.6 %) C_{16:1}ω7c alcohol (5.7 %) and summed feature 4 (1.7 %) were also detected and are different from strain SKP8-2^T (Table 2). The major fatty acids found in strain SKP8-2^T contained antesisio-C_{15:0} (36.3%), iso-C_{15:0} (19.2 %), iso-C_{16:0} (16.5 %) and antesisio-C_{17:0} (16.3 %). In addition, the other fatty acids, C_{16:0} (0.7 %), iso-C_{14:0} (4.4 %), iso-C_{17:0} (3.2 %), C_{16:1}ω7c alcohol (1.4 %) and summed feature 4 (0.6 %) were also detected (Table 2). The polar lipids of strain SKP4-8^T were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), three unknown phospholipids (PL1, PL2 and PL3) and unknown glycolipid (GL) (Supplementary Fig. S2). Strain SKP8-2^T showed major polar lipid similar to

strain SKP4-8^T which contained of PG, DPG, unknown glycolipid (GL), but showed two unknown phospholipids (PL1 and PL2) (Supplementary Fig. S3).

Table 2
Cellular fatty acids composition (%) of strains SKP4-8^T, SKP8-2^T and *A. halotolerans* LMG 24826^T. Data are determined in this study.

Fatty acids	SKP4-8 ^T	SKP8-2 ^T	LMG 24826 ^T
Saturated straight-chain			
C _{16:0}	0.8	0.7	0.7
Saturated branched-chain			
iso-C _{14:0}	1.8	4.4	5.7
iso-C _{15:0}	54.4	19.2	5.2
anteiso-C _{15:0}	11.9	36.3	52.4
iso-C _{16:0}	3.2	16.5	21.0
iso-C _{17:0}	11.6	3.2	0.4
anteiso-C _{17:0}	4.5	16.3	12.1
Unsaturated straight-chain			
C _{16:1} ω11c	0.6	-	-
C _{16:1} ω7c alcohol	5.7	1.4	0.8
Summed Feature 4	1.7	0.6	0.1
Fatty acids comprising less than 0.5 % are omitted. -, not detected.			
Summed feature 4 contained iso-C _{17:1} I and/or anteiso-C _{17:1} B.			

The draft genome sequence of strain SKP4-8^T was 2.59 Mb in size with an average in silico DNA G + C content of 38.8 mol%. The draft genome sequence of strain SKP8-2^T was 2.54 Mb in size with an average in silico DNA G + C content of 39.0 mol%. The draft genomic features of the strains SKP4-8^T and SKP8-2^T are described in Table 3. The ANIb and ANIm values of the draft genomes between strain SKP4-8^T and its closest related species, *A. halotolerans* LMG 24826^T (86.6 and 88.1 %, respectively) are shown in Table 4. The ANIb and ANIm values of the draft genomes between strain SKP8-2^T and its closest related species, *A. halotolerans* LMG 24826^T (87.1 and 88.3 %, respectively) are shown in Table 4. Strain SKP4-8^T exhibited 93.2 and 93.8 % of ANIb and ANIm with SKP8-2^T. Both ANI values are clearly lower than the 95–96 % cut-

off for species delineation (Meier-Kolthoff et al. 2013). The digital DNA-DNA hybridization (dDDH) value between the genomes of strain SKP4-8^T and *A. halotolerans* LMG 24826^T was 32.5 % (C.I. model 30.1–35.1 %). The digital DNA-DNA hybridization (dDDH) values between the genomes of the strain SKP8-2^T and *A. halotolerans* LMG 24826^T were 33.0 % (C.I. model 30.6–35.5 %) which are significantly lower than the threshold value of 70 % commonly used to delineate separated species status (Richter and Rosselló-Móra 2009) (Table 4), which are significantly lower than the threshold value of 70 % commonly used to delineate separated species status (Wayne et al. 1987). It is concluded from the above results that strains SKP4-8^T and SKP8-2^T should be recognized as a new species within the genus *Allobacillus*.

Table 3
Genomic statistics of strains SKP4-8^T (VMHE000000000), SKP8-2^T (JAGSIE000000000) and *A. halotolerans* LMG 24826^T (JAHLLZF000000000)

Attribute	SKP4-8 ^T	SKP8-2 ^T	LMG 24826 ^T
Genome size (bp)	2,595,246	2,543,747	2,726,708
G + C content (mol%)	38.8	39.0	39.5
Number of contigs	102	80	96
Total genes	2,666	2,622	2,820
Number of coding sequences	2,603	2,563	2,759
RNA genes	63	59	61

Table 4

ANib and ANIm values (%) and the digital (*in silico*) DNA-DNA hybridization (dDDH) values between the draft genomes of strains SKP4-8^T, SKP8-2^T and its closely related type strains.

Query genome	Reference genome	ANib	ANIm	% dDDH (Formula 2*)	Model C.I.	Distance	Prob. Digital DNA-DNA hybridization ≥ 70%	G+C difference
1	2	93.2	93.8	53.2	[50.5 - 55.9%]	0.0	28.9	0.1
1	3	86.6	88.1	32.5	[30.1 - 35.1%]	0.1	0.2	0.7
2	3	87.1	88.3	33.0	[30.6 - 35.5%]	0.1	0.3	0.5
Draft genomes: 1, Strain SKP4-8 ^T ; 2, SKP8-2 ^T ; 3, <i>A. halotolerans</i> LMG 24826 ^T . All data were determined in this study.								
*Recommended formula (identities/HSP length), which is liberated of genome length and is thus prosperous against the use of incomplete draft genome								

On the basis of 16S rRNA gene sequence analysis, strains SKP4-8^T (1471 nt) and SKP8-2^T (1441 nt) were closely related to *A. halotolerans* LMG 24826^T with 98.8 and 99.3 %, respectively (Fig. 1, Figs.S4 and S5). Strains SKP4-8^T exhibited 99.5 % 16S rRNA gene sequence similarity with SKP8-2^T. In addition, strains SKP4-8^T and SKP8-2^T were differentiated from *A. halotolerans* LMG 24628^T by growth in NaCl, nitrate reduction, aesculin hydrolysis, and acid production from D-fructose, D-galactose, D-glucose and D-mannose (Table 1), the amount of fatty acids of iso-C_{15:0} and anteiso-C_{15:0} (Table 2) and DNA-DNA relatedness. Strain SKP4-8^T and SKP8-2^T should represent the novel species of the genus *Allobacillus* for which the name *Allobacillus salarius* sp. nov. and *Allobacillus saliphilus* sp. nov. respectively, are proposed.

Description of *Allobacillus salarius* sp. nov.

Allobacillus salarius (sa.la'ri.us. L. fem. adj. *salarius* of, or belonging to salt).

Cells are strictly aerobic Gram-stain-positive rods (0.25–0.30 x 0.8–3.5 µm). Motile by peritrichous flagella. Spores with oval shape are located at terminal position. Colonies are circular, convex, entire and cream in color (1–2 mm in diameter). Growth occurs at 25–45°C, pH 6.0–9.0 and 1–16 % (w/v) NaCl (optimally at 37°C, at pH 7.0 and in 5–10 % NaCl). Positive for oxidase, catalase, urease and hydrolysis of aesculin, Tween 20, Tween 40, Tween 60, and Tween 80. Negative for nitrate reduction and hydrolysis of arginine, gelatin, skim milk, starch and acid production from L-arabinose, D-cellobiose, D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannitol, D-mannose, D-melibiose, D-raffinose, L-rhamnose, D-ribose, D-salicin, D-trehalose and D-xylose. In the API ZYM system positive reaction for alkaline phosphatase, C4 esterase, C8 esterase, C14 lipase, leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, while α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase. The polar lipid profile contains phosphatidylglycerol, diphosphatidylglycerol, three unknown phospholipids and one glycolipids. Contains *meso*-diaminopimelic acid in the peptidoglycan. The major cellular fatty acids are iso-C_{15:0}, anteiso-C_{15:0}, and iso-C_{17:0} and the major isoprenoid quinone is MK-7.

The type strain is SKP4-8^T (= KCTC 33905^T = LMG 30016^T = TISTR 2499^T), isolated from shrimp paste (*Ka-pi*) collected from a market in Samut Sakhon province, Thailand. The in silico DNA G + C content of the type strain is 38.8 mol%. The DDBJ accession numbers for the 16S rRNA gene sequence of strains SKP4-8^T is LC215448. The GenBank accession numbers of the draft genome of strain SKP4-8^T is VMHE00000000.

Description of *Allobacillus saliphilus* sp. nov.

Allobacillus saliphilus (sa.li.philus.L. n. sal, salis salt; Gr. adj. philos loving; N.L. masc. adj. *saliphilus* salt-loving)

Cells are strictly aerobic Gram-stain-positive, rods (0.2–0.3 x 0.6–4.9 µm). Motiles by peritrichous flagella. Colonies are convex, circular, entire and cream in color (1–2 mm in diameter). Spores with oval shape are

located at terminal position. Growth occurs at 25–45°C (optimum 37°C), pH 6.0–9.0 (optimum 8) and 0–20 % (w/v) NaCl (optimum 3–10 % NaCl). Positive for oxidase, catalase, nitrate reduction and hydrolysis of aesculin, Tween 20, Tween 40, Tween 60 and urea, but are negative for hydrolysis of arginine, gelatin, skim milk, starch, and Tween 80. Produces acid from D-fructose, D-galactose, D-glucose, D-mannose and sucrose but no acid from L-arabinose, D-cellobiose, lactose, maltose, D-mannitol, D-melibiose, D-raffinose, L-rhamnose, D-ribose, D-salicin, sorbitol, D-trehalose and D-xylose. In the API ZYM system, acid phosphatase, *N*-acetyl- β -glucosaminidase, alkaline phosphatase, cystine arylamidase, α -galactosidase and α -glucosidase (w) are positive while α -chymotrypsin, esterase lipase (C 4), esterase lipase (C 8), α -fucosidase, β -galactosidase, β -glucosidase, β -glucuronidase, leucine arylamidase, lipase (C14), α -mannosidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase are negative. The polar lipid profile contains phosphatidylglycerol, diphosphatidylglycerol, two unknown phospholipids and an unknown glycolipid. *Meso*-diaminopimelic acid is presented in the cell wall peptidoglycan. The major cellular fatty acids are antesio-C_{15:0}, iso-C_{15:0}, iso-C_{16:0} and antesio-C_{17:0}. The major isoprenoid quinone is MK-7.

The type strain is SKP8-2^T (= KCTC 33906^T = LMG 29682^T = TISTR 2558^T), isolated from shrimp paste (*Ka-pi*) collected from a market in Samut Sakhon province, Thailand. The in silico DNA G + C content of the type strain is 39.0 mol%. The DDBJ accession numbers for the 16S rRNA gene sequence of strains SKP8-2^T is LC215449. The GenBank accession numbers of the draft genome of strain SKP8-2^T is JAGSIE000000000.

Declarations

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Compliance with ethical standards

Conflicts of interest The authors declare that there are no conflicts of interest.

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Figures

Figure 1

Neighbor-Joining tree based on 16S rRNA gene sequences showing the relationships between strains SKP4-8T, SKP8-2T and *Alkalibacillus halotolerans* LMG 24826T. Bootstrap values (>50%) based on 1000 replications are given at branch nodes. *Bacillus subtilis* DSM 10T was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.

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