**Halobacterium piscisalsi** sp. nov., from fermented fish (**pla-ra**) in Thailand

Mongkol Yachai,¹ Somboon Tanasupawat,² Takashi Itoh,³ Soottawat Benjakul,¹ Wonnop Visessanguan⁴ and Ruud Valyasevi⁴

¹Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
²Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand
³Japan Collection of Microorganisms, RIKEN BioResource Center, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan
⁴National Center for Genetic Engineering and Biotechnology, 113 Thailand Science Park, Klong 1, Klong Luang, Pathumthani 12120, Thailand

A Gram-negative, motile, rod-shaped, extremely halophilic archaeon, designated strain HPC1-2ᵀ, was isolated from **pla-ra**, a salt-fermented fish product of Thailand. Strain HPC1-2ᵀ was able to grow at 20–60 °C (optimum at 37–40 °C), at 2.6–5.1 M NaCl (optimum at 3.4–4.3 M NaCl) and at pH 5.0–8.0 (optimum at pH 7.0–7.5). Hypotonic treatment with less than 1.7 M NaCl caused cell lysis. The major polar lipids of the isolate were C₂₀C₂₀ derivatives of phosphatidylglycerol, methylated phosphatidylglycerol phosphate, phosphatidylglycerol sulfate, triglycosyl diether, sulfated triglycosyl diether and sulfated tetraglycosyl diether. The G+C content of the DNA was 65.5 mol%. 16S rRNA gene sequence analysis indicated that the isolate represented a member of the genus **Halobacterium** in the family **Halobacteriaceae**. Based on 16S rRNA gene sequence similarity, strain HPC1-2ᵀ was related most closely to **Halobacterium salinarum** DSM 3754ᵀ (99.2 %) and **Halobacterium jilantaiense** JCM 13558ᵀ (97.8 %). However, low levels of DNA–DNA relatedness suggested that strain HPC1-2ᵀ was genotypically different from these closely related type strains. Strain HPC1-2ᵀ could also be differentiated based on physiological and biochemical characteristics. Therefore, strain HPC1-2ᵀ is considered to represent a novel species of the genus **Halobacterium**, for which the name **Halobacterium piscisalsi** sp. nov. is proposed. The type strain is HPC1-2ᵀ (=BCC 24372ᵀ=JCM 14661ᵀ=PCU 302ᵀ).

**Abbreviations:** PG, phosphatidylglycerol; PGP-Me, methylated phosphatidylglycerol phosphate; PGS, phosphatidylglycerol sulfate; STeGD, sulfated tetraglycosyl diether; STGD, sulfated triglycosyl diether; TGD, triglycosyl diether.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HPC1-2ᵀ is AB285020.

Maximum-parsimony and maximum-likelihood phylogenetic trees showing the relationships between strain HPC1-2ᵀ and related archaeal species based on 16S rRNA gene sequences are available with the online version of this paper.
Strain HPC1-2T was isolated from pla-ra samples collected from local markets in Thailand. Samples were plated on agar plates of halophilic medium [comprising (per litre distilled water): 250 g NaCl, 5 g Casamino acids, 5 g yeast extract, 1 g sodium glutamate, 2 g KCl, 3 g trisodium citrate, 20 g MgSO4.7H2O, 0.036 g FeCl3.4H2O, 0.00036 g MnCl2.4H2O, 20 g agar (pH 7.2)] and incubated at 37 °C for 1–2 weeks. A pure culture was obtained by repeated transfers of separate colonies on agar plates of the same medium. Hbt. salinarum JCM 8978T and Hbt. jilantaiense JCM 13558T (=NG4T) were used as reference strains in all tests except DNA base composition. Unless otherwise stated, strains were grown in liquid (with shaking at 200 r.p.m.) or on agar plates of the halophilic medium and cultivated at 37 °C for 1–2 weeks.

Phenotypic tests were performed in accordance with the proposed minimal standards for the description of new taxa in the order Halobacteriales (Oren et al., 1997). Growth at various temperatures (20–60 °C) was examined. NaCl requirement was determined in the above medium containing various NaCl concentrations (0–5.1 M). Similarly, the requirement of the strains for Mg2+ was tested in halophilic medium lacking MgSO4.7H2O but supplemented with 0–1.0 M MgCl2. Growth was determined by measuring culture turbidity at 600 nm. Cell motility and morphology were examined by phase contrast and transmission electron microscopy of liquid cultures grown for 7 days. Gram staining was carried out as described by Dussault (1955). Colony morphology was observed by growth on agar plates of the halophilic medium after incubation at 37 °C for 7 days. Anaerobic growth was tested on agar plates in the presence of nitrate (1 g l−1), L-arginine (1 g l−1) or DMSO (10 g l−1). Catalase and oxidase activities and the hydrolysis of casein, gelatin, starch and Tween 80 were tested according to the methods of Barrow & Feltham (1993). Casamino acids were omitted from the test medium for determination of hydrolysis of casein and gelatin. Additional enzyme activities were determined by using API test kits (API ZYM and API 20E) at 37 °C as recommended by the manufacturer (bioMérieux). Utilization of sugars, alcohols, amino acids and organic acids, and acid production from various substrates were determined in modified Leifson medium supplemented with 0.01% (w/v) yeast extract and 4.3 M NaCl, but lacking casitone and Tris-HCl (Leifson, 1963). Nitrate reduction, H2S formation and indole production were tested as described by Oren et al. (1997). Tests for DNase, urease, methyl red, the Voges–Proskauer reaction, lysine and ornithine decarboxylases were performed as described by Gerhardt et al. (1981). Determination of antibiotic susceptibility was tested according to the methods of Stan-Lotter et al. (2002). Menaquinones were analysed as described by Komagata & Suzuki (1987). Polar lipids were determined according to the method of Minnikin et al. (1984).

DNA was isolated and purified according to the method of Saito & Miura (1963). The G+C content was determined with the method of Tamaoka & Komagata (1984) by using reversed-phase HPLC. DNA–DNA hybridization was performed as reported by Ezaki et al. (1989) and levels of relatedness were determined by using the colorimetric method given by Tanasupawat et al. (2000). The 16S rRNA gene sequence of strain HPC1-2T, comprising 1375 bp, was PCR-amplified with primers D30F (5′-ATTCCGG-TTGATCTCTGC-3′; positions 6–12 according to the Escherichia coli numbering system) and D56R (5′-CTTGTACGACTT-3′; positions 1492–1509). The amplified DNA fragment was separated by agarose gel electrophoresis and was recovered by using a GenElute Minus EtBr Spin Column (Sigma). The sequence was determined by using the BigDye Terminator Cycle Sequencing Ready Reaction kit version 3.0 (Applied Biosystems) in an ABI PRISM 310 genetic analyser (Applied Biosystems) with the following primers: D30F, D33R (5′-TCGCGGCCTGCGCCCGGT-3′; positions 344–360), D34R (5′-GCTCTGCTCCTGCGCCGT-3′; positions 1096–1113) and D56R. The sequence was compared with reference 16S rRNA gene sequences available in the GenBank and EMBL databases obtained from the National Center for Biotechnology Information database by using BLAST searches. The alignment was subjected to phylogenetic analysis with the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood and maximum-parimony methods by using programs in the CLUSTAL_X and MEGA 4 packages (Thompson et al., 1997; Tamura et al., 2007). Confidence in the branching pattern was assessed by analysis of 1000 bootstrap replicates.

Cells of strain HPC1-2T were motile rods (0.5–1.0 × 1.0–2.0 μm) that possessed peritrichous flagella (Fig. 1). On halophilic agar medium, colonies of strain HPC1-2T were circular, smooth, translucent and red. Cell lysis occurred in 1.7 M NaCl solution. Cells stained Gram-negative. Strain HPC1-2T was capable of growing over a wide concentration range of NaCl, from 0.5 to 5.1 M. Under these conditions, the salt concentration optimal for growth was 3.0 M NaCl. Strain HPC1-2T was capable of using nitrate as the sole source of nitrogen, but the addition of nitrate to the medium was found to be detrimental to growth. The production of H2S was observed by growth on agar plates of the halophilic medium containing 0.01% (w/v) yeast extract and 4.3 M NaCl, but lacking casitone and Tris-HCl (Leifson, 1963). Nitrate reduction, H2S formation and indole production were tested as described by Oren et al. (1997). Tests for DNase, urease, methyl red, the Voges–Proskauer reaction, lysine and ornithine decarboxylases were performed as described by Gerhardt et al. (1981). Determination of antibiotic susceptibility was tested according to the methods of Stan-Lotter et al. (2002). Menaquinones were analysed as described by Komagata & Suzuki (1987). Polar lipids were determined according to the method of Minnikin et al. (1984).

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range of NaCl from 2.6 to 5.1 M. It grew optimally in the presence of 3.4–4.3 M NaCl, similar to most extremely halophilic Archaea (Grant, 2001). It also grew over a wide range of MgCl₂ concentrations from 0 to 1 M, with optimum growth at about 0.5–0.6 M. Strain HPC1-2T grew in the temperature range 20–60 °C (optimum 37–40 °C) and pH range 5.0–8.0 (optimum pH 7.0–7.5). Catalase and oxidase activity tests were positive. No indole formation was observed in the presence of tryptophan. Cells hydrolysed gelatin, casein and Tween 80, but not starch. Strain HPC1-2T showed anaerobic growth in the presence of DMSO, nitrate and L-arginine. Nitrate was not reduced and no gas formation was observed.

Two-dimensional TLC revealed that strain HPC1-2T possessed C₂₀C₂₀ derivatives of phosphatidylglycerol (PG), methylated phosphatidylglycerol phosphate (PGP-Me), phosphatidylglycerol sulfate (PGS), triglycosyl diether (TGD), sulfated triglycosyl diether (S-TGD) and sulfated tetraglycosyl diether (S-TeGD) and pigments. The overall polar lipid pattern was similar to those of *Hbt. salinarum* DSM 3754T (Ventosa & Oren, 1996) and *Hbt. jilantaiense* JCM 13558T (Yang et al., 2006). The presence of S-TGD can be used to distinguish the above three taxa from *Hbt. noricense* (Gruber et al., 2004). Strain HPC1-2T had menaquinones MK-8 (98 %) and MK-8 (H₂) (2 %). The DNA G+C content of strain HPC1-2T was 65.5 mol%, which was similar to that of

Table 1. Differential characteristics between strain HPC1-2T and recognized *Halobacterium* species

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>4</th>
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<tr>
<td>Cell morphology</td>
<td>Rods</td>
<td>Small rods</td>
<td>Rods</td>
<td>Irregular rods</td>
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<tr>
<td>Pigmentation</td>
<td>Red</td>
<td>Red</td>
<td>Red</td>
<td>Light red</td>
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<td>pH range for growth (M)</td>
<td>4.5–8.5</td>
<td>5.0–8.5</td>
<td>5.0–8.0</td>
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<td>Temperature range for growth (°C)</td>
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<td>20–60</td>
<td>20–60</td>
<td>28–50</td>
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<tr>
<td>Optimum NaCl concentration for growth (M)</td>
<td>3.4–4.3</td>
<td>3.4–4.3</td>
<td>3.4–4.3</td>
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<tr>
<td>Optimum MgCl₂ concentration for growth (M)</td>
<td>0.5–0.6</td>
<td>0.5–0.6</td>
<td>0.5–0.6</td>
<td>0.6–0.9</td>
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<tr>
<td>NaCl concentration (M) required to prevent cell lysis</td>
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<td>&gt;1.7</td>
<td>&gt;2.1</td>
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<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>−</td>
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<td>Hydrolysis of:</td>
<td></td>
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<tr>
<td>Casein</td>
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<td>+</td>
<td>+</td>
<td>ND</td>
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<tr>
<td>Gelatin</td>
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<td>+</td>
<td>+</td>
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<td>Tween 80</td>
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<td>Acid production from glycerol</td>
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<td>Citrate</td>
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<td>D-Xylose</td>
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<td>Enzyme assay (API ZYM)</td>
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<td>Acid phosphatase</td>
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<td>ND</td>
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<td>Leucine arylamidase</td>
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<td>Naphthol-AS-BI-phosphohydrolase</td>
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<td>+</td>
<td>+</td>
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<tr>
<td>Valine arylamidase</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>65.5</td>
<td>67.1–71.2*</td>
<td>64.2†</td>
<td>54.5</td>
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</table>

*Data from Grant (2001).
†Data from Yang et al. (2006).
Cells are rod-shaped (0.5–1.0 × 1.0–2.0 μm) and motile by means of peritrichous flagella. Cells stain Gram-negative and are chemo-organotrophic. Colonies on plates of halophilic agar medium are red, smooth, circular and elevated. Requires at least 2.6 M NaCl for growth; optimal growth occurs at 3.4–4.3 M NaCl. Cell lysis occurs upon hypotonic treatment with less than 1.7 M NaCl. Growth occurs at 0–1 M MgCl₂, optimally at around 0.5–0.6 M MgCl₂. The temperature and pH ranges for growth are 20–60°C (optimum at 37–40°C) and 5.0–8.0 (optimum at pH 7.0–7.5). Grows anaerobically in the presence of nitrate, L-arginine and DMSO. Catalase- and oxidase-positive. Nitrate and nitrite are not reduced. Methyl red, Voges-Proskauer reaction and indole production are also negative. Casein, gelatin and Tween 80 are hydrolysed, but starch is not. The following substrates are utilized for growth: lactose, melezitose, glycerol and citrate. Acid is produced from glycerol. Does not utilize L-arabinose, celllobiose, D-fructose, D-galactose, D-glucose, myo-inositol, inulin, maltose, D-mannitol, D-mannose, melibiose, raffinose, rhamnose, D-ribose, salicin, sorbitol, sucrose, trehalose, D-xylene, acetate, L-glycine, L-alanine, L-arginine, L-aspartate, L-glutamate or L-lysine. Produces alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase and naphthol-AS-BI-phosphohydrolase, but not leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, lysine decarboxylase, ornithine decarboxylase, urease or DNase. Sensitive to bacitracin (10 U), neomycin (30 μg).

**Description of Halobacterium piscisalsi sp. nov.**

*Halobacterium piscisalsi* (pis. ci. sal. ’si. L. n. piscis fish; L. adj. salsus salted, salt; N.L. gen. n. piscisalsi of salted fish).
and novobiocin (5 µg), but not to ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), rifampicin (30 µg), streptomycin (10 µg) or tetracycline (30 µg). The major polar lipids are C20:0-C26:0 derivatives of PG, PGP-Me, PGS, TGD, S-TGD and S-TeGD. The predominant menaquinone is MK-8. The G+C content of the DNA is 65.5 mol%.

The type strain, HPC1-2T (=BCC 24372T=JCM 14661T=PCU 302T), was isolated from pla-raft (salt-fermented fish product) in Thailand.

**Acknowledgements**

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**References**


