Haloplanus salinarum sp. nov., an extremely halophilic archaeon isolated from a solar saltern

Han-Bit Hwang,¹ Ye-Eun Kim,¹ Hyeon-Woo Koh,¹ Hye Seon Song,² Seong Woon Roh,² So-Jeong Kim,³ Seung Won Nam⁴ and Soo-Je Park¹,*

Abstract

An extremely halophilic archaeal strain SP28ᵀ was isolated from the Gomso solar saltern, Republic of Korea. Cells of the new strain SP28ᵀ were pleomorphic and Gram stain negative, and produced red-pigmented colonies. These grew in medium with 2.5–4.5 M NaCl (optimum 3.1 M) and 0.05–0.5 M MgCl₂ (optimum 0.1 M), at 25–50 °C (optimum 37 °C) and at a pH of 6.5–8.5 (optimum pH 8.0). Mg²⁺ was required for growth. A concentration of at least 2 M NaCl was required to prevent cell lysis. Polar lipids included phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and one glycolipid chromatographically identical to sulfated mannosyl glucosyl diether. 16S rRNA and rpoB gene sequence analyses showed that strain SP28ᵀ is closely related to Haloplanus ruber R35ᵀ (97.3 and 94.1 %), 16S rRNA and rpoB gene sequence similarity, respectively), Haloplanus litoreus GX21ᵀ (97.0 and 92.1 %), Haloplanus salinus YGH66ᵀ (96.0 and 91.9 %), Haloplanus vescus R05-8ᵀ (95.9 and 90.9 %), Haloplanus aerogenes TBN37ᵀ (95.6 and 90.3 %) and Haloplanus natans RE-101ᵀ (95.3 and 89.8 %). The DNA G+C content of the novel strain SP28ᵀ was 66.2 mol%, which is slightly higher than that of Hpn. litoreus GX21ᵀ (65.8 mol%) and Hpn. ruber R35ᵀ (66.0 mol%). DNA–DNA hybridization values between Hpn. ruber R35ᵀ and strain SP28ᵀ and between Hpn. litoreus GX21ᵀ and strain SP28ᵀ were about 24.8 and 20.7 %, respectively. We conclude that strain SP28ᵀ represents a novel species of the genus Haloplanus and propose the name Haloplanus salinarum sp. nov. The type strain is SP28ᵀ (=JCM 31424ᵀ=KCCM 43210ᵀ).

The genus Haloplanus was first proposed by Bardavid et al. [1] to accommodate the species Haloplanus natans, which was described based on three flat-shaped strains isolated from an experimental mesocosm filled with a mixture of water from the Dead Sea and the Red Sea, Israel [1]. In the following years, five novel species isolated from diverse environments (http://www.bacterio.net) were added to the genus Haloplanus. Haloplanus vescus was isolated from a solar saltern located in Jiangsu Province, P.R. China [2]. Haloplanus aerogenes was isolated from a marine solar saltern near Lianyungang city in Jiangsu Province, P.R. China [3]. Haloplanus salinus was isolated from a marine solar saltern near Sanya city in Hainan Province, P.R. China [4]. Haloplanus litoreus and Haloplanus ruber were isolated from a solar saltern and an aquaculture farm in P.R. China [5]. The most interesting characteristics of members of the genus Haloplanus is that their cells are pleomorphic and flat, Gram stain negative, and strictly aerobic, and in a static liquid medium usually float to the surface because of the presence of gas vesicles. The polar lipid composition of Haloplanus species includes phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and a glycolipid chromatographically identical to sulfated mannosyl glucosyl diether [2]. During our surveys of halophilic archaeal diversity in the Gomso solar saltern on the west coast of Korea, we found an extremely halophilic archaean. In this paper, we characterize this strain SP28ᵀ as a novel species of the genus Haloplanus.

Strain SP28ᵀ was isolated from brine samples from the Gomso solar saltern located in Gomso-ri, Buan, Republic of Korea (35° 35′ N, 126° 36′ E). The brine had a pH of 7.8, temperature of 35 °C and total salinity of 17–23 % (data from [6]). The brine samples were serially diluted and inoculated onto modified-SG medium [7], which contained the following ingredients (per litre): 7.5 g Casamino acids, 10 g yeast extract, 3 g trisodium citrate, 20 g MgSO₄·7H₂O, 2 g KCl,
0.05 g FeSO₄·7H₂O, 200 g NaCl and 0.1 g NH₄Cl. The pH was adjusted to 7.5 with 1 M NaOH solution. After three subcultures, the enrichment culture was plated onto modified-SG agar plates [containing 1.6 % (w/v) agar]. The plates were incubated in the dark at 37 °C for at least 2 weeks under aerobic conditions. A reddish colony was picked and streaked at least three times onto the same medium for purification; a single colony was subsequently designated strain SP28ᵀ.

Genomic DNA of halophilic archaeal strain SP28ᵀ was extracted using a commercial genomic DNA extraction kit (GeneAll). The 16S rRNA gene of strain SP28ᵀ was amplified by PCR from chromosomal DNA using a universal archaeal primer set: forward primer 20F (5'TTCCGTTGATC-CYGCCRG-3') and reverse primer 1492R (5'TACGGY-TACCTTGTAGCTT-3') [8]. PCR was performed in a thermal cycler (Bio-Rad T100) for 35 cycles (7 min denaturing at 96 °C in the first cycle; 30 s denaturing at 96 °C, 30 s annealing at 55 °C and 2 min elongation at 72 °C; final extension at 72 °C for 7 min). The PCR product was purified with a PCR purification kit (CosmoGenetech) and purified PCR products were sequenced by Macrogen using primers 20F, 340F, 958R and 1492R [8, 9]. The RNA polymerase subunit beta (rpoB) gene was amplified according to the method of Minegishi et al. [10]. The 16S rRNA and rpoB gene sequences of strain SP28ᵀ (1490 and 1827 bp, respectively) were obtained by assembling sequences using the Seqman program (DNASTAR). The 16S rRNA and rpoB gene sequences of related species were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov). 16S rRNA gene sequence similarities were calculated using the online EzBioCloud server [11], while the rpoB gene sequence similarity was calculated using MEGA6 [12].

Also, complete genome analysis revealed that strain SP28ᵀ has two copies of the 16S rRNA gene (Kim Y-E, Kim S-J, Park S-J, in preparation). The similarity of the two sequences is 99.5%. Phylogenetic trees were reconstructed based on the neighbour-joining [13], maximum-likelihood [14] and maximum-parsimony [15] methods using MEGA6 [12] with 1000 bootstrap replications for each. Based on the bootstrap results of these phylogenetic analyses (Fig. 1a), strain SP28ᵀ belonged to the genus Haloplanus in the family Halofacaceae within the order Halofacales [16]. Strain SP28ᵀ is related most closely to *Hpn. ruber* R35ᵀ (97.3 % 16S rRNA gene sequence similarity), *Hpn. litoreus* GX21ᵀ (97.0 %), *Hpn. salinus* YGH66ᵀ (96.0 %), *Hpn. vescus* RO5-8ᵀ (95.9 %), *Hpn. aerogenes* TBN37ᵀ (95.6 %) and *Hpn. natans* RE-101ᵀ (95.3 %). The similarities of the SP28ᵀ rpoB gene sequence to those of *Hpn. ruber* R35ᵀ, *Hpn. litoreus* GX21ᵀ, *Hpn. natans* RE-101ᵀ, *Hpn. vescus* RO5-8ᵀ, *Hpn. salinus* YGH66ᵀ and *Hpn. aerogenes* TBN37ᵀ were 94.1, 92.1, 91.9, 90.9, 90.3 and 89.8 %, respectively. Moreover, the identity of the RpoB amino acid sequence of strain SP28ᵀ ranged from 92.9 to 98.5 %. This result was also supported by the position of strain SP28ᵀ in the genus *Haloplanus* (Fig. 1b).

Based on their 16S rRNA and rpoB gene sequence similarities and phylogenetic analyses, the two type strains *Hpn. litoreus* JCM 17092ᵀ and *Hpn. ruber* JCM 17271ᵀ were selected as reference strains. Unless otherwise stated, strain SP28ᵀ and the reference strains were cultured using DBCM2 agar plates (JCM medium no. 574; containing per litre, 833 ml concentrated salt water (30 %, w/v), 1 ml FeCl₃ solution, 1 ml trace element solution, 0.25 g peptone (Oxoid), 0.05 g yeast extract (Difco), 5 ml 1 M NH₄Cl, 2 ml potassium phosphate buffer, 3 ml vitamin solution, 10 ml 1 M sodium pyruvate solution, 1.6 % (w/v) agar) in the dark at 37 °C.

Phenotypic characterizations were performed according to the proposed minimal standards for the description of new taxa in the order Halobacteriales [17]. Genomic DNA was used to determine the G+C content after the removal of RNA by incubation with a mixture of RNase A and T1 (20 U ml⁻¹ each) at 30 °C for 1 h as described by Gonzalez and Saiz-Jimenez [18]. DNA–DNA hybridization (DDH) experiments were performed with SP28ᵀ and the reference strains. DDH was performed in quintuplicate using the fluorometric method with photobiotin-labelled DNA probes [19]. Mean values from three independent DDH experiments were obtained. Polar lipids were extracted from freeze-dried cells [100 mg, early stationary-phase cultures (120 h post-incubation at 37 °C, pH 7.5)] from each strain using the chloroform and methanol method and analysed as described by Komagata and Suzuki [20]. The extracted lipids were separated by TLC on a silica gel glass plate [21] and detected using 10 % ethanolic molybdophosphoric acid for total lipid, α-naphthol-sulfuric acid for glycolipid, and molybdenum blue reagent for phospholipid [22].

The G+C content of the genomic DNA of strain SP28ᵀ was 66.2 mol%, a value that fell within the range of other species of *Haloplanus* (Table 1). The level of DNA–DNA relatedness of strain SP28ᵀ to *Hpn. ruber* JCM 17271ᵀ was about 24.8 %, while it was about 20.7 % to *Hpn. litoreus* JCM 17092ᵀ. The major polar lipids of strain SP28ᵀ were phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and one glycolipid chromatographically identical to sulfated mannosyl glucosyl diether (Fig. S1, available in the online Supplementary Material). This pattern is chromatographically similar to the polar lipid profiles of *Hpn. litoreus* JCM 17092ᵀ, *Hpn. ruber* JCM 17271ᵀ and *Hpn. natans* JCM 14081ᵀ, and supports the classification of strain SP28ᵀ in the genus *Haloplanus*.

Gram staining was performed as described by Dussault [23]. Cell morphology, cell size and presence of flagella were observed using transmission and scanning electron microscopy (Tecnai G2 Sprite; FEI) at the Korea Basic Science Institute in South Korea. Cell motility was examined by the modified semi-solid agar method (supplemented with 3.4 M sodium chloride) [24]. The minimum salt concentration to prevent cell lysis was determined by suspending washed cells in sterile saline solutions containing a range of NaCl concentrations, from 0 to 5 M. Cell stability was then detected by light microscopy. Optimal growth conditions and growth ranges were determined using modified-SG medium as a basal medium. The NaCl concentrations used were 1.5, 2.0, 2.5, 3.0,
3.1, 3.2, 3.3, 3.4, 3.5, 4.0, 4.5 and 5.0 M. The temperature range for cell growth was assessed at 5, 10, 15, 20, 25, 30, 37, 45, 50 and 55 °C in medium with the optimal NaCl concentration (3.4 M). The response to pH was determined in modified-SG medium adjusted to pH 4.5–10 at intervals of 0.5 pH units using the following buffers: 10 mM Homo-PIPES (pH 4.5–5.0), 10 mM MES (pH 5.0–6.5), 10 mM Bis-Tris propane (pH 7.0–8.5) and 10 mM CAPS (pH 9.0–10.0). The requirement for magnesium for growth was determined using modified-SG medium without MgSO₄·7H₂O and then adding MgSO₄·7H₂O at concentrations of 0, 0.05, 0.1, 0.2, 0.3 and 0.5 M. Anaerobic growth with nitrate, l-arginine and DMSO (all at 5 g l⁻¹) was tested using a 20 ml stoppered tube filled with liquid medium and incubated in the dark at 37 °C with shaking.

Fig. 1. Phylogenetic tree based on (a) 16S rRNA gene and (b) RpoB sequences, showing the positions of strain SP28ᵀ, species of the genus Haloplanus and representatives of related genera. Outgroups in each tree were (a) Natronoarchaeum rubrum GX48ᵀ and (b) Natronoarchaeum philippinense 294-194-5ᵀ. Bootstrap values of >60 % (based on 1000 replicates) were assigned to nodes based on neighbour-joining, maximum likelihood, and maximum parsimony methods. GenBank accession numbers are shown in parentheses. Bars: (a) 0.01 and (b) 0.02 substitutions per nucleotide position.
Table 1. Differential characteristics of strain SP28<sup>T</sup> and closely related species in the genus Haloplanus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt; required</td>
<td>+</td>
<td>–</td>
<td>+*</td>
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<tr>
<td>Anaerobic growth with arginine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Temperature range (°C)</td>
<td>25–50</td>
<td>25–45&lt;sup&gt;*&lt;/sup&gt;</td>
<td>25–50&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>Optimum (°C)</td>
<td>37</td>
<td>37&lt;sup&gt;*&lt;/sup&gt;</td>
<td>37&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>pH range</td>
<td>6.5–8.5</td>
<td>5.5–9.5&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6.0–8.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Optimum</td>
<td>8.0</td>
<td>6.5–7.0&lt;sup&gt;*&lt;/sup&gt;</td>
<td>6.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>NaCl range (M)</td>
<td>2.5–4.5</td>
<td>2.1–4.8&lt;sup&gt;*&lt;/sup&gt;</td>
<td>2.6–4.8&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>Optimum (M)</td>
<td>3.1</td>
<td>3.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.4&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt; range (M)</td>
<td>0.05–0.5</td>
<td>0–0.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.05–1.0&lt;sup&gt;*&lt;/sup&gt;</td>
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<tr>
<td>Optimum (M)</td>
<td>0.1</td>
<td>0.03&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.1&lt;sup&gt;*&lt;/sup&gt;</td>
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Utilization of (as sole carbon and energy source)

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<th>Sugar</th>
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<th>3</th>
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<tbody>
<tr>
<td>Maltose</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Succinate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DL-Malate</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Propionate</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Glycine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>DL-Alanine</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>L-Aspartate</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>L-Lysine</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>L-Serine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

H<sub>2</sub>S formation: +, Positive; –, Negative.

DNA G+C content (mol%): 66.2, 66.0<sup>*</sup>, 65.8<sup>*</sup>

*Data from Han and Cui [5].

for 4 weeks [25]. Anaerobic growth was then determined by turbidity [17]. The tests for activity of catalase, oxidase and urease, indole production, and the hydrolysis of casein, DNA and starch were conducted as described by Benson [26] using DBCM2 as the basal medium. The hydrolysis of Tween 20, 40 and 80 was tested as described by Gonzalez et al. [29]. All strains were Gram stain negative, and catalase and oxidase positive, produced red-pigmented colonies, were observed to lyse in distilled water, and were motile and pleomorphic. None could utilize DMSO or nitrate as electron acceptors under anaerobic conditions. All strains were negative for nitrate and nitrite reduction, and activity for urease, alkaline phosphatase, lysine and ornithine decarboxylases, hydrolysis of casein, gelatin, starch, DNA and Tween 20, 40 and 80, the formation of indole, and the utilization of D-fructose, adonitol, D-galactose, xylitol, D-glucose, raffinose, L-arginine and L-tryptophan as sole sources of carbon and energy. +, Positive; –, Negative.

was determined by the method of Gutiérrez et al. [30] on DBCM2 agar plates with antimicrobial compound discs incubated for 4 weeks in the dark at 37°C.

Cells of strain SP28<sup>T</sup> were motile and pleomorphic: both coccoid (diameter 0.5–0.9 µm) and rod-shaped (Fig. S2a, b; 0.6–2.3 µm in width and 0.6–3.5 µm in length by scanning and transmission electron microscopy, respectively). The gas vesicle was not observed by bright-field and differential interference contrast microscopy (ECLIPSE Ni-U; Nikon) under static liquid cultivation (Fig. S2c). The cells stained Gram negative, and their colonies were red-pigmented, smooth and round after incubation for 1 week on DBCM2 agar medium. The cell size of strain SP28<sup>T</sup> was observed to lyse in distilled water and at concentrations below 2 M NaCl. They were unable to grow under anaerobic conditions with nitrate, L-arginine and DMSO. Cells did not produce H<sub>2</sub>S from sodium thiosulfate, nor did they produce indole. Also, they did not hydrolyse casein, DNA, starch, gelatin or Tween 20, 40 or 80. Cells were positive for catalase and oxidase activities, but were negative for urease, alkaline phosphatase, and lysine and ornithine decarboxylases. Strain SP28<sup>T</sup> used maltose, succinate, D-xylene, propionate, DL-alanine and L-lysine as single carbon and energy sources. However, D-fructose, adonitol, D-galactose, xylitol, D-glucose, raffinose, sucrose, DL-malate, L-arginine, L-glycine, L-aspartate, L-serine, L-glutamine and L-tryptophan were not utilized. Acid was not produced from D-glucose, D-galactose, D-fructose or maltose. Cells were sensitive to the antimicrobials novobiocin (30 µg), anisomycin (30 µg), erythromycin (10 µg) and bacitracin (0.04 IU per disc), but resistant to streptomycin (10 µg), penicillin (10 IU per disc), tetracycline (30 µg), gentamicin (10 µg), ampicillin (10 µg), sulfamethoxazole/trimetoprim (23.75/1.25 µg), neomycin (30 µg), kanamycin (30 µg), chloramphenicol (10 µg) and rifampicin (10 µg). More detailed results for strain SP28<sup>T</sup> are presented in the species description and in a comparison with related species of the genus Haloplanus presented in Table 1.

On the basis of phylogenetic analysis, phenotypic characteristics and polar lipid composition, we propose that the isolated strain SP28<sup>T</sup> represents a novel species of the genus Haloplanus and suggest the name Haloplanus salinarum sp. nov.

**DESCRIPTION OF HALOPLANUS SALINARUM SP. NOV.**

*Haloplanus salinarum* (sa.li.na’rum. L. gen. pl. n. salinarum, of salt-works).

Cells are motile and pleomorphic (short-rod- and oval-shaped; 0.6–2.3 µm in width and 0.6–3.5 µm in length) under optimum growth conditions, and stain Gram negative. Cells are catalase and oxidase positive, but urease, alkaline phosphatase, lysine and ornithine decarboxylase negative. Growth...
occurs in 2.5–4.5 M NaCl (optimum 3.1 M), at 25–50°C (optimum 37°C), pH 6.5–8.5 (optimum pH 8.0) and with 0.05–0.5 M MgCl₂ (optimum 0.1 M). Colonies are red-pigmented, smooth and round. Cell lysis occurs in distilled water, requiring a minimal NaCl concentration of 2 M to prevent cell lysis. Under anaerobic conditions, H₂S is not produced from sodium thiosulfate and indole is not produced from tryptophan. Cells do not hydrolyse DNA, casein, gelatin, starch or Tween 20, 40 or 80. The following substrates are utilized as single carbon and energy sources for growth: maltose, succinate, D-xylene, propionate, DL-alanine and L-lysine. The following compounds are not used: D-fructose, adonitol, D-galactose, xyitol, D-glucose, raffinose, sucrose, DL-malate, L-arginine, L-glycine, L-aspartate, L-serine, L-glutamine and L-tryptophan. No acid is produced from D-glucose, D-galactose, D-fructose or maltose. The polar lipids are phosphatidylglycerol, phosphatidylglycerol phosphate methyl ester, phosphatidylglycerol sulfate and one glycolipid that is not yet identified. 

The type strain is SP28T (=JCM 31424T=KCCM 43210T) and was isolated from the Gosmo solar saltern located in Gomso-ri, Buan, Republic of Korea. The DNA G+C content of the type strain is 66.2 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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