Salipaludibacillus halalkaliphilus sp. nov., a moderately haloalkaliphilic bacterium from a coastal-marine wetland

Mohammad Ali Amoozegar, Azadeh Shahinpei, Somaye Makzum, Shokufeh Rafiyan, Mahdi Moshtagh Nikou, Cathrin Spröer and Antonio Ventosa

Abstract

A Gram-stain-positive, endospore-forming rod-shaped non-motile, moderately halophilic and alkaliphilic bacterium, strain GASy1T, was isolated from a water sample from Gomishan, a marine wetland in Iran. GASy1T required at least 0.5 % (w/v) NaCl for growth and was able to grow at NaCl concentrations of up to 15 % (w/v), with optimum growth occurring at 5 % (w/v) NaCl. The optimum pH for growth was pH 8.5–9.0 and 30 °C, respectively, while it was able to grow over a pH range and a temperature range of 7.5–10.0 and 4–40 °C, respectively. GASy1T was catalase-positive and oxidase-negative. Analysis of 16S rRNA gene sequences revealed that GASy1T represents a member of the genus Salipaludibacillus, family Bacillaceae within the order Bacillales, showing 97.4 % sequence similarity to Salipaludibacillus neizhouensis DSM 071004T, and 96.2 and 95.7 % sequence similarity to Salipaludibacillus agaradhaerens AC 13T and Salipaludibacillus aurantiacus S9T, respectively. The DNA G+C content of GASy1T was 38.8 mol%. The polar lipids of the strain were phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and two unidentified phospholipids and its major cellular fatty acids were anteiso-C15:0, C16:0 and iso-C15:0. The isoprenoid quinone was MK-7. DNA–DNA hybridization experiments revealed a low level of relatedness between GASy1T and Salipaludibacillus neizhouensis IBRC-M 10892T (18 %). On the basis of a combination of phenotypic, chemotaxonomic and phylogenetic features, GASy1T represents a novel species of the genus Salipaludibacillus, for which the name Salipaludibacillus halalkaliphilus sp. nov. is proposed. The type strain of Salipaludibacillus halalkaliphilus is GASy1T (=IBRC M 10902T=LMG 28385T).

The genus Salipaludibacillus, a member of the family Bacillaceae within the order Bacillales, class Bacilli was proposed by Sultanpuram and Mothe [1] for Gram-stain-positive, endospore-forming rod-shaped organisms with MK-7 as the predominant respiratory quinone. They are halotolerant to moderately halophilic, alkalitolerant to alkaliphilic and their cell wall contains meso-diaminopimelic acid. The G+C content of their DNA is 39.3–42.4 mol% and the major fatty acids are anteiso-C15:0, C16:0 and iso-C15:0. At the time of writing, this genus comprises three species with validly published names: Salipaludibacillus aurantiacus (type species), isolated from a salt lake in India [1], Salipaludibacillus neizhouensis, isolated from sea anemone in the South China Sea [2] and Salipaludibacillus agaradhaerens, isolated from soil [3].

Here we describe the taxonomic properties of a halophilic and alkaliphilic bacterial strain, designated GASy1T, which was isolated from Gomishan wetland (37° 04′ 06.3″ N 54° 00′ 24.5″ E), located in the north of Iran and we propose that it represents a novel species of the genus Salipaludibacillus. The Gomishan wetland is an alkaline thassohaline, coastal–marine wetland with 3–5 % salinity and a pH of about 9.0 by the eastern shore of the Caspian Sea. In our previous studies on microbial diversity of this coastal-marine wetland, five novel taxa of Gram-stain-negative bacteria have been isolated [4–8].

The novel strain was isolated by diluting a soil sample taken from the Gomishan wetland, in sterile 3.0 % (w/v) salt solution and plating on modified alkaliphilic halophile agar (MAHA) with 5 % (w/v) total salts. This medium contains NaCl, 30.0; peptone, 5.0; yeast extract, 2.0; meat extract, 1.0; tri-sodium citrate, 0.12; KCl, 0.08; MgSO4.7H2O, 0.04; FeSO4.7H2O, 2.0 mg; MnCl2.4H2O, 0.36 mg [9]. Sodium sesquicarbonate solution ([g l−1] Na2CO3, 10.6; and

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Abbreviations: HPLC, high-performance liquid chromatography; MIDI, microbial identification system.

One supplementary table and three supplementary figures are available with the online version of this article.
The phylogenetic position was also confirmed in trees generated using the EzBiocloud sequence similarity were achieved using the CLUSTAL-X software package. Clustering was performed using three different methods, the neighbour-joining [16] the minimum-evolution [17] and the maximum-likelihood [18, 19] algorithms. Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by performing 1000 resamplings [20].

An almost complete 16S rRNA gene sequence (1494 nt) of GASy1 was obtained. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzBiocloud server (https://www.ezbiocloud.net/) [21]. The 16S rRNA gene sequence phylogenetic analysis using the neighbour-joining algorithm clearly indicated that the position of GASy1 was within the genus Salipaludibacillus (Fig. 1). The phylogenetic position was also confirmed in trees generated using the minimum-evolution and maximum-likelihood algorithms (see Figs S1 and S2, available in the online version of this article). The 16S rRNA gene sequence similarities revealed that GASy1 represents a member of the family Bacillaceae and the genus Salipaludibacillus; the similarities between GASy1 and closely related taxa; Salipaludibacillus neizhouensis JSM 071004 T, Salipaludibacillus agaradhaerens AC 13 T and Salipaludibacillus aurantiacus S9 T were 97.4, 96.2 and 95.7 %, respectively.

Cell morphology and motility were examined using a BX51 microscope (Olympus) equipped with phase-contrast optics using cells from exponentially growing cultures. Cell morphology was observed on agar medium under optimal growth conditions after incubation at 30 °C for two days. GASy1T was non-motile, rod-shaped and stained Gram-positive. The strain also formed central or sub-terminal endospores (Fig. S3). Colonies formed on agar plates were circular with entire margins, convex and smooth, translucent, small and cream-pigmented.

Physiological and biochemical tests were conducted using liquid or solid medium as mentioned above, unless stated otherwise. Liquid cultures were incubated at 30 °C on a shaking incubator at 150 r.p.m. Growth rates were determined by monitoring the increase in OD_{600}. Growth temperature range was examined in liquid 5 % MH medium at temperatures 0, 4, 10, 15, 20, 25, 30, 35, 40, 45 and 50 °C. For growth at different pH values a range of 5.0–11.0 was tested; the buffers sodium acetate/acetate acid (pH 5–6.0), Tris/HCl (pH 6.5–8.5) and glycine/sodium hydroxide (pH 9–11) were added at a concentration of 50 mM. The requirements for NaCl for growth were determined in media containing 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.5, 10.0, 12.5, 15.0 and 20.0 % (w/v) NaCl.

GASy1T grew at a temperature range of 4–40 °C (optimum 30 °C) and a pH range of 7.5–10 (optimum pH 8.5–9.0). The strain was capable of growing over a wide range of NaCl concentrations [0.5 to 15 % (w/v)]. It grew optimally in the presence of 5 % (w/v) NaCl. This strain was a moderately halophilic and alkaliphilic bacterium.

Acid production from substrates was tested in unbuffered medium and was determined by measuring the initial and final pH of the medium. The culture was considered positive for acid production if the pH decreased by at least 1 unit. Tests for the utilization of carbon sources, were performed as recommended by Ventosa et al. [10]. The anaerobic growth of GASy1T was tested in the presence of nitrate by adding 0.1 % (w/v) KNO_{3} to the medium with 5 % (w/v) NaCl in filled stoppered tubes in an anaerobic chamber [23]. Catalase and oxidase, nitrate reduction, hydrolysis of aesculin, production of indole and H_{2}S were tested as recommended by Smibert and Krieg [24], using media with 5 % (w/v) NaCl. Tween hydrolysis activity was detected as described by Gutiérrez and González [25]. Hydrolysis of gelatin, casein, starch, urease and DNase activity were examined as described by Mata et al. [26]. Antimicrobial susceptibility tests were performed on Mueller–Hinton agar plus 5 % (w/v) sea salts [10] seeded with a bacterial suspension containing 1.5 × 10^{6} c.f.u. ml^{-1} using discs (HiMedia) impregnated with various antimicrobial compounds. The plates were incubated at 30 °C for 48 h and the inhibition zone was interpreted according to the manufacturer’s recommendations.
manual. Other physiological and biochemical tests were performed as described previously [10, 26, 27].

GASy1<sup>T</sup> was catalase-positive, oxidase-negative and facultatively anaerobic. The strain was sensitive to ampicillin (10 µg), bacitracin (10 µg), cephalothin (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), neomycin (30 µg), novobiocin (5 µg), nitrofurantoin (300 µg), penicillin G (10 U), polymyxin B (300 U) and streptomycin (10 µg) but resistant to erythromycin (15 µg), kanamycin (5 µg) and nalidixic acid (30 µg). The detailed physiological and biochemical characteristics of GASy1<sup>T</sup> are listed in Table 1 and in the species description.

For determination of the DNA base composition, cells were disrupted by using a Constant Systems TS 0.75 kW (IUL Instruments) and the DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. [28]. The DNA G+C content was determined by the HPLC method [29]. The DNA G+C content of GASy1<sup>T</sup> was 38.8 mol%, which is slightly lower than the range described for members of the genus Salipaludibacillus (39.3–42.4 mol%) [1–3]. DNA–DNA hybridization was carried out as described by De Ley et al. [30] with the modifications described by Huss et al. [31] using a Cary 100 Bio UV/VIS spectrophotometer fitted with a Peltier-thermostat-equipped 6 × 6 multicell changer and a temperature controller with an in situ temperature probe (Varian). The DNA–DNA reassociation value of the novel strain to Salipaludibacillus neizhouensis IBRC-M 10892<sup>T</sup> was 18 %. According to the 70 % threshold proposed by Wayne et al.
[32] for the delineation of species using DNA–DNA relatedness, the results confirmed that the isolate represented a novel species, so the value is low enough to differentiate GASy1T from its closest relative Salipaludibacillus neizhouensis IBRC-M 10892T. Cell biomass for cell wall peptidoglycan, isoprenoid quinone, polar lipids and fatty acids analyses was obtained by cultivation on 5% MH medium at pH 8.5 and 30°C. Cells were harvested during the mid-exponential growth phase. Preparation and hydrolysis of the cell-wall was carried out using the method described by Schleifer [33] and the interpeptide bridge in the cell-wall peptidoglycan was analysed by using the method described by Schleifer and Kandler [34]. Cell-wall hydrolysates were separated by one- or two-dimensional chromatography on cellulose thin-layer plates (Merck). GASy1T contained meso-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan which is in accordance with the results reported for members of the genus Salipaludibacillus [35].

The polar lipids and respiratory quinones of the type strain were analyzed as described by Groth et al. [36]. Phosphatidylglycerol, diphasphatidylglycerol, phosphatidylethanolamine and two unknown phospholipids were present in GASy1T (Fig. 2). The polar lipid pattern is similar to that of other species of the genus Salipaludibacillus in that phosphatidylglycerol, diphasphatidylglycerol and phosphatidylethanolamine are the major polar lipids. However, the polar lipid profile of the novel strain was more similar to that of Salipaludibacillus neizhouensis JSM 071004T, which also contained unidentified phospholipids [1–3]. MK-7 was the only respiratory quinone present in GASy1T, in contrast to the other members of the genus Salipaludibacillus, which contain MK-7 as the major isoprenoid quinone and MK-6 as a minor menaquinone [1–3].

The whole-cell fatty acid composition of the novel isolate was determined according to the standard protocol of the Microbial Identification System (MIDI, Version 6.1; Identification Library TSBA 5.0, Microbial ID). Extracts were analysed using a model HP6890A gas chromatograph (Hewlett Packard) equipped with a flame-ionization detector as described by Kämpfer and Kroppenstedt [37]. The cellular fatty acid profile of GASy1T was characterized by the fatty acids anteiso-C15:0 (51.0%), C16:0 (14.2%), iso-C15:0 (12.6%), anteiso-C17:0 (6.1%) and iso-C17:0 (4.7%), as the major fatty acids. The fatty acids profile of the strain was very similar to that of Salipaludibacillus aurantiacus KCTC 33633T, with anteiso-C15:0, C16:0, iso-C15:0 and anteiso-C17:0 as major fatty acids, while the pattern for fatty acids of GASy1T was different from those of the other closely related

**Table 1. Differential characteristics between GASy1T and phylogenetically related species of the genus Salipaludibacillus**

<table>
<thead>
<tr>
<th>Taxa</th>
<th>GASy1T (Salipaludibacillus halalkaliphilus sp. nov.)</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony pigmentation</td>
<td>Cream</td>
<td>Pale-yellow</td>
<td>White</td>
<td>Orange</td>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Salinity range [% (w/v) NaCl]</td>
<td>0.5–15</td>
<td>0.5–10</td>
<td>0–15</td>
<td>0.5–22</td>
</tr>
<tr>
<td>Growth temperature (°C):</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Range</td>
<td>4–40</td>
<td>4–30</td>
<td>10–45</td>
<td>20–45</td>
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<tr>
<td>Optimum</td>
<td>30</td>
<td>25</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>pH growth range</td>
<td>7.5–10</td>
<td>6.5–10</td>
<td>7.5–10</td>
<td>8–11</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Citrate utilization</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Anaerobic growth</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Hydrolysis of:</td>
<td></td>
<td></td>
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<tr>
<td>Aesculin</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Tween 20</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>Acid production from:</td>
<td></td>
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<tr>
<td>D-Fructose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Polar lipids*</td>
<td>DPG, PG, PE, PL1, PL2</td>
<td>DPG, PG, PE</td>
<td>DPG, PG, PE, PL1</td>
<td>DPG, PG, PE, PL1, L</td>
</tr>
<tr>
<td>DNA G+C content (mol%)*</td>
<td>38.8</td>
<td>39.8</td>
<td>39.5</td>
<td>42.4</td>
</tr>
</tbody>
</table>

*DPG, diphasphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid; L, unidentified polar lipid.
†Data for the DNA G+C contents of the reference strains were obtained from Chen et al. [2] and Sultanpuram and Mothe [1].
species. However, the presence of anteiso-C15:0 as the most common major fatty acid is similar for all species of the genus *Salipaludibacillus* (Table S1).

In conclusion, the phylogenetic analysis based on the 16S rRNA gene sequences revealed that GASy1T represents a member of the genus *Salipaludibacillus*. However, low levels of 16S rRNA gene sequence similarity with *Salipaludibacillus neizhouensis* JSM 071004T and other species of the genus *Salipaludibacillus* and low DNA–DNA hybridization as well as several phenotypic features, such as NaCl concentration range and optimum for growth, temperature range and optimum for growth, pH range and optimum for growth, acid production from carbohydrates, oxidase activity, colony pigmentation and hydrolysis of aesculin (Table 1) support the view that GASy1T represents a novel species of the genus *Salipaludibacillus*, for which we propose the name *Salipaludibacillus halalkaliphilus* sp. nov.

**DESCRIPTION OF *SALIPALUDIBACILLUS HALALKALIPHILUS* SP. NOV.**

*Salipaludibacillus halalkaliphilus* (hal.al.ca.li’phi.lus. Gr. n. hals salt; N.L. n. alkali alkali; Gr. adj. philos loving; N.L. masc. adj. halalkaliphilus loving salty and alkaline environments).

Cells are Gram-stain-positive and form ellipsoidal endospores at central or sub-terminal positions in swollen sporangia. They are non-motile, single or short-chain rods, 0.3–0.4 \( \times \) 1.2–1.9 \( \mu \)m in size. Colonies are small, circular with entire margins, convex and smooth, translucent and cream pigmented. Facultatively anaerobic, moderately halophilic and alkaliophilic, growing over a wide range of NaCl concentrations (from 0.5 to 15% w/v NaCl), with optimal growth at 5% (w/v) NaCl and pH 7.5–10.0 (optimally at pH 8.5–9.0). Grows at 4–40 °C (optimally at 30 °C). Catalase-positive but oxidase-negative. Nitrate and nitrite are not reduced and H2S is not produced. Indole is not produced from tryptophan. Aesculin is hydrolyzed, whereas casein, DNA, gelatin, urea, starch, Tween 20, Tween 40, Tween 60, Tween 80 and tyrosine are not. D-glucose, D-fructose, D-arabinose, D-galactose, maltose, D-mannitol, D-sorbitol, sucrose, D-xylene, lactose, ribose, acetate, citrate, L-alanine, L-arginine, L-glycine, L-histidine, L-proline, L-methionine and L-serine are not utilized as sole sources of carbon and energy. Acid is produced from D-glucose, D-fructose, D-mannose, maltose and sucrose but not from D-galactose, D-mannitol, lactose, myoinositol, cellobiose, D-ribose, raffinose, D-rhamnose and D-sorbitol. Methyl red and Voges–Proskauer tests are negative. Polar lipids are phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine and two unidentified phospholipids. The predominant fatty acids are anteiso-C15:0, C16:0 and iso-C15:0.

The type strain, GASy1T (=IBRC M 10902T =LMG 28385T), was isolated from Gomishan wetland, Iran. The DNA G+C content of the type strain is 38.8 mol% (as determined by HPLC).

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

No experimental work with animals or human has been performed for this article.

**References**


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