Aliidiomarina iranensis sp. nov., a haloalkaliphilic bacterium from a coastal-marine wetland

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A novel Gram-stain-negative, straight rod-shaped, non-pigmented, slightly halophilic and alkaliphilic bacterium, designated strain GBPy7⁷, was isolated from a sample of the coastal-marine wetland Gomishan in Iran. Cells of strain GBPy7⁷ were motile. Growth occurred on media with 1–15 % (w/v) NaCl (optimum 3 %), at pH 7–10 (optimum pH 8.5) and at 4–45 °C (optimum 37 °C). Phylogenetic analysis based on 16S rRNA gene sequence comparison indicated that strain GBPy7⁷ belonged to the family Idiomarinaceae. Its closest relatives were Aliidiomarina shirensis AIST (98.1 % 16S rRNA gene sequence similarity) and other Aliidiomarina species (95.9–94.2 %), together with Idiomarina seosinensis CL-SP19⁷ (94.3 %) and Idiomarina fontislapidosi F23⁷ (94.3 %). The major cellular fatty acids of the isolate were iso-C15 : 0, iso-C17 : 0, iso-C17 : 1ω9c and C18 : 1ω7c and its polar lipid profile comprised phosphatidylglycerol, diphosphatidylglycerol, phosphatidylethanolamine, one unknown phospholipid and one unknown aminophospholipid. Cells of strain GBPy7⁷ contained ubiquinone Q-8. The G+C content of the genomic DNA of this strain was 51.6 mol%. The level of DNA–DNA relatedness between strain GBPy7⁷ and A. shirensis IBRC-M 10414⁷ was 21 %. The physiological, biochemical, genotypic and phylogenetic differences between strain GBPy7⁷ and other previously described taxa indicate that the strain represents a novel species of the genus Aliidiomarina within the family Idiomarinaceae, for which the name Aliidiomarina iranensis sp. nov. is proposed. The type strain is GBPy7⁷ (=IBRC-M 10763⁷=CECT 8339⁷).

The family Idiomarinaceae, within the class Gammaproteobacteria, was proposed by Ivanova et al. (2004). Members of this family are aerobic or facultatively anaerobic chemo-organotrophs, use carbohydrates poorly as sole carbon and energy sources and require sodium ions for growth. Members of the family have been isolated from open and deep-sea waters. The fatty acids of this family are dominated by iso-C15 : 0 and iso-C17 : 0 (Ivanova et al., 2004; Jean et al., 2006) and the major isoprenoid quinone is ubiquinone 8 (Q-8) (Taborda et al., 2009). The family currently comprises two genera: Idiomarina (Ivanova et al., 2000) and Aliidiomarina (Huang et al., 2012), as species of the genus Pseudidiomarina (Jean et al., 2006) were reassigned to the genus Idiomarina (Taborda et al., 2009). The genera Idiomarina and Aliidiomarina cannot be differentiated on the basis of phenotypic characteristics, and signature nucleotides in the 16S rRNA gene sequences have been defined for the family Idiomarinaceae (Taborda et al., 2009). The type species of the genus Aliidiomarina, Aliidiomarina taiwanensis, was isolated from a seawater sample collected in the shallow coastal region of Bitou Harbour, Taiwan (Huang et al., 2012). To date, four other species have been described in this genus, Aliidiomarina haloalkalitolerans (Srinivas et al., 2012), ‘Aliidiomarina sanensis’ (Wang et al., 2013), Idiomarina maris which was reclassified within the genus Aliidiomarina as Aliidiomarina
To characterize strain GBPy7T phenotypically, standard and supplemented with 30 % (v/v) glycerol. (MAHA) with 5 % (w/v) total salts: (g l \(^{-1}\)) NaCl, 30.0; peptone, 5.0; yeast extract, 2.0; meat extract, 1.0; trisodium citrate, 0.12; KCl, 0.08; MgSO\(_4\), 7H\(_2\)O, 0.04; FeSO\(_4\) \(\cdot\) 7H\(_2\)O, 2.0 mg; MnCl\(_2\) \(\cdot\) 4H\(_2\)O, 0.36 mg; and agar, 15.0 (Atlas, 2005). Sodium sesquicarbonate solution \([\text{g} \text{l}^{-1}\text{Na}_2\text{CO}_3, 10.6; \text{and NaHCO}_3, 8.42]\) was added to obtain alkaline conditions; it was added after sterilization in an autoclave and the plates were incubated at 34 °C for 2 weeks. The pH of this medium was adjusted to pH 9.5. The strain was subsequently purified three times by plating on the same medium. In addition, the optimal media for growth were examined and found to be MH agar (Ventosa et al., 1982) with 3.0 % total salts of pH 8.5 and R2A medium (Reasoner & Geldreich, 1985) of pH 8.5. The strain was maintained on the mentioned medium and also at (80 °C in R2A medium without agar and supplemented with 30 % (v/v) glycerol.

To characterize strain GBPy7T phenotypically, standard phenotypic tests were selected according to the recommendations of the notes on the characterization of prokaryote species of the genus *Alidiomarinana*. The strain was isolated by diluting a sample taken from exponentially growing cultures. Gram staining was performed with an Olympus BX51 microscope equipped with phase-contrast optics using cells of pH 8.5. The strain was subsequently purified three times by plating on the same medium. In addition, the optimal media for growth were examined and found to be MH agar (Ventosa et al., 1982) with 3.0 % total salts of pH 8.5 and R2A medium (Reasoner & Geldreich, 1985) of pH 8.5. The strain was maintained on the mentioned medium and also at (80 °C in R2A medium without agar and supplemented with 30 % (v/v) glycerol.

Cell morphology was examined with an Olympus BX51 microscope equipped with phase-contrast optics using cells from exponentially growing cultures. Gram staining was performed by the Burke method (Murray et al., 1994). Motility was analysed by the wet-mount method (Murray et al., 1994). Catalase and oxidase activities, nitrate reduction, hydrolysis of aesculin, production of indole and Voges–Proskauer tests were carried out as recommended by Smibert & Krieg (1994), using media with 3 % (w/v) NaCl. Hydrolysis of Tweens 20, 40 or 80 was examined as described by Harrigan & McCance (1976) on media with 3 % (w/v) NaCl. Tests for determination of acid production from carbohydrates, as well as utilization of carbon sources, were performed as recommended by Ventosa et al. (1982). Antimicrobial susceptibility tests were performed on Mueller–Hinton agar plus 3 % (v/v) sea salts (Ventosa et al., 1982) seeded with a bacterial suspension containing 1.5 × 10\(^8\) c.f.u. ml\(^{-1}\) using discs (HiMedia) impregnated with various antimicrobial compounds. The plates were incubated at 37 °C for 48 h and the inhibition zone was interpreted according to the manufacturer’s manual.

To determine the optimal temperature and pH for growth of the strain, R2A broth with 3 % (v/v) NaCl was incubated at 0, 4, 10, 15, 20, 25–37 (at intervals of 1.0 °C), 40, 45 and 50 °C and at pH 5–10.5 at intervals of 0.5 pH units. pH values below 6, pH 6–9 and pH values above 9 were obtained using sodium acetate/acetic acid, Tris/HCl and glycine/sodium hydroxide buffers, respectively. Growth at different NaCl concentrations (0.5, 1.0, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 7.5, 10.0, 12.5, 15.0 and 20.0 %, w/v) was tested in R2A broth at pH 8.5. Anaerobic growth was tested in R2A broth medium with 3 % (w/v) NaCl in an anaerobic chamber by substituting air with nitrogen gas. Growth was monitored by turbidity at OD\(_{600}\) using a spectroscopic method (model UV-160 A; Shimadzu). Other physiological and biochemical tests were performed as described previously (Ventosa et al., 1982; Quesada et al., 1984; Mata et al., 2002).

Strain GBPy7T was Gram-stain-negative, motile and facultatively anaerobic. Cells were straight rods with a width of 0.6–0.9 μm and length of 1.8–2.2 μm. This isolate was a slightly halophilic bacterium, growing in media containing 1–15 % (w/v) NaCl and optimally in media containing 3 % (w/v) NaCl. Strain GBPy7T grew between pH 7.0 and 10.0 and optimally in media of pH 8.5. It was sensitive to amoxicillin (30 μg), ampicillin (10 μg), carbenicillin (100 μg), cepoxitin (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), kanamycin (30 μg) nalidixic acid (30 μg), nitrofurantoin (300 μg), penicillin G (10 U), polymyxin B (300 U), rifampicin (5 μg), streptomycin (10 μg), tetracycline (30 μg), tobramycin (10 μg), gentamicin (10 μg) and neomycin (30 μg). Details of other phenotypic features are included in Table 1 and the species description.

Genomic DNA of strain GBPy7T was extracted using the method described by Marmur (1961). The 16S rRNA gene was amplified using the bacterial universal primers 27F and 1492R (Lane, 1991). The purified PCR product was sequenced in both directions using an automated sequencer by the Macrogen Company. 16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig et al., 2004). The 16S rRNA gene sequence was aligned with the published sequences of closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were reconstructed using three different methods, the maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981; Stamatakis, 2006) algorithms, integrated in the ARB
software for phylogenetic inference. The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1. An almost-complete 16S rRNA gene sequence (1413 bp) of strain GBPy7T was obtained and used for BLAST searches in GenBank and phylogenetic analysis. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net; Kim et al., 2012). 16S rRNA gene sequence analysis showed that strain GBPy7T is a member of the genus Aliidiomarina. The closest relative of strain GBPy7T was A. shirensis AIST, with a 16S rRNA gene sequence similarity of 98.1 %. Other species phylogenetically related to strain GBPy7T were A. haloalkalitolerans AK5T (95.9 %), A. maris CF12-14T (95.5 %), A. taiwanensis AIT1T (94.4 %), ‘A. sanyensis’ GYP-17 (94.2 %), Idiomarina seosinensis CL-SP19T (94.3 %), Idiomarina fontislapidosi F23T (94.3 %) and Idiomarina abyssalis KMM 227T (93.9 %). Due to the difficulties in differentiating the two genera Idiomarina and Aliidiomarina based on phenotypic characteristics, signature nucleotides in the 16S rRNA gene sequences were examined in this study. Strain GBPy7T exhibited the signature nucleotides that are distinctive for the family Idiomarinaceae (Ivanova et al., 2004; Jean et al., 2006), i.e. positions 143 (A), 662 (A), 682 (A), 830 (T) and 856 (A). However, the isolate also exhibited nucleotides at positions 240 (A), 286 (T), 762 (A) and 811 (T) that distinguished members of the genus Aliidiomarina from other members of the family Idiomarinaceae, which exhibited G, C, T and C at these positions, respectively. Phylogenetic analysis using the neighbour-joining algorithm revealed that strain GBPy7T represents a separate lineage. The phylogenetic position was also confirmed by trees generated using the maximum-parsimony and maximum-likelihood algorithms (Fig. 1). Based on sequence divergence, it was evident that strain GBPy7T constituted a different taxon separated from other species of the genus Aliidiomarina.

For determination of DNA base composition, cells were disrupted by using a Constant Systems TS 0.75 kW French press (IUL Instruments) and the DNA in the

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5⁺</th>
<th>6†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
<td>0.6–0.9×1.8–2.2</td>
<td>0.8–1.7×0.4–0.5</td>
<td>0.8–1.0×1.0–1.5</td>
<td>0.3–0.6×0.8–2.4</td>
<td>0.4–0.8×1.5–3.0</td>
<td>0.4–0.5×0.7–2.0</td>
</tr>
<tr>
<td>Salinity range [‰ (w/v) NaCl]</td>
<td>1.0–15.0</td>
<td>0.5–15</td>
<td>0.5–12</td>
<td>0.1–15.0</td>
<td>0.5–10</td>
<td>1–10</td>
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<td>Growth temperature (°C):</td>
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<td>Range</td>
<td>4–45</td>
<td>1–45</td>
<td>10–40</td>
<td>4–42</td>
<td>4–45</td>
<td>10–45</td>
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<td>pH growth range</td>
<td>7–10</td>
<td>5–9.5</td>
<td>7–11</td>
<td>6–11.5</td>
<td>7–9</td>
<td>7–9</td>
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<tr>
<td>Nitrate reduction</td>
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<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>Anaerobic growth</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>ND</td>
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<tr>
<td>Indole production</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Acid production from:</td>
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<td>Arabinose</td>
<td>+</td>
<td>–</td>
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<td>Utilization of:</td>
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<tr>
<td>D-Galactose</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>D-Glucose</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Maltose</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Mannitol</td>
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<td>–</td>
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<td>–</td>
<td>+</td>
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<td>Glycerol</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>PG, DPG, PE, PL1, APL1</td>
<td>PG, DPG, PE, PL1–PL4</td>
<td>PG, DPG, PE</td>
<td>PG, DPG, PE</td>
<td>PE, PL1–PL3, L</td>
<td></td>
</tr>
<tr>
<td>DNA G+C content (mol%)§</td>
<td>51.6</td>
<td>45.8</td>
<td>54.7</td>
<td>50.4</td>
<td>51.5</td>
<td>53.6</td>
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</table>

*Data are from Huang et al. (2012). †Data are from Wang et al. (2013). §Data for the DNA G+C content of the reference species were obtained from Chiu et al. (2014), Srinivas et al. (2012), Zhang et al. (2012), Huang et al. (2012) and Wang et al. (2013).
crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). The DNA G+C content was determined by reversed-phase HPLC of nucleosides according to the protocol of Mesbah et al. (1989). The G+C content of the DNA of strain GBPy7T was 51.6 mol%. This value is within the range described for the family Idiomarinaceae (45.0–54.7 mol%) and higher than that of the type strain of A. shirensis (45.8 mol%) (Huang et al., 2012; Wang et al., 2013; Chiu et al., 2014). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) with the modifications described by Huss et al. (1983), using a Cary 100 Bio UV/VIS spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multiecell changer and a temperature controller with an in situ temperature probe (Varian). DNA–DNA hybridization experiments between strain GBPy7T and its closest relative A. shirensis IBRC-M 10414T revealed a value of 21 %. According to the 70 % threshold proposed by Wayne et al. (1987) for the discrimination of prokaryotic species using DNA–DNA relatedness, this result confirmed that the new isolate constitutes a novel species.

Fig. 1. The phylogenetic relationships between strain GBPy7T and related species of the family Idiomarinaceae obtained by comparison of the 16S rRNA gene sequences and using the neighbour-joining algorithm. Desulfosarcina cetonica DSM 7267T (AJ237603) was used as the outgroup. Bootstrap values above 50 %, based on 1000 resamplings, are shown at branch points. Filled circles indicate branches that were also obtained using the maximum-parsimony and maximum-likelihood algorithms. Bar, 0.02 sequence divergence in the neighbour-joining tree.
Cell biomass for fatty acid, isoprenoid quinone and polar lipid analyses was obtained by cultivation on 3% MH medium at pH 8.5 and 37 °C. Cells were harvested in the mid-exponential growth phase. The whole-cell fatty acid composition of strain GBPy7T was characterized according to the standard protocol of the Microbial Identification System (MIDI, Version 6.1; Identification Library TSBA40 4.1; Microbial ID). Extracts were analysed using a Hewlett Packard model HP6890A gas chromatograph equipped with a flame-ionization detector as described by Kämpfer & Kroppenstedt (1996). Fatty acid peaks were identified using the TSBA40 database. The cellular fatty acid profile of strain GBPy7T was unique for strain GBPy7T in comparison with other Aliidiomarina species.

Strain GBPy7T shared some phenotypic features and similar chemotaxonomic characteristics such as quinone composition and fatty acid composition with species of the genus Aliidiomarina. However, 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, anaerobic growth and nitrate reduction as well as differences in signature nucleotides in the 16S rRNA gene sequence and genomic DNA G+C content can be used to distinguish this strain from phylogenetically related taxa (Table 1). Therefore, on the basis of data from this polyphasic taxonomic study, we propose that strain GBPy7T represents a novel species of the genus Aliidiomarina, for which the name Aliidiomarina iranensis sp. nov. is proposed.

**Description of Aliidiomarina iranensis sp. nov.**

*Aliidiomarina iranensis* (i.ran.en’sis. N.L. fem. adj. *iranensis* from Iran, where the organism was isolated).

Cells are Gram-stain-negative, motile, straight rods with a width of 0.6–0.9 μm and length of 1.8–2.2 μm. Heterotrophic and facultatively anaerobic. Colonies grown for 24 h at 37 °C on R2A medium with 3% (w/v) NaCl are circular with entire margin (3–6 mm in diameter), convex, non-pigmented, buttery and opaque. Growth occurs at 4–45 °C (optimum 37 °C), at pH 7.0–10.0 (optimum pH 8.5) and with 1–15% (w/v) NaCl (optimum 3%). Positive for oxidase and catalase activities, but negative for arginine dihydrolase, ornithine decarboxylase, lysine decarboxylase, Voges–Proskauer and ONPG tests. Indole and H2S are not produced and nitrate is not reduced. Aesculin, urea, tyrosine, starch and casein are hydrolysed, while gelatin, Tween 20, 40 and 80 and DNA are not. Acid production from arabinose is positive but acid production from D-glucose, D-galactose sucrose, fructose, lactose, ribose, D-mannose, trehalose, D-mannitol, glycerol, D-xylene, starch, cellobiose and maltose is negative. L-Arginine and D-arabinose are utilized as sole source of carbon and energy. D-Glucose, sucrose, trehalose, D-galactose, raffinose, melibiose, fructose, lactose, ribose, starch, citrate, D-mannose, D-mannitol, glycerol, D-xylene, cellobiose, maltose, L-rhamnose and L-phenylalanine are not utilized as sole source of carbon and energy. Polar lipids present are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, one unknown phospholipid and one unknown aminophospholipid (Fig. 2), whereas its closest relative, *A. shirensis* AIST, contained only diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as polar lipids, like the type species *A. taiwanensis* AIT1T (Huang et al., 2012; Chiu et al., 2014). However, the polar lipid profile of strain GBPy7T was more similar to that of *A. halalkalitolerans*, which also contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unknown phospholipid. The unknown aminophospholipid was unique for strain GBPy7T in comparison with other Aliidiomarina species.

**Fig. 2.** Polar lipids of strain GBPy7T after two-dimensional TLC and detection with molybdenophosphoric acid and heating at 200 °C for 10 min. PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PL, unknown phospholipid; APL, unknown aminophospholipid.
isoprenoid quinone is Q-8. Major cellular fatty acids are iso-C₁₅ : ₀, iso-C₁₇ : ₀, iso-C₁₇ : ₀9c and C₁₈ : ₁₀7c.

The type strain is GBPy7T (=IBRC-M 10763T = CECT 8339T), isolated from a sample of the coastal-marine Gomishan wetland in Iran. The DNA G+C content of the type strain is 51.6 mol% (HPLC).

Acknowledgements

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