Aquaticitalea lipolytica gen. nov., sp. nov., isolated from Antarctic seawater

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A Gram-stain-negative, rod-shaped bacterium, designated Ar-125T, was isolated from Antarctic seawater. It produced carotenoid-like pigments and did not produce Bchl a. Ar-125T was positive for hydrolysis of DNA, aesculin, gelatin, starch, Tween 40 and Tween 60. The sole respiratory quinone was MK-6. The major polar lipids were phosphatidylethanolamine, one unidentified aminolipid, one unidentified glycolipid and two unidentified lipids. The principal fatty acids were branched-chain fatty acids, including iso-C15:0, iso-C15:1ω7c, summed feature 3 (iso-C16:1ω7c and/or C16:1ω6c), iso-C17:0, 3-OH, iso-C16:0 3-OH and iso-C15:0 3-OH, as well as C15:0. The genomic DNA G+C content was 31.8 mol%. On the basis of 16S rRNA gene sequence analysis, Ar-125T is closely related to the species of the genera Bizonia (with 16S rRNA gene pairwise sequence similarity of 93.7–96.5%), Formosa(94.3–95.8%), Gaetbulibacter(94.2–95.7%), Geojedonia(95.5%), Gelidibacter (93.3–94.4%), Meridianmaribacter(95.3%) and Psychroserpen(94.8–95.3%), of the family Flavobacteriaceae. Phylogenetic analysis indicated that it represented an independent lineage and that the closest relatives were members of the genus Gelidibacter. Differential phenotypic properties and chemotaxonomic differences, together with phylogenetic distinctiveness, revealed that Ar-125T could be differentiated from members of closely related genera. Therefore, it is proposed that Ar-125T represents a novel species in a new genus, for which the name Aquaticitalea lipolytica gen. nov., sp. nov. (type strain Ar-125T =CGMCC 1.15295T =JCM 30876T) is proposed.

The family Flavobacteriaceae, belonging to the phylum Bacteroidetes, is phenotypically, metabolically and ecologically diverse (McBride et al., 2014). The family was proposed by Jooste (1985) and emended by Bernardet et al. (1996) based on the phylogenetic analysis of 16S rRNA gene sequences. Currently the family consists of more than 100 genera with validly published names (http://www.bacterio.net/classification.html). Most species are aerobic, have rod-shaped cells (with some of them exhibiting long filamentous cells), and possess menaquinone 6 (MK-6) as the major or sole respiratory quinone (McBride et al., 2014). Some species are able to degrade macromolecules, such as polysaccharides, lipids and proteins (McBride et al., 2014). In this paper, we present a polyphasic study describing a novel bacterium, strain Ar-125T, isolated from Antarctic seawater.

The seawater sample was collected from the western part of Prydz Bay, near Cape Darnley, Antarctica (latitude 70° 30’ 54” E, latitude 68° 00’ 42” S, water depth 496 m) at depth of 200 m during the 25th Chinese National Antarctic Research Expedition in February of 2009. Aboard the ship, all samples were subsampled aseptically and stored at 4 °C. Approximately 100 μl seawater was spread on modified marine agar 2216 (natural seawater 1 l) with peptone (0.5 g), yeast extract (0.1 g) and ornithine (10 g), final pH 7.2) by using the standard ten-fold dilution plating technique. After three days of aerobic incubation at 28 °C, one yellow colony, designated Ar-125T, was picked. The isolate was purified by repeated streaking. Purity was confirmed by the uniformity of cell morphology. Unless otherwise stated, Ar-125T was routinely cultured on marine agar 2216 (MA; BD, Difco) at 30°C and preserved as a glycerol suspension (30% v/v) at −80°C or by lyophilization.

Abbreviations: Bchl a, Bacteriochlorophyll a; MK-6, Menaquinone 6.
The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of 19 strain Ar-125T is JX844498.
Two supplementary tables and one supplementary figure are available with the online Supplementary Material.
Strain Ar-125T did not grow in Luria-Bertani and TSB (BD, Difco) media. All phenotypic tests were carried out in MB or on MA at 30°C, except where indicated otherwise. The temperature range for growth was determined by incubating at 4, 15, 20, 25, 28, 30, 35, 37, 40, 42 and 45°C. The pH range for growth was determined in MB that was adjusted to pH 5.0–10.5 (in 0.5 pH-unit intervals) using appropriate buffers (MES for pH 5.0–6.5, PIPES for pH 6.5–7.0, Tricine for pH 7.5–8.5 and CAPSO for pH 9.0–10.5) at a concentration of 50 mM. Evaluation of the pH values after autoclaving revealed only minor changes. The optimal conditions for growth were tested by using NaCl-free MB (prepared according to the MB formula, but without NaCl) with different NaCl concentrations (0, 0.5, 1.0, 3.0, 5.0, 7.5, 10.0, 12.0, 15.0 and 20.0% as final concentration, w/v). The requirement of Ar-125T for artificial sea salts was evaluated in YP medium [yeast extract (5 g l⁻¹) and peptone (1 g l⁻¹)] with the addition of 0, 1, 2, 3, 5, 7, 9, 10, 11, 12 or 15% (w/v) sea salts (Sigma). Cell morphology and motility were examined using optical microscopy (BX40; Olympus), confocal laser scanning microscopy (TCS SP5; Leica) and transmission electron microscopy (JEM-1230; JEOL).

Gram reaction and spore formation were tested by using the methods described by Dong & Cai (2001). Catalase activity was determined by bubble production in 3% (v/v) H₂O₂ solution. Oxidase activity was determined by oxidation of 1% (w/v) p-aminodimethylaniline oxalate. Anaerobic growth was carried out with AnaeroPack (Mitsubishi) using potassium nitrate and sodium nitrite as potential electron acceptors. The pigment absorption spectrum analysis was performed by the method described by Hildebrand et al. (1994), using a Beckman DU 800 Spectrophotometer (absorption spectrum from 300–1000 nm). Pigments were extracted with methanol. The carotenoid composition was determined on an HPLC system (eluted with water:methanol:acetoniitrile, 2:5:3; flow rate 1 ml min⁻¹, absorption at 476 nm). Commercial astaxanthin (CAS: 472–61–7, Sigma) was used as a standard. Tests for hydrolysis of aesculin, DNA, gelatin, starch, Tween 40 and Tween 60 were performed according to the methods of Dong & Cai (2001). Acid production was tested using the marine oxidation–fermentation (MOF) medium supplemented with 0.5% sugars (Leifson, 1963). The utilization of carbohydrates as sole carbon and energy sources was determined in BM medium (Farmer et al., 2005) and basic MB medium (formula of MB without yeast extract and peptone). The corresponding filter-sterilized complex nutrients [yeast extract, peptone and tryptone, (0.2%, w/v)], sugars (0.2%, w/v), alcohols (0.2%, w/v), organic acids (0.1%, w/v) or amino acids (0.1%, w/v) were added into the medium. Basic biochemical tests and carbon-source oxidation tests were also performed using GN2 MicroPlates (Biolog) according to the manufacturer’s instructions. Sensitivity to antimicrobial agents was determined with a two-layer plate method on MA at 30°C according to the methods of Wu et al. (2015). Additional enzyme activities and biochemical characteristics were determined using API 20 NE, API 20E and API ZYM kits (bioMérieux) at 30°C. Enzyme activities were tested using the API ZYM kit as recommended by the manufacturer. Strips were inoculated with a heavy bacterial suspension (MacFarland 5 standard) in AUX medium supplemented with 2% (w/v) sea salts (Sigma) according to the protocols of Park et al. (2005). Bizonia echini KCTC 22015T and Gelidibacter salicidans IC162T were used as reference strains in the above tests.

Genomic DNA was obtained by using the method described by Marmur (1961). The G+C content of the resulting deoxyribonucleosides was determined by reversed-phase HPLC and calculated from the deoxyguanosine:thymidine ratio (Mesbah & Whitman, 1989). The quadrant streak method was used to dilute the inoculum so that quadrant 3 exhibiting confluent growth (at the late exponential phase) along the streaking axis. Cellular fatty acid methyl esters were obtained from cells grown in MA at 30°C from quadrant 3 and analyzed according to the instructions of the Microbial Identification System (MIDI). Isoprenoid quinones were extracted from freeze-dried cells (200 mg) with chloroform and analyzed by HPLC-MS. Total lipids were extracted by the modified method of Kamekura & Kates (1988) and identified by two-dimensional TLC. Molybdocphosphoric acid and sulfuric acid were used for the detection of all lipids, ninhydrin reagent for lipids containing free aminolipids, ammonium molybdate reagent for phosphorus-containing lipids and anisaldehyde reagent for glycolipids (Tindall et al., 2007).

The 16S rRNA gene was amplified and analyzed as described previously (Xu et al., 2007). PCR products were cloned into vector pMD 19-T (TaKaRa) and then sequenced to determine the almost-complete sequence of 16S rRNA gene. The sequence was compared with closely related sequences of reference organisms from the EzTaxon-e service (Kim et al., 2012). Based on EzTaxon-e results, phylogenetic trees were reconstructed including the eight most closely related genera (16S rRNA similarity higher than 95%) and performed using the software tool MEGA 5. Sequence data were aligned with CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Fitch, 1971) and maximum-likelihood methods (Felsenstein, 1981) with the MEGA 5 program package (Tamura et al., 2011). Evolutionary distances

![Fig. 1. Transmission electron micrographs showing the cell morphology and ultrastructure of strain Ar-125T. Bars, 0.2 µm.](image-url)
were calculated according to the algorithm of the Kimura two-parameter model (Kimura, 1980) for the neighbour-joining method.

Cells of Ar-125T were Gram-stain-negative, aerobic and rod-shaped (0.2–0.3 µm in width and 1.5–2.5 µm in length) without a flagellum (Fig. 1). Colonies are yellow, circular, rod-shaped (0.2 cm in width and 1.5–2 mm in diameter after five days incubation at 30°C on MA. Ar-125T was positive for catalase activity, oxidase activity and hydrolysis of DNA, aesculin, gelatin, starch, Tween 40 and Tween 60, susceptible to (µg per disc unless otherwise stated) ampicillin (10), amoxicillin (20), cefalexin (30), ceftriaxone (30), chloramphenicol (30), erythromycin (15), nitrofurantoin (300), novobiocin (30), rifampicin (5), tetracycline (30) and vancomycin (30), and resistant to gentamicin (10), kanamycin (30), minocycline (30), neomyacin (30), penicillin G (10 IU), polymyxin B (300 IU), streptomycin (10) and tobramycin (10). Ar-125T can use yeast extract, peptone or tryptone as sole carbon and energy sources in the BM medium and the basic MB medium. The Biolog GN2 tests showed that Ar-125T was positive for 46 kinds of substrates. Detailed phenotypic characteristics are given in the species description, Table 1 and Table 2.

The almost-complete 16S rRNA gene sequence (1439 nt) of Ar-125T was obtained. The strain was most closely related to members of the genera Bizonia (with 16S rRNA gene pairwise sequence similarity of 93.7–96.5%), Formosa (94.3–95.8%), Gaetbulibacter (94.2–95.7%), Geojedonia (95.5%), Gelidibacter (93.3–95.4%), Meridianimaribacter (95.3%) and Psychroserpens (94.8–95.3%) within the family Flavobacteriaceae. The neighbour-joining, maximum likelihood and maximum-parsimony phylogenetic tree topologies reconstructed by MEGA 5 showed that Ar-125T clustered with the genus Gelidibacter and formed an independent clade which could not be associated with any recognized genera (Fig. 2). Bizonia echini and Gelidibacter salicanalis were the most closely related species within the genera Bizonia and Gelidibacter to Ar-125T.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationships of the novel isolate and related taxa. Bootstrap values (>70%) based on 1000 replications are shown at branch nodes. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and maximum-parsimony algorithms. Cellulophaga lytica NBRC 14961T (GenBank accession number is ABS17706) was used as an outgroup. Bar, 0.005 substitutions per nucleotide position.
The chemotaxonomic data support the results of the phylogenetic analysis, suggesting that Ar-125T represents a member of the family Flavobacteriaceae. The major respiratory quinone found in Ar-125T was MK-6, consistent with all members of the family Flavobacteriaceae (Bernardet, 2010), including the closely related genera (Bizonia and Gelidibacter). The principal fatty acids (>5%) are iso-C15:0, iso-C17:0 3-OH, and anteiso-C15:0 3-OH. In comparison with the reference strains, Ar-125T contained a high percentage of branched fatty acids in cellular fatty acids (73.2%), which was similar to the phenotypes of the reference strains (74.9–75.8%, Table S1, available in the online Supplementary Material). The major polar lipids are phosphatidylethanolamine, one unidentified aminolipid, one unidentified glycolipid and two unidentified lipids. Ar-125T and the two reference strains possessed PE as the major polar lipid (Fig. S1, available in the online Supplementary Material).

The chemotaxonomic data results show clear differences in fatty acid composition and polar lipid profile compared with closely related genera. The percentage of anteiso-fatty acids (including anteiso-C15:0, anteiso-C17:0 3-OH, anteiso-C15:1 A and anteiso-C17:1ω9c) of strain Ar-125T (1.7%) is strikingly lower than that of Gelidibacter salicanalis IC162T (25.4%). The percentages of iso-C16:1 H and C17:0 2-OH of strain Ar-125T (1.5% and < 0.3%) are lower than those of Gelidibacter salicanalis IC162T (7.4 and 8.7%). In addition, the percentage of iso-C17:1ω9c of Ar-125T (0.8%) was lower than that of the two reference strains (4.7% and 5.3%, for Gelidibacter salicanalis IC162T and Bizonia echini KCTC 22015T, respectively). However, the percentage of iso-C14:0 of Ar-125T (4.1%) was higher than that of the reference strains (0.5% and 0.8%, for Gelidibacter salicanalis IC162T and Bizonia echini KCTC 22015T, respectively). The differences in polar lipid profile between Ar-125T and the reference strains are showed in the Fig. S1. Both PL3 and L9 were present in Gelidibacter salicanalis IC162T, but were not detected in Ar-125T (Fig. S1a & b). GL1 was the major polar lipid of Ar-125T, but was not found in Bizonia echini KCTC 22015T (Fig. S1a & c). Conversely, both PL3 and L8 were major polar lipids of Bizonia echini KCTC 22015T, but were not observed in Ar-125T. In addition, moderate to minor amounts of AL3, PL1, L6 and L7 were only detected in Ar-125T.

Ar-125T could also be distinguished from the type strains of closely related species by phenotypic characteristics differences such as oxidase, temperature range, optimum temperature, acid production, enzyme activities (Table 1) and GN2 microplate system results (Table S2). For instance, the optimum temperate for growth of strain Ar-125T is 30°C, while that of Gelidibacter salicanalis IC162T is 20°C. It also could be phenotypically differentiated from the related genera on the basis of colony color, motility and enzyme activities (Table 2).

<table>
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<tr>
<th>Characteristics</th>
<th>1</th>
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<td>0–18</td>
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<td>34.4‡</td>
<td>42‡</td>
<td>36–38</td>
<td>34.7–34.9</td>
<td>32.7</td>
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*Result in this study was negative, but was positive in the original paper.
†Data from Nedashkovskaya et al., 2010.
‡Data from Bowman & Nichols, 2005.
Table 2. Differential phenotypic characteristics of *Aquaticitalea lipolytica* Ar-125<sup>T</sup> and closely related genera

<table>
<thead>
<tr>
<th>Characteristics</th>
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<tr>
<td>Colony color*</td>
<td>Y</td>
<td>GY, VY, O, YO</td>
<td>LY, Y</td>
<td>OY, Y</td>
<td>Y, GY</td>
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<td>Motility</td>
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<td>−</td>
<td>v(+)</td>
<td>v(−)</td>
<td>(+)</td>
<td>−</td>
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<td>v(−)</td>
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<td>Gelatin</td>
<td>+</td>
<td>(+)</td>
<td>v(+)</td>
<td>v(−)</td>
<td>NA(+)</td>
<td>−</td>
<td>+</td>
<td>NA(+)</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>(−)</td>
<td>v(+)</td>
<td>v(−)</td>
<td>v(+)</td>
<td>+</td>
<td>+</td>
<td>v(−)</td>
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<tr>
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<td>+</td>
<td>v(+)</td>
<td>(−)</td>
<td>NA(−)</td>
<td>v(−)</td>
<td>NA</td>
<td>NA</td>
<td>v(−)</td>
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<tr>
<td>Tween 60</td>
<td>+</td>
<td>NA(+)</td>
<td>NA(+)</td>
<td>(−)</td>
<td>v(−)</td>
<td>NA</td>
<td>NA</td>
<td>v(−)</td>
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<td>DNA G+C content (mol%)</td>
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<td>32.4–38.1</td>
<td>36–42</td>
<td>35.7</td>
<td>32.7</td>
<td>27–33.5</td>
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</table>

*Y, Yellow; GY, Gold yellow; VY, Vivid-yellow; LY, light yellow; O, Orange; YO, Yellowish orange; OY, orange yellow.

In addition, the carotenoid composition of Ar-125<sup>T</sup> differs from that of the type strains of closely related species. Methanol extracts showed absorption maxima at 449 and 475 nm, probably due to the presence of carotenoid-like pigments. In the HPLC system, commercial astaxanthin eluted at 16.98 min (data not shown). The crude pigment isolated from the cells of Ar-125<sup>T</sup> was composed of at least three pigments. The major pigment constituents of strain Ar-125<sup>T</sup> are in peak 3 (retention time 18.44 min) (data not shown). Compared with the reference strains, the retention time of major pigment were identical between strain Ar-125<sup>T</sup> and *Bizonia echini* KCTC 22015<sup>T</sup> (18.44 min), but were different between strain Ar-125<sup>T</sup> and *Gelidibacter salicanalis* IC162<sup>T</sup> (16.71 min). The elution profile of HPLC indicated the absence of astaxanthin in strain Ar-125<sup>T</sup>.

On the basis of the phylogenetic analysis and chemotaxonomic data, as well as phenotypic characteristics, obtained in this study, following the guidelines given by Bernardet et al. (2002) for the description of new taxa of the family Flavobacteriaceae, strain Ar-125<sup>T</sup> represents a novel species in a new genus, for which the name *Aquaticitalea lipolytica* gen. nov., sp. nov. is proposed.

**Description of *Aquaticitalea gen. nov.*

*Aquaticitalea* (A.qua.ti.ca.l.e.a. L. adj. aquaticus of the water; L. fem. n. talea a rod; N.L. fem. n. *Aquaticitalea* a rod from the water, i.e., isolated from seawater).

Gram-stain-negative, rod-shaped and non-spore-forming. Catalase- and oxidase-positive. Aerobic chemoheterotrophs. The major polar lipids are phosphatidylethanolamine, unidentified aminolipid, unidentified glycolipid and unidentified lipid. The principal fatty acids (>10%) are iso-C<sub>15:0</sub> and iso-C<sub>15:1</sub> G. The predominant respiratory quinone is MK-6. The type species is *Aquaticitalea lipolytica*.

**Description of *Aquaticitalea lipolytica* sp. nov.

*Aquaticitalea lipolytica* (li.po.lyti.ca. Gr. n. lipos fat; N.L. fem. adj. *lytica* from Gr. fem. adj. lytkē able to dissolve; N.L. fem. adj. *lipolytica* fat-dissolving, referring to the property of being able to hydrolyse lipids).

Cells are Gram-stain-negative, aerobic, non-motile by gliding and rod-shaped, 0.2–0.3 μm in width and 1.5–2.5 μm in length. Colonies are yellow, circular, convex, smooth and 1–2 mm in diameter after five days incubation at 30 °C on MA. Requires natural seawater or artificial sea-salts for growth. Growth occurs on NaCl-free MB supplemented with 0.5–5.0 % (w/v) NaCl (optimum 0.5–1.0 %), but not in TSB without sea salts. The pH and temperature range for growth are pH 6.5–8 and 15–37 °C (optimum at pH 7.0 and 30 °C). No growth is detected at 4 °C or above 40 °C. Contains carotenoid-like pigments. Bchl a is absent. No anaerobic growth occurs on MA supplemented with potassium nitrate and sodium nitrite. Positive for catalase, oxidase, citrate utilization and Voges–Proskauer reaction. Negative for arginine.
dihydrolase, fermentation of glucose, lysine and ornithine decarboxylase, tryptophan deaminase, nitrate reduction, indole formation, H₂S production and urease. DNA, ascuscin, gelatin, starch, Tween 40 and Tween 60 are hydrolyzed. Acid and alkali phosphatases, \( \alpha \)-chymotrypsin, cysteine arylamidase, esterase (C4), esterase lipase (C8), and alkaline phosphatases, gelatin, starch, Tween 40 and Tween 60 are hydrolyzed. Acid and maltose. Acid is not produced from

\[ \text{lipase (C14) and naphthol-AS-BI-phosphohydrolase activities} \]

The type strain, Ar-125 was isolated from Antarctic seawater.

\[ \text{D-} \]

| 2662 |

Maripatay and others

\[ \text{References} \]


International Journal of Systematic and Evolutionary Microbiology 66

Acknowledgements

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The type strain, Ar-125\(^{T}\) (=CMGCC 1.15295\(^{T}\) =JCM 30876\(^{T}\)), was isolated from Antarctic seawater.


