Planktosalinus lacus gen. nov., sp. nov., a member of the family Flavobacteriaceae isolated from a salt lake

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A Gram-staining-negative bacterium, strain X14M-14T, was isolated from a salt lake (Lake Xiaochaidan) in Qaidam basin, Qinghai Province, China. Its taxonomic position was determined by using a polyphasic approach. Cells of strain X14M-14T were non-spore-forming, non-motile rods. Strain X14M-14T was strictly heterotrophic and aerobic, catalase-positive and oxidase-negative. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain X14M-14T belonged to the family Flavobacteriaceae and formed a distinct lineage that was independent of the most closely related genera: Aequorivita (16S rRNA gene sequence similarities, 91.8–93.1 %) and Salinimicrobium (91.5–92.4 %). Strain X14M-14T contained MK-6 as the major respiratory quinone, and phosphatidylethanolamine and two unknown lipids as the major polar lipids. The major cellular fatty acids were iso-C15 : 0, iso-C15 : 1G and iso-C17 : 03-OH. The presence of iso-C15 : 1G as a predominant fatty acid could distinguish this strain clearly from the most closely related genera in the family Flavobacteriaceae. The DNA G+C content was 36.6 mol%. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain X14M-14T represents a novel genus and species of the family Flavobacteriaceae, for which the name Planktosalinus lacus gen. nov., sp. nov. is proposed. The type strain is X14M-14T (=CGMCC 1.12924T=KCTC 42675T).

Being the largest family within the phylum Bacteroidetes (formerly Cytophaga-Flavobacterium-Bacteroides group) (Garrity & Holt, 2001), the family Flavobacteriaceae was proposed by Jooste (1985), the name validly published by Reichenbach (1992) and the description emended by Bernardet et al. (1996, 2002). At the time of writing, this family consisted of about 120 genera with validly published names, most members of which are characterized as aerobic, having rod-shaped cells and containing MK-6 as the major respiratory quinone (McBride, 2014). Members of the family Flavobacteriaceae are distributed globally in various environments, such as freshwater (Zhang et al., 2014), seawater (Park et al., 2013), sediment (Kim et al., 2008), sand (Zhang et al., 2013), plant tissue (Liu et al., 2013), fish (Shakeela et al., 2015) and so on. During a survey on the bacterial diversity of a salt lake (Lake Xiaochaidan), a novel Flavobacteriaceae-like strain, designated X14M-14T, was obtained. In this study, its taxonomic position was investigated by a polyphasic approach.

The water sample was collected at 0.3 m beneath the surface of Lake Xiaochaidan [GPS coordinates 37° 29' 14" N 95° 32' 39" E; salinity 9.6 % (w/w), dissolved oxygen 4.9 mg l⁻¹, pH 8.3 and temperature 10.5 °C] in Qaidam basin, Qinghai Province, China. Strain X14M-14T was isolated by the standard dilution plating technique on marine agar 2216 (MA; Difco) at 30 °C. It could also grow well in marine broth 2216 (MB; Difco), but not in Luria–Bertani (LB) broth or trypticase soy broth (TSB; Bacto), despite supplementing with 2.0 % (w/v) NaCl. The strain was preserved as glycerol stocks at −80 °C.
The 16S rRNA gene of strain X14M-14\(^T\) was amplified with universal primers 27F and 1492R (Weisburg et al., 1991), and cloned into the pEASY-T1 vector and sequenced by Sinogenomax, China, with primers M13f and M13r. The almost-complete 16S rRNA gene sequence (1479 nt) was obtained and compared with available sequences in the GenBank database using the BLAST program (Altschul et al., 1990) at NCBI (http://www.ncbi.nlm.nih.gov) and also on the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/) by using identity analysis (Kim et al., 2012). Strain X14M-14\(^T\) was closely related to species within the family Flavobacteriaceae and showed <93.1% sequence similarities to them (data not shown).

With Flavobacterium aquatile LMG 4008\(^T\) as the outgroup, a total of 30 16S rRNA gene sequences of strain X14M-14\(^T\) (1479 nt) and related taxa were aligned with the CLUSTAL X program 2.0 (Larkin et al., 2007). Maximum-likelihood (Felsenstein, 1981), neighbour-joining (Saitou & Nei, 1987) and maximum-parsimony (Fitch, 1971) trees were reconstructed using the MEGA program version 5 (Tamura et al., 2011). Evolutionary distances were calculated by Kimura's two-parameter model (Kimura, 1983) and the gaps/missing data treatment option chosen was pairwise-deletion. The maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences is shown in Fig. 1. Strain X14M-14\(^T\) formed a distinct lineage that was independent of the most closely related genera: Aequorivita, Vitellibacter, Salinimicrobium, Aquimarina, Mariniflexile and Mesoflavibacter, to which strain X14M-14\(^T\) showed 91.8–93.1%, 91.6–92.4%, 91.5–92.4%, <92.3%, <92.2% and <92.1% 16S rRNA gene sequence similarities, respectively. Less than 92.1% sequence similarities were found to other species of the family Flavobacteriaceae. These phylogenetic data suggested that strain X14M-14\(^T\) cannot be assigned to any known genus and represents a novel genus and species of the family Flavobacteriaceae. Therefore, the type strains Aequorivita viscosa CGMCC 1.11023\(^T\) and Salinimicrobium catena CGMCC 1.6101\(^T\) were obtained from China General Microbiological Culture Collection Center (CGMCC) and used as reference strains for phenotypic tests and chemotaxonomic analyses, and all experiments were conducted in triplicate. Both reference strains could grow well on MA (Difco) and in the corresponding broth (Difco) at 30 °C.

Cell morphology was observed by optical microscopy (BH-2, Olympus) and transmission electron microscopy (H-600, Hitachi) after negative staining with 1% (w/v) phosphotungstic acid. Gram reaction and detection of intracellular granules of poly-β-hydroxybutyrate were performed according to the methods of Dong & Cai (2001). Motility was determined by the hanging-drop technique (Bernardet et al., 2002) and a motility agar stab (Dong & Cai, 2001). Growth at 0, 4, 10, 15, 20, 25, 30, 35 and 40 °C was measured in MB. Growth at pH 6.0–10.5 (at intervals of 0.5 pH units) was determined in MB at 30 °C. The pH was adjusted with two different buffers (final concentration, 50 mM): phosphate buffer (for pH 6.0–8.0) and Tris/HCl buffer (for pH 8.0–10.5), and adjusted again with sterile buffers after autoclaving. Tolerance to NaCl was examined in modified MB with final NaCl concentrations of 0–13.0% (w/v, at intervals of 0.5%) as described previously (Zhong et al., 2014). The requirement for oxygen was tested in an anaerobic system (Anaero-Gen, OXOID, UK) and microaerobic system (AnaeroPack-MicroAero, 2.5 l, MGC, Japan). Production of hydrogen sulfide was assessed with lead acetate paper (Dong & Cai, 2001). Degradation of CM-cellulose (1%, w/v) was detected by growing on MA for 2 weeks and identified by formation of clear zones (Dong & Cai, 2001). Catalase and oxidase activities, and degradation of crystalline cellulose (filter paper), casein, starch and Tween 20, 40, 60 and 80 were determined according to the methods of Dong & Cai (2001). Utilization of carbon substrates (0.5%, w/v) was tested according to the protocol of Dong & Cai (2001) with artificial seawater instead of distilled water; 0.01% (w/v) yeast extract was added as a growth factor. The artificial seawater contained (per litre distilled water): 24.0 g NaCl, 5.1 g MgCl\(_2\), 4 g Na\(_2\)SO\(_4\), 1.1 g CaCl\(_2\), 0.7 g KCl, 0.2 g NaHCO\(_3\), 0.1 g KBr, 0.027 g H\(_2\)BO\(_3\), 0.024 g SrCl\(_2\) and 0.003 g NaF. The presence of flexirubin-type pigments was investigated using 20% (w/v) KOH solution (Reichenbach, 1992). The pigments of strain X14M-14\(^T\) were extracted and analysed according to the method of Biebl et al. (2007) using a UV/visible spectrophotometer (model UV7200, UNICO). Additionally, API ZYM and API 20NE systems (bioMérieux) were used for tests of enzyme activities and other physiological and biochemical traits according to the manufacturer’s instructions. Susceptibility to antibiotics was determined by the disc diffusion method using filter-paper discs (Beijing Pharmaceutical), including (μg per disc) streptomycin (10), gentamicin (10), sulfamethoxazole (300), erythromycin (15), chloramphenicol (30), tetracycline (30), ampicillin (10), clarithromycin (15), kanamycin (30), clindamycin (2), vancomycin (30) and norfloxacin (10). Growth inhibition effects were observed and estimated according to the protocol of Nokhal & Schlegel (1983) after incubation on MA (pH 7.5) for 3 days at 30 °C. Inhibition zones were measured from the edges of susceptibility discs to the edges of the clear zones: <2 mm, 2–5 mm and >5 mm inhibition zones represented resistance, weak resistance and susceptibility, respectively (Nokhal & Schlegel, 1983).

The phenotypic characteristics of strain X14M-14\(^T\) are given in the genus and species descriptions as well as in Table 1. The data obtained in this study for reference strains Aequorivita viscosa CGMCC 1.11023\(^T\) and S. catena CGMCC 1.6101\(^T\) are included in Table 1 and corresponded to the original descriptions (Liu et al., 2013; Ying et al., 2007).

Biomass for chemotaxonomic and genotypic analyses of strain X14M-14\(^T\), as well as the reference strains, was obtained from cultivation on MA or in MB at 30 °C for 3 days to late exponential phase. Isoprenoid quinones were extracted and analysed using reversed-phase (RP)
HPLC with UV detection as described by Komagata & Suzuki (1987). Isoprenoid quinones were dissolved with acetone. The mobile phase for RP-HPLC was methanol/isopropyl ether (3:1, v/v) with the flow rate being controlled at 1 ml min$^{-1}$. The major respiratory quinone was MK-6 (accounting for 51.6%), which is typical of members within the family Flavobacteriaceae (McBride, 2014). Other respiratory quinones of strain X14M-14$^{T}$ were MK-5 (29.2%) and MK-7 (19.2%).

Cellular fatty acids were analysed using the standard protocol of the MIDI Sherlock Microbial Identification System (version 6.0), and peaks were identified on an Agilent 6890N Network GC system using the TSBA6 peak-naming table (Sasser, 1990). The detailed fatty acid profiles of strain X14M-14$^{T}$ and the type strains of species of the most closely related genera are shown in Table 2. It is noted that the fatty acid profiles obtained in this study for the reference strains were similar to those given in the original descriptions (Chen et al., 2008; Liu et al., 2013; Ying et al., 2007) in terms of the major fatty acids, despite some differences in their proportions. The major fatty acids of strain X14M-14$^{T}$ were iso-C$\text{\textsubscript{15}:0}$ (23.6%), iso-C$\text{\textsubscript{15}:1}$G (21.8%) and iso-C$\text{\textsubscript{17}:0}$ 3-OH (20.1%). The presence of iso-C$\text{\textsubscript{15}:1}$G as a major fatty acid for strain X14M-14$^{T}$ was different from all members of the most closely related genera: Aequorivita (Bowman & Nichols, 2002; Liu et al., 2013; Park et al., 2009) and Salinimicrobium (Chen et al., 2008; Lee et al., 2012; Lim et al., 2008; Nedashkovskaya et al., 2010; Subhash et al., 2014; Ying et al., 2007). The lack of summed features 3 (C$\text{\textsubscript{16}:1\omega 7c}$ and/or C$\text{\textsubscript{16}:1\omega 6c}$) and 9 (iso-C$\text{\textsubscript{17}:0\omega 9c}$ and/or C$\text{\textsubscript{16}:0$ 10-methyl}) in strain X14M-14$^{T}$ could differentiate strain X14M-14$^{T}$ clearly from the type strains of species of the genera Aequorivita and Salinimicrobium (Table 2).

**Fig. 1.** Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic position of strain X14M-14$^{T}$ and representatives of the most closely related genera. Bootstrap values (expressed as percentages of 1000 replications) >70 % are shown at branch points. Filled circles indicate branches that were also recovered in both the neighbour-joining and maximum-parsimony trees. *F. aquatile* LMG 4008$^{T}$ was used as an outgroup. Bar, evolutionary distance ($K_{\text{nuc}}$) of 0.01.
representatives of phylogenetically related genera

strains are negative for Gram staining, motility, indole production, and from the study, except for morphological properties, growth tests and DNA G+C contents of the reference strains, which are taken from Liu et al. (2013) and Ying et al. (2007). All strains are negative for Gram staining, motility, indole production, and from some members of the genus Aequorivita by the absence of some minor polar lipids.

Genomic DNA was extracted using a bacterial genomic kit (D3350-1, Omega Bio-Tek). The DNA base composition was determined by the thermal denaturation method (Marmur & Doty, 1962), with genomic DNA of Escherichia coli K-12 as a reference. The DNA G+C content of strain X14M-14T was 36.6 mol%, which fell within the range of DNA G+C contents of members of the most closely related genus Aequorivita, but was slightly lower than those of members of the genus Salinimicrobium (Table 1).

Combining the above phenotypic, chemotaxonomic and genotypic results, it is concluded that strain X14M-14T represents a novel genus and species of the family Flavobacteriaceae, for which the name Planktosalinus lacus gen. nov., sp. nov. is proposed.

### Table 1. Differential characteristics of strain X14M-14T and representatives of phylogenetically related genera

<table>
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<th>Characteristic</th>
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<th>3</th>
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</thead>
<tbody>
<tr>
<td>Cell size (µm)</td>
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<td></td>
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</tr>
<tr>
<td>Width</td>
<td>0.4–0.8</td>
<td>0.2–2.2</td>
<td>0.3–1.0</td>
</tr>
<tr>
<td>Length</td>
<td>1.4–3.2</td>
<td>0.5–20.0</td>
<td>0.5–6.0</td>
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<tr>
<td>Growth with/at:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0 % (w/v) NaCl</td>
<td>+</td>
<td>−</td>
<td>v</td>
</tr>
<tr>
<td>12 % (w/v) NaCl</td>
<td>+</td>
<td>−</td>
<td>v</td>
</tr>
<tr>
<td>4 ºC</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>H2S production</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Flexirubin pigment</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Hydrolisis of Tween 20</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>α-Glucosidase</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>activity (API ZYM)</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Assimilation of D-glucose</td>
<td>−</td>
<td>v</td>
<td>+</td>
</tr>
<tr>
<td>Major polar lipids*</td>
<td>PE, L1, L2</td>
<td>PE, L1, L2</td>
<td>PE, L2</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>36.6</td>
<td>33.8–38.5</td>
<td>40.9–47.5</td>
</tr>
</tbody>
</table>

*PE, phosphatidylethanolamine; L1 and L2, two unknown lipids.

Polar lipids of strain X14M-14T were extracted using a chloroform/methanol system and identified using two-dimensional TLC, as described by Kates (1986). Merck silica gel 60 F254 aluminium-backed thin-layer plates were used in the TLC analysis. Lipids were characterized by spraying 10 % ethanolic molybdatophosphoric acid reagent (Gunstone & Jacobsberg, 1972), phosphate stain reagent (Kates, 1986), ninhydrin reagent, α-naphthol and sulfuric acid/ethanol reagent, and clorox-benzidine reagent (Bischel & Austin, 1963) to detect total lipids, phospholipids, aminolipids, glycolipids and sphingolipids, respectively. The polar lipid profile of strain X14M-14T contained phosphatidylethanolamine (PE) and two unknown lipids as the major polar lipids, and contained three unidentified aminolipids and two additional unknown lipids as the minor lipids (Fig. S1, available in the online Supplementary Material). Glycolipids and sphingolipids were not detected (data not shown). The presence of PE and an uncharacterized lipid as the major lipids was in agreement with the results reported for members of the genera Aequorivita (Liu et al., 2013) and Salinimicrobium (Lee et al., 2012; Subhash et al., 2014). However, strain X14M-14T could still be distinguished from all members of the genus Salinimicrobium by the presence of a second unknown lipid as a major polar lipid (Table 1) and from some members of the genus Aequorivita by the absence of some minor polar lipids.

<table>
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<th>Fatty acids</th>
<th>1</th>
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<tbody>
<tr>
<td>C16 : 0</td>
<td>1.6</td>
<td>TR</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C15 : 0</td>
<td>23.6</td>
<td>32.2</td>
<td>16.1</td>
</tr>
<tr>
<td>iso-C15 : 1 G</td>
<td>21.8</td>
<td>6.9</td>
<td>6.0</td>
</tr>
<tr>
<td>anteoiso-C15 : 0</td>
<td>7.8</td>
<td>5.8</td>
<td>8.2</td>
</tr>
<tr>
<td>anteoiso-C15 : 1 A</td>
<td>1.9</td>
<td>−</td>
<td>TR</td>
</tr>
<tr>
<td>iso-C16 : 0</td>
<td>2.6</td>
<td>TR</td>
<td>5.0</td>
</tr>
<tr>
<td>iso-C16 : 1 H</td>
<td>−</td>
<td>−</td>
<td>1.9</td>
</tr>
<tr>
<td>iso-C15 : 0 3-OH</td>
<td>5.2</td>
<td>4.4</td>
<td>5.7</td>
</tr>
<tr>
<td>iso-C16 : 0 3-OH</td>
<td>4.8</td>
<td>2.8</td>
<td>5.6</td>
</tr>
<tr>
<td>iso-C17 : 0 3-OH</td>
<td>20.1</td>
<td>25.0</td>
<td>14.8</td>
</tr>
<tr>
<td>C15 : 0 2-OH</td>
<td>2.8</td>
<td>1.0</td>
<td>4.0</td>
</tr>
<tr>
<td>C15 : 0 3-OH</td>
<td>TR</td>
<td>1.7</td>
<td>3.2</td>
</tr>
<tr>
<td>C17 : 0 2-OH</td>
<td>3.3</td>
<td>1.4</td>
<td>6.9</td>
</tr>
<tr>
<td>C15 : 10:6c</td>
<td>−</td>
<td>−</td>
<td>3.0</td>
</tr>
<tr>
<td>C17 : 10:6c</td>
<td>−</td>
<td>−</td>
<td>1.9</td>
</tr>
<tr>
<td>Summed feature 3*</td>
<td>−</td>
<td>4.7</td>
<td>7.5</td>
</tr>
<tr>
<td>Summed feature 9*</td>
<td>−</td>
<td>9.3</td>
<td>3.7</td>
</tr>
</tbody>
</table>

*Summed features are groups of two or three fatty acids that are treated together for the purpose of evaluation in the MIDI system and include both peaks with discrete equivalent chain lengths (ECLs) as well as those where the ECLs are not reported separately. Summed feature 3 was listed as C16 : 10:7c and/or C16 : 10:9c. Summed feature 9 was listed as iso-C17 : 10:6c and/or C16 : 0 10-methyl.

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Description of Planktosalinus gen. nov.

Planktosalinus (Plank.to.sa.lin’us. Gr. adj. planktos planktonic; N.L. adj. salinus saline; N.L. masc. n. Planktosalinus a bacterium from surface water of a salt lake).

Strictly heterotrophic and aerobic. Oxidase-negative and catalase-positive. Cells are Gram-staining-negative, non-spore-forming, non-motile rods. Contains MK-6 as the major respiratory quinone, and iso-C15 : 0, iso-C15 : 1 G and iso-C17 : 0 3-OH as the major fatty acids. The major polar lipids are phosphatidylethanolamine and two unidentified lipids. The DNA G+C content of the type strain of the type species is 36.6 mol%. The genus is a member of the family Flavobacteriaceae. The type species is Planktosalinus lacus.

Description of Planktosalinus lacus sp. nov.

Planktosalinus lacus (la’cus. L. masc. gen. n. lacus of a lake, indicating the isolation source of this organism).

Possesses the following characteristics in addition to those described for the genus. Cells are 0.4–0.8 μm wide and 1.4–3.2 μm long (Fig. S2). Colonies are circular, smooth, glistening, 0.2–0.5 mm in diameter and yellow after cultivation on MA (pH 7.5) at 30 °C for 4 days. Grows on MA and in MB, but not in LB or TSB. Growth occurs at 4–35 °C (optimum, 25–30 °C) and pH 6.5–10.0 (optimum, pH 7.5–8.0) in MB, and in the presence of 0–12.0 % (w/v) NaCl (optimum, 2.0 %), but not at 0 °C or 40 °C, pH 6.0 or pH 10.5, or in the presence of > 12.5 % (w/v) NaCl in modified MB. Grows poorly under microaerobic conditions. Positive for production of H₂S, naphthol-AS-BI-phosphohydrolase; negative for lipase, trypsin, a-galactosidase, a-glucosidase, -mannosidase and -fucosidase. Resistant to streptomycin, ampicillin, tetracycline, chloramphenicol, norfloxacin. The polar lipid profile consists of phosphatidylethanolamine and two unknown lipids as the major polar lipids, and three unidentified aminolipids and two additional unknown lipids as the minor lipids.

The type strain is X14M-14 T (= CGMCC 1.12924 T = KCTC 42675 T), isolated from a surface water sample of Lake Xiaochaidan in Qaidam basin, Qinghai Province, China. The DNA G+C content of the type strain is 36.6 mol%.

Acknowledgements

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