Salinispirillum marinum gen. nov., sp. nov., a haloalkaliphilic bacterium in the family ‘Saccharospirillaceae’

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A novel Gram-staining-negative, motile, non-pigmented, facultatively anaerobic, spirillum-shaped, halophilic and alkaliphilic bacterium, designated strain GCWy1T, was isolated from water of the coastal–marine wetland Gomishan in Iran. The strain was able to grow at NaCl concentrations of 1–10 % (w/v) and optimal growth was achieved at 3 % (w/v). The optimum pH and temperature for growth were pH 8.5 and 30 °C, while the strain was able to grow at pH 7.5–10 and 4–40 °C. Phylogenetic analysis based on the comparison of the 16S rRNA gene sequence placed the isolate within the class Gammaproteobacteria as a separate deep branch, with 92.1 % or lower sequence similarity to representatives of the genera Saccharospirillum and Reinekea and less than 91.0 % sequence similarity with other remotely related genera. The major cellular fatty acids of the isolate were C18:1v7c, C16:0 and C17:0, and the major components of its polar lipid profile were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The cells of strain GCWy1T contained the isoprenoid quinones Q-9 and Q-8 (81 % and 2 %, respectively). The G+C content of the genomic DNA of this strain was 52.3 mol%. On the basis of 16S rRNA gene sequence analysis in combination with chemotaxonomic and phenotypic data, strain GCWy1T represents a novel species in a new genus in the family ‘Saccharospirillaceae’, order Oceanospirillales, for which the name Salinispirillum marinum gen. nov., sp. nov. is proposed. The type strain of the type species is GCWy1T (=IBRC-M 10765T = CECT 8342T).

The family ‘Saccharospirillaceae’ (Labrenz et al., 2003) belongs to the order Oceanospirillales (Garrity et al., 2005), class Gammaproteobacteria and, at the time of writing, comprises two genera: Saccharospirillum (Labrenz et al., 2003) and Reinekea (Romanenko et al., 2004). The genus Saccharospirillum accommodates Gram-staining-negative, spirillum-shaped bacteria that are chemoheterotrophic, catalase- and oxidase-positive, aerobic and motile by means of monopolar flagella, and which have Q-8 as the major respiratory quinone (Choi et al., 2011). The genus Reinekea was defined as Gram-staining-negative, chemoheterotrophic, aerobic or facultatively anaerobic, non-pigmented, motile, rod-shaped bacteria (Romanenko et al., 2004). The species of the family ‘Saccharospirillaceae’ have been isolated from various habitats, such as tidal flat sediment, hypersaline environments, subterranean brine, marine and marine coastal sediments (Chen et al., 2009; Choi & Cho, 2010; Choi et al., 2011; Labrenz et al., 2003; Pinhassi et al., 2007).

Gomishan wetland is an alkaline thalassohaline, coastal–marine wetland, located along the eastern shore of the Caspian Sea, in Golestan province, Iran. During a study of the diversity of prokaryotic micro-organisms in the Gomishan wetland, several halophilic and halotolerant
bacteria with alkaliphilic and alkali-tolerant behaviour were isolated. In this paper we characterize and describe a novel haloalkaliphilic bacterial strain from Gomishan wetland that is a member of the Gammaproteobacteria, and which is related to the order Oceanospirillales. Our results indicate that this bacterium represents a new genus and novel species, for which we propose the name Salinispirillum marinum gen. nov., sp. nov. Strain GCWy1T was isolated from a water sample (pH 8.8, 45 g dissolved salts l\(^{-1}\)) collected from the Gomishan wetland in Iran (37° 09’ 00.4” N 054° 00’ 15.3” E). The novel strain was isolated by plating the water sample directly onto modified alkaliphilic halophile agar medium (MAHA) with 5% (w/v) total salt: (g l\(^{-1}\)) NaCl, 30.0; peptone, 5.0; yeast extract, 2.0; meat extract (Merck Millipore), 1.0; trisodium citrate, 0.12; KCl, 0.08; MgSO\(_4\), 7H\(_2\)O, 0.04; FeSO\(_4\), 7H\(_2\)O, 0.002; MnCl\(_2\), 4H\(_2\)O, 0.036 and agar, 15.0 (Atlas, 2005). Sodium sesquisulfonate carbonate (g l\(^{-1}\)) Na\(_2\)CO\(_3\), 10.6, NaHCO\(_3\), 8.42, was added after autoclaving to obtain alkaline conditions. The pH of this medium was adjusted to pH 9.5. The inoculated medium was incubated at 34 °C for 2 weeks. The strain was subsequently purified three times by plating on the same medium. In addition, the best medium for growth was examined and MH medium with 3.0% total salts, pH 8.5 was selected as the optimized medium (Ventosa et al., 1980). The strain was maintained on the MH medium with 3% salts and also at -80 °C in MH medium without agar and supplemented with 30% (v/v) glycerol. The characterization of the strain was achieved by following a polyphasic approach, including the investigation of phenotypic features, chemotaxonomy (polar lipid profile, fatty acids composition and quinone analyses) and 16S rRNA gene sequence analysis. For the phenotypic characterization, the standard methods were used, after supplementation with salts to provide suitable conditions for the growth of haloalkaliphilic bacteria. Saccharosirrillium impatiens CECT 5721T was used as a reference strain for comparison in our study. It was cultured following the recommendations of the culture collection.

Cell morphology was examined with a BX51 microscope (Olympus) equipped with phase-contrast optics using cells from exponentially growing cultures. Gram staining was performed by the Burke method (Murray et al., 1994) and by the KOH test (Baron & Finegold, 1990). Motility was observed by the wet-mount method (Murray et al., 1994). Catalase and oxidase activities, nitrate reduction, hydrolysis of aesculin, starch, gelatin, casein, tyrosine and urea, production of indole and H\(_2\)S tests were done as recommended by Smibert & Krieg (1994), using media with 3% (w/v) NaCl and pH 8.5. Hydrolysis of Tween 20 and 80 was examined as described by Harrigan & McCance (1976) on media with 3% NaCl at pH 8.5. Determination of acid production from carbohydrates, as well as utilization of carbon sources, was performed as recommended by Ventosa et al. (1982). Antimicrobial susceptibility tests were performed on Mueller–Hinton agar plus 3% (w/v) sea salts at pH 8.5 (Ventosa et al., 1982) 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 manual.

To determine the optimal temperature and pH for growth of the strain, 3.0% MH broth was incubated at 0, 4, 10, 15, 20, 25–37 (at intervals of 1.0 °C), 40 and 45 °C and at pH 5–10 at intervals of 0.5 pH units. pH values below pH 6, pH 6–9 and pH values above pH 9 were obtained using sodium acetate/acetate acid, Tris/HCl and glycine/sodium hydroxide buffers, respectively. Growth with 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 and 15.0%, w/v) was tested on MH broth at pH 8.5. Growth was monitored by measuring turbidity (OD\(_{600}\)) using a spectrophotometric method (model UV-160 A; Shimadzu). Other physiological and biochemical tests were performed as described previously (Mata et al., 2002; Quesada et al., 1984; Ventosa et al., 1982).

Strain GCWy1T was Gram-staining-negative, motile and facultatively anaerobic. Cells were spirillum-shaped with a width of 0.3–0.5 μm and length of 2.5–5.9 μm. When grown for 48 h at 30 °C on MH medium with 3% salts, the creamy-beige colonies were circular, convex and smooth, had entire margins, and were translucent and 1–3 mm in diameter. This isolate was a slightly halophilic bacterium, growing in media containing 1–10.0% (w/v) NaCl and optimally in media containing 3% (w/v) NaCl. GCWy1T grew in media with alkalinites between pH 7.5 and 10.0 and optimally in media with pH 8.5. The strain was sensitive to polymyxin B (300 μg), chloramphenicol (30 μg), streptomycin (10 μg), erythromycin (15 μg) and bacitracin (10 μg) and resistant to tetracycline (30 μg). The detailed phenotypic features are included in the species description and Table 1.

The genomic DNA of the novel strain was extracted using the method described by Marmur (1961). The 16S rRNA gene was then 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 Macrogen, Seoul, Rep. of Korea. Phylogenetic analysis was performed by using the software package MEGA version 5 (Tamura et al., 2011) after multiple alignments of 16S rRNA gene sequences were made with CLUSTAL_X (Thompson et al., 1997). Clustering was performed using the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and minimum-evolution (Rzhetsky & Nei, 1992) methods. Bootstrap analysis with 1000 resamplings (Felsenstein, 1985) was used to evaluate the topology of the phylogenetic trees.

An almost-complete 16S rRNA gene sequence (1417 bp) of strain GCWy1T 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). The 16S rRNA gene sequence analysis showed
that strain GCWy1T is a member of the family ‘Saccharospirillaceae’ but there were low similarities to the type species of members of this family. The closest relative of strain GCWy1T was Saccharosipillum impatiens EL-105T with a sequence similarity of 92.1%. Other strains closely related to strain GCWy1T were Reinekea marinisedimentorum DSM 15388T (91.2%), Neptunomonas japonica JAMM 0745T (90.9%), Marinomonas ostreistagni UST010306-043T (90.9%) and Marinobacterium lutimaris AN9T (90.7%). Phylogenetic analysis using the neighbour-joining algorithm revealed that strain GCWy1T represents a novel separate lineage within the family ‘Saccharospirillaceae’ (Fig. 1). The phylogenetic position was also confirmed by trees generated using the minimum-evolution and maximum-likelihood algorithms (Figs S1 and S2 available in the online Supplementary Material).

For determination of 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. (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 GCWy1T was 52.3 mol%. This value is within the range for members of the family ‘Saccharospirillaceae’ but is lower than that of Saccharosipillum impatiens CECT 5721T and higher than the values reported for R. marinisedimentorum DSM 15388T, N. japonica JAMM 0745T and Marinomonas ostreistagni UST010306-043T (Table 1).

Cell biomass for fatty acid, isoprenoid quinone and polar lipid analyses was obtained by cultivation in 3% salt MH broth medium with shaking at 150 r.p.m. and a temperature of 30 °C. Cells were harvested during the mid-exponential growth phase. The whole-cell fatty acid composition of strain GCWy1T was determined 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 GCWy1T was characterized by the fatty acids C₁₈:₁ω₇c (41.9%), C₁₆:₀ (23.8%), C₁₇:₀ (5.6%), C₁₆:₁ω₁₁c (4.6%) as the major fatty acids and C₁₇:₁ω₁₀c, C₁₆:₁ω₁₀c, C₁₅:₀ iso-C₁₆:₀, C₁₈:₀, C₁₇:₁ω₁₁c and C₁₄:₀ in smaller amounts. The isolate could be distinguished by the absence of 3-hydroxy fatty acids unlike the type species of the genera Saccharosipillum and Reinekea. The fatty acids profile was mostly in agreement with those of other related species of the family ‘Saccharospirillaceae’, except for their abundances (Labrenz et al., 2003; Romanenko et al., 2004).

Polar lipids were analysed as described by Groth et al. (1996). Isoprenoid quinone analysis was carried out as described by Monciardini et al. (2003). Strain GCWy1T contained Q-9 as the major isoprenoid quinone (81%) and Q-8 as a minor quinone (2%), while ubiquinones of the most closely related species were predominantly Q-8 (94–95%) with Q-9 (4–6%) present in minor amounts.

### Table 1. Differential characteristics between strain GCWy1T and species of phylogenetically related genera

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>3</th>
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<tr>
<td><strong>Cell morphology</strong></td>
<td>Spirillum</td>
<td>Spirillum</td>
<td>Rod</td>
<td>Rod</td>
<td>Rod</td>
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<td>Cell size (μm)</td>
<td>0.3–0.5 × 2.5–5.9</td>
<td>0.5–0.9 × 3.8–6.2</td>
<td>0.4–0.5 × 1.5–1.7</td>
<td>0.8–1.0 × 1.6–1.8</td>
<td>0.4–0.6 × 0.6–3.6</td>
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<td>Facultatively anaerobic</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>Coccoid body formation</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<td>ND</td>
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<td>–</td>
<td>+</td>
<td>–</td>
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<td>+</td>
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<td>4 °C</td>
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<td>–</td>
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<td>10% NaCl</td>
<td>+</td>
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<td>–</td>
<td>–</td>
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<td>Aesculin</td>
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<td>Tween 80</td>
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<td>+</td>
<td>W</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Starch</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Major respiratory lipoquinone</td>
<td>Q-9</td>
<td>Q-8*</td>
<td>Q-8</td>
<td>Q-8</td>
<td>ND</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>52.3</td>
<td>54.5*</td>
<td>51.1</td>
<td>43.6</td>
<td>49.8</td>
</tr>
</tbody>
</table>

*Data from Labrenz et al. (2003).
The major polar lipids of strain GCWy1T were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, one unknown lipid and two unknown aminophospholipids (Fig. 2). The polar lipid profile of strain GCWy1T was similar to those of *Saccharospirillum impatiens* EL-105T and *R. marinisedimentorum* KMM 3655T (Labrenz et al., 2003; Romanenko et al., 2004), except for the absence of phosphatidylmonomethylamine that was present in *Saccharospirillum impatiens* EL-105T, phosphatidylinositol and an unknown

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**Fig. 1.** Phylogenetic tree based on 16S rRNA gene sequence composition showing the relationship of strain GCWy1T with related genera of the class Gammaproteobacteria. The phylogenetic tree was reconstructed using the neighbour-joining algorithm. Bootstrap values for 1000 replicates are shown. The sequence of *Terasakiella pusilla* NBRC 13613T (AB006768) was used as an outgroup. Bar, 2% estimated sequence divergence.
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Description of Salinispirillum marinum sp. nov.

Salinispirillum marinum (ma.rí’num. L. neut. adj. mar-inum belonging to the sea, marine).

Cells are Gram-staining-negative, motile and spirillum-shaped with a width of 0.3–0.5 μm and length of 2.5–5.9 μm. Colonies grown for 48 h on MH medium with 3% NaCl. Facultative anaerobe. Positive for oxidase and catalase activities. Hydrolyzes casein, tyrosine, gelatin and Tween 20 but aesculin, starch, Tween 80, DNA and urea are not hydrolysed. Indole and H2S are not produced and nitrate is not reduced. Acid production from adonitol, cellobiose, D-arabitol D-galactose, D-glucose, D-mannitol, D-sorbitol, D-xyllose, melibiose, myo-inositol and sucrose are negative. D-Arabitol, cellobiose, D-galactose, D-glucose, D-mannitol, maltose, melibiose, myo-inositol, sucrose, L-alanine, L-serine and L-ornithine are utilized as sole carbon and energy sources. Citrate, adonitol, D-sorbitol, D-xyllose, L-glutamic acid, L-hydroxyproline and L-threonine are not utilized as sole sources of carbon and energy. The cellular fatty acid profile is characterized by the fatty acids C18:1ω7c, C16:0, C17:0 and C16:1ω11c. The major respiratory quinone is Q-9. This genus represents a main phylogenetic sublineage within the family 'Saccharospirillaceae', showing remote relatedness to other marine and non-marine members of the class Gammaproteobacteria. The type species is Salinispirillum marinum.

Description of Salinispirillum gen. nov.

Salinispirillum gen. nov. (Sa.li.ni.spi.ril’lum. L. n. salina a saltern; L. neut. n. spirillum a screw; N.L. neut. n. Salinispirillum a screw (shaped bacterium) from a saltern).

Gram-staining-negative, heterotrophic, oxidase- and catalase-positive, spirillum-shaped and motile. The temperature range for growth is 4–40 °C. The cells have an absolute requirement for NaCl and grow at salinities in the range of 1.0–10.0% (w/v) NaCl. The pH range for growth is 7.5–10.0. Facultatively anaerobic. Anaerobic growth occurs on glucose in the absence of nitrate. Grow on various carbohydrates. Diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, one unknown lipid and two unknown aminophospholipids are present. The predominant cellular fatty acids are C18:1ω7c, C16:0, C17:0 and C16:1ω11c. The major respiratory quinone is Q-9. This genus represents a main phylogenetic sublineage within the family 'Saccharospirillaceae', showing remote relatedness to other marine and non-marine members of the class Gammaproteobacteria. The type species is Salinispirillum marinum.

In summary, the phylogenetic analysis revealed that GCWy1T clustered within the Gammaproteobacteria, which encompasses the genera Saccharospirillum and Reinekeia (Fig. 1), but showed very low 16S rRNA gene sequence similarity with respect to the species of the related genera (equal or lower than 92.1%), which supports the placement of the novel isolate within a separate genus. Also, some differences with respect to the chemotaxonomic and phenotypic characteristics with respect to the closely related taxa were observed (Table 1). The novel isolate could be distinguished from members of the genus of the family 'Saccharospirillaceae' by its morphology, quinones and fatty acid composition. Some phenotypic properties delineating GCWy1T were also observed with respect to those that characterize members of the related species. We should note that the remote phylogenetic relationship observed on the basis of the 16S rRNA gene sequence similarity to the most closely related species of the novel bacterium was strongly supported by phenotypic and chemotaxonomic features. Therefore, based on these lines of evidence, we propose that strain GCWy1T be classified as a representative of a novel genus and species, Salinispirillum marinum gen. nov., sp. nov.

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