Marinospirillum celere sp. nov., a novel haloalkaliphilic, helical bacterium isolated from Mono Lake

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Two strains of a Gram-negative, helical, haloalkaliphilic bacterium were isolated from Mono Lake (USA). Both strains were mesophilic and grew between 13 and 55 °C, with optimum growth at 35–45 °C. The optimum pH for growth was 9.5. Growth was observed at NaCl concentrations of 0.5–12 % (w/v), with optimum growth at 2 % NaCl. Both isolates were motile by means of bipolar tuft flagella, coccoid body-forming and strictly aerobic. It was concluded that they belong to the same species, based on DNA–DNA hybridization values (95 % DNA relatedness). DNA G+C contents of the novel strains were 52.1 and 52.3 mol%. On the basis of 16S rRNA gene sequence similarity, both strains were shown to be related closely to the members of the genus *Marinospirillum* (family *Oceanospirillaceae*, class *Gammaproteobacteria*). Sequence similarity of strain v1c_Sn-red^T to the type strains of *Marinospirillum alkaliphilum*, *Marinospirillum minutulum*, *Marinospirillum megaterium* and *Marinospirillum insulare* was 95.0, 92.7, 91.8 and 91.8 %, respectively. Chemotaxonomic data [major ubiquinone, Q8; major fatty acids, C_{18:1}(n-7) and C_{16:0}] and physiological and biochemical tests supported the affiliation of the novel strains to the genus *Marinospirillum* as members of a novel species, for which the name *Marinospirillum celere* sp. nov. is proposed, with the type strain v1c_Sn-red^T (=LMG 24610^T=VKM 2416^T).

The genus *Marinospirillum* was created by Satomi *et al.* (1998) and at present it comprises four species: *Marinospirillum minutulum* (Watanabe, 1959; Satomi *et al.*, 1998; type species of the genus), *Marinospirillum megaterium* (Satomi *et al.*, 1998), *Marinospirillum alkaliphilum* (Zhang *et al.*, 2002) and *Marinospirillum insulare* (Satomi *et al.*, 2004). All species that belong to this genus are motile, helical, halophilic and Gram-negative. Organotrophic growth occurs on complex organic substrates such as yeast extract and peptone and on organic acids. Carbohydrates are not utilized. Sorokin *et al.* (2007) isolated an alkaliphilic strain, *Marinospirillum* sp. ANL-isoa, utilizing amides and carboxylic acids in an isobutyronitrile-degrading consortium, but this strain was not validly described as representing a novel species.

Strain v1c_Sn-red^T was isolated from a combined water–sediment slurry sample taken from a hot spring (40 °C, pH 9.3, salinity 25 g l⁻¹) on Paoha island on Mono Lake

(CA, USA). Strain Spir-10 was isolated from a water sample (10 m depth, pH 9.5, salinity 90 g l⁻¹) of Mono Lake. Samples were collected in August 2000. Subsamples (1 ml) were inoculated into 20 ml aerobic medium containing (l⁻¹): KH₂PO₄, 0.5 g; NH₄Cl, 0.5 g; KCl, 0.5 g; NaCl, 25 g; Na₂SO₄, 0.5 g; MgSO₄·7H₂O, 0.2 g; yeast extract, 1 g; peptone, 2 g; trace element solution, 1 ml (Pfennig & Lippert, 1966); vitamin B₁₂, 15 µg. After autoclaving, 0.05 g CaCl₂·2H₂O l⁻¹, 5 g NaHCO₃ l⁻¹ and 5 g Na₂CO₃ l⁻¹ were added from sterile (10 %, w/v) stock solutions. Enrichment cultures of the strains were incubated at 40 °C (v1c_Sn-red^T) or 25 °C (Spir-10). For strain isolation, samples of water were transferred onto plates that contained 2 % agar. Growth was followed by monitoring the increase in OD₅₅₀ in Hungate tubes (anaerobically) or in plain tubes (aerobically). All runs were done in duplicate. The pH was adjusted to 9.5 by varying the amounts of NaHCO₃ and Na₂CO₃ and addition of 1 M HCl and 1 M NaOH. After determination of optimal growth conditions, both strains were cultivated at 35 °C, pH 9.5 and 2 % NaCl (w/v). Substrate-utilization tests were

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain v1c_Sn-red^T is EU375807.

performed at optimal temperature and in a pH-optimal carbonate-buffered medium of the following composition: mineral stock plus vitamin solution were the same as above; yeast extract, 0.05 g l⁻¹; substrate, 1 g l⁻¹. All substrates were prepared as anaerobic stock solutions in distilled water. Reduction of various inorganic compounds was tested anaerobically on a medium containing pyruvate, peptone and yeast extract (all at 1 g l⁻¹) as described previously (Gorlenko *et al.*, 2004). Poly- β -hydroxybutyrate accumulation, catalase and oxidase tests, casein, gelatin, hippurate and starch hydrolysis and production of DNase, RNase, urease, lipase, phosphatase, indole and H₂S were determined as described by Gerhardt *et al.* (1994).

Gram staining was performed as described by Gerhardt *et al.* (1994). Cell morphology was observed under an Olympus BX-41 phase-contrast microscope at $\times 1000$ with cells grown overnight. Micrographs were taken by C-7070 (Olympus) photo attachment on slides coated with 1% (w/v) ultrapure agar. The fine structure of cells was studied after fixation of cells with a 1% (w/v) solution of OsO₄ by the method of Ryter & Kellenberger (1958). Ultrathin sections were obtained on an LKB-NOVA ultramicrotome and contrasted with a 2% (w/v) aqueous solution of uranyl acetate with subsequent treatment with lead citrate (Reynolds, 1963). A JEM-100CXII electron microscope was used at magnifications of $\times 20\,000$ – $27\,000$. Negative staining of cells was achieved with 0.1% uranyl acetate and cells were examined under a transmission electron microscope. Membrane fatty acids were extracted from freeze-dried cells with methanol/chloroform and analysed by GC-MS as described by Zhilina *et al.* (1997). Analysis of isoprenoid quinones was performed as described by Collins (1985).

DNA was isolated according to Marmur (1961). The G + C content was determined by the thermal-denaturation method of Marmur & Doty (1962). DNA–DNA hybridization was performed spectrophotometrically and initial renaturation rates were recorded as described by De Ley *et al.* (1970). Genomic DNA extraction and PCR-mediated amplification of the 16S rRNA gene were done as described by Rainey *et al.* (1996). PCR products were sequenced by using a CEQ DTCS kit and run on a CEQ 2000XL DNA sequencing system (Beckman-Coulter). The sequences were aligned by using the CLUSTAL_X software (Thompson *et al.*, 1997). An evolutionary-distance matrix was calculated by using the algorithm of Jukes & Cantor (1969). The phylogenetic tree was constructed by using the TREECON package (Van de Peer & De Wachter, 1994) and the neighbour-joining algorithm (Saitou & Nei, 1987). Bootstrap analyses were based on 500 resamplings.

Physiological and biochemical characteristics of the strains studied are listed in the species description and in Table 1. Both strains require NaCl, with growth being observed at NaCl concentrations of 0.5–12% (w/v) with an optimum at 2% for both strains. Growth of both strains was seen at 13–55 °C, with a wide temperature optimum of 35–45 °C. The pH range for growth of both strains is 8.0–10.5, with an optimum at 9.5. Best growth occurred on capriate (up to 3×10^8 cells ml⁻¹). The shortest doubling time on capriate-containing medium under optimal conditions was 20 min. At the early stages of development, cells were motile with a swimming speed of up to 60 $\mu\text{m s}^{-1}$. After about 4 days cultivation, thin-walled coccoid bodies are observed, but no spores are produced. In old cultures, cells become shorter and thinner and a protuberance arises from

Table 1. Characteristics of *Marinospirillum* species and strain v1c_Sn-red^T

Strain designations: 1, v1c_Sn-red^T (data from this study); 2, *M. alkaliphilum* Z4^T (data from Zhang *et al.*, 2002); 3, *M. minutulum* ATCC 19193^T (Watanabe, 1959; Satomi *et al.*, 1998); 4, *M. megaterium* JCM 10129^T (Satomi *et al.*, 1998); 5, *M. insulare* K^T (Satomi *et al.*, 2004). +, Positive; –, negative; (+), weakly positive; ND, no data; Mi, microaerophilic; Ae, aerophilic.

Characteristic	1	2	3	4	5
Cell length (μm)	1.4–15	2.7–4.0	2–2.8	5–15	2.5–7.5
Cell diameter (μm)	0.2–0.4	0.2–0.3	0.3–0.4	0.8–1.2	0.1–0.2
Colony formation	+	+	+	–	+
Oxygen requirement	Ae	Ae	Ae	Mi	Ae
Temperature for growth (°C)					
Range	13–55	8–49	11–37	4–25	4–37
Optimum	35–45	37	30	20–25	25–30
pH for growth					
Range	8.0–10.5	7.0–11.0	7.0–10.5	7.5–9.0	6.5–10.0
Optimum	9.5	9.5	9.0	8.0	8.0
NaCl range for growth (% w/v)	0.5–12	0.2–5.0	0.2–8.0	0.5–9.0	0.5–10.0
Catalase	+	+	+	– or (+)	+
Nitrate reduction	–	+	+	–	+
Isoprenoid quinone	Q8	Q8, MK6	Q8	Q8	Q8
Major fatty acids	C _{18:1} (n-7), C _{16:0}	ND	C _{16:0} , C _{18:1} (n-7), C _{16:1}	ND	C _{16:0} , C _{18:1} (n-7), C _{16:1}
DNA G + C content (mol%)	52.3	46.8	42.5	44–45	42–43

the outer membrane at the end of the cell. The protuberance enlarges and eventually forms a single coccoid body as the helical cell is absorbed (Fig. 1). Similarities in phenotypic characteristics support the inclusion of the studied strains in the genus *Marinospirillum*. However, the new strains clearly differed from other species by a higher growth-temperature range and optimum and an inability to grow at pH values <8.0.

Strain v1c_Sn-red^T contains ubiquinones that consist mainly of Q8. Menaquinone and methylmenaquinone were not detected. Other *Marinospirillum* species also have Q8 as the major isoprenoid quinone (Satomi *et al.*, 1998, 2004; Zhang *et al.*, 2002). The fatty acid profile of strain v1c_Sn-red^T is composed of C_{18:1}(n-7) (48.52%), C_{16:0} (36.22%), C_{16:1}(n-7) (8.05%), C_{14:1}(n-3) (3.16%) and C_{14:0} (1.07%). This novel strain shows a combination of fatty acids found in other species of the genus *Marinospirillum*.

A DNA–DNA hybridization experiment revealed that the new isolates are related closely to each other (95% DNA relatedness), suggesting that they belong to the same species. The G+C contents of the new isolates v1c_Sn-red^T and Spir-10 were respectively 52.3 and 52.1 mol%, i.e. 5–10 mol% higher than those of other *Marinospirillum* species. Strains v1c_Sn-red^T and Spir-10 have a high level of 16S rRNA gene sequence similarity to each other (99.6%) and form a stable phylogenetic cluster, with a

bootstrap value of 100%, within the genus *Marinospirillum* (Fig. 2). Sequence-similarity calculations after neighbour-joining analysis indicated that the closest relatives of strain v1c_Sn-red^T were *M. alkaliphilum* Z4^T (95.0%), *M. minutulum* ATCC 19193^T (92.7%), *M. megaterium* JCM 10129^T (91.8%) and *M. insulare* K^T (91.8%). Lower sequence similarities (<89.5%) were found with other Gram-negative spirilla.

On the basis of the low 16S rRNA gene sequence-similarity values (<95%) to other species of the genus *Marinospirillum*, a higher G+C content and their distinct physiological characteristics compared with other species of this genus, strains v1c_Sn-red^T and Spir-10 represent a novel species. We propose the name *Marinospirillum celere* sp. nov. for this taxon, with the type strain v1c_Sn-red^T.

Sequence-similarity calculations, a higher G+C content and higher pH and temperature optima show that both novel strains are related more closely to *M. alkaliphilum* than to other *Marinospirillum* species. Although *M. minutulum*, *M. megaterium* and *M. insulare* were isolated previously from an ocean environment and artificial brines (Satomi *et al.*, 1998, 2004), the novel strains and *M. alkaliphilum* were isolated from soda lakes. The last two species could form a novel genus in the family *Oceanospirillaceae*, but we believe that it is better to wait until more haloalkaliphilic *Marinospirillum* species have been isolated before naming this taxon.

Description of *Marinospirillum celere* sp. nov.

Marinospirillum celere (ce'le.re. L. neut. adj. *celere* rapid, indicating the rapid growth on nutrient media and high motility).

Gram-negative, helical, aerobic and chemoheterotrophic. Motile by bipolar flagella. Cells are 0.2–0.4 µm in diameter and 1.4–15 µm in length. Coccoid bodies are observed after

Fig. 1. (a, b) Electron micrographs of ultrathin-sectioned cells of strain v1c_Sn-red^T from (a) the exponential-growth phase, showing the invagination of the cell membrane during cell division (arrow), and (b) the stationary-growth phase, showing the formation of a coccoid body. (c) Phase-contrast photograph of cells of strain v1c_Sn-red^T from the exponential-growth phase. Bars, 0.5 µm (a, b); 2 µm (c).

Fig. 2. Phylogenetic tree constructed by the neighbour-joining method based on 16S rRNA gene sequences of the new isolates v1c_Sn-red^T and Spir-10 and type strains of related species. Bar, 0.02 genetic distance. Numbers at nodes are bootstrap-support values (percentages of 500 resamplings). The type strain of *Alkalispirillum mobile* was used as the outgroup.

4 days culture, but no spores are produced. The helix type is counterclockwise. Halophilic, NaCl is required for growth; growth occurs at NaCl concentrations of 0.5–12 % (w/v) and is optimal at 2 % NaCl. Temperature range for growth is 13–55 °C; optimal temperature is 35–45 °C. Alkaliphilic, pH range for growth is 8.0–10.5; optimal pH is 9.5. Colonies are circular, smooth, opalescent and 1 mm in diameter after overnight culture. Catalase- and oxidase-positive. Able to utilize caprilate, acetate, pyruvate, lactate, Casamino acids, yeast extract and peptone. D-Ribose, D-glucose, sucrose, rhamnose, cellulose, glycerol, citrate, glycolate and cysteine are not utilized. Casein, gelatin, hippurate and starch are not hydrolysed. Indole, H₂S, DNase, RNase, phosphatase, urease and lipase are not produced. Reduction of thiosulfate, sulfate, sulfite, polysulfide, sodium selenite, ferric citrate, ferric hydroxide and elemental sulfur is not observed. Nitrate reduction and denitrifying activity are negative. Does not grow autotrophically on H₂+CO₂. Intracellular poly- β -hydroxybutyrate is formed. Isoprenoid quinone type is Q8. Whole-cell fatty acids consist mainly of C_{18:1}(n-7) and C_{16:0}. DNA G+C content of the type strain is 52.3 mol%.

The type strain is v1c_Sn-red^T (=LMG 24610^T=VKM 2416^T), isolated from Mono Lake (CA, USA).

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