Balneola alkaliphila sp. nov., a marine bacterium isolated from the Mediterranean Sea

Laurent Urios,[1] Laurent Intertaglia,[1] Françoise Lesongeur[2] and Philippe Lebaron[1]

Correspondence
Philippe Lebaron
lebaron@obs-banyuls.fr

[1]Observatoire Océanologique, Laboratoire d'Océanographie Biologique de Banyuls, Université Pierre et Marie Curie (Paris VI), Institut National des Sciences de l'Univers (INSU), CNRS UMR 7621, BP44, 66651 Banyuls-sur-Mer Cedex, France

[2]Laboratoire de Microbiologie des Environnements Extrêmes, UMR 6197, IFREMER, Centre de Brest, BP 70, 29280 Plouzané, France

A novel aerobic, Gram-negative bacterium, designated strain CM41_14b^T, was isolated from surface waters in the coastal north-western Mediterranean Sea. Cells were non-motile, straight rods, 2.6 μm long and 0.7 μm wide and formed pale-orange colonies on marine agar medium. The G+C content of the genomic DNA was 39 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence placed the strain within the genus *Balneola* (phylum *Bacteroidetes*). On the basis of 16S rRNA gene sequence comparisons, and physiological and biochemical characteristics, the isolate represents a novel species for which the name *Balneola alkaliphila* sp. nov. is proposed. The type strain is CM41_14b^T (=DSM 19538^T=CIP 109603^T=OOB 103^T).

Stoecker *et al.* (2006) have provided molecular evidence that the genus *Crenothrix* belongs to the order *Methylococcales*. Consequently, other genera originally assigned to the family *Crenotrichaceae* within the order *Sphingobacteriales* are currently considered *Sphingobacteriales genera incertae sedis*, including the genera *Rhodothermus* (Alfredsson *et al.*, 1988; Sako *et al.*, 1996) and *Salinibacter* (Antón *et al.*, 2002), which include species that exhibit extremophilic characteristics. *Rhodothermus* strains are thermophilic and their optimal growth temperatures are between 60 and 80 °C. *Salinibacter* strains are extreme halophiles that require at least 150 g salt l^{-1} for growth. Recently, a new genus that was phylogenetically related to *Rhodothermus* and *Salinibacter* was described and named *Balneola* (Urios *et al.*, 2006). This genus was defined by a single species, *Balneola vulgaris*, which was isolated from a seawater sample from the bay of Banyuls-sur-Mer (France) and which does not exhibit extremophilic characteristics. Optimal growth of *B. vulgaris* DSM 17893^T was found to occur at pH 8.0 and 30 °C and with 20 g NaCl l^{-1}. Therefore, *B. vulgaris* did not exhibit any of the extreme features found in *Rhodothermus* and *Salinibacter* species. Here we describe a novel strain that was isolated from seawater in the same area as *B. vulgaris*. The strain presented characteristics that allowed the proposition of a novel species of the genus *Balneola*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CM41_14b^T is EU008564.

Graphs showing the effects of temperature, salinity and pH on the growth of strain CM41_14b^T are available as a supplementary figure with the online version of this paper.

Samples were collected in February 2004 in the bay of Banyuls-sur-Mer (42° 29′ N 3° 08′ E) by submerging a sterile bottle and opening it at a depth of 3 m. Subsamples were spread onto marine agar plates (MA 2216; Difco) and incubated at 25 °C for 2 weeks. Colonies were picked and purified after at least three subcultures. Among these colonies, an isolate that formed pale-orange colonies was obtained and designated strain CM41_14b^T.

Microscopic observations (Olympus AX70) showed that cells of strain CM41_14b^T were non-motile rods, approximately 2.6±0.1 μm long and 0.7±0.05 μm wide. The Ryu KOH reaction (Powers, 1995) led to immediate cell lysis that was confirmed by phase-contrast microscopy (Olympus AX70). This positive reaction indicated that the cells were Gram-negative.

Strain CM41_14b^T was grown in marine broth medium (MB 2216; Difco). To determine the salinity range for growth, MB 2216 was prepared according to the composition provided by the manufacturer, with the appropriate NaCl concentration. To determine the pH range for growth, MES, PIPES, AMPSO or MOPS (Sigma) was added to MB 2216 to reach the required pH. Cultures were incubated at 30 °C under aerobic conditions. The methods used for the determination of growth parameters were as reported by Wery *et al.* (2001b). Growth was observed at 15–37 °C, with optimum growth occurring at 25 °C (see Supplementary Fig. S1a, available in IJSEM Online). Growth rates were 10-fold lower at 15 and 44 °C, compared with the optimal growth rate at 25 °C. Strain CM41_14b^T grew at NaCl concentrations ranging from 10 to 80 g l^{-1}; an optimum

concentration for growth could be defined at 30 g l^{-1}, but growth rates at 20 and 40 g l^{-1} were only 10 % lower than that at 30 g l^{-1} (Supplementary Fig. S1b). Growth occurred at pH 6.0–10.0, with optimum growth between pH 8.0 and 9.0. An approximately linear increase in growth rate was observed to occur between pH 6.0 and 8.0 (Supplementary Fig. S1c). The ability of strain CM41_14b^T to use different substrates was investigated using Biolog GN2 MicroPlates (Tang *et al.*, 1998), according to the manufacturer's instructions. Positive reactions were observed for D-fructose, turanose, acetate, citrate, D-glucosaminic acid and inosine (Table 1). Weak positive reactions were observed for cellobiose, D-glucose, itaconate, glycyl L-aspartic acid and γ-aminobutyric acid (Table 1).

Enzymic activities were investigated using the API ZYM system (bioMérieux), according to the manufacturer's instructions. Alkaline phosphatase, leucine arylamidase, valine arylamidase and trypsin exhibited positive reactions, whereas reactions for chymotrypsin and acid phosphatase were weak. Catalase and oxidase tests were performed; strain CM41_14b^T was found to be catalase-positive and oxidase-negative. The presence of flexirubin pigments was investigated according to McCammon & Bowman (2000); a negative reaction was observed.

Analysis of the fatty acid methyl esters was performed by the Identification Service of the Deutsche Sammling von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The fatty acid composition of strain CM41_14b^T was as follows: 15 : 0 iso (45.2 %), 15 : 0 iso 2-OH (7.5 %), 17 : 1ω8*c* (1.8 %), 15 : 0 (1.6 %), 15 : 1ω6*c* (4.3 %), 13 : 0 iso (14.2 %), 17 : 1ω9*c* iso (9.7 %), 15 : 0 anteiso (3.3 %), 15 : 1 iso (2.7 %), 17 : 1ω6*c* (1.0 %), 14 : 0 iso (1.0 %), 16 : 0 iso (0.6 %), 17 : 0 iso (2.0 %), 16 : 0 (0.6 %), 15 : 1ω8*c* (1.4 %) and 16 : 1ω5*c* (1.2 %). The major fatty acids were generally similar to those of *B. vulgaris* DSM 17893^T (Urios *et al.*, 2006); however, the amounts of 15 : 0 iso and 13 : 0 iso were twice those of *B. vulgaris* DSM 17893^T, the amount of 15 : 1ω6*c* was 2.5-fold lower and 17 : 1ω8*c* and 15 : 0 were both 6-fold lower in strain CM41_14b^T compared with *B. vulgaris* DSM 17893^T. Fatty acids 17 : 0 and 15 : 1 iso were not detected in *B. vulgaris* DSM 17893^T.

Genomic DNA was extracted as described by Wery *et al.* (2001a). The G+C content was determined by thermal denaturation using the method of Marmur & Doty (1962) and the conditions described by Raguénès *et al.* (1997). The G+C content of the genomic DNA of strain CM41_14b^T was 39±1 mol%. The 16S rRNA gene was amplified and sequenced as described by Agogué *et al.* (2005) and the sequence was analysed as described by Urios *et al.* (2006). Strain CM41_14b^T was phylogenetically affiliated to the genus *Balneola* (Fig. 1). The closest relative was *B. vulgaris* DSM 17893^T, with a 16S rRNA gene sequence similarity of 95 %.

Some characteristics of strain CM41_14b^T differed from those of its closest relative: lower optimal temperature

Table 1. Characteristics that distinguish strain CM41_14b^T from its most closely related species, *B. vulgaris*

The two strains were isolated from seawater samples from the bay of Banyuls-sur-Mer, France. +, Positive; –, negative; (+), weakly positive. Data for *B. vulgaris* are from Urios *et al.* (2006).

Characteristic	*B. vulgaris* DSM 17893^T	Strain CM41_14b^T
DNA G+C content (mol%)	41.8	38.7
Colony colour	Orange	Pale orange
Cell dimensions (μm)	2–3 × 0.2	2.6 × 0.7
Motility	+	−
Temperature range (optimum) for growth (°C)	10–40 (30)	15–37 (25)
Salinity range (optimum) for growth (g NaCl l^{-1})	0–50 (20)	10−80 (30)
pH range (optimum) for growth	5–10 (8)	6−10 (8−9)
Substrates utilized		
Turanose	−	+
D-Glucose	+	(+)
Cellobiose	−	(+)
Lactose	(+)	−
Maltose	+	−
Mannitol	(+)	−
Sorbitol	+	−
Citrate	−	+
D-Glucosaminic acid	−	+
Itaconate	−	(+)
Inosine	−	+
Glycyl L-aspartic acid	−	(+)
γ-Aminobutyric acid	−	(+)

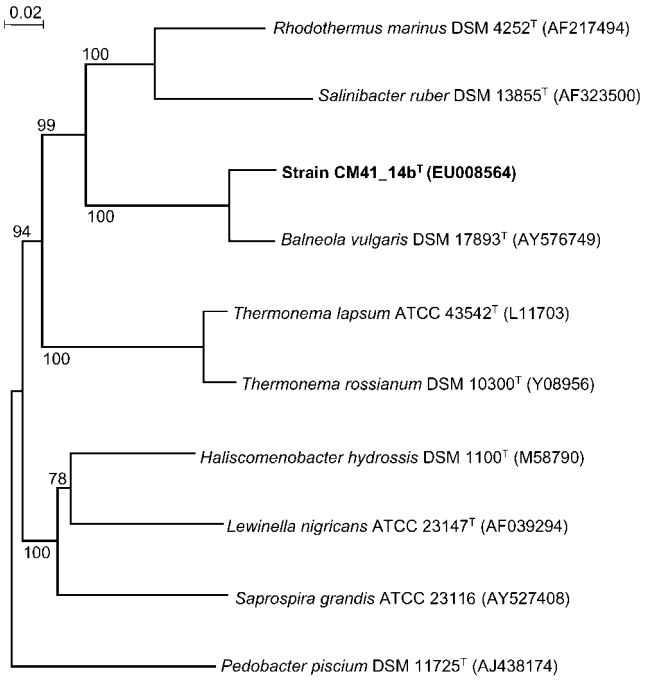

Fig. 1. Unrooted neighbour-joining phylogenetic tree (Kimura corrections), based on 16S rRNA gene sequences, showing the position of strain CM41_14b^T. The sequence of *Pedobacter piscium* DSM 11725^T was used as the outgroup. GenBank accession numbers are given in parentheses. Bootstrap percentages (based on 1000 replications) are shown at branch points. Bar, 2 substitutions per 100 nucleotide positions.

(5 °C), more-limited temperature range for growth, higher salinity range for growth (up to 30 g NaCl l^{-1}) and a slightly higher pH optimum for growth of 8.0–9.0 (Table 1). Cells of strain CM41_14b^T were non-motile, unlike those of *B. vulgaris* DSM 17893^T. The G+C content of strain CM41_14b^T was lower (3 mol%) than that of *B. vulgaris* DSM 17893^T.

Based on the phenotypic and genotypic differences between strain CM41_14b^T and its closest relative, we propose that strain CM41_14b^T represents a novel species of the genus *Balneola*. Because of the differences in pH range and optimum between *B. vulgaris* and strain CM41_14b^T, the name *Balneola alkaliphila* sp. nov. is proposed.

Description of *Balneola alkaliphila* sp. nov.

Balneola alkaliphila (al.ka.li.phi′la. N.L. n. *alkali* alkali; Gr. adj. *philos* loving; N.L. fem. adj. *alkaliphila* loving alkaline conditions).

Forms pale-orange colonies on MA 2216. Cells are non-motile rods. Growth occurs at 15–37 °C (optimum, 25 °C), at pH 6.0–10.0 (optimum, 8.0–9.0) and at salinities of 10–80 g NaCl l^{-1} (optimum, 30 g NaCl l^{-1}). Positive reactions with Biolog GN2 plates are obtained for D-fructose, turanose, acetate, citrate, D-glucosaminic acid and inosine. Positive reactions (API ZYM) are obtained for alkaline phosphatase, leucine arylamidase, valine arylamidase and trypsin. The DNA G+C content of the type strain is 39 mol%.

The type strain, CM41_14b^T (=DSM 19538^T=CIP 109603^T=OOB 103^T), was isolated from a water column in the bay of Banyuls-sur-Mer.

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