Rhodonellum psychrophilum gen. nov., sp. nov., a novel psychrophilic and alkaliphilic bacterium of the phylum *Bacteroidetes* isolated from Greenland

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A novel alkaliphilic and psychrophilic bacterium was isolated from the cold and alkaline ikaite tufa columns of the Ikka Fjord in south-west Greenland. According to 16S rRNA gene sequence analysis, strain GCM71^T belonged to the family 'Flexibacteraceae' in the phylum *Bacteroidetes*. Strain GCM71^T, together with five related isolates from ikaite columns, formed a separate cluster with 86–93 % gene sequence similarity to their closest relative, *Belliella baltica*. The G + C content of the DNA from strain GCM71^T was 43.1 mol%, whereas that of *B. baltica* was reported to be 35 mol%. DNA–DNA hybridization between strain GCM71^T and *B. baltica* was 9.5 %. The strain was red pigmented, Gram-negative, strictly aerobic with non-motile, rod-shaped cells. The optimal growth conditions for strain GCM71^T were pH 9.2–10.0, 5 °C and 0.6 % NaCl. The fatty acid profile of the novel strain was dominated by branched and unsaturated fatty acids (90–97 %), with a high abundance of iso-C_{17:1}ω9c (17.5 %), iso-C_{17:0} 3-OH (17.5 %) and summed feature 3, comprising iso-C_{15:0} 2-OH and/or C_{16:1}ω7c (12.6 %). Phylogenetic, chemotaxonomic and physiological characteristics showed that the novel strain could not be affiliated to any known genus. A new genus, *Rhodonellum* gen. nov., is proposed to accommodate the novel strain. Strain GCM71^T (=DSM 17998^T=LMG 23454^T) is proposed as the type strain of the type species, *Rhodonellum psychrophilum* sp. nov.

Introduction

Bacteria of the phylum *Bacteroidetes* often occur in marine and freshwater environments where they may comprise a large fraction of the bacterioplankton community and play an important role in the degradation of organic matter (Cottrell & Kirchman, 2000; Brettar *et al.*, 2004). In this study, we describe a member of the *Bacteroidetes* that was isolated from cold and alkaline submarine ikaite columns in the Ikka Fjord, south-west Greenland (Buchardt *et al.*, 1997, 2001; Stockmann *et al.*, 2000). The columns are formed by

the meta-stable cold-water mineral ikaite (CaCO₃·6H₂O) and stabilized by a microbial biofilm. A rich microbial community of several hundred different bacterial species exists inside the porous ikaite columns (Stougaard *et al.*, 2002; Schmidt *et al.*, 2006). The ikaite columns form a freshwater environment that provides a stable ecological niche for particle-associated bacteria. These bacteria are able to degrade organic matter from the bacteria and higher eukaryotes that inhabit the columns or are trapped in the ikaite during column growth. Members of the phylum *Bacteroidetes* have been estimated to constitute 7–12 % of the total bacterial diversity inside ikaite columns based on 16S rRNA gene fragment clone libraries and culture analysis (Schmidt *et al.*, 2006).

In this study, we isolated five bacterial strains that form a separate lineage within the family 'Flexibacteraceae' of the phylum *Bacteroidetes*. One of these ikaite isolates, strain GCM71^T, was studied in more detail with respect to morphology, growth characteristics, biofilm production, pigmentation, cellular fatty acid content and phylogeny. Our phylogenetic, chemotaxonomic and physiological results lead us to propose a new genus within the phylum

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The GenBank/EMBL/DDJB accession number for the 16S rRNA gene sequence of *Rhodonellum psychrophilum* sp. nov. GCM71^T is DQ112660.

Tables detailing carbon source utilization, the results of API 20NE and API ZYM tests and the fatty acid content of the strains analysed in this study and a graph showing the absorbance spectra of strain GCM71^T when grown for six weeks with or without light are available as supplementary material in IJSEM Online.

Bacteroidetes, *Rhodonellum* gen. nov., with the novel species *Rhodonellum psychrophilum* sp. nov.

Methods

Strain GCM71^T was isolated from ikaite tufa columns collected from the Ikka Fjord, SW Greenland (61° 11' N 48° 01' W) in 2002. The columns were conserved in 15 % glycerol and kept at -20 °C. The strain was isolated on agar plates containing R2A medium diluted 10-fold (1/10 R2A) as described by Schmidt *et al.* (2006). Physiological features were determined using R2 broth medium diluted 10-fold (1/10 R2B) containing the same ingredients as 1/10 R2A except for the agar. The 10-fold diluted R2 broth also contained 1.0 ml l⁻¹ Vogel's trace elements (Hoekstra, 1996) and 1.0 ml l⁻¹ vitamin solution (containing the following ingredients l⁻¹: 0.1 g thiamine hydrochloride, 0.03 g vitamin B₁₂ and 0.03 g D-biotin).

For determination of the optimum pH, 1/10 R2 broth was adjusted to one of the following pH values: 6.5, 7.5, 8.0, 9.2, 9.5, 10.0 or 10.7 using sodium phosphate buffer (pH 6.5–8.0) or sodium carbonate buffer (pH 9.2–10.7). Media adjusted to each of the pH values were filter-sterilized through a 0.22 µm pore filter and incubated at different temperatures. The temperatures applied were 0, 5, 10, 15, 22, 30, 37 and 45 °C. The medium used for carbon utilization analysis was 1/10 R2B adjusted to pH 10, except it was not supplemented with glucose and soluble starch. Even though this medium still contains low levels of yeast extract and peptone, no growth was observed when no other carbon sources were added and therefore this medium was used for the carbon utilization experiment. The individual carbon sources were added to a final concentration of 20 mM (see Supplementary Table S1 in IJSEM Online) and the inoculated cultures were incubated at 10 °C. Growth requirements were also tested with the API 20NE and API ZYM (bioMérieux) commercial systems. The manufacturer's guidelines were followed with the following exceptions: cells from a 25 ml culture were collected and used for the assay, the AUX medium was adjusted to pH 9.2 using a 1 M carbonate/bicarbonate buffer and the assay was incubated at 10 °C. The API 20NE test was read after 24 and 48 h as instructed, while the API ZYM was read after 5 days.

Salinity tolerance and requirement were determined in 1/10 R2B containing 0, 0.1, 0.3, 0.6, 1.0, 1.5, 3.0 and 6.0 % (w/v) NaCl with pH adjusted to 10.0 at 10 °C. All growth experiments were performed with six replicates and growth in broth cultures was measured as OD₆₀₀ in flat-bottomed 96-well microtitre plates using a ELX808 RIU reader (Biotek).

The novel strains were tested for a number of characteristics using standard procedures such as Gram staining, oxidase test (Microbiology Bactident oxidase-strips), catalase test (H₂O₂) and cell size and morphology (phase-contrast microscopy and scanning electron microscopy).

Photosynthetic growth was determined on full strength R2A (aerobic) and in R2 broth (anaerobic, atmosphere: 20 % CO₂, 80 % N₂) in glass jars sealed with rubber stoppers. Inoculated agar plates and glass jars were incubated at 5 °C and illuminated at 20–40 µE m⁻² s⁻¹. Control cultures were grown in darkness at 5 °C.

Identification of biofilm production was performed using a modified version of the methods described by O'Toole & Kolter (1998), Prigent-Combaret *et al.* (2001) and Dorel *et al.* (1999). Colonies isolated on R2A media were precultured in R2 broth before inoculation in flat-bottomed 96-well microtitre plates with 200 µl per well. Plates were sealed with Parafilm and incubated at 5 °C with 75 r.p.m. shaking for 4 days. To correlate biofilm formation to planktonic cell growth in each well, the planktonic cell fraction was transferred to a new microtitre plate and OD₆₀₀ was measured in a Wallac-Victor² 1420 Multilabel Counter. Biofilm formation by attached cells was analysed with crystal violet staining as previously reported by O'Toole & Kolter (1998). Crystal violet bound to biofilm was measured as absorbance at A₆₀₀ using the Wallac-Victor² 1420 Multilabel Counter. Absorbance measurements obtained were related to the OD₆₀₀ of the planktonic cell fraction.

DNA was extracted from the strains using the DNeasy tissue kit (Qiagen) following the manufacturer's instructions for Gram-negative bacteria. 16S rRNA gene fragment amplification was carried out using primers 27F and 1492R, while primers 27F, 519R, 532F, 907R, 926F and 1492R (Lane, 1991) were used in the sequencing reaction. Sequence analysis was performed using an automatic DNA sequencer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems), applying the BigDye Terminator Cycle sequencing kit (Applied Biosystems) following the manufacturer's protocols. Alignment of sequences was carried out using the CLUSTAL W algorithm from the EMBL-EBI homepage (<http://www.ebi.ac.uk/clustalw>) and BioEdit 7.0.4.1 software (Hall, 1999). A phylogenetic tree was created by neighbour-joining analysis (bootstrap = 100) using TREECON 1.3b software (Van de Peer & De Wachter, 1994).

DNA–DNA hybridization and the DNA G + C content of strain GCM71^T were analysed at DSMZ, Braunschweig, Germany. DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), incorporating the modifications described by Huß *et al.* (1983), using a Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in situ* temperature probe (Varian). The DNA G + C content was determined by HPLC and the ratio was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT) according to the method of Mesbah *et al.* (1989).

For fatty acid analysis, the novel strain was grown on full-strength R2A, pH 10 at 5 °C. Fatty acid extraction was

performed using standard procedures for GC fatty acid analysis as described by Sasser (1990) and fatty acid analysis was performed as described by Mansfeld-Giese *et al.* (2002).

For pigment analysis, the novel strain was grown on full-strength R2A, pH 10 at 5 °C. Spectral reflectance features of individual bacterial colonies were measured with a fibre-optic spectrometer (USB2000; Ocean Optics) operated with a PC running the data acquisition software, OOIBase32 (Ocean Optics; USA). The flat-cut end of a multimode optical fibre (fused silica, 600 µm diameter, numerical aperture 0.22) was fixed in a hypodermic needle on a syringe. This was then mounted on a micromanipulator. The other end of the fibre was connected to the spectrometer via a standard SMA connector. A Petri dish containing the bacterial strain was illuminated vertically from above with a fibre-optic halogen lamp with the heat filter removed and equipped with a collimating lens. The Petri dish was placed on top of a white reflectance standard. Before each measurement, the fibre probe was carefully positioned at the surface of the agar plate at an angle of about 45° relative to the vertical. After initial recording of spectra in darkness, a reference spectrum, $R_0(\lambda)$, of the reflectance from agar without bacteria was recorded. Subsequent measurements of spectral reflectance from bacterial colonies, $R_b(\lambda)$, were expressed as quasi-absorbance spectra, $A(\lambda) = \log_{10} [(R_0(\lambda)/R_b(\lambda))]$.

Results and discussion

Cells of strain GCM71^T were Gram-negative, oval to straight rods of 0.8–3 µm in length and 0.7–1 µm in width. Motility was not observed by phase-contrast microscopy. Cells were observed to multiply by binary division and occasionally chains of up to eight cells were observed. Colonies grown in darkness were circular (0.5–3.0 mm in diameter), smooth and bright red. When grown under light conditions, colonies were smaller (0.1–1.0 mm in diameter), irregular and transparent to light red.

Strain GCM71^T was strictly aerobic, oxidase-negative and catalase-positive. It grew in the range 0–22 °C, while the optimal growth temperature was 5–10 °C (Fig. 1). Strain GCM71^T had a growth range between pH 7.5 and pH 10.7. The broadest pH range (pH 7.5–10.7) was observed at the optimal growth temperature, i.e. at 5–10 °C. Above and below the optimal growth temperature, the pH range was narrower (pH 9.2–10.0). Strain GCM71^T grew best in the range of 0–1.5 % NaCl (w/v), with an optimum around 0.6 % NaCl (Table 1). No growth was detected above 3 % NaCl. The strain may need Na⁺ as an adaptation to living in an alkaline environment (Krulwich *et al.*, 1997), whereas Cl[−] seems not to be essential. In the R2-based carbon-assimilation assay, several compounds were utilized as sole carbon source when grown chemotrophically (aerobic/dark) (see Supplementary Table S1 in IJSEM Online). Strain GCM71^T showed activity in the API 20NE system with positive results for β-glucosidase and β-galactosidase activities and for the assimilation of glucose, mannose, *N*-acetylglucosamine and maltose. Furthermore, strain GCM71^T showed good activity in the API ZYM assay, where only tests for lipase (C14), β-glucuronidase and α-fucosidase were negative (see Supplementary Table S2 in IJSEM Online).

Strain GCM71^T was not capable of anaerobic growth, either chemo- nor phototrophically. When grown at 5 °C, strain GCM71^T showed weak biofilm formation during a 4-day growth period.

Approximately 1400 bases of 16S rRNA gene sequences were determined from strain GCM71^T. The 16S rRNA gene sequences showed that the strain was affiliated to the family 'Flexibacteraceae' of the phylum Bacteroidetes. The closest recognized relative to strain GCM71^T was *Belliella baltica* (Brettar *et al.*, 2004) (Fig. 2). Furthermore, strain GCM71^T forms a cluster with the previously reported, but as yet uncharacterized, ikaite isolates ikaite c9 (GenBank accession no. AJ431335) (Stougaard *et al.*, 2002), GCM63

Fig. 1. Optimal growth temperature and pH for strain GCM71^T. Standard error ($n=6$) is indicated on each bar.

Table 1. Differential characteristics of *Rhodonellum psychrophilum* sp. nov. GCM71^T and *Belliella baltica* BA134^T

Data for *B. baltica* are from Brettar *et al.*, 2004. ND, Not determined.

Characteristic	GCM71 ^T	<i>Belliella baltica</i> BA134 ^T
Cell morphology	Rods, straight	Rods, straight
Cell size (µm)	0.7–1 × 0.8–3	0.3–0.4 × 1.5–3.0
Colony colour	Pink/red	Pink/orange
DNA G + C content (mol%)	43.1	35.3–35.5
Pigments:		
Carotenoids	Yes	Yes
Salinity (%):		
Growth range	0–3.0	0–6
Optimum	0–0.6	0–3.4
Temperature (°C):		
Growth range	0–22	4–37
Optimum	5	25–30
pH:		
Growth range	7.5–10.7	6–10
Optimum	9.2–10.0	ND
Catalase activity	+	+
Cytochrome <i>c</i> oxidase activity	–	+

(DQ028355) (Schmidt *et al.*, 2006) and three additional ikaite isolates GCM68, GCM69 and GCM70 (GenBank accession nos DQ112662, DQ112661 and DQ112663, respectively). Sequences in this cluster shared an 86–93 % gene sequence similarity to their closest relative, *B. baltica*. The unidentified Hailaer soda lake bacterium Z3 (GenBank accession no. AF275712) shared 86–91 % gene sequence similarity with the ikaite isolates (91 % to strain GCM71^T), whereas bacteria of the genera *Cyclobacterium* and *Algoriphagus* only showed 90 % gene sequence similarity to strain GCM71^T. No species of the genera *Cyclobacterium* and *Algoriphagus* are known to be alkaliphilic. However *Algoriphagus antarcticus* from Lake Reid, Antarctica, is reported to be a psychrophilic bacterium (Van Trappen *et al.*, 2004).

DNA–DNA hybridization was 2.6–9.5 % between strain GCM71^T and *B. baltica*. One other isolate from ikaite columns, GCM70, showed between 66.5 and 69.6 % DNA–DNA hybridization with strain GCM71^T. This indicates that these isolates may represent two different species, since the threshold value to separate two species is 70 % (Wayne *et al.*, 1987). Growth physiological data support this fact, but other data, from fatty acid profiles, API tests and 16S rRNA gene sequence similarity, indicate that these two strains may belong to the same species.

The DNA G + C content of strain GCM71^T was 43.1 mol%, whereas those of *B. baltica* and *Cyclobacterium marinum* have been reported to be 35.3–35.5 mol% and 34–38 mol%, respectively (Brettar *et al.*, 2004).

Fig. 2. Phylogenetic dendrogram based on comparison of 16S rRNA gene sequences from strains isolated from ikaite and some of their closest phylogenetic relatives. The tree was created by neighbour-joining analysis. Bootstrap ($n=100$) values below 50 are not shown. *Algoriphagus antarcticus* was used as the outgroup. Bar, 0.1 substitutions per nucleotide position.

Predominant fatty acids of strain GCM71^T were iso-C_{15:1} G (6.3%), iso-C_{15:0} (7.6%), iso-C_{16:1} H (7.3%), iso-C_{17:1} ω9c (17.5%), C_{17:1} ω6c (6.8%), iso-C_{17:0} 3-OH (17.5%) and summed feature 3, comprising C_{16:1} ω7c and/or iso-C_{15:0} 2-OH (12.6%), and summed feature 4, comprising C_{14:0} 2-OH and/or iso-C_{15:0} 2-OH (6.5%), which could not be distinguished by the method applied (see Supplementary Table S3 in IJSEM Online). The cellular fatty acid content of the strain was dominated by branched and unsaturated fatty acids which make up around 90% of the total fatty acids if summed feature 4 is not included and around 97% of the total fatty acids if summed feature 4 is included. Summed feature 3 was included because it consists of a branched and/or an unsaturated fatty acid. The large amount of branched and unsaturated fatty acids in the cell walls is a characteristic adaptation of both alkaliphilic and psychrophilic bacteria as these fatty acids increase membrane fluidity and hence increase cellular activity and transport processes at low temperatures and elevated pH (Nichols *et al.*, 2002; Feller & Gerday, 2003; Furusawa & Koyama, 2004). When the fatty acid profile of strain GCM71^T is compared with that of the phylogenetically closest relative, *B. baltica*, both are dominated by iso-C_{15:0}, iso-C_{17:1} ω9c, C_{17:1} ω6c and summed feature 3 (C_{16:1} ω7c and/or iso-C_{15:0} 2-OH). The most remarkable difference is the lower content of C₁₅ fatty acids together with the higher amount of iso-C_{17:1} ω9c and iso-C_{17:0} 3-OH in strain GCM71^T as compared with *B. baltica*.

Strain GCM71^T was not capable of phototrophic growth under anaerobic conditions. Under aerobic conditions, colonies subjected to light (20–40 μE m⁻² s⁻¹) were small (0.1–0.5 mm in diameter), irregular and light red to transparent. Colonies grown aerobically under dark conditions were larger (0.5–3.0 mm in diameter), more regular in shape and bright red in colour. An absorbance spectrum for both strains showed a broad peak with maximum absorbance at 489 nm with two shoulder peaks at 460 and 517 nm, which suggest the presence of carotenoids (Supplementary Fig. S1, IJSEM Online). The closest relative, *B. baltica*, showed a similar carotenoid absorption spectrum with absorption peaks at 450, 475 and 505 nm (Brettar *et al.*, 2004). The absorbance at these wavelengths was 3.5–4 times higher in isolates grown under dark conditions than in isolates grown in the light. Furthermore, both strains had four smaller peaks above 670 nm. The isolates grown under dark conditions had peaks at 694, 745, 815 and 907 nm, while isolates grown in the light had peaks at 676, 727, 788 and 853 nm.

A novel alkaliphilic and psychrophilic bacterium, strain GCM71^T, was isolated from the permanently alkaline and cold ikaite columns in the Ikka Fjord in south-west Greenland. The strain is phylogenetically related to bacteria of the phylum *Bacteroidetes* with *B. baltica* as the closest relative. DNA–DNA hybridization analysis and DNA G + C content determination, as well as differences in cell morphology, growth physiology and fatty acid profiles,

support the notion that strain GCM71^T is a novel species belonging to a new genus. We propose a new genus, *Rhodonellum* gen. nov., comprising the species *Rhodonellum psychrophilum* sp. nov. Strain GCM71^T is the type strain of *R. psychrophilum* sp. nov.

Description of *Rhodonellum* gen. nov.

Rhodonellum (Rho.do.nell.um. Gr. neut. n. *rhodon* a rose; L. neut. dim. ending *-ellum*; N.L. neut. n. *Rhodonellum* a small rose, referring to the red colour of the colonies).

Cells are Gram-negative, rod-shaped, oxidase-negative and catalase-positive. Growth is heterotrophic, aerobic and chemoheterotrophic. Predominant fatty acids are iso-C_{17:1} ω9c, iso-C_{17:0} 3-OH (12.5–18.5%) and summed feature 3. Cells contain red pigment in the form of carotenoids. Optimal growth occurs above pH 9. NaCl is not required for growth, but growth is enhanced by the presence of up to 0.6% NaCl. The genus *Rhodonellum* belongs to the class 'Sphingobacteria' and the family 'Flexibacteraceae'. The type species is *Rhodonellum psychrophilum*.

Description of *Rhodonellum psychrophilum* sp. nov.

Rhodonellum psychrophilum (psy.chro'phi.lum. Gr. adj. *psychros* cold; Gr. adj. *philos* loving; N.L. neut. adj. *psychrophilum* cold-loving).

Colonies are smooth, circular and red due to the presence of carotenoids when grown under low light intensities. Colonies are white to light red when grown at light intensities of 20–40 μE m⁻² s⁻¹. Cells are Gram-negative, rod-shaped, 0.8–3 μm in length and 0.7–1 μm in width and are oxidase-negative and catalase-positive. Growth occurs at temperatures from 0 to 22 °C, with an optimum at around 5 °C. Growth occurs from pH 7.5 to above pH 10.7, with an optimum at pH 9.2–10.0. At optimal growth temperature, the range of tolerated pH is largest, while below and above the optimal growth temperature, a narrower pH range is tolerated. NaCl is not required for growth, but up to 3% (w/v) NaCl is tolerated. Optimal growth occurs around 0.6% (w/v) NaCl. Strains are able to use a wide spectrum of carbon sources such as galactose, lactose, maltose, mannose, starch, glycerol and sorbitol. DNA G + C content of the type strain is 44.2 mol%.

The type strain is GCM71^T (=DSM 17998^T =LMG 23454^T).

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