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Psychroflexus sediminis sp. nov., a mesophilic bacterium isolated from salt lake sediment in China

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A novel Gram-negative, mesophilic, slightly halophilic, catalase- and oxidase-positive, obligately aerobic bacterium, designated strain YIM-C238^T, was isolated from a sediment sample from a salt lake in the Qaidam Basin, north-west China. Cells were non-sporulating, non-motile, straight to slightly curved rods. Coccoid bodies and filaments of varying length developed in older cultures. Growth occurred with 0.5-6% (w/v) NaCl [optimum, 2-3% (w/v) NaCl], at pH 6.0-10.0 (optimum, pH 7.0-8.0) and at 10-40 °C (optimum, 25-30 °C). The major cellular fatty acids were anteiso- $C_{15:0}$, 3-OH iso- $C_{16:0}$ and anteiso- $C_{15:1}\omega 10c$, and menaquinone 6 was the sole respiratory quinone. Non-diffusible carotenoid pigments were produced. Flexirubin pigments were absent. The genomic DNA G+C content was 35.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain YIM-C238^T should be assigned to the genus Psychroflexus. The sequence similarities between the isolate and the type strains of members of the genus Psychroflexus were in the range 95.5-97.0%. The combination of phylogenetic analysis, DNA-DNA hybridization data, phenotypic characteristics and chemotaxonomic differences supported the view that strain YIM-C238^T represents a novel species of the genus Psychroflexus, for which the name Psychroflexus sediminis sp. nov. is proposed. The type strain is YIM-C238^T (=CCTCC AA 207030^T=KCTC 22166^T).

The genus *Psychroflexus*, in the family *Flavobacteriaceae*, was first proposed by Bowman *et al.* (1998) with the description of *Psychroflexus torquis* as well as the reclassification of *Flavobacterium gondwanense* (Dobson *et al.*, 1993) as *Psychroflexus gondwanensis*. The genus was defined as Gram-negative, catalase- and oxidase-positive, strictly aerobic, heterotrophic, non-motile or motile via gliding motility, slightly or moderately halophilic, psychrophilic or psychrotrophic rods, with menaquinone 6 (MK-6) as the predominant isoprenoid quinone and a DNA G+C content range of 32–36 mol% (Bowman *et al.*, 1998). *Psychroflexus tropicus* (Donachie *et al.*, 2004) was the first mesophilic member of the genus. In a recent study of the microbial diversity of the Qaidam Basin in Qinghai

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain YIM-C238^T is EU135715.

A table showing the fatty acid compositions of strain YIM-C238^T and other *Psychroflexus* species is available as supplementary material with the online version of this paper.

Province, north-west China, a novel strain, designated YIM-C238^T, was isolated from a sediment sample collected from the Dachaidamu salt lake. The lake is located at 37° 46′ N–37° 55′ N and 95° 22′ E–95° 33′ E. The water temperature was 18 °C, pH 6.4–7.8 and had a salinity of 27.4 % (w/v). Based on the results of a polyphasic taxonomic study, strain YIM-C238^T is proposed to represent a novel species, *Psychroflexus sediminis* sp. nov.

Strain YIM-C238^T was isolated from a sediment sample by plating 1:10 serial dilutions of the sample on Difco marine agar 2216 (MA; pH 7.5) followed by incubation at 28 °C for 14 days. After primary isolation and purification, the isolate was preserved both on MA slants at 4 °C and in Difco marine broth 2216 (MB) supplemented with 20 % (v/v) glycerol at -80 °C. Unless otherwise indicated, morphological and physiological studies were performed with cells grown on MA (pH 7.5) at 28 °C. The reference strain *Psychroflexus tropicus* DSM 15496^T was obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen

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und Zellkulturen). Cell morphology was examined by using light microscopy (model BH 2; Olympus). Gram staining was carried out using the standard Gram reaction combined with the KOH lysis test method (Gregersen, 1978). Motility was observed as described previously (Chen et al., 2008). Growth was tested at various temperatures in the range 5-45 °C (using increments of 5 °C) on MA and at pH values in the range 5.0-11.0 (in increments of pH 0.5) in MB. Tolerance and requirement for salts was determined in nutrient broth (3.0 g beef extract l^{-1} and 5.0 g peptone l^{-1} ; Difco) supplemented with modified artificial sea water containing (1^{-1}) : 0–15% (w/v) NaCl, 5.94 g MgSO₄.7H₂O, 4.53 g MgCl₂.6H₂O₃, 0.64 g KCl and 1.3 g CaCl₂ (Lim et al., 2005). Other media were used as controls, i.e. MA and ISP medium 2 agar (Shirling & Gottlieb, 1966). Hydrolysis of aesculin and nitrate reduction were determined as described by Lányí (1987). Hydrolysis of casein, DNA, gelatin, starch, Ltyrosine, xanthine and Tweens 20, 40, 60 and 80 and urease activity were determined as described by Cowan & Steel (1965). Growth under anaerobic conditions and catalase and oxidase activities were detected as described previously (Chen et al., 2007, 2008). Other enzyme activities were also assayed using API ZYM strips (bioMérieux) according to the manufacturer's instructions. Acid production from carbohydrates was determined by employing the API 50CH system (bioMérieux), according to the manufacturer's instructions. Oxidation of carbohydrates, alcohols, organic acids, amino acids and nucleosides as single carbon sources was determined using Biolog GN2 MicroPlates, according to the manufacturer's instructions. All suspension media were supplemented with 2% (w/v) NaCl, and all commercial systems were incubated at 28 °C. The results of the phenotypic tests are given in the species description and in Table 1.

DNA was isolated according to Hopwood et al. (1985) and the DNA G+C content was determined by using the thermal denaturation method (Mandel & Marmur, 1968) with a Shimadzu UV-visible spectrophotometer (UV1601). Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were done as described previously (Cui et al., 2001). Phylogenetic analysis was performed using the software package MEGA version 3.1 (Kumar et al., 2004) after multiple alignment of sequence data by CLUSTAL X (Thompson et al., 1997). Distances (corrected using Kimura's two-parameter model; Kimura, 1980) were calculated and clustering was performed using the neighbour-joining method (Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximumparsimony (Kluge & Farris, 1969) trees (not shown) were generated using the treeing algorithms contained in the PHYLIP package (Felsenstein, 1993). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985). DNA-DNA hybridization was carried out using photobiotin-labelled probes in microplate wells as described by Ezaki et al. (1989). A microplate spectrofluorimeter

Table 1. Characteristics that differentiate strain YIM-C238^T from other *Psychroflexus* species

Strains: 1, YIM-C238^T (*Psychroflexus sediminis* sp. nov.; data from this study); 2, *P. tropicus* LA1^T (Donachie *et al.*, 2004); 3, *P. torquis* ACAM 623^T (Bowman *et al.*, 1998); 4, *P. gondwanensis* ACAM 44^T (Bowman *et al.*, 1998). +, Positive; –, negative.

Characteristic	1	2	3	4
Mobility	Non-motile	Gliding	Gliding	Non-motile
NaCl range (%, w/v)	0.5–6	1–20	1.5–4.6	0–17.5
NaCl optimum (%, w/v)	2–3	7.5–10	2.3	5.8
Temperature range ($^{\circ}$ C)	10–40	4–43	-16 to 20	-5 to 30
β-Glucuronidase activity	+	_	_	_
Hydrolysis of:				
Aesculin	+	_	_	+
Gelatin	+	_	_	_
Tween 80	+	_	+	+
Acid production from:				
Arabitol	_	+	_	_
D-Fructose	_	+	_	_
D-Lactose	+	_	_	_
Maltose	+	_	+	+
D-Mannitol	_	+	_	_
D-Sorbitol	_	+	_	_

(GeminiXPS; Molecular Devices) was employed for fluorescence measurements.

The DNA G+C content of strain YIM-C238^T was 35.8 mol%. An almost-complete 16S rRNA gene sequence (1475 bp) was determined. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain YIM-C238^T was closely related to the type strains of the three recognized species of the genus Psychroflexus (Bowman et al., 1998). The four strains formed a robust cluster in the phylogenetic tree (bootstrap value, 100 %), in which strain YIM-C238^T was phylogenetically most closely related to P. tropicus ATCC BAA-734^T (Donachie et al., 2004), and the two strains formed a distinct subclade with significant bootstrap support (96%) (Fig. 1). The sequence similarities between the isolate and the type strains of members of the genus Psychroflexus were 97.0% (P. tropicus ATCC BAA-734^T), 96.5 % (P. gondwanensis ACAM 44^T) and 95.5 % (*P. torquis* ACAM 623^T). To establish the precise taxonomic position of strain YIM-C238^T, DNA-DNA hybridizations were performed between the novel isolate and the reference strain P. tropicus DSM 15496^T; the level of DNA-DNA relatedness between the two strains was 8.4%, which was far below the threshold value of about 70% recommended by Wayne et al. (1987) for assigning strains to the same species. Therefore, based on phylogenetic analysis and the DNA-DNA hybridization data, it is

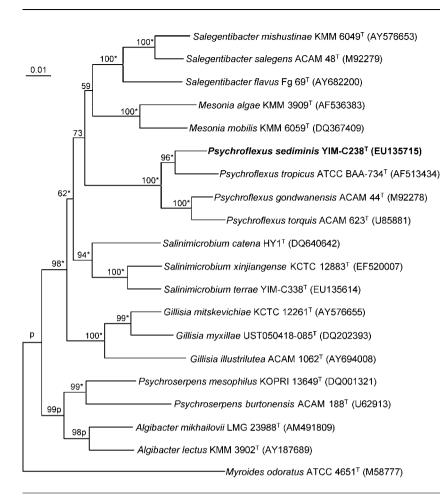


Fig. 1. Phylogenetic dendrogram based on 16S rRNA gene sequence analysis and constructed using the neighbour-joining method showing the phylogenetic positions of strain YIM-C238^T and related taxa. The 'p' labels indicate branches that were also found with the maximum-parsimony (Kluge & Farris, 1969) algorithm; asterisks indicate branches that were also recovered using the maximum-likelihood (Felsenstein, 1981) and maximum-parsimony algorithms. Numbers at nodes are bootstrap values (>50 %) based on a neighbour-joining analysis of 1000 resampled datasets. Bar, 1 substitution per 100 nt.

evident that strain YIM-C238^T represents a novel species of the genus *Psychroflexus* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Isoprenoid quinones were analysed by HPLC as described by Groth et al. (1996). Fatty acids were determined as described by Sasser (1990) using the Microbial Identification System (MIDI; Microbial ID) with cells grown in MB in flasks on a rotary shaker at 200 r.p.m at 30 °C for 3 days. The bathochromic shift test with 10 % (w/v) KOH was used to detect flexirubin pigments. The test was performed on a small mass of cells collected with a loop and deposited on a glass slide placed on a white background; another similar mass of cells was deposited on the slide as a control (Bernardet et al., 2002). Carotenoid pigments were extracted using methanol according to Schmit et al. (1994). Absorption spectra were determined between 250 and 700 nm using a Shimadzu UV-visible spectrophotometer (UV1601). Chemotaxonomic data for strain YIM-C238^T were compatible with its assignment to the genus Psychroflexus (Bowman et al., 1998). MK-6 was the sole respiratory quinone detected. The fatty acid profile of strain YIM-C238^T was similar to those of the type strains of the genus Psychroflexus (see Supplementary Table S1, available in IJSEM Online). The major fatty acids of strain YIM-C238^T were anteiso- $C_{15:0}$ (29.4 %), 3-OH iso- $C_{16:0}$

(16.1%) and anteiso- $C_{15:1}\omega 10c$ (10.7%). The strain produced carotenoid pigments with absorbance peaks at 455 and 480 nm. Flexirubin pigments were absent.

The results of the phylogenetic analysis and chemotaxonomic studies supported the view that strain YIM-C238^T should be assigned to the genus Psychroflexus (Bowman et al., 1998). However, the strain could be distinguished by using a number of phenotypic properties (Table 1) and by its discriminative fatty acid profile (Supplementary Table S1), although the latter may partly result from different culture conditions. The presence of noticeable amounts of unbranched monounsaturated fatty acids (making up 5.0% of the total), whereas the absence of saturated straight-chain fatty acids in the fatty acid pool of strain YIM-C238^T (Supplementary Table S1), together with its ability to produce acid from D-lactose, to hydrolyse gelatin and to exhibit β -glucuronidase activity, as well as the inability to grow at less than 10 °C (Table 1), differentiated strain YIM-C238^T markedly from the three recognized Psychroflexus species. In addition to all the differences presented here, strain YIM-C238^T could be clearly distinguished from its closest phylogenetic neighbour P. tropicus by some other discriminative taxonomic markers, such as its ability to hydrolyse aesculin and Tween 80 and to produce acids from maltose, as well as its ~3.5-fold

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increment in the ratio of anteiso- to iso-branched fatty acids (Table 1 and Supplementary Table S1) and the low level of DNA–DNA relatedness between the species. Overall, the results of the polyphasic taxonomic study presented above allowed us to assign the novel isolate as representing a novel species, for which we propose the name *Psychroflexus sediminis* sp. nov.

Description of Psychroflexus sediminis sp. nov.

Psychroflexus sediminis (sed.i'min.is. L. gen. n. sediminis of sediment).

Cells are Gram-negative, straight to slightly curved rods, approximately 0.4-1.0 µm wide and 3.0-6.0 µm long, and occur singly. Coccoid bodies and filaments of varying length develop in older cultures. Endospores are not formed. Devoid of flagella and gliding motilities. Strictly aerobic, catalase- and oxidase-positive. Colonies are orange-pigmented, flat and non-translucent with glistening surfaces and circular/slightly irregular margins, and 2-3 mm in diameter after incubation for 3-5 days at 28 °C on MA. Non-diffusible orange carotenoid pigments are produced. Flexirubin pigments are absent. Mesophilic and slightly halophilic, growth occurs at 10-40 °C (optimum, 25–30 °C) with 0.5–6 % (w/v) NaCl [optimum, 2–3 % (w/v) NaCl]. Growth occurs at pH 6.0-10.0, with optimum growth at pH 7.0-8.0. Aesculin, gelatin and Tweens 20 and 80 are hydrolysed, but casein, cellulose (carboxymethylcellulose and filter paper), chitin, DNA, starch, L-tyrosine, xanthine or Tweens 40 and 60 are not hydrolysed. Nitrate is reduced to nitrite. Urease is not produced. Acids are produced from gentiobiose, glycogen, D-lactose, maltose, sucrose, turanose and trehalose (API 50CH). The following carbon sources are oxidized (Biolog GN2 MicroPlate): Lalanyl glycine, L-aspartic acid, dextrin, glycogen, L-fucose, gentiobiose, maltose, D-mannitol, methyl β -D-glucoside, Dpsicose, L-serine, succinic acid, sucrose, trehalose and Tween 80. Constitutive enzymes expressed include acid phosphatase, alkaline phosphatase, cystine arylamidase, esterase (C4), β -glucuronidase, leucine arylamidase, valine arylamidase and naphthol-AS-BI-phosphohydrolase (API ZYM). Major fatty acids are anteiso- $C_{15:0}$, 3-OH iso- $C_{16:0}$ and anteiso- $C_{15:1}\omega 10c$. MK-6 is the sole respiratory quinone. The DNA G+C content of the type strain is 35.8 mol%.

The type strain, YIM-C238^T (=CCTCC AA 207030^T= KCTC 22166^T), was isolated from a sediment sample collected from the Dachaidamu salt lake in the Qaidam Basin in Qinghai Province, north-west China.

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