Hymenobacter psychrotolerans sp. nov., isolated from the Qinghai–Tibet Plateau permafrost region

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A psychrotolerant bacterium, designated strain Tibet-IIU11^T, was isolated from the Qinghai–Tibet Plateau permafrost region, China. A taxonomic study was conducted using a polyphasic approach, with determination of physiological and biochemical properties and phylogenetic analysis based on 16S rRNA gene sequences. The novel isolate was found to belong to the genus *Hymenobacter* and was distinct from the recognized species of this genus. The major fatty acids were iso-C_{15:0} (24.5%), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 20.0%), summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B; 14.0%), C_{16:1}ω5c (12.7%) and anteiso-C_{15:0} (8.4%). Phosphatidylethanolamine and an unknown aminophospholipid were predominant in the polar lipid profile. The quinone system consisted exclusively of menaquinone MK-7 and sym-homospermidine was the major polyamine present. These chemotaxonomic traits are in good agreement with the characteristics of the genus *Hymenobacter*. The assignment of the novel isolate to this genus was further supported by a DNA G + C content of 60 mol%. On the basis of the polyphasic evidence presented in this study, it is proposed that strain Tibet-IIU11^T (=DSM 18569^T=CGMCC 1.6365^T) represents a novel species, *Hymenobacter psychrotolerans* sp. nov.

The Qinghai–Tibet Plateau represents a unique and important permafrost environment, being a result of high elevation and low latitude caused by land uplift in the order of 3000 m over the last 2 million years (Wang & French, 1995). Such a unique permafrost environment might accommodate an original microbial assemblage. In the course of a study on microbial diversity in the Qinghai–Tibet Plateau permafrost region (Zhang *et al.*, 2007b), a bacterial strain, designated Tibet-IIU11^T, was isolated and subjected to detailed characterization in order to clarify its taxonomic status.

Strain Tibet-IIU11^T was isolated from a permafrost sediment sample collected from the Beilu river basin (34° 50' N 92° 56' E, 4676 m above sea level) in the Qinghai–Tibet Plateau region of China, in August 2002 by

using the standard dilution plating technique. Isolation was achieved after incubation on PYGV (Staley, 1968) at 4 °C for 30 days. The isolate was routinely cultured with liquid R2A medium (Oxoid) and maintained as a glycerol suspension (15%, w/v) at –80 °C.

Genomic DNA was extracted and purified as described by Sambrook *et al.* (1989). The almost complete 16S rRNA gene (*Escherichia coli* positions 8–1510) of strain Tibet-IIU11^T was amplified and sequenced as described previously (Zhang *et al.*, 2007a). The gene sequence of strain Tibet-IIU11^T was aligned (CLUSTAL W 1.81; Thompson *et al.*, 1997) with the sequences of type strains of species exhibiting the highest sequence similarities as obtained from the Ribosomal Database Project (Cole *et al.*, 2003) and the European Molecular Biology Laboratory (EMBL). Phylogenetic dendrograms were constructed by the neighbour-joining method (Saitou & Nei, 1987) and tree topologies were evaluated by performing bootstrap analysis (Felsenstein, 1985) of 1000 resamplings by use of the MEGA2 package (Kumar *et al.*, 2001).

Abbreviations: SEM, scanning electron microscopy/micrograph; TEM, transmission electron microscopy/micrograph.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain Tibet-IIU11^T is DQ177475.

Preliminary sequence comparisons with 16S rRNA gene sequences held in GenBank indicated that the novel isolate was closely related to members of the genus *Hymenobacter*. Strain Tibet-IIU11^T showed the highest 16S rRNA gene sequence similarity to *Hymenobacter rigui* WPCB131^T (96.6%), *Hymenobacter actinosclerus* CCUG 39621^T (95.5%) and *Hymenobacter gelipurpurascens* Txg1^T (95.3%); these values are well below the threshold generally accepted for the delineation of bacterial species (Stackebrandt & Goebel, 1994). The 16S rRNA gene sequence similarities of strain Tibet-IIU11^T to those of *Hymenobacter xinjiangensis* X2-1g^T, *Hymenobacter aerophilus* I/26-Cor1^T, *Hymenobacter chitinivorans* Txc1^T, *Hymenobacter norwichensis* NS/50^T, *Hymenobacter ocellatus* Myx 2105^T and *Hymenobacter roseosalivarius* AA-718^T were 95.0, 93.9, 94.9, 94.2, 90.5 and 93.4%, respectively, values that were within the range found among the recognized species of the genus (Buczolits *et al.*, 2006). The relationship of strain Tibet-IIU11^T to the other species of the genus *Hymenobacter* was also evident from its position in the phylogenetic tree. Strain Tibet-IIU11^T formed a robust clade together with *H. gelipurpurascens* Txg1^T, *H. xinjiangensis* X2-1g^T and *H. rigui* WPCB131^T in the neighbour-joining tree (Fig. 1). These results suggest that strain Tibet-IIU11^T represents a novel species within the genus *Hymenobacter*.

Growth on various standard bacteriological media was tested using tryptone soy agar (TSA), MacConkey agar, Czapek–Dox agar and R2A agar (Oxoid). Cells grown on R2A agar at 18 °C for 2–5 days were used for physiological and biochemical tests. Cell motility was examined by phase-contrast microscopy using wet mounts. Cell morphology was observed under a transmission electron microscope (TEM) (JEM-1230; JEOL) by negative staining. For scanning electron microscopy (SEM), a bacterial sample was dehydrated with a graded series of acetone, critical point-dried with CO₂ and sputter-coated with gold before examination (JSM-6380; JEOL). Gram behaviour was determined by the KOH-lysis test (Moaledj, 1986) and by staining as described by Smibert & Krieg (1994). Cell sizes were estimated using an Olympus BX-60 microscope by DAPI staining according to Porter & Feig (1990), as

modified by Sherr *et al.* (1992), from cells grown in R2A at 18 °C for 72 h. Conventional biochemical tests were performed as described by Smibert & Krieg (1994), including tests for oxidase, catalase, nitrate reduction, H₂S production, citrate utilization, indole production and urease activity. Carbon-source utilization tests and qualitative enzyme tests were performed with microtitre plates as described previously (Kämpfer *et al.*, 1991). Antimicrobial susceptibility testing was conducted as described by Buczolits *et al.* (2002). Growth at various pH values (pH 4 to 12) was analysed by modifying the pH of R2A medium. Optical densities at 540 nm were monitored after growing cells in a shaker at 20 °C for 24 h. The temperature range for growth was tested between 4 to 37 °C using R2A agar. The effect of NaCl on growth was tested by supplementing R2A medium with 0.5, 1, 2, 3 and 5% (w/v) NaCl and incubating for 1 week at 20 °C. Enzyme activities were tested using the API ZYM kit (bioMérieux) following the manufacturer's instructions. Detailed results are shown in Table 1 and are given in the species description. It is evident from Table 1 that there are at least five physiological characteristics that readily differentiate strain Tibet-IIU11^T from other species of the genus *Hymenobacter*.

Cells grown on R2A agar were collected for whole cellular fatty acid analysis. Cellular fatty acids of strain Tibet-IIU11^T were analysed as methyl esters by GLC according to the instructions of the Microbial Identification System (MIDI). The G+C content of the DNA was determined according to the thermal denaturation method (Marmur & Doty, 1962) on a UV spectrophotometer (UV-1700 PharmaSpec; Shimadzu). Strain Tibet-IIU11^T contained a complex fatty acid profile consisting of predominantly branched fatty acids with minor amounts of straight-chain fatty acids (Table 2). Most of the fatty acids that are characteristic of other *Hymenobacter* species (Hirsch *et al.*, 1998; Buczolits *et al.*, 2002, 2006; Baik *et al.*, 2006; Zhang *et al.*, 2007c) were detected, but differences in the quantities present were useful for differentiation of the species (Table 2). The DNA G+C content of strain Tibet-IIU11^T was 60 mol%.

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain Tibet-IIU11^T and related taxa within the phylum *Bacteroidetes*. The numbers at the nodes indicate the percentages of bootstrap sampling derived from 1000 replications. The sequence of *Flavobacterium aquatile* ATCC 11947^T (M62797) was used as an outgroup. Bar, 2 inferred nucleotide substitutions per 100 nucleotides.

Table 1. Characteristics that distinguish strain Tibet-IIU11^T from the type strains of other species of the genus *Hymenobacter*

Strains: 1, Tibet-IIU11^T (this study); 2, *H. roseosalivarius* DSM 11622^T (Hirsch *et al.*, 1998); 3, *H. actinoscleris* CCUG 39621^T (Collins *et al.*, 2000); 4, *H. aerophilus* DSM 13606^T (Buczolits *et al.*, 2002); 5, *H. rigui* WPCB131^T (Baik *et al.*, 2006); 6, *H. chitinivorans* Txc1^T; 7, *H. gelipurpurascens* Txgl^T; 8, *H. ocellatus* Myx 2105^T; 9, *H. norwichensis* NS/50^T (Buczolits *et al.*, 2006); 10, *H. xinjiangensis* X2-1g^T (Zhang *et al.*, 2007c). +, Positive; (+), weakly positive; –, negative; ND, not determined; pNA, *p*-nitrophenyl; pNP, *p*-nitroanilide. All strains are negative for assimilation of L-rhamnose and maltitol but no results were reported for *H. xinjiangensis* X2-1g^T.

Characteristic	1	2	3	4	5	6	7	8	9	10
Growth at/in:										
4 °C	+	+	–	+	+	+	(+)	–	(+)	+
8 °C	(+)	–	+	+	+	+	+	+	+	+
37 °C	–	–	+	–	+	–	–	+	–	(+)
1 % NaCl	+	+	ND	+	+	(+)	(+)	+	–	–
3 % NaCl	–	ND	ND	(+)	–	(+)	(+)	+	–	–
Assimilation of:										
<i>N</i> -Acetyl-D-glucosamine, gluconate	–	–	(+)	–	–	–	–	–	–	(+)
<i>p</i> -Arbutin, D-ribose	–	–	(+)	–	–	–	–	–	(+)	–
D-Cellobiose	–	–	(+)	–	+	–	–	–	(+)	+
D-Xylose	–	–	–	+	–	–	–	–	(+)	ND
D-Glucose	+	–	(+)	(+)	+	–	–	–	(+)	–
D-Mannose	+	–	(+)	(+)	–	–	–	–	(+)	(+)
Sucrose	+	–	–	(+)	+	–	–	–	–	+
Trehalose	–	–	–	–	+	–	–	–	(+)	+
Salicin, maltose, D-mannitol	–	–	–	–	–	–	–	–	(+)	(+)
Acetate	–	(+)	(+)	(+)	–	–	–	–	–	+
Propionate	(+)	(+)	(+)	(+)	–	–	–	–	–	+
Glutarate	–	(+)	(+)	–	–	–	–	–	–	–
Pyruvate	–	(+)	(+)	–	–	–	–	–	–	–
<i>cis</i> -Aconitate, adipate, citrate, fumarate, DL-3-hydroxybutyrate, L-malate, itaconate, DL-lactate, mesaconate, oxoglutarate, suberate, L-alanine, L-proline, L-phenylalanine	–	–	(+)	–	–	–	–	–	–	–
Hydrolysis of:										
pNP β-D-glucuronide	–	–	–	–	ND	–	–	+	–	ND
pNP phosphate, pNP α-D-glucopyranoside, L-proline pNA	(+)	(+)	(+)	(+)	ND	–	+	+	(+)	ND
pNP β-D-glucopyranoside	–	–	–	–	ND	–	–	+	(+)	ND
bis-pNP phosphate	(+)	(+)	(+)	(+)	ND	–	–	+	(+)	ND
pNP phenyl-phosphonate	–	–	(+)	(+)	ND	–	–	–	(+)	ND
L-Alanine pNA	(+)	(+)	(+)	(+)	ND	+	+	+	(+)	ND
L-Glutamate-γ-3-carboxy pNA	–	–	(+)	(+)	ND	–	–	–	–	ND

Growth conditions for production of biomass and the techniques for the extraction and analyses of the polyamines, quinone system and polar lipids were all as reported previously (Busse & Auling, 1988; Tindall, 1990a, b; Altenburger *et al.*, 1996; Stolz *et al.*, 2007). The polyamine pattern of strain Tibet-IIU11^T contained mainly *sym*-homospermidine (2.0 μmol g^{–1} dry weight) and minor amounts of putrescine (0.3 μmol g^{–1} dry weight), spermidine (0.3 μmol g^{–1} dry weight) and spermine (0.1 μmol g^{–1} dry weight). The main quinone was menaquinone-7 (MK-7). The polar lipid profile (Fig. 2) consisted mainly of phosphatidylethanolamine (PE) and an unknown aminophospholipid (APL3). Two unknown polar lipids (L1 and L2) occurred at moderate levels. Three unidentified aminophospholipids (APL1, 2, 4) and an unknown aminolipid (AL1) were also present. The majority of the chemotaxonomic characteristics were in

good agreement with those reported for other species of the genus *Hymenobacter* (Buczolits *et al.*, 2002, 2006), but the polar lipid profile contained some compounds that may be useful for the differentiation of the novel strain from other species of the genus.

The results of phylogenetic investigations, as well as major chemotaxonomic characteristics, demonstrate that strain Tibet-IIU11^T is a member of the genus *Hymenobacter*. However, the complete polar lipid profile and the relative amounts of certain fatty acids, as well as several physiological characteristics, clearly distinguish this new isolate from other recognized species of the genus *Hymenobacter* (Tables 1 and 2). Therefore, it is concluded that strain Tibet-IIU11^T should be classified as a novel species within the genus *Hymenobacter*, for which the name *Hymenobacter psychrotolerans* sp. nov. is proposed.

Table 2. Relative amounts (%) of fatty acids of strain Tibet-IIU11^T and the type strains of species of the genus *Hymenobacter*

Strains: 1, Tibet-IIU11^T (this study); 2, *H. roseosalivarius* DSM 11622^T (Hirsch *et al.*, 1998); 3, *H. actinosclerulus* CCUG 39621^T (Collins *et al.*, 2000); 4, *H. aerophilus* DSM 13606^T (Buczolits *et al.*, 2002); 5, *H. rigui* WPCB131^T (Baik *et al.*, 2006); 6, *H. chitinivorans* Txc1^T; 7, *H. gelipurpurascens* Txg1^T; 8, *H. ocellatus* Myx 2105^T; 9, *H. norwichensis* NS/50^T (Buczolits *et al.*, 2006); 10, *H. xinjiangensis* X2-1g^T (Zhang *et al.*, 2007c). Values are relative percentages of total fatty acids. Strain Tibet-IIU11^T was grown on R2A agar at 18 °C for 48 h prior to analysis. For unsaturated fatty acids, the position of the double bond can be located by counting from the methyl (ω) end of the carbon chain; *cis* isomers are indicated by the suffix *c*. Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed features: 1, iso-C_{15:1} I and/or C_{13:0} 3-OH; 2, C_{16:1} ω 7*c* and/or iso-C_{15:0} 2-OH; 3, iso-C_{17:1} I and/or anteiso-C_{17:1} B.

Fatty acid	1	2	3	4	5	6	7	8	9	10
iso-C _{13:0}	—	—	—	—	—	0.6	—	0.6	—	—
C _{14:0}	0.8	—	—	—	—	—	—	—	1.3	1.3
Unknown 16.535	0.6	—	—	—	—	0.5	0.8	—	—	—
iso-C _{14:0}	—	—	—	—	—	0.5	—	1.5	0.8	—
iso-C _{15:1} G	1.8	—	—	—	—	0.6	0.8	—	—	2.0
anteiso-C _{15:1} A	0.6	—	—	—	—	—	1.1	—	—	—
Summed feature 1	1.5	—	2.3	0.8	—	2.1	—	2.7	0.8	2.2
iso-C _{15:0}	24.5	8.3	22.3	10.8	34.8	31.1	17.3	36.7	27.3	19.5
anteiso-C _{15:0}	8.4	—	25.8	22.3	5.9	3.7	23.1	3.9	10.6	3.7
iso-C _{15:0} 3-OH	2.7	2.7	1.6	1.6	—	4.1	2.3	4.3	2.2	2.2
C _{15:0} 2-OH	—	—	0.8	0.9	—	—	0.9	—	—	—
C _{15:1} ω 6 <i>c</i>	0.6	—	0.8	0.8	—	—	—	—	0.5	—
C _{15:0}	—	—	—	—	—	—	—	—	0.5	—
iso-C _{16:1} H	1.2	2.7	1.5	1.5	—	2.0	3.7	1.3	1.4	1.8
C _{16:1} ω 7 <i>c</i> alcohol	—	0.7	—	—	—	—	—	—	—	—
iso-C _{16:0} 3-OH	—	1.0	—	—	—	—	—	—	—	—
C _{16:1} ω 5 <i>c</i>	12.7	23.3	3.7	7.9	15.0	8.9	11.3	2.0	13.6	10.6
Summed feature 3	20.0	29.8	13.1	21.4	13.8	13.9	17.6	5.4	23.6	20.2
iso-C _{16:0}	2.1	2.1	—	0.8	—	1.4	2.7	2.9	1.2	1.0
C _{16:0}	2.3	1.1	—	1.7	6.4	—	—	0.6	2.2	6.2
C _{16:0} 3-OH	1.4	1.2	—	—	—	0.5	—	—	0.6	—
iso-C _{17:0}	0.4	1.7	1.8	4.5	5.0	2.6	1.7	2.8	1.6	1.8
anteiso-C _{17:0}	—	—	0.7	2.3	—	—	2.0	—	—	<1
iso-C _{17:0} 3-OH	3.5	5.8	3.1	3.5	3.1	6.2	3.7	6.8	3.0	3.4
C _{17:0} 2-OH	—	—	2.0	1.3	—	—	1.7	—	—	—
C _{17:1} ω 6 <i>c</i>	—	1.1	0.7	—	—	—	—	1.2	—	—
iso-C _{17:1} ω 9 <i>c</i>	—	—	—	—	—	0.7	—	0.6	—	—
Summed feature 4	14.0	18.5	19.9	17.7	14.4	20.8	9.4	26.9	8.3	8.5

Description of *Hymenobacter psychrotolerans* sp. nov.

Hymenobacter psychrotolerans (psy.chro.to'ler.ans. Gr. adj. *psychros* cold; L. part. adj. *tolerans* tolerating; N.L. adj. *psychrotolerans* tolerating cold temperature).

Cells are rod-shaped (Fig. 3), aerobic, non-spore-forming and produce water-insoluble light pink pigment. Cells are approximately 0.8–1.7 × 0.5 µm. Motility is not observed. Colonies on R2A agar are translucent, low-convex, circular, smooth and slimy; diameter is up to 4.0 mm after 5 days at 18–20 °C (pH 7). Growth occurs in 0–1.5% (w/v) NaCl (optimum 0%), pH 5–10 (optimum pH 7) and at 4–28 °C (optimum 18 °C). Cells are Gram-negative according to Gram-staining and the KOH (3%) test. Growth occurs on TSA and R2A agar, but not on Czapek–Dox agar or MacConkey agar. Oxidase- and catalase-positive. Nitrate

reduction is positive without production of gas. Negative for production of indole from tryptophan, arginine dihydrolase or urease. Positive in the API ZYM system for alkaline phosphatase, esterase C4, esterase lipase C8, leucine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and valine arylamidase; negative for lipase C14, trypsin, chymotrypsin, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -galactosidase, α -mannosidase and α -fucosidase. Other characteristics are listed in Table 1. Sensitive to bacitracin (10 U), chloramphenicol (30 mg), colistin sulphate (10 mg), erythromycin (15 mg), fusidic acid (10 mg), gentamicin (10 mg), kanamycin (30 mg), penicillin G (10 U), vancomycin (30 mg), polymyxin B sulphate (300 U) and tetracycline (10 mg). The fatty acid profile contains predominantly methyl-branched acids of the iso- and anteiso-types. The complete fatty acid profile is given in Table 2.

Fig. 2. Polar lipid profile of strain Tibet-IIU11^T after separation in two-dimensional TLC. APL1–4, unknown aminophospholipids 1–4; AL1, unknown aminolipid; L1, L2, unknown polar lipids.

The type strain, Tibet-IIU11^T (=DSM 18569^T=CGMCC 1.6365^T), was isolated from a permafrost sediment sample, Beilu river basin (34° 50' N 92° 56' E, 4676 m above sea level) in the Qinghai–Tibet Plateau, China. The DNA G+C content of strain Tibet-IIU11^T is 60 mol%.

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Fig. 3. TEM (a) and SEM (b) images of cells of strain Tibet-IIU11^T. Bars, 0.5 µm.

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