Hymenobacter psychrophilus sp. nov., a psychrophilic bacterium isolated from soil

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A non-motile, rod-shaped, red-pigmented bacterium, designated strain BZ33r^T, was isolated from soil of an industrial site. Cells stained Gram-negative and were aerobic and psychrophilic, showing good growth at 1–20 °C. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BZ33r^T was related to members of the genus *Hymenobacter* and had highest sequence similarity with *Hymenobacter aerophilus* DSM 13606^T (97.5 %). The predominant cellular fatty acids were anteiso-C_{15:0} (20.3 %), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 20.2 %), iso-C_{15:0} (20.0 %), summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B; 8.2 %) and C_{16:1}ω5c (7.9 %). Strain BZ33r^T had MK-7 as the major menaquinone. The polyamine pattern contained sym-homospermidine as the major compound with moderate amounts of spermidine. Phosphatidylethanolamine, three unknown aminophospholipids, two unknown aminolipids, two unknown glycolipids and five unknown polar lipids were present in the polar lipid profile. The G + C content of the DNA was 61.6 mol%. On the basis of phenotypic characteristics, phylogenetic analysis and DNA–DNA relatedness data, strain BZ33r^T is considered to represent a novel species of the genus *Hymenobacter*, for which the name *Hymenobacter psychrophilus* sp. nov. is proposed. The type strain is BZ33r^T (=DSM 22290^T =CGMCC 1.8975^T =LMG 25548^T).

The genus *Hymenobacter*, which belongs to the phylum *Bacteroidetes*, order *Sphingobacteriales*, family *Cytophagaceae*, was proposed by Hirsch *et al.* (1998) to accommodate aerobic, Gram-stain-negative, rod-shaped, red-pigmented bacteria that contain menaquinone MK-7 and have phosphatidylethanolamine as the main phospholipid. According to an emended description (Buczolits *et al.*, 2006), members of the genus *Hymenobacter* contain sym-homospermidine as the major compound in the polyamine profile and, compared with other taxa of the *Bacteroidetes*, a high DNA G + C content (55–65 mol%). The fatty acid profile consists predominantly of branched components. At the time of writing, the genus *Hymenobacter* comprised 14 recognized species: *Hymenobacter roseosalivarius* (type species of the genus; Hirsch *et al.*, 1998), *H. actinosclerus* (Collins *et al.*, 2000), *H. aerophilus* (Buczolits *et al.*, 2002), *H. chitinivorans*, *H. gelipurpurascens*, *H. norwichensis* and *H. ocellatus*

(Buczolits *et al.*, 2006), *H. rigui* (Baik *et al.*, 2006), *H. xinjiangensis* (Zhang *et al.*, 2007), *H. soli* (Kim *et al.*, 2008), *H. psychrotolerans* (Zhang *et al.*, 2008), *H. tibetensis* (Dai *et al.*, 2009), *H. deserti* (Zhang *et al.*, 2009) and *H. daecheongensis* (Xu *et al.*, 2009). Many members of the genus have been isolated from environmental samples such as soil (Hirsch *et al.*, 1998; Buczolits *et al.*, 2006; Zhang *et al.*, 2007, 2009; Kim *et al.*, 2008; Dai *et al.*, 2009), permafrost sediment (Zhang *et al.*, 2008), stream sediment (Xu *et al.*, 2009), wetland freshwater (Baik *et al.*, 2006) and air (Buczolits *et al.*, 2002, 2006). Three species, *H. actinosclerus*, *H. xinjiangensis* and *H. tibetensis*, have been reported to be resistant to UV radiation (Dai *et al.*, 2009). In this study, we report the characterization of a novel bacterium of the genus *Hymenobacter* isolated from an industrial site.

Strain BZ33r^T was isolated from soil containing high levels of heavy oil and heavy metals at an industrial site in Bozen, South Tyrol (Italy). Soil was collected under sterile conditions in spring 2008. A sample of 10 g was shaken (150 r.p.m.) with 90 ml sterile 1 % sodium pyrophosphate for 20 min. Appropriate dilutions, prepared with sterile saline solution (0.9 % NaCl), were plated (0.1 ml) on R2A

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BZ33r^T is GQ131579.

A supplementary figure and a supplementary table are available with the online version of this paper.

agar (0.05 % yeast extract, 0.05 % peptone, 0.05 % Casamino acids, 0.05 % glucose, 0.05 % starch, 0.03 % sodium pyruvate, 0.03 % K_2HPO_4 , 0.005 % $MgSO_4$ and 1.5 % agar, pH 7; Reasoner & Geldreich, 1985) and incubated at 20 °C. One of the purified cultures was red-pigmented and was designated strain BZ33r^T. *H. aerophilus* DSM 13606^T was routinely grown on nutrient agar (0.5 % peptone, 0.3 % beef extract and 1.5 % agar, pH 7.0) at 20 °C and used as a reference strain.

DNA was extracted and purified as described by Sambrook *et al.* (1989). The gene encoding 16S rRNA was amplified by PCR with two universal primers (Zhang *et al.*, 2006). PCR products were cloned in pGEM-T vectors by using the pGEM-T easy vector systems (Promega) according to the manufacturer's instructions. Sequencing reactions were performed as described by Zhang *et al.* (2006). The nearly complete 16S rRNA gene sequence (1511 bp) of strain BZ33r^T was analysed by using the FASTA algorithm. Phylogenetic analysis with the program MEGA version 3.0 (Kumar *et al.*, 2004) showed that strain BZ33r^T grouped with members of the genus *Hymenobacter* and formed a distinct cluster with *H. aerophilus* DSM 13606^T (97.5 % 16S rRNA gene sequence similarity) and *H. actinosclerus* CIP 106628^T (96.9 %) with 100 % bootstrap support in the neighbour-joining tree (Fig. 1). In addition, a similar topology was found in the tree generated with the maximum-likelihood method via the PHYLIP 3.69 package (Felsenstein, 2009) after sequence alignment by using CLUSTAL X, version 1.8 (Thompson *et al.*, 1997).

Strain BZ33r^T was routinely cultured on R2A agar medium at 20 °C and maintained as a suspension in skimmed milk (10 %, w/v) at –80 °C. Cell morphology was examined by phase-contrast microscopy ($\times 1000$; Leitz Diaplan) of cells grown on R2A agar plates at 20 °C. Colonial morphology was also observed on R2A agar plates. The API M system was used to evaluate cell motility. The Gram reaction was tested by Gram staining and was confirmed by the KOH lysis test. Catalase activity was determined by bubble production in 3 % (v/v) H_2O_2 and oxidase activity was determined by using 1 % (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. API strips (API 20 E, API 20 NE and

API ZYM; bioMérieux) incubated at 20 °C were used according to the manufacturer's instructions to determine the physiological and biochemical characteristics and enzyme activities of strain BZ33r^T and of the reference strain *H. aerophilus* DSM 13606^T. Activities of β -galactosidase, amylase and protease were additionally tested by using R2A agar plates supplemented with appropriate substrates (Margesin *et al.*, 2003). Growth at 1–30 °C was assessed on R2A agar plates and in R2A liquid medium with shaking at 150 r.p.m. Growth at pH 5–9 and in the presence of 0–5 % (w/v) NaCl was determined on R2A agar plates. Susceptibility to antibiotics was determined on R2A agar plates supplemented with various antibiotics and incubated at 20 °C. The features that serve to differentiate strain BZ33r^T from the phylogenetically most closely related members of the genus *Hymenobacter* are given in Table 1. Morphological, physiological and biochemical characteristics of strain BZ33r^T are given in the species description and in Table 1.

Respiratory quinones were extracted and purified according to Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989), by using menaquinone MK-7 from *H. aerophilus* DSM 13606^T as a reference. Strain BZ33r^T contained MK-7 as the major menaquinone.

Cellular polar lipids were extracted and analysed on silica gel plates (Kieselgel 60 F; Merck) by TLC (Kates, 1986). Phosphatidylethanolamine, three unknown aminophospholipids, two unknown aminolipids, two unknown glycolipids and five unknown polar lipids were detected in the polar lipid profile (Supplementary Fig. S1, available in IJSEM Online).

For analysis of fatty acid methyl esters, strain BZ33r^T and *H. aerophilus* DSM 13606^T were grown on trypticase soy agar plates at 20 °C for 3 days. Analysis was performed according to the standard protocol of the MIDI (Microbial Identification) system (Sasser, 1990). The predominant cellular fatty acids were anteiso-C_{15:0} (20.3 %), summed feature 3 (C_{16:1} ω 7c and/or iso-C_{15:0} 2-OH; 20.2 %), iso-C_{15:0} (20.0 %), summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B; 8.2 %) and C_{16:1} ω 5c (7.9 %). The detailed fatty acid profile of strain BZ33r^T was in agreement with

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic position of strain BZ33r^T, recognized members of the genus *Hymenobacter* and representatives of some related taxa. Asterisks indicate nodes that were also found in the maximum-likelihood tree. *Flavobacterium aquatile* ATCC 11947^T was used as an outgroup. Bootstrap values are shown as percentages of 1000 replicates and are shown for branches with ≥ 50 % support. GenBank accession numbers are given in parentheses. Bar, 2 % sequence divergence.

Table 1. Phenotypic characteristics that differentiate strain BZ33r^T from the type strains of phylogenetically related *Hymenobacter* species

Strains: 1, BZ33r^T (data from this study); 2, *H. aerophilus* DSM 13606^T (this study); 3, *H. actinosclerus* CIP 106628^T (Collins *et al.*, 2000; Buczolits *et al.*, 2002); 4, *H. gelipurpurascens* Txg1^T (Buczolits *et al.*, 2006); 5, *H. psychrotolerans* DSM 18569^T (Zhang *et al.*, 2008). +, Positive; –, negative; w, weakly positive; ND, no data available. All strains are positive for activities of alkaline phosphatase and leucine arylamidase. All strains are negative for activities of lipase (C14), trypsin, α -chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, β -glucosidase, α -mannosidase, α -fucosidase and urease, indole production and assimilation of adipate, gluconate, citrate and *N*-acetylglucosamine (except for *H. actinosclerus* CIP 106628^T, which assimilates these compounds weakly).

Characteristic	1	2	3	4	5
Isolation source	Soil	Air	Irradiated pork	Soil	Permafrost sediment
Growth at/in presence of:					
5 °C	+	+	–	+	+
25 °C	w	+	+	+	+
30 °C	–	+	+	ND	–
37 °C	–	–	+	–	–
2 % (w/v) NaCl	w	+	ND	w	–
Nitrate reduction	+	–	–	w	+
Enzyme activity (API ZYM)					
Acid phosphatase	+	+	+	–	+
<i>N</i> -Acetyl- β -glucosaminidase	+	+	+	–	–
α -Glucosidase	+	+	ND	–	–
Esterase (C4)	–	+	ND	w	+
Esterase lipase (C8)	w	+	+	w	+
Valine arylamidase	–	+	+	w	+
Cystine arylamidase	+	+	+	–	+
Assimilation of (API 20 NE):					
D-Glucose	w	w	w	–	+
D-Mannose	–	–	w	–	+
D-Mannitol	w	–	–	–	–
Maltose	w	+	–	–	–
Malate	w	–	w	–	–

those of recognized *Hymenobacter* species (Buczolits *et al.*, 2002, 2006; Zhang *et al.*, 2008) (Supplementary Table S1).

For polyamine analysis, strain BZ33r^T was grown in PYE medium (0.3 % yeast extract, 0.3 % peptone, pH 7). Polyamines were extracted as described by Busse & Auling (1988) and were analysed as described by Busse *et al.* (1997) and Stolz *et al.* (2007). The polyamine pattern of strain BZ33r^T consisted of *sym*-homospermidine [39.9 μmol (g dry cell mass)^{–1}] as the major compound, moderate amounts of spermidine [6.5 μmol (g dry cell mass)^{–1}] and spermine [3.2 μmol (g dry cell mass)^{–1}] and a trace amount of putrescine [0.2 μmol (g dry cell mass)^{–1}]. This polyamine pattern is in good agreement with that given in the emended description of the genus *Hymenobacter* (Buczolits *et al.*, 2006).

The DNA G+C content was determined by the thermal denaturation method with *Escherichia coli* K-12 as reference, and DNA–DNA hybridization experiments were performed by the liquid renaturation method (De Ley *et al.*, 1970) as modified by Huß *et al.* (1983). The two experiments were carried out by using a model Lambda 35 UV/Vis spectrometer equipped with a temperature program controller (Perkin–Elmer). The DNA G+C

content of strain BZ33r^T was 61.6 mol%, within the range given for the genus. The level of DNA–DNA relatedness between strain BZ33r^T and *H. aerophilus* DSM 13606^T was as low as 39.2 %, much lower than the threshold (70 %) recommended for species demarcation (Wayne *et al.*, 1987). These data clearly demonstrate that the novel strain represents a species distinct from *H. aerophilus*. Levels of 16S rRNA gene sequence similarity of <97.0 % were found between strain BZ33r^T and the type strains of recognized species of the genus *Hymenobacter*. It has been suggested that, in bacterial strains sharing <97 % 16S rRNA gene sequence similarity, levels of DNA–DNA relatedness are <70 % (Stackebrandt & Goebel, 1994; Stackebrandt & Ebers, 2006). Therefore, strain BZ33r^T is genotypically distinct from all recognized species of the genus *Hymenobacter*.

The results presented above demonstrate that strain BZ33r^T is a psychrophilic member of the genus *Hymenobacter* (16S rRNA gene sequence analysis, quinone system and polyamine pattern), showing good growth at 1–20 °C (highest cell yields at 1–10 °C), weak growth at 25 °C and no growth at 30 °C. The strain can be differentiated easily from its closest phylogenetic neighbours, *H. aerophilus* DSM 13606^T and *H. actinosclerus* CIP 106628^T, by its

ability to reduce nitrate, inability to grow at 30 °C and inability to produce valine arylamidase, as well as by its polar lipid profile (two unknown aminolipids and two unknown glycolipids) and the relative amounts of certain fatty acids (iso-C_{15:0}, summed feature 3 and summed feature 4). Therefore, based on the phenotypic, phylogenetic and genomic evidence presented, strain BZ33r^T is considered to represent a novel species of the genus *Hymenobacter*, for which the name *Hymenobacter psychrophilus* sp. nov. is proposed.

Description of *Hymenobacter psychrophilus* sp. nov.

Hymenobacter psychrophilus (psy.chro'phi.us. Gr. adj. *psychros* cold; Gr. adj. *philos* liking, loving; N.L. masc. adj. *psychrophilus* cold-loving).

Cells are aerobic, Gram-stain-negative, non-motile and rod-shaped (1–1.1 × 2–7 µm after 6 days at 20 °C on R2A agar plates). Colonies on R2A agar are red, round, convex and with entire margins; colony diameter is 1 mm after 6 days at 20 °C. Good growth occurs in liquid R2A medium and on agar plates at 1–20 °C, with highest cell yields in liquid culture at 1–10 °C. Growth is weak at 25 °C and absent at 30 °C. Growth at 25 °C is absent in nutrient broth. On R2A agar plates, grows in the presence of 0–1 % (w/v) NaCl; growth in the presence of 2 % (w/v) NaCl is weak. Produces catalase and cytochrome oxidase. Negative for production of indole, H₂S and urease, utilization of citrate and hydrolysis of aesculin. Positive for nitrate reduction. Negative for activities of arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, tryptophan deaminase, trypsin, α-chymotrypsin, lipase (C14), esterase (C4), valine arylamidase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase and α-fucosidase. Positive for activities of acid phosphatase, alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, protease, amylase, cystine arylamidase, α-glucosidase and N-acetyl-β-glucosaminidase. Activity of esterase lipase (C8) is weak. In the API 20 NE system, assimilates D-glucose, D-mannitol, maltose and malic acid weakly. Does not assimilate L-arabinose, D-mannose, N-acetylglucosamine, gluconate, citrate, capric acid, adipic acid or phenylacetic acid. Negative for fermentation of glucose, ribose, xylose, mannitol, maltose, lactose, sucrose, glycogen, inositol, sorbitol, rhamnose, melibiose, amygdalin and L-arabinose. Resistant to cyclosporin A (100 µg ml⁻¹), but sensitive to ampicillin (50 µg ml⁻¹), kanamycin, streptomycin, rifampicin, tetracycline, penicillin G, chloramphenicol and trimethoprim (each at 100 µg ml⁻¹). The predominant cellular fatty acids are anteiso-C_{15:0}, summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH), iso-C_{15:0}, summed feature 4 (iso-C_{17:1} I and/or anteiso-C_{17:1} B) and C_{16:1}ω5c. MK-7 is the major menaquinone. Phosphatidylethanolamine, three unknown aminophospholipids, two unknown aminolipids, two unknown glycolipids and five unknown polar lipids are present in the polar lipid profile. The polyamine pattern is

composed of *sym*-homospermidine as the major compound, moderate amounts of spermidine and spermine and a trace amount of putrescine. The DNA G + C content of the type strain is 61.6 mol%.

The type strain, BZ33r^T (=DSM 22290^T =CGMCC 1.8975^T =LMG 25548^T), was isolated from soil from an industrial site in Bozen, South Tyrol, Italy.

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