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

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A Gram-negative, aerobic bacterium, designated strain BZ26^T, was isolated from hydrocarbon-contaminated soil. The strain was psychrophilic, showing good growth over a temperature range of 1–20 °C. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BZ26^T was related to members of the genus *Dyadobacter* and had highest 16S rRNA gene sequence similarity to *Dyadobacter alkalitolerans* 12116^T (98.1%), *Dyadobacter koreensis* KCTC 12537^T (97.5%) and *Dyadobacter ginsengisoli* Gsoil 043^T (97.2%). Strain BZ26^T had MK-7 as the major menaquinone and summed feature 3 (C_{16:1}ω7c/iso-C_{15:0} 2-OH), C_{16:1}ω5c and iso-C_{15:0} as major fatty acids. The genomic DNA G+C content of strain BZ26^T was 48.9 mol%. On the basis of phenotypic characteristics and genotypic analysis, strain BZ26^T is considered to represent a novel species of the genus *Dyadobacter*, for which the name *Dyadobacter psychrophilus* sp. nov. is proposed. The type strain is BZ26^T (=DSM 22270^T =CGMCC 1.8951^T).

The genus *Dyadobacter* was proposed by Chelius & Triplett (2000) to accommodate bacteria of the phylum *Bacteroidetes*, class *Sphingobacteria*, that show straight to curved cell arrangements, occurring in pairs in young cultures and forming chains of coccoid cells in old cultures, and produce a non-diffusible, yellow, flexirubin-like pigment (Reddy & Garcia-Pichel, 2005). At the time of writing, the genus *Dyadobacter* comprises seven recognized species: *Dyadobacter fermentans* (Chelius & Triplett, 2000), *D. crusticola* (Reddy & Garcia-Pichel, 2005), *D. hamtensis* (Chaturvedi *et al.*, 2005), *D. ginsengisoli* (Liu *et al.*, 2006), *D. koreensis* (Baik *et al.*, 2007), *D. beijingensis* (Dong *et al.*, 2007) and *D. alkalitolerans* (Tang *et al.*, 2009). All species of the genus, except *D. hamtensis* and *D. fermentans*, are able to grow at low temperatures, down to 4 °C. The type strains of *Dyadobacter* species have been isolated from various sources, such as soil, desert sand, plant material, fresh water and glacial habitats. In the present study, we report on the characterization of a novel, psychrophilic *Dyadobacter*-like bacterium, strain BZ26^T, isolated from soil from an industrial site contaminated with hydrocarbons.

Strain BZ26^T was isolated from soil containing large amounts of heavy oil in Bozen, South Tyrol, Italy. Soil was collected under sterile conditions in spring 2008. A 10 g soil sample was shaken with 90 ml sterile 1% sodium

pyrophosphate for 20 min at 150 r.p.m. Appropriate dilutions, prepared with sterile saline solution (0.9% NaCl), were plated on R2A agar (0.05% yeast extract, 0.05% peptone, 0.05% Casamino acids, 0.05% glucose, 0.05% starch, 0.03% sodium pyruvate, 0.03% K₂HPO₄ and 0.005% MgSO₄, pH 7) and incubated at 10 °C. One of the pure cultures obtained was yellow-pigmented and was designated strain BZ26^T.

D. ginsengisoli LMG 23409^T, *D. koreensis* DSM 19938^T and *D. alkalitolerans* CCTCC AB 207176^T were obtained from the Laboratory of Microbiology Gent Bacteria Collection, the Deutsche Sammlung von Mikroorganismen und Zellkulturen and the China Center for Type Culture Collection, respectively, and were used as reference strains.

DNA was extracted and purified as described by Sambrook *et al.* (1989). The gene encoding the 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 system (Promega) according to the manufacturer's instructions. Sequencing reactions were carried out by using an ABI BigDye 3.1 sequencing kit (Applied BioSystems) and an automated DNA sequencer (model ABI3730; Applied BioSystems). The nearly complete 16S rRNA gene sequence (1501 bp) of strain BZ26^T was submitted to GenBank and EMBL to search for similar sequences via the BLAST algorithm. The phylogenetic tree was constructed by using Kimura's two-parameter and pairwise-deletion model implemented in the program

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BZ26^T is GQ131577.

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic position of strain BZ26^T, recognized members of the genus *Dyadobacter* and representatives of some related taxa. Numbers at nodes represent percentage levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers are given in parentheses. Bar, 2% sequence divergence.

MEGA version 3.0 (Kumar *et al.*, 2004). The resulting tree topologies were evaluated by bootstrap analysis based on 1000 replicates. Phylogenetic analysis (Fig. 1) showed that strain BZ26^T was grouped with members of the genus *Dyadobacter* in the neighbour-joining tree. A similar topology was found in the tree generated with the maximum-parsimony algorithm (not shown).

Strain BZ26^T was routinely cultivated on low-strength R2A agar medium at 20 °C and was maintained as a suspension in skimmed milk (10%, w/v) at –80 °C. Cell morphology was examined by phase-contrast microscopy (×1000) after growth on R2A agar plates at 20 °C. Colony morphology was also observed on R2A agar plates. The Gram reaction was tested by using classical Gram staining and was confirmed by the KOH lysis test. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂; oxidase activity was determined by using 1% (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The API M system (bioMérieux) was used to evaluate cell motility. API strips (API 20 E, API 20 NE, API ZYM; bioMérieux) incubated at 20 °C were used according to the manufacturer's instructions to determine physiological and biochemical characteristics as well as enzyme activities. The assimilation of a number of carbon sources was additionally tested in liquid culture by supplementing 10 ml of a pH-neutral phosphate-buffered mineral salts medium with trace elements (Margesin *et al.*, 2002) and various carbohydrates (final concentration 0.2%, w/v) as the sole carbon source. Activities of β-galactosidase, amylase and protease were tested by using R2A agar plates supplemented with X-Gal, starch or skimmed milk (Margesin *et al.*, 2003). Growth at 1–37 °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. Antibiotic susceptibility was determined on R2A agar plates supplemented with various antibiotics and incubated at 20 °C. The morphological, physiological and biochemical characteristics of strain BZ26^T are given in the species description and in Table 1. The characteristics that serve to differentiate strain BZ26^T from the most closely related members of the genus *Dyadobacter* are given in Table 1.

Respiratory quinones were extracted and purified according to Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989), with MK-7 from *D. koreensis* DSM 19938^T as a reference. Strain BZ26^T contained MK-7 as the major menaquinone.

Table 1. Differential phenotypic characteristics between strain BZ26^T and the type strains of recognized *Dyadobacter* species

Strains: 1, BZ26^T; 2, *D. alkalitolerans* 12116^T (data from Tang *et al.*, 2009); 3, *D. ginsengisoli* KCTC 12589^T (Liu *et al.*, 2006); 4, *D. koreensis* NBRC 101116^T (Baik *et al.*, 2007); 5, *D. hamtensis* HHS11^T (Chaturvedi *et al.*, 2005). All strains produce light-yellow to yellow colonies on agar plates. All are positive for aerobic growth, growth at pH 6–8, growth in the presence of 1% (w/v) NaCl, catalase and oxidase and assimilation of glucose and sucrose. All are negative for the Gram reaction, motility, hydrolysis of starch and urea, H₂S production, activities of tryptophan deaminase (indole production) and arginine dihydrolase and utilization of D-mannitol, inositol and sorbitol as sole carbon sources. +, Positive; d, delayed positive; –, negative; w, weakly positive; NA, no data available.

Characteristic	1	2	3	4	5
DNA G + C content (mol%)	48.9	46.3	48.0	44.0	49.0
Growth temperature range (°C)	1–30*	4–30	4–30	4–30	10–37
Nitrate reduction	–	+	+	–	–
Hydrolysis of aesculin	d	+	–	+	–
Hydrolysis of gelatin	d	–	–	–	–
β-Galactosidase activity	+	–	NA	+	+
Voges–Proskauer test	–	+	NA	–	–
Fermentative metabolism	–	+	–	–	+
Assimilation of:					
Maltose	–	+	+	NA	–
D-Mannose	–	+	+	+	–
L-Arabinose	–	–	+	+	+
L-Rhamnose	–	–	+	w	–†
Sodium acetate	–	–	–	NA	+

*Weak growth at 25–30 °C.

†Assimilation of D-rhamnose was tested.

For analysis of fatty acid methyl esters, cell mass of strain BZ26^T was harvested from R2A agar plates after incubation at 20 °C for 4 days. Fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI (Microbial Identification) system (Sasser, 1990). The predominant cellular fatty acids of strain BZ26^T were summed feature 3 (C_{16:1}ω7c/iso-C_{15:0} 2-OH; 52.6%), C_{16:1}ω5c (19.0%) and iso-C_{15:0} (17.0%). The fatty acid profiles of strain BZ26^T and the type strains of recognized *Dyadobacter* species are shown in Table 2.

The DNA G+C content of strain BZ26^T, determined by using the thermal denaturation method of Marmur & Doty (1962) with *Escherichia coli* K-12 as reference, was 48.9 mol%. DNA–DNA relatedness tests were performed by using the optical renaturation method (De Ley *et al.*, 1970) as modified by Huß *et al.* (1983). Tests were performed on a model Lambda 35 UV/VIS spectrometer equipped with a temperature programme controller (Perkin–Elmer). Levels of DNA–DNA relatedness between strain BZ26^T and *D. alkalitolerans* CCTCC AB 207176^T, *D. ginsengisoli* LMG 23409^T and *D. koreensis* DSM 19938^T were 21.6, 15.9 and 10.5 %, respectively.

Strain BZ26^T is a psychrophilic (cold-adapted) representative of the genus *Dyadobacter*, showing good growth at 1–20 °C, weak growth at 25–30 °C and no growth at 37 °C. Growth was fast at 10–15 °C, and highest cell yields were observed at 1–5 °C (Fig. 2). The strain could be differentiated clearly from its closest phylogenetic neighbour, *D. alkalitolerans*, by its ability to produce

β-galactosidase and its inability to grow under highly alkaline conditions, to reduce nitrate and to produce acetoin (Voges–Proskauer reaction) and by the absence of fermentative metabolism. Based on the phenotypic, phylogenetic and genomic evidence presented, strain BZ26^T is considered to represent a novel species of the genus *Dyadobacter*, for which the name *Dyadobacter psychrophilus* sp. nov. is proposed.

Description of *Dyadobacter psychrophilus* sp. nov.

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

Cells are Gram-negative, aerobic and non-motile. Cells in young cultures (3 days at 20 °C on R2A agar plates) are rod-shaped (0.9–1.2 × 1.5–3 µm) and often occur in pairs, whereas cells in older cultures (4–6 days at 20 °C) are short rod-shaped to coccoid (diameter 1.0–1.3 µm) and form chains. Colonies on R2A agar are yellow, mucoid, round, convex and smooth with an entire margin; colony diameter is 1 mm after 3 days and 3–5 mm after 6 days at 20 °C on R2A agar. By contrast, growth on nutrient agar is delayed and colonies are smaller. Growth occurs in liquid R2A medium and on agar plates at 1–30 °C, with fast growth at 10–15 °C and with highest cell yields at 1–5 °C. Growth is weak at 25–30 °C and absent at 37 °C (Fig. 2). On R2A agar plates, growth is observed at pH 6–8 and in the presence of 1% (w/v) NaCl. Produces catalase and cytochrome oxidase; hydrolysis of aesculin and gelatin is delayed. Positive for alkaline phosphatase, acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase, β-glucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase and α-mannosidase. Negative for nitrate reduction, Voges–Proskauer reaction

Table 2. Comparison of the cellular fatty acid profiles of strain BZ26^T and recognized *Dyadobacter* species

Strains: 1, BZ26^T; 2, *D. alkalitolerans* 12116^T (data from Tang *et al.*, 2009); 3, *D. beijingsensis* A54^T (Dong *et al.*, 2007); 4, *D. crusticola* CP183-8^T (Reddy & Garcia-Pichel, 2005); 5, *D. fermentans* NS114^T (Chelius & Triplett, 2000); 6, *D. hamtensis* HHS11^T (Chaturvedi *et al.*, 2005); 7, *D. ginsengisoli* KCTC 12589^T (Liu *et al.*, 2006); 8, *D. koreensis* NBRC 101116^T (Baik *et al.*, 2007). Values are percentages of total fatty acids. ND, Not detected/not reported; NA, not applicable.

Fatty acid	1	2	3	4	5	6	7	8
C _{14:0}	0.8	1.5	0.6	1.4	ND	1.2	ND	0.7
iso-C _{15:1}	1.5	ND	0.5	1.5	ND	3.7	ND	ND
iso-C _{15:0}	17.0	16.6	19.2	13.4	16.8	24.7	20.3	24.2
C _{16:1} ω5c	19.0	8.3	10.3	21.4	17.5	19.8	7.9	11.0
C _{16:1} ω7c	52.6*	37.2*	17.5	41.2	43.5*	14.8	44.6	34.8
C _{16:0}	1.0	4.2	2.9	12.4	4.8	8.6	11.0	9.4
iso-C _{15:0} 2-OH	NA	NA	23.4	ND	NA	ND	ND	ND
iso-C _{15:0} 3-OH	2.1	2.6	2.6	2.4	2.7	ND	3.9	2.8
C _{16:0} 3-OH	0.6	2.0	3.1	2.2	4.7	2.5	2.3	2.5
C _{18:0}	ND	4.0	ND	0.2	ND	ND	ND	ND
C _{18:1}	ND	ND	ND	0.8	ND	2.5	ND	ND
iso-C _{17:0} 3-OH	2.1	9.7	12.4	2.9	7.4	22.2	7.6	9.5

*As summed feature 3 (comprising C_{16:1}ω7c and/or iso-C_{15:0} 2-OH).

Fig. 2. Effect of temperature on growth of strain BZ26^T in R2A medium. Data are mean values of two replicates.

(acetoin production), production of indole and H₂S, citrate utilization, and hydrolysis of urea, starch and Tween 80. Negative for arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, lipase (C14), trypsin, α -chymotrypsin and α -fucosidase. Weakly positive for cystine arylamidase activity. Utilizes D-glucose and sucrose (delayed growth), but not L-arabinose, citrate, malate, maltose, D-mannose, D-mannitol, N-acetylglucosamine, potassium gluconate, L-rhamnose, sodium acetate, *myo*-inositol, sorbitol, capric acid, adipic acid or phenylacetic acid as sole carbon sources. Negative for fermentation of glucose, ribose, xylose, mannitol, maltose, lactose, sucrose, glycogen, inositol, sorbitol, rhamnose, melibiose, amygdalin and L-arabinose. Sensitive to ($\mu\text{g ml}^{-1}$) penicillin G (100), ampicillin (100), streptomycin, tetracycline, chloramphenicol (each 30) and rifampicin (10), but resistant to kanamycin (100), cyclosporin A (100) and trimethoprim (100). MK-7 is the major menaquinone. The predominant cellular fatty acids are summed feature 3 (C_{16:1 ω 7c}/iso-C_{15:0} 2-OH), C_{16:1 ω 5c} and iso-C_{15:0}. The DNA G + C content of the type strain is 48.9 mol%.

The type strain, BZ26^T (=DSM 22270^T =CGMCC 1.8951^T), was isolated from hydrocarbon-contaminated soil in Bozen, South Tyrol, Italy.

Acknowledgements

This research work was supported by a research grant from the Autonome Provinz Bozen, Südtirol. We thank P. Thurbichler for technical assistance.

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