Agromyces bauzanensis sp. nov., isolated from soil

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A Gram-stain-positive, aerobic bacterium, designated strain BZ41^T, was isolated from hydrocarbon-contaminated soil. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BZ41^T was related to members of the genus *Agromyces* and showed highest similarity with the type strain of *Agromyces ramosus* (96.8 %). The morphological, biochemical and chemotaxonomic characteristics of the new isolate were consistent with the description of the genus *Agromyces*. The cell-wall peptidoglycan of strain BZ41^T was of type B2 γ and contained the amino acids 2,4-diaminobutyric acid, alanine, glycine and glutamic acid in an approximate molar ratio of 1.8 : 0.7 : 1.1 : 1.0. The predominant cell-wall sugars were galactose, glucose, mannose and rhamnose. Strain BZ41^T had MK-12 and MK-11 as major menaquinones and contained anteiso-C_{15:0} and anteiso-C_{17:0} as major fatty acids. The genomic DNA G + C content of strain BZ41^T was 69.7 mol%. On the basis of phenotypic characteristics and genotypic analysis, strain BZ41^T is considered to represent a novel species of the genus *Agromyces*, for which the name *Agromyces bauzanensis* sp. nov. is proposed. The type strain is BZ41^T (=DSM 22275^T =CGMCC 1.8984^T).

The genus *Agromyces* was proposed by Gledhill & Casida (1969) and its description was emended by Zgurskaya *et al.* (1992). At the time of writing, the genus comprises 19 recognized species, and many members of the genus are widespread in soil (Takeuchi & Hatano, 2001; Li *et al.*, 2003; Jurado *et al.*, 2005a; Yoon *et al.*, 2008). In the present study, we report on the characterization of a novel *Agromyces*-like bacterium isolated from soil from an industrial site contaminated with hydrocarbons.

Strain BZ41^T was isolated from soil from an industrial site containing high levels of heavy oil located in Bozen, South Tyrol, Italy. Soil was collected under sterile conditions in spring 2008. Ten grams of soil 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 (0.1 ml) on R2A agar plates (Reasoner & Geldreich, 1985; 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) that were incubated at 20 °C.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BZ41^T is FJ972171.

Results of two-dimensional TLC of the phospholipids and glycolipid of strain BZ41^T are available as supplementary material with the online version of this paper.

One of the pure cultures was yellow pigmented and was designated strain BZ41^T. *Agromyces ramosus* DSM 43045^T and *Agromyces humatus* CD5^T (kindly provided by Dr Cesáreo Saiz-Jimenez, Instituto de Recursos Naturales y Agrobiología, Seville, Spain) 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.*, 2007). PCR products were cloned by using the pGEM-T easy vector systems (Promega) according to the manufacturer's instructions. Sequencing reactions and phylogenetic analysis were performed as described by Zhang *et al.* (2007). Phylogenetic analysis (Fig. 1) showed that strain BZ41^T grouped with members of the genus *Agromyces* in the neighbour-joining tree. Levels of 16S rRNA gene sequence similarity between strain BZ41^T and other strains used in the phylogenetic analysis were less than 96.8 %. Similar tree topologies were found in the tree generated with the maximum-parsimony algorithm (data not shown).

Strain BZ41^T was routinely cultured in low-strength R2A agar medium and was maintained as a suspension in skimmed milk (10 %, w/v) at –80 °C. Cell morphology was examined by phase-contrast microscopy ($\times 1000$) of cells grown on R2A agar plates at 25 °C. Colony morphology was also observed on R2A agar plates. The Gram reaction was

Fig. 1. Phylogenetic relationship between strain BZ41^T and recognized members of the genus *Agromyces* based on 16S rRNA gene sequences. *Microbacterium lacticum* IFO 14135^T served as an outgroup. The tree was constructed by using the neighbour-joining method. Numbers at nodes represent levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled datasets. GenBank accession numbers of 16S rRNA gene sequences are given in parentheses. Bar, 1% sequence divergence.

tested via classical Gram staining and was confirmed by the KOH lysis test. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂, and oxidase activity was determined by using 1% (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; these activities were tested by using cells grown for 24 h at 25 °C on R2A agar plates. API strips [API 20 E, API 20 NE, API 50CH (B/E), API ZYM; bioMérieux] incubated at 25 °C were used according to the manufacturer's instructions to determine the physiological and biochemical characteristics as well as enzyme activities of strain BZ41^T and of the reference strains *A. ramosus* DSM 43045^T and *A. humatus* CD5^T. 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, protease and esterase lipase were additionally tested by using R2A agar plates supplemented with the appropriate enzyme substrates (Margesin *et al.*, 2003). The API M system was used to evaluate cell motility. Growth at different temperatures (1–37 °C) was assessed on R2A agar plates and in R2A liquid medium. Growth at pH 5–9 and salt tolerance (0–5%, w/v, NaCl) were determined on R2A agar plates. Susceptibility to antibiotics was determined on R2A agar plates supplemented with various antibiotics; plates were incubated at 25 °C. Growth under anaerobic conditions was determined after 3 days incubation at 25 °C in an anaerobic jar [containing Anaerocult A (Merck) to produce anaerobic conditions] on R2A agar plates as well as on R2A agar plates

supplemented with 10 mM KNO₃. Growth under micro-aerophilic conditions was assessed after 3 days at 25 °C on R2A agar plates that were incubated in a microaerophilic atmosphere containing 8–10% (v/v) carbon dioxide and 5–7% (v/v) oxygen; this atmosphere was generated in sealed jars containing Anaerocult C (Merck). Morphological, physiological and biochemical characteristics of strain BZ41^T are given in the species description and in Table 1. The strain was characterized by its inability to assimilate a wide range of carbon sources, although it shared with phylogenetically related members of the genus *Agromyces* the ability to hydrolyse aesculin and starch.

For analysis of the peptidoglycan structure and cell-wall sugars, cells of strain BZ41^T were cultured in shaken flasks of medium 830 (<http://www.dsmz.de>) at 28 °C for 3 days. Purified peptidoglycan preparations were obtained after disruption of cells by shaking with glass beads and subsequent trypsin digestion, according to the method of Schleifer (1985). The peptidoglycan structure was determined by using hydrolysates of purified cell walls according to Schleifer (1985).

Amino acids and peptides were separated by two-dimensional ascending TLC on cellulose plates with the solvent systems given by Schleifer & Kandler (1972). The molar ratio of amino acids was determined by GC as described by MacKenzie (1987). The peptidoglycan of strain BZ41^T contained the amino acids 2,4-diaminobutyric acid, alanine, glycine and glutamic acid in an approximate molar ratio of 1.8:0.7:1.1:1.0. The quantitative amino acid composition of the peptidoglycan of strain BZ41^T was consistent with peptidoglycan type B2 γ according to Schleifer & Kandler (1972), which is usually found in members of the genus *Agromyces*. According to Sasaki *et al.* (1998), all recognized species of the genus *Agromyces* contain the L-isomer of diaminobutyric acid almost exclusively.

For analysis of cell-wall sugars, cell walls were hydrolysed in 0.5 M H₂SO₄ at 100 °C for 2 h. H₂SO₄ was removed by shaking with 20% *N,N*-diethylmethylamine in chloroform according to Whiton *et al.* (1985) and the sugars in the hydrolysate were analysed by TLC on cellulose plates according to Stanek & Roberts (1974). The main cell-wall sugars of strain BZ41^T were galactose, glucose, mannose and rhamnose. Smaller amounts of xylose were also detected.

For analysis of fatty acid methyl esters, cell mass of strain BZ41^T was harvested from trypticase soy agar plates (3% trypticase soy broth, 0.3% yeast extract, 1.5% agar) after incubation at 28 °C for 4 days. Fatty acid methyl esters were prepared and extracted according to the standard protocol of the MIDI (Microbial Identification) system (Sasser, 1990). The predominant cellular fatty acids of strain BZ41^T were anteiso-C_{15:0} (46.4%), anteiso-C_{17:0} (21.1%), iso-C_{15:0} (17.6%), iso-C_{16:0} (7.0%), C_{16:0} (4.4%) and iso-C_{17:0} (2.8%). The fatty acid profile of strain BZ41^T differed from those of recognized *Agromyces*

Table 1. Phenotypic characteristics that differentiate strain BZ41^T from the type strains of recognized *Agromyces* species

Strains: 1, BZ41^T; 2, *A. albus* VKM Ac-1800^T (data from Dorofeeva *et al.*, 2003; Jurado *et al.*, 2005b); 3, *A. humatus* CD5^T (Jurado *et al.*, 2005b); 4, *A. neolithicus* 23-23^T (Jurado *et al.*, 2005a); 5, *A. ramosus* IMET 11027^T (Gledhill & Casida, 1969; Groth *et al.*, 1996; Dorofeeva *et al.*, 2003). All strains are positive for hydrolysis of aesculin and starch and all often form branching hyphae. All are negative for endospore formation and motility, for hydrolysis of urea and Tween 80, for assimilation of citrate and for fermentative acid production from lactose and turanose. +, Positive; –, negative; w, weakly positive; v, variable; NA, no data available.

Characteristic	1	2	3	4	5
Isolation source	Contaminated soil	Plant leaves	Wall of tomb	Soil	Soil
DNA G + C content (mol%)	69.7	69.0	70.6	65.3	68.9
Cell-wall sugars*	Gal, Glc, Man, Rha	Rha, Gal, Glc, Man	Glc, Gal, Rha, Man†	Glc, Gal, Man†	Gal, Glc, Man, Rha, Xyl
Optimum growth temperature (°C)	25–30	26–28	28	28	30
Growth at 10 °C	+‡	+	+‡§	–	+‡§
Oxidase reaction	–	+	–§	v	–§
Catalase reaction	+	+	+§	+	–§
Nitrate reduction	–	–	+§	+	–§
H ₂ S production	–	+	+	+	+
Hydrolysis of:					
Casein	–	+	+	+	–
Gelatin	–	–	+§	+	–§
Enzyme assays (API ZYM)					
α-Glucosidase	+	–	w§	NA	+§
N-Acetyl-β-glucosaminidase	–	+	–§	+	+§
α-Chymotrypsin	–	–	w§	+	–§
α-Galactosidase	–	–	–§	+	–§
β-Galactosidase	–	+	+§	–	w§
β-Glucuronidase	–	–	w§	+	–§
α-Mannosidase	–	–	–§	+	–§
Assimilation (API 20NE) of:					
Glucose	–	+	+§	NA	+§
Maltose	–	+	+§	NA	+§
Mannitol	–	+	+§	NA	w§
N-Acetylglucosamine	–	NA	+§	NA	–§
Sodium gluconate	–	NA	+§	NA	–§
Malate	–	+	+§	–	+§
Fermentative acid production (API 50CH B/E) from:					
Amygdalin	–	–	–§	–	+§
D-Arabinose	–	+	–§	+	+§
D-Mannitol	–	+	+§	+	w§
Inulin	–	–	+§	–	+§
L-Fucose	–	–	–§	+	+§
L-Rhamnose	–	+	+§	–	+§

*Gal, Galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; Xyl, xylose.

†Whole-cell sugars.

‡Data obtained on R2A agar plates.

§Data from the present study using *A. humatus* CD5^T and *A. ramosus* DSM 43045^T.

species by the larger proportion of iso-C_{15:0} and smaller proportion of iso-C_{16:0} (Takeuchi & Hatano, 2001; Li *et al.*, 2003; Jurado *et al.*, 2005a, b).

Respiratory quinones were extracted and purified according to Collins (1985) and were analysed by HPLC (Wu *et al.*, 1989). Cellular polar lipids were extracted and analysed on silica gel plates (Kieselgel 60 F; Merck) by TLC (Kates,

1986). Strain BZ41^T contained MK-12 (61 %) and MK-11 (34 %) as the major menaquinones, with a minor amount of MK-13. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and an unidentified glycolipid (see Supplementary Fig. S1, available in IJSEM Online).

The DNA G + C content of strain BZ41^T, as determined with the thermal denaturation method (Sly *et al.*, 1986)

with *Escherichia coli* K-12 DNA as reference, was 69.7 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). On the basis of 16S rRNA gene sequence similarity, strain BZ41^T was related most closely to *A. ramosus* DSM 43045^T (96.8%). However, the level of DNA–DNA relatedness between this reference strain and strain BZ41^T was only 26.6%.

Based on the phenotypic, phylogenetic and genomic evidence presented, strain BZ41^T is considered to represent a novel species of the genus *Agromyces*, for which the name *Agromyces bauzanensis* sp. nov. is proposed.

Description of *Agromyces bauzanensis* sp. nov.

Agromyces bauzanensis (bau.zan.en'sis. M.L. masc. adj. *bauzanensis* referring to Bauzanum, the medieval Latin name of Bozen/Bolzano, a city in South Tyrol, Italy, from where the type strain was isolated).

Cells form branching hyphae (0.3–0.5 µm long) that break up into irregular and rod-like, diphtheroid, non-motile fragments. Cells are Gram-stain-positive and aerobic; no growth occurs under anaerobic or microaerophilic conditions. Colonies on R2A agar are lemon yellow, round, convex, opaque and smooth with an entire margin; colony diameter is approx. 1 mm. Growth on nutrient agar is weak; good growth occurs in R2A medium at pH 7–8 and at 10–30 °C, with fastest growth rates at 25–30 °C; at 37 °C, growth occurs on R2A agar plates but is very weak in liquid medium. No growth occurs at 42 °C; growth at 1–5 °C is delayed and weak. Grows in the presence of 0–2% (w/v) NaCl. Catalase-positive; cytochrome oxidase is not produced. Negative for nitrate reduction and production of indole, H₂S and urease. Positive for hydrolysis of aesculin and starch and for activities of α-galactosidase and leucine arylamidase. Negative for activities of gelatinase, arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, tryptophan deaminase, alkaline phosphatase, lipase (C14), α-galactosidase, β-galactosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, trypsin and α-chymotrypsin. Activities of esterase (C4), esterase lipase (C8) and valine arylamidase are weak. Sensitive to ampicillin and rifampicin (10 µg ml⁻¹) as well as to penicillin G, kanamycin, streptomycin, tetracycline and chloramphenicol (30 µg ml⁻¹). The following substrates are not assimilated: D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, maltose, gluconate, citrate, malic acid, capric acid, adipic acid and phenylacetic acid. Negative for fermentative acid production from glucose, erythritol, D- and L-arabinose, ribose, L-xylose, adonitol, D-galactose, lactose, fructose, mannose, L-sorbose, rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, turanose, D-lyxose, D-tagatose, D- and L-fucose, D-arabitol, potassium gluconate and 2-ketogluconate; positive for fermentative

acid production from aesculin and weakly positive for fermentative acid production from glycerol, D-xylose, salicin, cellobiose, maltose, starch, glycogen and 5-ketogluconate. The cell-wall peptidoglycan is of type B2γ and contains the amino acids 2,4-diaminobutyric acid, alanine, glycine and glutamic acid. The predominant cell-wall sugars are galactose, glucose, mannose and rhamnose. The predominant cellular fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{15:0} and iso-C_{16:0}. The major menaquinones are MK-12 and MK-11; a minor amount of MK-13 is also found. The major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and an unidentified glycolipid. The DNA G + C content of the type strain is 69.7 mol%.

The type strain, BZ41^T (=DSM 22275^T =CGMCC 1.8984^T), was isolated from hydrocarbon-contaminated soil in Bozen, South Tyrol, Italy.

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