Pseudomonas bauzanensis sp. nov., isolated from soil

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A Gram-negative, aerobic, motile rod, designated BZ93^T, was isolated from soil from an industrial site. The strain grew at 5–30 °C. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BZ93^T was related to members of the genus *Pseudomonas* and was related most closely to *Pseudomonas xiamenensis* C10-2^T (97.8 % 16S rRNA gene sequence similarity) and *Pseudomonas pertucinogena* IFO 14163^T (97.4 %). The predominant cellular fatty acids of strain BZ93^T were C_{18:1ω7c} (54.8 %), summed feature 3 (C_{16:1ω7c} and/or iso-C_{15:0} 2-OH; 10.3 %), C_{16:0} (9.9 %) and C_{17:0} cyclo (7.4 %). The major quinone was ubiquinone 9. The major phospholipids were phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and an unknown phospholipid. The genomic DNA G+C content was 61.8 mol%. On the basis of phenotypic characteristics, phylogenetic analysis and DNA–DNA relatedness, a novel species, *Pseudomonas bauzanensis* sp. nov., is proposed. The type strain is BZ93^T (=DSM 22558^T =CGMCC 1.9095^T =LMG 26048^T).

The genus *Pseudomonas* was originally created by Migula (1894). Bacteria of the genus *Pseudomonas* belong to the *Gammaproteobacteria* and are aerobic, chemo-organotrophic, non-spore-forming, motile, Gram-negative rods with polar flagella and a respiratory rather than a fermentative metabolism (Palleroni, 1984; Timmis, 2002). Over the years, species of the genus have been reclassified and revised several times (Sneath *et al.*, 1981; Oyaizu & Komagata, 1983; Vancanneyt *et al.*, 1996; Anzai *et al.*, 2000). Members of the genus *Pseudomonas* are widely distributed in soil, water, air, plants and many other natural habitats (Palleroni, 1992). Some representatives of this genus colonize cold habitats (Yumoto *et al.*, 2001; Reddy *et al.*, 2004; Bozal *et al.*, 2007; Margesin *et al.*, 2008). The present study deals with the characterization of a novel strain of the genus *Pseudomonas* that was isolated from soil and is able to grow at low temperatures.

Strain BZ93^T was isolated from soil from an industrial site (containing high amounts of heavy oil and heavy metals) in Bozen, South Tyrol, Italy, as described previously (Zhang *et al.*, 2010). The soil was sampled and processed under sterile conditions in spring 2008. A sample (10 g) was shaken with 90 ml sterile 1 % sodium pyrophosphate for 20 min at

150 r.p.m. Appropriate dilutions were prepared with sterile 0.9 % NaCl and 0.1 ml aliquots were spread 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, 0.005 % MgSO₄, 1.5 % agar; pH 7; Reasoner & Geldreich, 1985) and incubated at 20 °C. A white colony was purified and designated BZ93^T. Strain BZ93^T was routinely cultured on R₂A medium at 20 °C and maintained as a suspension in skimmed milk (10 % w/v) at –80 °C. *Pseudomonas pertucinogena* LMG 1874^T and *Pseudomonas xiamenensis* CGMCC 1.6446^T were routinely grown on R₂A agar at 25 °C and used as reference strains.

DNA was extracted and purified as described by Sambrook *et al.* (1989). The 16S rRNA gene was amplified by PCR using universal primers (27F, 5'-AGAGTTTGATCCTGG-CTCAG-3'; 1541R, 5'-AAGGAGGTGATCCAGCCGCA-3'). PCR products were cloned into the pGEM-T vector (Promega) according to the manufacturer's instructions. FASTA similarity searches were done using the GenBank and EMBL databases. A pairwise comparison of the 16S rRNA gene sequences was analysed using EzTaxon (Chun *et al.*, 2007). The 16S rRNA gene sequence of strain BZ93^T showed 97.8 and 97.4 % similarity with *P. xiamenensis* C10-2^T and *P. pertucinogena* IFO 14163^T, respectively. Similarities between strain BZ93^T and other members of the genus *Pseudomonas* were <96.5 %.

Phylogenetic analysis was performed using MEGA version 4.0 (Tamura *et al.*, 2007) and PHYLIP version 3.69

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BZ93^T is GQ161991.

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

(Felsenstein, 2009) after multiple alignment of data using CLUSTAL X version 1.8 (Thompson *et al.*, 1997). For neighbour-joining analysis (Saitou & Nei, 1987), genetic distances were calculated using Kimura's two-parameter model (Kimura, 1980). Strain BZ93^T fell within the genus *Pseudomonas* and formed a distinct cluster with *P. xiamenensis* C10-2^T and *P. pertucinogena* IFO 14163^T (Fig. 1). The relative position of strain BZ93^T was also confirmed in the maximum-likelihood tree.

Cell morphology was examined by phase-contrast microscopy ($\times 1000$) of cells grown on R₂A agar at 20 °C. Colony morphology was also observed on R₂A agar at 20 °C. Motility of cells grown at 20 °C was evaluated on motility agar (API M; bioMérieux) and under a light microscope ($\times 1000$).

All of the following tests were done with strain BZ93^T and the reference strains. The Gram reaction was tested by Gram staining and confirmed by the KOH-lysis test.

Pigment analysis was performed as described by Hildebrand *et al.* (1994). To test for the production of a cellular (methanol-extractable) pigment, cells were grown for 4 days at 25 °C on King's B agar and nutrient agar. To test for the presence of a diffusible pigment, cells were grown for 48 h at 25 °C and 200 r.p.m. in King's B broth and nutrient broth. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂. Oxidase activity was determined using 1% (w/v) *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. The API 20 E, API 20 NE and API ZYM systems (bioMérieux) and EcoPlates (Biolog) were used at 25 °C to determine physiological and biochemical characteristics and enzyme activities. β -Galactosidase, amylase and lipase esterase activities were additionally tested using R₂A agar supplemented with appropriate substrates (Margesin *et al.*, 2003). Growth at 1–45 °C was assessed on R₂A agar and in R₂A liquid medium at 150 r.p.m. Growth at pH 5–9 and with 0–20% (w/v) NaCl was determined on R₂A agar at 20 °C. Susceptibility to antibiotics was determined at 25 °C using antibiotic

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences, showing the phylogenetic position of strain BZ93^T within the genus *Pseudomonas*. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Asterisks indicate that the corresponding nodes were also recovered in the maximum-likelihood tree. *Halomonas elongata* ATCC 33173^T was used as an outgroup. Bar, 0.01 nucleotide substitutions per site.

discs (bioMérieux). The morphological, physiological and biochemical characteristics of strain BZ93^T are given in the species description and Table 1. The features that serve to differentiate strain BZ93^T from *P. xiamenensis* CGMCC 1.6446^T and *P. pertucinogena* LMG 1874^T are given in Table 1.

Respiratory quinones were extracted and purified according to Collins (1985) and were analysed by HPLC (Wu

et al., 1989). The quinone system consisted predominantly of ubiquinone Q-9.

For fatty acid methyl ester analysis, cell mass of strain BZ93^T and the reference strains was harvested from trypticase soy agar after incubation at 25 °C for 3 days. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the Sherlock Microbial Identification System (MIDI; Sasser, 1990). The predominant cellular fatty acids of strain BZ93^T were C_{18:1}ω7c (54.8%), summed feature 3 (C_{16:1}ω7c and/or iso-C_{15:0} 2-OH; 10.3%), C_{16:0} (9.9%) and C_{17:0} cyclo (7.4%). The fatty acid profiles of strain BZ93^T, *P. xiamenensis* CGMCC1.6446^T and *P. pertucinogena* LMG 1874^T are shown in Supplementary Table S1 (available in IJSEM Online).

The cellular polar lipids were extracted and analysed on silica gel plates (Kieselgel 60 F; Merck) by TLC (Kates, 1986) and staining with molybdatophosphoric acid. The polar lipid profile of strain BZ93^T consisted of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and an unknown phospholipid. An unknown aminophospholipid and 11 unknown polar lipids were also detected (Supplementary Fig. S1, available in IJSEM Online).

The G + C content was determined by the thermal denaturation method (Marmur & Doty, 1962) with *Escherichia coli* K-12 as the reference strain, and DNA–DNA hybridization was done by the liquid renaturation method (De Ley *et al.*, 1970) as modified by Huß *et al.* (1983). Both experiments were carried out using a model Lambda 35 UV/VIS spectrometer equipped with a temperature program controller (Perkin-Elmer). The DNA G + C content of strain BZ93^T was 61.8 mol%. DNA–DNA relatedness between strain BZ93^T and *P. xiamenensis* CGMCC 1.6446^T and *P. pertucinogena* LMG 1874^T was 32.5 and 26.6 %, respectively.

The results presented above demonstrate that strain BZ93^T is a member of the genus *Pseudomonas*. Strain BZ93^T could be easily differentiated from its closest phylogenetic neighbours by its ability to grow well at 5 °C and with 10 % (w/v) NaCl and to utilize a number of carboxylic acids (pyruvic acid methyl ester, itaconic acid and α-ketobutyric acid). In addition, strain BZ93^T could be differentiated from *P. xiamenensis* CGMCC 1.6446^T by its inability to reduce nitrate and to grow at pH 9 and its ability to assimilate adipic acid, as well as by having higher amounts of the cellular fatty acid C_{18:1}ω7c and smaller amounts of C_{19:0} cyclo ω8c. Strain BZ93^T could be differentiated from *P. pertucinogena* LMG 1874^T by its inability to grow at 37 °C, ferment glucose and utilize 4-hydroxybenzoic acid, by the absence of cystine arylamidase, trypsin and α-chymotrypsin and its ability to assimilate trisodium citrate and grow with 7 % (w/v) NaCl and at 10 °C, as well as by having higher amounts of the cellular fatty acids C_{18:1}ω7c, C_{17:0} cyclo and C_{19:0} cyclo ω8c and smaller amounts of summed feature 3.

Table 1. Phenotypic characteristics that differentiate strain BZ93^T from its closest phylogenetic neighbours

Strains: 1, *Pseudomonas bauzanensis* sp. nov. BZ93^T; 2, *P. xiamenensis* CGMCC 1.6446^T; 3, *P. pertucinogena* LMG 1874^T. All data were taken from this study. All strains are positive for catalase, cytochrome oxidase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and assimilation of capric acid and malic acid. All strains are negative for α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase, arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, citrate utilization, H₂S production, urease production, tryptophan deaminase, indole production, gelatinase, aesculin hydrolysis and utilization of L-arabinose, D-mannose, D-mannitol, maltose, N-acetylglucosamine, phenylacetic acid, gluconate (API 20 NE), D-glucosaminic acid, D-galacturonic acid, γ-hydroxybutyric acid, cellobiose, α-lactose, methyl β-D-glucoside, D-xylose, i-erythritol, glucose 1-phosphate, DL-α-glycerol phosphate, D-galacturonic acid, γ-lactone, L-arginine, L-asparagine, L-phenylalanine, L-threonine, glycyl L-glutamic acid, phenylethylamine, putrescine, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, α-cyclodextrin and glycogen (EcoPlates). +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3
Growth at/with:			
5 °C	+	–	–
10 °C	+	+	–
37 °C	–	–	+
pH 9	–	+	–
7 % (w/v) NaCl	+	+	–
10 % (w/v) NaCl	+	–	–
Nitrate reduction	–	+	–
Enzyme activities (API ZYM)			
Cystine arylamidase	–	–	+
Trypsin	–	–	+
α-Chymotrypsin	–	–	+
Glucose fermentation	–	–	+
Utilization (API 20 NE)			
Adipic acid	+	–	+
Trisodium citrate	+	+	–
Utilization (EcoPlates)			
Pyruvic acid methyl ester	+	–	–
4-Hydroxybenzoic acid	–	–	+
Itaconic acid	+	–	–
α-Ketobutyric acid	+	–	–
L-Serine	w	–	–

Therefore, on the basis of phenotypic, phylogenetic and genomic evidence, strain BZ93^T was identified as a representative of a novel species of the genus *Pseudomonas*, for which the name *Pseudomonas bauzanensis* sp. nov. is proposed.

Description of *Pseudomonas bauzanensis* sp. nov.

Pseudomonas bauzanensis (bau.za.nen'sis. N.L. fem. adj. *bauzanensis* referring to *Bauzanum* medieval Latin name of Bozen/Bolzano, a city in South Tyrol, Italy, where the species was first isolated).

Cells are aerobic, Gram-negative, motile, short rods (0.7–0.9 × 1.1–1.7 µm after 4 days at 20 °C on R₂A agar). Grows on R₂A agar, nutrient agar, trypticase soy agar and King's B agar. Colonies on R₂A agar are white, round, convex, smooth and with entire margins (1.5–2 mm in diameter after 6 days at 20 °C). Negative for production of a cellular (methanol-extractable) or diffusible fluorescent pigment. Good growth occurs in R₂A liquid medium at 5–30 °C (highest cell yields at 10–20 °C); growth at 1 and 37 °C is absent in R₂A liquid culture and on R₂A agar plates. On R₂A agar, growth occurs at pH 7–8 and with 0–7 % (w/v) NaCl; growth is delayed with 10 % (w/v) NaCl and absent with 15 % (w/v) NaCl. Produces catalase and cytochrome oxidase. Negative for production of indole, H₂S and urease, utilization of citrate, reduction of nitrate and hydrolysis of aesculin. Positive for acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase and leucine arylamidase; weakly positive for valine arylamidase and lipase (C14); and negative for gelatinase, amylase, arginine dihydrolase, lysine dihydrolase, ornithine dihydrolase, tryptophan deaminase, trypsin, α-chymotrypsin, cystine arylamidase, *N*-acetyl-β-glucosaminidase, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, α-mannosidase and α-fucosidase. With API 20 NE, assimilates capric acid, adipic acid, malic acid and trisodium citrate, but not D-glucose, D-mannitol, maltose, L-arabinose, D-mannose, *N*-acetylglucosamine, gluconate or phenylacetic acid. With EcoPlates, utilizes pyruvic acid methyl ester, itaconic acid, α-ketobutyric acid, D-malic acid, Tweens 40 and 80 and weakly utilizes L-serine, but does not utilize D-glucosaminic acid, D-galacturonic acid, γ-hydroxybutyric acid, cellobiose, α-lactose, methyl β-D-glucoside, D-xylose, i-erythritol, D-mannitol, *N*-acetyl-D-glucosamine, glucose 1-phosphate, DL-α-glycerol phosphate, D-galacturonic acid, γ-lactone, L-arginine, L-asparagine, L-phenylalanine, L-threonine, glycyl L-glutamic acid, phenylethylamine, putrescine, 2-hydroxybenzoic acid, 4-hydroxybenzoic acid, α-cyclodextrin or glycogen. Does not ferment glucose, mannitol, sucrose, inositol, sorbitol, rhamnose, melibiose, amygdalin or L-arabinose. Resistant to (µg per disc) tetracycline (30) and erythromycin (15), and sensitive to kanamycin (30), amikacin (30), gentamicin (10), streptomycin (10), ampicillin (10), nalidixic acid (30), chloramphenicol (30) and rifampicin (30). The predominant cellular fatty acids are C_{18:1}ω7c, summed feature 3 (C_{16:1}ω7c and/or

iso-C_{15:0} 2-OH), C_{16:0} and C_{17:0} cyclo. The quinone system consists predominantly of ubiquinone Q-9. The polar lipid profile consists of phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol and an unknown phospholipid; an unknown aminophospholipid and 11 unknown polar lipids are also present. The DNA G+C content of the type strain is 61.8 mol%.

The type strain is BZ93^T (=DSM 22558^T =CGMCC 1.9095^T =LMG 26048^T) and was isolated from soil contaminated with hydrocarbons and heavy metals in Bozen, South Tyrol, Italy.

Acknowledgements

This work was supported by the Autonome Provinz Bozen, Südtirol. We thank P. Thurnbichler and J. Mair for technical assistance.

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