Brevibacillus gelatini sp. nov., isolated from a hot spring

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Two Gram-stain-positive, moderately thermophilic, endospore-forming, rod-shaped, motile bacteria designated PDF4^T and PDF10, were isolated from Camkoy hot spring in the provinces of Aydın, Turkey and were characterized in order to determine their phylogenetic position. 16S rRNA gene sequence analysis revealed that the two strains belonged to the genus *Brevibacillus*. Strain PDF4^T showed highest 16S rRNA gene sequence similarity to strain PDF10 (99.5 %), *Brevibacillus brevis* DSM 30^T (98.9 %), *Brevibacillus parabrevis* DSM 8376^T (98.6 %) and *Brevibacillus formosus* DSM 9885^T (98.5 %); similarities to other species of the genus *Brevibacillus* were less than 98.5 %. The predominant fatty acids of strain PDF4^T were anteiso-$C_{15:0}$ (60.0 %) and iso-$C_{15:0}$ (22.3 %). The polar lipids of strain PDF4^T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, an unknown phospholipid, two unknown lipid, an unknown aminophospholipid and two unknown aminolipids. MK-7 was detected as a sole respiratory quinone, and the cell wall of strain PDF4^T contained *meso*-diaminopimelic acid. The DNA G+C content of strain PDF4^T was 51.7 mol%. DNA–DNA hybridization showed less than 60 % relatedness between strain PDF4^T and type strains of the most closely related species given above. Based on these data, the two strains are considered to represent a novel species of the genus *Brevibacillus*, for which the name *Brevibacillus gelatini* sp. nov. is proposed. The type strain is PDF4^T (=NCCB 100559^T=DSM 100115^T).

Bacillus brevis was first described in 1900 (Migula, 1900) and was reclassified as a novel species (*Brevibacillus brevis*) of the novel genus *Brevibacillus*, along with nine other species of the *Bacillus brevis* group (Shida *et al.*, 1996). At the time of writing, the genus *Brevibacillus* comprised 19 species with validly published names (http://www.bacterio.net/brevibacillus.html). Members of this genus are Gram-stain-positive or Gram-stain-variable, motile, strictly aerobic or facultatively anaerobic, rod-shaped, produce oval endospores in swollen sporangia and possess menaquinone-7 (MK-7) as the major respiratory quinone (Shida *et al.*, 1996). Distinguishing between species of this genus is extremely difficult, as most strains have poor reactivity in conventional identification tests and few have useful differential phenotypic characteristics (Goto *et al.*, 2004). Even with molecular analyses, such as amplified rRNA restriction analysis, discriminating between all of the species is not always simple (Logan *et al.*, 2002). However, more recently, hypervariable (HV) region sequence is a powerful index for rapid and convenient identification and grouping of *Brevibacillus* (Goto *et al.*, 2004). In members of the genus *Brevibacillus*, the HV region sequence is highly conserved within a species and has diverged sufficiently between species, thus enabling identification and grouping of species of the genus *Brevibacillus* by sequence comparisons of the HV region (Goto *et al.*, 2004; Allan *et al.*, 2005).

In the present study, two strains (PDF4^T and PDF10) were isolated from a hot spring and taxonomically characterized based on data obtained using a polyphasic approach, including 16S rRNA gene sequence analysis, phenotypic properties, chemotaxonomic and DNA–DNA hybridization data. The results indicated that strains PDF4^T and PDF10 represent a novel species of the genus *Brevibacillus*.

Abbreviation: HV, hypervariable.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequences of strains PDF4^T and PDF10 are KP899808 and KP899809, respectively.

Four supplementary figures and two supplementary tables are available with the online Supplementary Material.

Strains PDF4^T and PDF10 were isolated from water samples collected from the Camkoy hot spring, which is located 150 m above sea level from the Aydın coast, Turkey. The method used for isolation has been described previously (Belduz *et al.*, 2003). The water temperature of this hot spring is around 40–60 °C. The water sample was filtered within 2 h of sampling through Millipore membrane filters with a pore size of 0.45 μm. The filters were deposited on Degryse 162 agar and incubated aerobically at 45 °C for 3 days (Inan *et al.*, 2012). Cream colonies were picked from the filters (which otherwise contained mostly white and yellow bacterial colonies) and were purified by streaking on the same medium. The purity of the isolates was assessed by using colony morphology and microscopy. Strains PDF4^T and PDF10 were routinely cultured on Degryse 162 agar and preserved at −80 °C as a suspension in Degryse 162 broth supplemented with 20 % (v/v) glycerol. Growth was tested on various media including Degryse 162 agar, nutrient agar (NA), Luria–Bertani (LB) agar and trypticase soy agar (TSA). Maximum growth for the strains was observed on Degryse 162 agar. Growth was abundant on TSA, but weak on NA and LB agar.

The 16S rRNA genes of strains PDF4^T and PDF10 were selectively amplified from purified genomic DNA by using the oligonucleotide primer pair UNI16S-L and UNI16S-R (Belduz *et al.*, 2003). PCR conditions were as described by Beffa *et al.* (1996). PCR products were cloned into pGEM-T easy vector systems and then 16S rRNA gene sequences were determined with an Applied Biosystems model 373A DNA sequencer by using the ABI PRISM cycle sequencing kit (Macrogen). The results of 16S rRNA gene sequencing were analysed using the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). The 16S rRNA gene sequences of related taxa were obtained from EzTaxon-e server and edited by using the BioEdit program (Hall, 1999) and multiple alignments were performed with the CLUSTAL X program (Thompson *et al.*, 1997). Phylogenetic analyses were performed using the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods with MEGA software version 6.0 (Tamura *et al.*, 2013).

The best-fit evaluative model for 16S rRNA gene sequences was selected by the AKAIKE information criterion implemented in the jMODELTEST 0.1.1 (Posada & Crandall 1998; 2001). The model selected as the best-fit for the 16S rRNA gene dataset was the TrN + I + G model. Phylogenetic trees were inferred with the neighbour-joining method using Kimura's two-parameter model (Kimura, 1980). The bootstrap analysis was performed with 1000 replicates. The rate variation among sites was considered to have a γ-distributed rate (α=1). The other default parameters (e.g. Poisson distance) were not changed. Maximum-parsimony analyses were performed with 1000 replicates of heuristic search with random addition of sequences and subsequent tree bisection-reconnection (TBR) branch swapping. Analyses were performed with gaps treated as missing data. Bootstrap analysis with 1000 replicates was performed in the same way but using 10 rounds of random sequence addition and subsequent TBR branch swapping during each bootstrap replicate. The maximum-likelihood tree was obtained using Kimura's two-parameter model as the nucleotide substitution model. The nearest-neighbour-interchange was used as the maximum-likelihood heuristic method. The Gamma-distributed with Invariant-sites rate was considered and number of discrete gamma categories was five. Bootstrap analysis based on 1000 replicates was also conducted in order to obtain confidence levels for the branches (Felsenstein, 1985).

The almost-complete 16S rRNA gene sequences of strains PDF4^T (1354 nt) and PDF10 (1354 nt) were obtained and used for the phylogenetic analyses. The phylogenetic tree based on the neighbour-joining algorithm showed that strains PDF4^T and PDF10 were members of the genus *Brevibacillus* (Fig. 1). The other two tree-making algorithms (maximum-likelihood and maximum-parsimony) resulted in trees showing similar topologies (Figs S1 and S2, available in the online Supplementary Material). Phylogenetic analyses showed that strains PDF4^T and PDF10 formed a monophyletic lineage (99.5 % 16S rRNA gene sequence similarity), and formed a well-separated branch with *Brevibacillus brevis* DSM 30^T (98.9 %), *Brevibacillus parabrevis* DSM 8376^T (98.6 %), *Brevibacillus formosus* DSM 9885^T (98.5 %), *Brevibacillus agri* DSM 6348^T (98.4 %), *Brevibacillus nitrificans* DSM 26674^T (98.2 %), *Brevibacillus limnophilus* DSM 6472^T(98.1 %), *Brevibacillus choshinensis* DSM 8552^T (98.1 %) and *Brevibacillus reuszeri* DSM 9887^T (97.9 %) in the neighbour-joining phylogenetic tree (Fig. 1). 16S rRNA gene sequence similarities between the two isolates and all other members of the genus *Brevibacillus* ranged from 94.5 to 97.6 %.

HV region corresponded to the 5′ end of 16S rRNA (nucleotide positions 70–344; *Bacillus subtilis* numbering). The HV region sequence is a useful marker for rapid and convenient identification and grouping of species of the genus *Brevibacillus*. HV region sequences of PDF4^T, PDF10 and the type strains of species of the genus *Brevibacillus* were compared using the same method as a dendrogram, based on a comparison of 263 common nucleotide positions (Fig. S3). Strain PDF4^T clustered with strain PDF10 and 16S rRNA HV sequence similarity within this cluster was 100 %. However, 16S rRNA HV sequence similarity of strain PDF4^T to the type strains of species of the genus *Brevibacillus* was in range of 96.6–85.9 %. Goto *et al.* (2004) determined that strains of each species of the genus *Brevibacillus* clustered with their individual type strains with 100 % sequence similarity. Thus, strain PDF4^T could be regarded as an independent species.

The reference strains used in this study for side-by-side analyses, *Brevibacillus formosus* DSM 9885^T, *Brevibacillus choshinensis* DSM 8552^T, *Brevibacillus reuszeri* DSM 9887^T, *Brevibacillus brevis* DSM 30^T, *Brevibacillus parabrevis* DSM

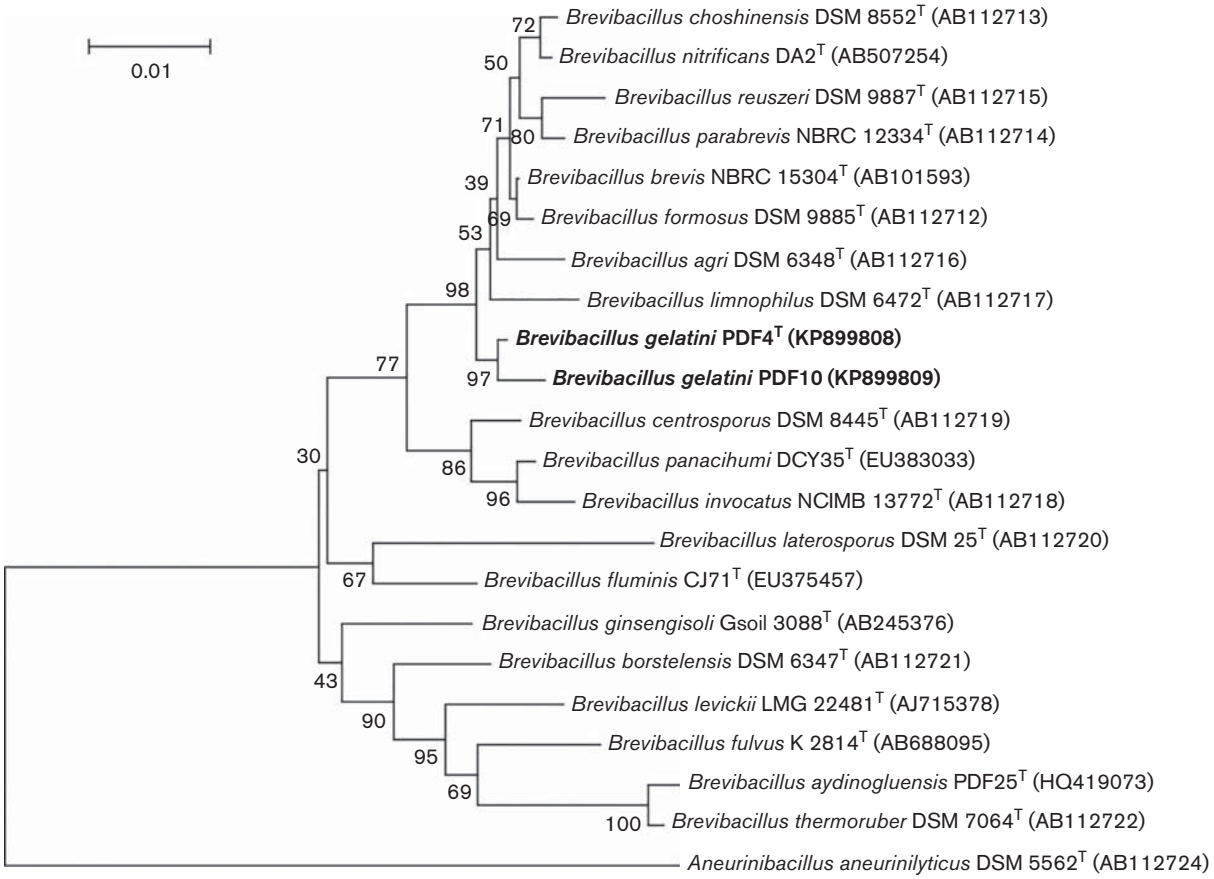

Fig. 1. Neighbour-joining phylogenetic tree, reconstructed from a comparative analysis of nearly complete 16S rRNA gene sequences, showing the position of strains PDF4^T and PDF10 among related taxa within the genus *Brevibacillus*. Bootstrap values based on 1000 replications are listed as percentages at branching points. *Aneurinibacillus aneurinilyticus* DSM 5562^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

8376^T, *Brevibacillus limnophilus* DSM 6472^T, *Brevibacillus nitrificans* DSM 26674^T, *Brevibacillus agri* DSM 6348^T were obtained from the Leibniz Institut Deutsche Sammlung von Micro-organismen und Zellkulturen (DSMZ; Braunschweig, Germany).

For determination of the G+C contents, genomic DNAs were extracted and purified using the method of Marmur (1961). Genomic DNA G+C contents were determined from the midpoint value (melting temperature, T_m) of the thermal denaturation profile (Mandel & Marmur, 1968) using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with *in situ* temperature probe (Varian). Thermal denaturation was performed with approximately 20 μg DNA ml^{-1} from each sample in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Thermal conditions consisted of a ramp from 55 °C to 90 °C at 0.5 °C min^{-1} on a Bio UV/VIS-spectrophotometer. The thermal melt curve of each sample DNA was generated in triplicate. The DNA G+C contents for strains PDF4^T and PDF10 were calculated using linear regression analyses of T_m against the G+C content of the standard DNA (i.e. *Escherichia coli* K-12 DNA). The DNA G+C contents of strains PDF4^T and PDF10 were 51.7 mol% and 51.4 mol%, respectively, which were within the range reported for recognized species of the genus *Brevibacillus* (42.8–57.4 mol%).

DNA–DNA hybridization was performed spectrophotometrically by DNA reassociation kinetics as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983). A Cary 100 Bio UV/VIS-spectrophotometer was used to measure renaturation. Hybridization was replicated five times for each sample; the highest and lowest values for each sample were excluded and the remaining three values were used for calculation of the mean DNA–DNA relatedness value. Strain PDF4^T exhibited a high level of DNA–DNA relatedness with strain PDF10 (92 %), supporting that they belong to the same species. Strain PDF4^T exhibited relatively low levels of DNA–DNA relatedness with the closely related type strains *Brevibacillus formosus* DSM 9885^T (32 %),

Brevibacillus choshinensis DSM 8552^T (30 %), *Brevibacillus reuszeri* DSM 9887^T (18 %), *Brevibacillus brevis* DSM 30^T (40 %), *Brevibacillus parabrevis* DSM 8376^T (46 %), *Brevibacillus limnophilus* DSM 6472^T (34 %), *Brevibacillus nitrificans* DSM 26674^T (36 %) and *Brevibacillus agri* DSM 6348^T (59 %). These values are clearly below the 70 % cut-off point recommended for the delineation of genomic species (Stackebrandt & Goebel, 1994; Wayne *et al.*, 1987). Thus, the results support the placement of strain PDF4^T as a separate and previously unrecognized species.

For cellular fatty acid analyses, 40 mg wet cells were scraped from Degryse 162 agar plates after incubation at 40 °C for 24 h in the late-exponential growth phase, and fatty acid methyl esters were obtained by saponification, methylation and extraction using minor modifications of described methods (Kuykendall *et al.*, 1988; Miller, 1982). Fatty acid methyl ester mixtures were separated using the Sherlock Microbial Identification System (MIDI) with an Agilent model 6890N gas chromatograph equipped with a 5 % phenylmethyl silicone capillary column (0.2 mm × 25 m), a flame-ionization detector, an Agilent model 7683A automatic sampler and a Hewlett Packard computer with the MIDI database (Aerobic bacteria library, TSBA6 version 6.10). Peaks were automatically integrated and percentages were calculated using the MIS Standard Software (Microbial ID).

The fatty acid composition of strains PDF4^T, PDF10 and related species of the genus *Brevibacillus* are given in Table S1. The fatty acid profile of strain PDF4^T was very similar to that of PDF10, and the predominant fatty acids of the two isolates were anteiso-$C_{15:0}$ and iso-$C_{15:0}$, which are also the predominant fatty acids in all members of the genus *Brevibacillus* (Baek *et al.*, 2006).

The polar lipids of strains PDF4^T and PDF10 were extracted from 100 mg freeze-dried cells and were separated by two-dimensional thin-layer chromatography on silica gel plates (no. 818 135; Macherey-Nagel) according to the method of Tindall *et al.* (2007). The first direction was developed with chloroform/methanol/water (65 : 25 : 4, by vol.) and the second direction with chloroform/methanol/acetic acid/water (80 : 12 : 15 : 4, by vol.). Detection was performed using 5 % ethanolic molybdophosphoric acid for total lipids, molybdenum blue for phospholipids, ninhydrin for aminolipids, and α-naphthol for glycolipids (Tindall *et al.*, 2007). Strains PDF4^T and PDF10 showed similar patterns for polar lipids. The polar lipids of strain PDF4^T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine, an unknown phospholipid, two unknown lipids, an unknown aminophospholipid and two unknown aminolipids (Table S2).

Isoprenoid quinones were extracted using methanol/hexane (Tindall, 1990a, b), followed by phase separation into hexane. This analysis was performed on strains PDF4^T and PDF10. The sole isoprenoid quinone found in strains PDF4^T and PDF10 was MK-7, the same as found in recognized members of the genus *Brevibacillus* (Shida *et al.*, 1996, Baek *et al.*, 2006). The composition of amino acids in the cell wall was determined as previously described (Komagata & Suzuki, 1987). The cell walls of strains PDF4^T and PDF10 contained *meso*-diaminopimelic acid.

To characterize strains PDF4^T and PDF10 phenotypically, standard phenotypic tests were performed. Vegetative cells of strains PDF4^T and PDF10 were Gram-stained using the method of Dussault (1955) and the Gram type was also determined using the KOH test (Powers, 1995). Cell morphology and motility were examined by using phase-contrast microscopy (Eclipse E600; Nikon) on an exponentially growing liquid culture. The morphology, size and pigmentation of colonies were observed under optimal growth conditions on Degryse 162 agar after incubation for 2 days at 40 °C. The temperature range and optimum for growth were tested at 10–60 °C (in 5 °C intervals) in Degryse 162 broth. The pH range for growth, at 40 °C, was determined in Degryse 162 broth that had been adjusted to pH 4.0–11.0 (in increments of 0.5 pH unit) by addition of HCI or Na_2CO_3; the pH was verified after autoclaving. Growth was assessed by measuring the OD_{600} after incubation for 3 days. Salt tolerance was tested in Degryse 162 liquid medium supplemented with 0–10 % NaCl (w/v, at intervals of 1 %) for 7 days at 40 °C. Anaerobic growth was tested using a GasPak pouch (Becton Dickinson). Catalase and oxidase activities were assessed using standard methods (Tindall *et al.*, 2007). Hydrolysis of casein was determined by observing clear zones surrounding colonies on skimmed milk agar (Atlas, 1993). Hydrolysis of starch, aesculin, Tween 80 and tyrosine was investigated according to previously described methods (Smibert & Krieg, 1994). Other phenotypic characterizations were conducted using the API 20E and API 50CH kits (bioMérieux) at 40 °C for 3 days. Utilization of carbon sources was tested using Vitek2 Bacilli Identification Card (BCL) microtest systems (bioMérieux), according to the manufacturer's instructions. Sporulation was tested on solid maintenance medium and sporangial morphology was assessed by the Schaeffer and Fulton staining method (Smibert & Krieg, 1994).

The physiological and biochemical characteristics of strains PDF4^T and PDF10 are given in the species description and Table 1. Strains PDF4^T and PDF10 grew in the presence of 0–2.0 % (w/v) NaCl, at pH 6.0–9.0 (optimum pH 7.0) and at 20–50 °C (optimum 40 °C). Endospores of strains PDF4^T and PDF10 were ellipsoidal and occurred subterminally or centrally in swollen sporangia (Fig. S4). Strain PDF4^T could only be distinguished from strain PDF10 based on the activity of leucine arylamidase. Strains PDF4^T and PDF10 exhibited a number of phenotypic similarities with respect to species of the genus *Brevibacillus*, including being facultatively anaerobic, endospore-forming, motile and having rod-shaped cells. However, Strains PDF4^T and PDF10 were distinguishable from related species of the genus *Brevibacillus* by differences in

Table 1. Differential phenotypic characteristic of strains PDF4^T and PDF10 and their closest phylogenetic relatives

Strains: 1, PDF4^T; 2, PDF10; 3, *Brevibacillus formosus* DSM 9885^T; 4, *Brevibacillus choshinensis* DSM 8552^T; 5, *Brevibacillus reuszeri* DSM 9887^T; 6, *Brevibacillus brevis* DSM 30^T; 7, *Brevibacillus parabrevis* DSM 8376^T; 8, *Brevibacillus limnophilus* DSM 6472^T; 9, *Brevibacillus nitrificans* DSM 26674^T; 10, *Brevibacillus agri* DSM 6348^T. All data were obtained in this study. +, Positive; −, negative; W, weakly positive.

Characteristic	1	2	3	4	5	6	7	8	9	10
Nitrate reduction	−	−	+	−	−	+	+	−	+	−
Hydrolysis of:										
Gelatin	+	+	+	W	+	W	+	−	+	+
Aesculin	−	−	+	−	W	+	+	−	+	W
Utilization of:										
D-Glucose	−	−	+	W	−	−	+	−	+	+
Trehalose	−	−	+	−	−	+	+	+		+
N-Acetylglucosamine	−	−	+	+	+	+	+	−	+	+
D-Mannitol	−	−	+	−	+	+	+	−	+	+
Acid production from:										
D-Glucose	−	−	+	−	−	−	+	−	−	+
D-Ribose	−	−	−	+	−	−	−	−	+	−
D-Galactose	−	−	−	−	−	+	−	−	−	−
D-Fructose	−	−	+	−	−	−	−	−	−	−
D-Mannitol	−	−	+	−	−	+	+	−	+	+
Glycerol	−	−	+	−	−	−	+	−	−	+
Aesculin ferric citrate	−	−	−	−	−	−	−	−	−	W
Cellobiose	−	−	−	−	−	+	+	−	−	−
Maltose	−	−	−	−	−	−	+	−	−	−
Sucrose	−	−	−	−	−	−	−	+	−	−

several phenotypic characteristics, which were determined under the same conditions and methods (Table 1). The distinctive characteristics of strains PDF4^T and PDF10 from the type strains tested were as follows: hydrolysis of aesculin, acid production from D-mannitol, D-glucose and glycerol, and utilization of D-glucose, D-mannitol, trehalose and *N*-acetylglucosamine.

On the basis of morphological, physiological and chemotaxonomic characteristics, together with data from 16S rRNA gene sequence comparisons, and DNA–DNA hybridization experiments, strains PDF4^T and PDF10 represent a novel species of the genus *Brevibacillus*, for which the name *Brevibacillus gelatini* sp. nov. is proposed.

Description of *Brevibacillus gelatini* sp. nov.

Brevibacillus gelatini (ge.la.ti′ni. N.L. neut. gen. n. *gelatini* from gelatin).

Cells are Gram-stain-variable, motile, facultatively anaerobic rods, 0.8–1.0 μm in diameter and 2.0–3.5 μm long on Degryse 162 agar. After incubation for 3 days at 40 °C, colonies on Degryse 162 agar are circular, convex, smooth, creamish-white and 0.5–1.0 mm in diameter. Endospores are ellipsoidal, occurring subterminally or centrally in swollen sporangia. Growth occurs in the presence of 0–2.0 % (w/v) NaCl, at pH 6.0–9.0 (optimum pH 7.0) and at 20–50 °C (optimum 40 °C). Positive for oxidase, catalase and hydrolysis of gelatin, but negative for hydrolysis of starch, casein, tyrosine and Tween 80. Arginine dihydrolase, urease, ornithine, and lysine decarboxylase activities are absent. Aesculin hydrolysis, nitrate reduction, indole production, glucose fermentation, citrate utilization and H_2S production are negative. In the Vitek2 BCL gallery, L-proline arylamidase, L-pyrrolydonyl arylamidase, leucine arylamidase and Ala-Phe-Pro arylamidase activities are present, but L-aspartate arylamidase, α-mannosidase, phenylalanine arylamidase, β-galactosidase, α-galactosidase, tyrosine arylamidase, β-xylosidase, L-lysine arylamidase, alanine arylamidase, β-*N*-acetylglucosaminidase, phosphorylcholine hydrolase, α-glucosidase, glycine arylamidase, β-glucosidase and β-mannosidase activities are absent. D-Galactose, glycogen, maltotriose, cyclodextrin, D-mannose, palatinose, D-glucose, D-ribose, *myo*-inositol, methyl D-xyloside, D-mannitol, melezitose, pyruvate, ellman, L-rhamnose, D-tagatose, trehalose, inulin and *N*-acetyl-D-glucosamine are not utilized as sole sources of carbon and energy. In the API 50CH gallery, acid is not produced from aesculin ferric citrate, glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-mannose, *N*-acetylglucosamine, amygdalin, cellobiose, salicin, maltose, lactose, melibiose, starch, glycogen, gentiobiose, D-fructose, ferric citrate, D-mannitol, D-sorbitol, sucrose, trehalose, inulin, raffinose, potassium gluconate, potassium 5-ketogluconate, D-arabinose, L-xylose, methyl D-xylopyranoside, L-sorbose, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, arbutin, melezitose, turanose, D-lyxose, D-tagatose, D- and L-fucose, erythritol, D-adonitol, L-rhamnose, dulcitol, inositol,

xylitol, D-and L-arabitol or potassium 2-ketogluconate. The major fatty acids are anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The major respiratory quinone is MK-7. The polar lipids consist of diphosphatidylglycerol, phosphatidylglycerol, phosphatidyletha-nolamine, phosphatidylmonomethyletha-nolamine, an unknown phospholipid, two unknown lipids, an unknown aminophospholipid and two unknown aminolipids.

The type strain is PDF4^T (=NCCB 100559^T=DSM 100115^T), isolated from Camkoy hot spring in the provinces of Aydın, Turkey. The DNA G+C content of type strain is 51.7 mol%. An additional strain of the species is PDF10.

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