Anoxybacillus bogrovensis sp. nov., a novel thermophilic bacterium isolated from a hot spring in Dolni Bogrov, Bulgaria

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A novel moderately thermophilic bacterium, designated strain BT 13^T, was isolated from a geothermal water source in Dolni Bogrov, near Sofia, Bulgaria. The isolate was spore-forming, Gram-positive, facultatively anaerobic, alkalitolerant and heterotrophic, and was able to ferment a wide variety of carbon sources including D-glucose, sucrose, L-arabinose, L-rhamnose, starch, sorbitol and glycogen. Strain BT 13^T grew optimally at pH 8.0 and 65 °C. Intracellular amylolytic activity was registered with glucose as the main product of starch hydrolysis. Phylogenetic analysis based on the 16S rRNA gene revealed that the strain belonged to the genus *Anoxybacillus*, the closest relatives being *Anoxybacillus flavithermus* and *Anoxybacillus kamchatkensis*. The DNA G+C content was 44.1 mol%. The fatty acid profile with a content of iso-branched fatty acids of around 80 % of the total fatty acids is similar to that of recognized *Anoxybacillus* species. On the basis of genotypic differentiation and significant differences in phenotypic characteristics, it was concluded that strain BT 13^T represents a novel species of the genus *Anoxybacillus*, for which the name *Anoxybacillus bogrovensis* sp. nov. is proposed. The type strain is BT 13^T (=DSM 17956^T=NBIMCC 8427^T).

The genus *Anoxybacillus*, with the type species *A. pushchinoensis*, was proposed and *Bacillus flavothermus* (Heinen *et al.*, 1982) was reclassified as *Anoxybacillus flavithermus* by Pikuta *et al.* (2000). At present, the genus comprises 10 species with validly published names. Although representatives of the genus *Anoxybacillus* were identified initially as anaerobes, an additional amendment referred to them as aerotolerant anaerobes (Pikuta *et al.*, 2003). Most of the species were found to be facultative anaerobes (*Anoxybacillus voinovskiensis*, *A. flavithermus*, *A. gonensis*, *A. amylolyticus*, *A. contaminans*, *A. kamchatkensis*) but, for some species, anaerobic growth was registered only under certain conditions (Dulger *et al.*, 2004; Poli *et al.*, 2006). *Anoxybacillus rupiensis* was described as a strict aerobe (Derekova *et al.*, 2007). In the present study, we report on the phylogenetic, physiological and biochemical characteristics of a novel thermophilic, facultative anaerobic micro-organism with amylolytic activity, strain BT 13^T, which was isolated from a geothermal spring in the village of Dolni Bogrov, Sofia region, Bulgaria. The properties determined indicated that strain BT 13^T represents a novel species of the genus *Anoxybacillus*.

Strain BT 13^T was isolated from a mud sample with a pH of 8.5 and a temperature of 56 °C, taken from a geothermal spring near the village Dolni Bogrov, Sofia region, Bulgaria. Samples were enriched in PY medium (pH 7.0), containing (l⁻¹): 2 g peptone and 1 g yeast extract, and plated on PY agar (pH 7.0). The cultures were incubated for 18 h at 60 °C and at 240 r.p.m. Pure strains were isolated after at least three repeated cultivations on agar. The cultures were considered to be pure when only one type of bacterium per culture was observed microscopically.

Morphological properties of strain BT 13^T were determined using light and electron microscopy of ultrathin sections of cells after cultivation for 24–48 h on agar medium (Boone *et al.*, 1995). The cells were typically endospore-producing, Gram-positive (Gram colour staining set; Merck), regular, non-motile rods, 1.4–4.0 µm long and 0.3–0.6 µm wide (Fig. 1). A distal formation of ellipsoidal endospores was observed using electron microscopy. The novel isolate differed from its closest relatives (Table 1) with respect to its lack of motility.

Physiological and biochemical characteristics of the isolate were investigated as described by Smibert & Krieg (1981). The fermentative properties of the novel isolate were examined using minimal salt medium, consisting of (l⁻¹):

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BT 13^T is AM409184.

Fig. 1. Transmission electron micrograph of cells of strain BT 13^T (24 h cultivation) showing typical endospore formation and free spores. Bar, 0.5 µm.

1 g (NH₄)₂HPO₄, 0.2 g KCl, 0.1 g MgSO₄, 1 µg thiamin and 0.0001 g bromothymol blue, with the addition of 0.01 % (w/v) yeast extract. Carbohydrates were added to a final

concentration of 0.1 % (w/v). Sugars tested were D-glucose, sucrose, D-lactose, D-ribose, D-fructose, L-arabinose, D-laevulose, D-galactose, raffinose, L-rhamnose, D-mannose, D-xylose and cellobiose. Polyols tested were inositol, ribitol, mannitol, sorbitol and galactitol. The following polysaccharides were used: dextran, glycogen, inulin, salicin and starch. Catalase activity was assayed by mixing a pellet of a freshly centrifuged culture with a drop of 6 % hydrogen peroxide. Anaerobic growth was tested on PY agar at 60 °C in a jar, with Anaerocult A (Merck) as a reducing agent. Growth was also determined in tubes with PY agar slants under optimal growth conditions, and stab cultures with a paraffin film on top. Growth experiments were carried out in triplicate. Anaerobic growth was observed only when Anaerocult A was used. Other tests were performed using an API 20E kit (bioMérieux) at 55 °C, according to the manufacturer's instructions. The major physiological and biochemical properties of the novel isolate differed from those of its closest relatives (Table 1) in the spectrum of carbohydrate utilization. Strain BT 13^T differed from its nearest neighbour, *A. flavithermus* (16S rRNA gene sequence similarity of 96.8 %) in its smaller dimensions, more-restricted temperature range for growth, alkaline pH values,

Table 1. Differential characteristics of strain BT 13^T and its closest relatives, *A. flavithermus*, *A. gonensis*, *A. ayderensis* and *A. kamchatkensis*

Strains: 1, BT 13^T (*A. bogrovensis* sp. nov.); 2, *A. flavithermus* DSM 2641^T (data from Pikuta *et al.*, 2000); 3, *A. gonensis* NCIMB 13933^T (Belduz *et al.*, 2003); 4, *A. ayderensis* NCIMB 13972^T (Dulger *et al.*, 2004); 5, *A. kamchatkensis* JW/VK-KG4^T (Kevbrin *et al.*, 2005). All strains are facultative anaerobes. +, Positive; –, negative; w, weak response; ND, not determined.

Characteristic	1	2	3	4	5
Source of isolation	Geothermal water (Bulgaria)	Geothermal water (India)	Geothermal water (Turkey)	Geothermal water (Turkey)	Geothermal waters (Geyser valley, Russian Kamchatka)
Cell size [length/width] (µm)	1.37–4.03 × 0.34–0.57	0.85 × 2.30–7.10	0.75 × 5.00	0.55 × 4.60	2.5–8.8 × 1.0
Motility	–	+	+	+	+
Catalase formation	+	+	w	+	–
Temperature range for growth (optimum) (°C)	40–69 (65)	30–72 (60–65)	40–70 (55–60)	30–70 (50)	38–67 (57–62)
pH range for growth (optimum)	6.5–10.0 (8.0)	5.5–9.0 (7.0)	6.0–10.0 (7.5–8.0)	6.0–11.0 (7.5–8.5)	5.7–9.9 (6.8–8.5)
NaCl range (%)	0.5–1.5	0.0–2.5	0.0–4.0	0.0–2.5	ND
DNA G + C content (mol%)	44.1	42.3	57.0	54.0	42.3
Nitrate reduction	–	+	–	+	ND
Substrates utilized					
L-Arabinose	+	w	–	+	–
Xylose	–	–	+	–	–
D-Glucose	+	–	+	+	+
D-Mannose	–	+	–	+	–
Sucrose	+	ND	–	+	+
Glycogen	+	–	+	ND	ND
Hydrolysis of:					
Gelatin	+	–	+	+	–
Casein	–	+	ND	ND	–
Starch	+	–	+	+	–

lower NaCl tolerance, fermentation of glucose, lack of nitrate reduction, and hydrolysis of glycogen and gelatin. The final products of amylolytic action on starch of strain BT 13^T were assayed using both TLC and HPLC. The products of hydrolysis of various α -glucans were developed using TLC Silicagel 60 precoated plates (25 × 25 cm; Merck). An amylolytic activity assay (Pantshev *et al.*, 1981; Miller, 1959) revealed that the enzyme synthesis was constitutive and was located in the cytosol. Final products of the enzyme reaction were predominantly glucose, with traces of maltose (data not shown), suggesting an exo-mechanism of enzyme action. Amylolytic activity has been observed for *A. amylolyticus* (Poli *et al.*, 2006) and the enzyme synthesized was α -amylase, whereas the enzyme from strain BT 13^T had an exo-mechanism of action.

Partial (1–501 nt; *Escherichia coli* numbering) and full sequence (8–1513 nt; *E. coli* numbering) analysis of the 16S rRNA gene of strain BT 13^T was performed at Novozymes, Denmark, as described by Johansen *et al.* (1999). The phylogenetic tree was constructed using the Fast DNA ML tool in the ARB program package (Strunck & Ludwig, 1995). The results of the 16S rRNA gene sequence analysis placed the novel isolate in the genus *Anoxybacillus* (Fig. 2). The closest phylogenetic neighbours were *A. flavithermus*, *A. kamchatkensis* and *A. ayderensis* (sequence similarities of 96.8, 96.8 and 96.5 %, respectively). Other recognized species of the genus *Anoxybacillus* had sequence similarities of less than 96 %. The significant phylogenetic distance from all these micro-organisms clearly indicated that strain BT 13^T represents a separate taxon, with an evolutionary distance greater than the level (3 % distance) at which strains are attributed to separate species (Stackebrandt & Goebel, 1994).

Extraction and purification of the genomic DNA and determination of the G + C content for the novel isolate were performed at DSMZ (Braunschweig, Germany), using the HPLC method (Mesbah *et al.*, 1989). The DNA G + C

content of strain BT 13^T was 44.1 mol%, in the range of values for recognized *Anoxybacillus* species (Table 1).

Cultivation, harvesting, preparation and analysis of cellular fatty acid methyl esters from whole-cell fatty acids were performed at DSMZ, according to the Sherlock Microbial Identification System manual (version 4.0; MIDI). The fatty acid pattern of strain BT 13^T was identified by comparison with patterns stored in the MIDI database (MIS; USA) using the MIDI V. 4.0 library. The results from the fatty acid analysis revealed that strain BT 13^T synthesized mainly iso- and anteiso-branched saturated fatty acids, similar to thermophilic *Bacillus* and *Thermus* species. Good correspondence with the general features of fatty acid profiles of recognized *Anoxybacillus* species was demonstrated, with the main fatty acids being iso-C_{15:0} and iso-C_{17:0}. The iso-branched fatty acids comprised about 80 % of the total fatty acids. The major fatty acid of strain BT 13^T and the closest relatives *A. ayderensis*, *A. flavithermus* and *A. gonensis* was iso-C_{15:0}, followed by iso-C_{17:0} and anteiso-C_{17:0}. However, lower contents of shorter fatty acids such as C_{14:0} (0.4 %), iso-C_{14:0} (0.25 %), C_{15:0} (0.4 %), iso-C_{15:0} (40.1 %) and anteiso-C_{15:0} (1.8 %) were observed. Instead, the contents of fatty acids with longer chains such as iso-C_{17:0} (36.1 %), anteiso-C_{17:0} (8.98 %), C_{18:0} (0.5 %) and iso-C_{19:0} (0.5 %) were higher for strain BT 13^T. In addition, the contents of iso-C_{16:0} and C_{16:0} were determined to be 3.9 and 6.3 %, respectively.

On the basis of its 16S rRNA gene sequence, strain BT 13^T was placed within the genus *Anoxybacillus*. Its physiological characteristics, 16S rRNA gene sequence, and the DNA G + C content were similar to those of other recognized *Anoxybacillus* species. The specific phenotypic properties and sequence similarities of less than 97 % with the closest relatives indicated that strain BT 13^T represents a novel species. Therefore we propose the name *Anoxybacillus bogrovensis* sp. nov. for the novel isolate.

Fig. 2. Phylogenetic position of strain BT 13^T within the genus *Anoxybacillus*, based on 16S rRNA gene sequence comparison. *Geobacillus stearothermophilus* was included as the type species for *Geobacillus* and *Bacillus subtilis* as the out-group. Bar, 0.02 nucleotide substitutions per position.

Description of *Anoxybacillus bogrovensis* sp. nov.

Anoxybacillus bogrovensis (bo.gro.ven'sis. N.L. masc. adj. *bogrovensis*, pertaining to Bogrov region, referring to the place of isolation of the type strain).

Cells are Gram-positive, rod-shaped, single or in long chains, facultatively anaerobic, non-motile and $1.4\text{--}4.0 \times 0.3\text{--}0.6\text{ }\mu\text{m}$. Endospores are ellipsoidal, and located terminally in non-swollen sporangia. After cultivation for 24 h on PY agar, colonies are pale white–yellowish in colour, with an irregular edge, high conical surface and viscous consistency. Moderately thermophilic (temperature range for growth is $40\text{--}69\text{ }^{\circ}\text{C}$, with optimum growth at $65\text{ }^{\circ}\text{C}$) and alkalitolerant (pH range for growth is $6.0\text{--}10.0$, with an optimum at pH 8.0). Doubling time is 28 min. Catalase-positive and grows at NaCl concentrations up to 1.5 %, with optimum growth at 0.5 %. Chemo-organotrophic, and is able to ferment a wide spectrum of carbon sources: D-glucose, fructose, sucrose, maltose, cellobiose, L-rhamnose, L-arabinose, melibiose, amygdalin, mannitol, salicin, glycogen and starch. The Voges–Proskauer reaction is negative. Starch and gelatin are hydrolysed, but casein is not. Acetoin, H_2S and indole are not formed, phenylalanine and urea are not decomposed, and nitrates are not reduced. Arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase tests are negative; β -galactosidase is positive. Cells mainly contain iso-branched saturated fatty acids (content of about 80 % of the total fatty acids). The major fatty acid is iso- $\text{C}_{15:0}$, followed by iso- $\text{C}_{17:0}$ and anteiso- $\text{C}_{17:0}$. The G + C content of the DNA of the type strain is 44.1 mol%.

The type strain, BT 13^T (=DSM 17956^T=NBIMCC 8427^T), was isolated from a hot spring near Dolni Bogrov village, Sofia region, Bulgaria.

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