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ORIGINAL PAPER

Anoxybacillus rupiensis sp. Nov., a novel thermophilic bacterium isolated from Rupi basin (Bulgaria)

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Abstract Three strains of a novel thermophilic, strictly aerobic, Gram-positive, spore-forming hemo-organotrophic bacterium were isolated from three hot springs in the region of Rupi basin, Bulgaria as producers of amylolytic enzymes. Their 16S rRNA gene sequences (first 500 nucleotides) were very similar (99.8%). Strains were able to ferment a wide spectrum of carbohydrates such as sugars, polyols, and polysaccharides like xylan, glycogen and starch. Optimal growth was observed at 55–58°C, and pH at 6.0–6.5. Phylogenetic analysis of the whole 16S rRNA gene sequence clustered the strain R270^T with the representatives of the genus *Anoxybacillus* and with *Geobacillus tepidamans*. The G + C content of the genomic DNA was 41.7%. DNA–DNA hybridization analysis revealed low homology with the closest relatives (32.0 mol% homology to *Geobacillus tepidamans*). Fatty acid profile (major fatty acids iso-C15:0 and iso-C17:0) confirmed the affiliation of the strain to the genus *Anoxybacillus*. On the basis of the data presented here, we propose that strain R270^T, represents a new species of the genus *Anoxybacillus* for which, we recommend the name *Anoxybacillus rupiensis* sp. nov. (=DSM 17127^T = NBIMCC 8387^T). The 16S rRNA gene sequence data of a strain R270^T have been deposited in the EMBL databases under the accession number AJ879076.

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Introduction

The first representative of the genus *Anoxybacillus, A. pushchinoensis* was described by Pikuta et al. (2000) as strictly anaerobic and an emended description of the species was published later on (Pikuta et al. 2003) according to which this species should be considered as aerotolerant anaerobe and the genus *Anoxybacillus* should be emended to aerotolerant anaerobes and facultative anaerobes. In the next few years, new representatives of the genus *Anoxybacillus* have been described and it comprises eight species at the time of writing of this paper: *A. pushchinoensis* (Pikuta et al. 2000), *A. flavithermus* (Pikuta et al. 2000), *A. gonensis* (Belduz et al. 2003), *A. contaminans* (De Clerck et al. 2004), *A. ayderensis* (Dulger et al. 2004), *A. kestanbolensis* (Dulger et al. 2004), *A. voinovskiensis* (Yumoto et al. 2004), *A. kamchatkensis* (Kevbrin et al. 2005), and *A. amylolyticus* (Poli et al. 2006). Although the name of the genus *Anoxybacillus* means ''without oxygen Bacillus'', according to the authors (Pikuta et al. 2000), most of the species described grow well aerobically and even for some species anaerobic growth was registered only under certain conditions (Yumoto et al. 2004).

Bulgaria is a country rich in geothermal water sources, largely varying in their temperatures (ranging from 45 to 100°C) and pH. Although thermophilic producers of biotechnologically valuable enzymes were isolated from Bulgarian hot springs (Kambourova and Emanuilova 1992; Dimitrov et al. 1997; Emanuilova et al. 2000; Uzunova

et al. 2001), still there is a lack of thorough phylogenetic information on their representatives.

In this study, we describe phylogenetic, physiological and biochemical properties of a new species and propose to classify the isolate as a novel species in the genus *Anoxybacillus*, for which we propose the name *Anoxybacillus rupiensis* sp. nov.

Methods

Sample collection and enrichment

Water, soil and algobacterial mat samples were collected from different springs in the area of Rupi basin. The temperature measured at the sampling sites varied between 57 and 85°C and pH varied between 6.5 and 9.0. One-liter water from each place was filtered by sterile filter (Millipore, 0.22 μm), the filter was kept for 2 h on Petri dish and growth on Petri dish determined after overnight cultivation. The cells grown were collected with sterile medium and used for inoculation of 20 ml medium in 100 ml Erlenmeyer flasks. Soil and algobacterial mats (about 1 g each) were used also for inoculation of the liquid medium. Before inoculation, all samples were treated at 80°C for 10 min in order to isolate only the spore-forming microorganisms. The cultures were cultivated at liquid medium and after that tested on Petri dishes for degradation of Azure cross-linked (AZCL) amylose. Cultures showing some carbohydrate-degrading activity were further purified by streaking samples on agar for at least three times. Single colonies were then subcultured until a pure culture was obtained. The subcultures were considered pure after microscopic observation of one type bacterium per culture.

Culture medium and growth conditions

The samples were enriched in PY medium, containing (g l^{-1}): peptone (Difco), 2 and yeast extract (Difco), 1. pH of the medium was adjusted before autoclaving to 7.0 using 1 M NaOH. Cultures were incubated overnight at 60°C with shaking at 240 rpm. The same medium supplied with 2% agar was used for obtaining of pure cultures. The ability of mixed cultures and after that of purified strains to degrade carbohydrates was shown in PY agar medium containing 0.05% AZCL amylose.

Phenotypic characterization

Growth was determined either by measuring the increase in OD_{600}, or by direct cell counts using Bürker counting chamber. Gram staining was carried out using standard procedures. Spore formation was observed by light and phase contrast microscopy of Gram-stained cultures. Samples of the cultures were streaked on agar medium and cultivated overnight.

The effect of pH on growth was determined in the area 5.0–9.0 with 0.5 pH steps using acetate (for pH 5–5.5), phosphate (for pH 5.5–8.0) or glycine–NaOH (for pH 8.0–9.0) buffers at concentration 0.05 M. pH values were adjusted at room temperature. The influence of temperature on growth was determined at pH 7.0 at different temperature with 5°C steps under shaking. Methods described by Smibert and Krieg (1981) were used for physiological characterization of the strains. Anaerobic growth was tested in PY agar medium under paraffin. Catalase activity was assayed by mixing a pellet of freshly centrifuged 6 h culture with a drop of 6% hydrogen peroxide. Their ability to utilize different carbohydrates was examined in a minimal salt medium (MSM) consisting of (g l^{-1}): $(NH_4)_2HPO_4$, 1.0; KCl, 0.2; $MgSO_4$, 0.1; thiamine, 1.10^{-6}; bromthymol blue, 1.10^{-4}. Carbohydrates were added at final concentration of 0.1% (w/v). Sugars tested were: L-arabinose, ribose, xylose, fructose, glucose, galactose, manose, L-rhamnose, sucrose, lactose, maltose and raffinose. Polyols examined were: adonitol, dulcitol, inositol, mannitol and sorbitol. The following polysaccharides were used: glycogen, inulin, pectin, salicin and xylan. Sugars were sterilized separately and thiamin was sterilized by filtration. Selected compounds were tested as nitrogen sources: peptone, yeast extract, caseine, gelatine, urea and $(NH_4)_2HPO_4$. They were added in the concentration of 0.1% in ammonium free mineral medium (MSM without $(NH_4)_2HPO_4$ and bromthymol blue) with 2 g l^{-1} glucose as a carbon source.

Electron microscopy

The electron microscopic examination included samples of the strain taken at 12 and 24 h of cultivation. The cells were treated as described by Götz et al. (2002) using standard glutaraldehyde/osmium peroxide and LR White embedding regimens for thin sectioning (Beveridge et al. 1994).

Antibiotic sensitivity

Sensitivity to ampicillin, oxacillin, penicillin G, tetracycline, gentamicin, streptomycin sulfate, erythromycin, carbenicillin, chloramphenicol and nalidixic acid (Sigma) each at concentration of 100 μg ml^{-1} was tested. One milliliter aliquot of exponentially growing cultures was transferred to 100 ml fresh PY medium containing filter-sterilized antibiotic. The cultures were incubated at 55°C and pH 6.5.

Phylogenetic analysis of the rRNA genes

16S rRNA gene sequencing and data analysis were performed according to Johansen et al. (1999). For phylogenetic analysis were sequences for type strains in the genera *Geobacillus* and *Anoxybacillus* and the closest relatives found by BLAST downloaded from public databases and imported into the ARB program package (Strunck and Ludwig 1995). The sequences were aligned using the ARB aligner followed by manual alignment. Phylogenetic trees were calculated using the Neighbour joining program and the Fast ML program in the ARB program package.

G + C content analysis

G + C content was determined at the Identification Service of Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany. DNA was purified on hydroxyapatite according to the procedure of Cashion et al. (1977). G + C content was determined according to Mesbah et al. (1989) by using HPLC. Non-methylated Lambda-DNA (Sigma), with GC-content 49.858 mol% was used as a reference DNA. GC content was calculated from the ratio of deoxyguanosine (dG) and thymidine (dT).

DNA–DNA hybridization studies

DNA–DNA homology experiments were performed at DSMZ, Braunschweig, Germany. DNA–DNA hybridization was carried out as described by De Ley et al. (1970) with the modification described by Huss et al. (1983).

Cellular fatty acids

Cultivation, harvesting, preparation and analysis of cellular fatty acid methyl esters from whole-cell fatty acids were performed at the Identification Service of DSMZ, Braunschweig, Germany according to the method described in the Sherlock Microbial Identification System manual (version 4.0; MIDI). The fatty acid pattern of a strain R270^T was identified by comparing with patterns stored in the database of the Microbial Identification System (MIS, MIDI Del. USA).

Nucleotide sequence accession number

The nucleotide sequence of the 16S rRNA gene of strain R270^T has been deposited in the Gene Bank database under accession no AJ879076.

Results and discussion

Enrichment and isolation

Several samples collected from Bulgarian hot springs gave a positive reaction after enrichment followed by cultivation on AZCL amylose Petri dishes. They were used for further isolation of pure spore-forming amylolytic strains by serial dilutions and picking single colonies. Three of them belonged to the same species based on a partial 16S rRNA gene sequence analysis. These strains were isolated from different springs in the area of Rupi basin. Some of their properties were characterized and the isolate R270^T was studied in detail.

Phenotypic and physiological characteristics

Colonies of strain R270^T in PY agar medium were small (diameter 0.5 mm), whitish, glistening, with umbonate surface and undulate edge. R268 and R273 colonies were similar in size with a lens shape and convex surface. Cells of the three strains were relatively large, morphologically similar, straight or slightly curved spore-forming rods, Gram-positively stained and highly motile. Cells of strain R270^T appeared approximately 3.3–7.0 μm long and 0.7–1.5 μm wide. Their electron microscopic examination revealed typical Gram-positive structure of the cell wall. In the stationary growth phase, a polymorphism in cell shape was observed—typical protoplasts were observed in parallel with rods. Electron micrographs of spores showed the presence of exosporium, spore coat, cortex and core.

The three novel isolates (R268, R270^T and R273) were aerobic microorganisms and anaerobic growth was not detected under conditions described above. The temperature range for growth of the strains R268, R270^T and R273 was 35–37 to 67°C. The maximum growth rate (μ_{max}) for R270^T was observed at 55°C. The highest cell yield at the end of the exponentially phase was obtained at the same temperature. The pH range for growth (adjusted at 20°C) of the same strain was 5.5–8.5. Maximal growth rate was registered at pH 6.0–6.5 (1.87–1.84 h^{-1}) and the highest cell yield was reached at pH 6.5. pH 6.0–6.5 was considered as optimum area for growth. The shortest doubling time registered in PY medium at optimal conditions was 28 min and the highest cell concentration observed was 2.8×10^8 cells ml^{-1}.

Strains R268, R270^T and R273 were heterotrophic. Growth was observed both on carbohydrates (including sugars, polysaccharides and polyols) and on proteinaceous substrates used as the sole carbon sources in MSM. Acid but no gas was produced from glucose, fructose, xylose, ribose, maltose, manitol and starch. Growth on galactose, L-rhamnose, sucrose, lactose, raffinose, ribitol, galactitol,

sorbitol, inulin and citrate was not observed. Some physiological differences were registered between the three strains. Unlike other two strains, R270^T grew on arabinose, mannose and glycogen. Inositol supported growth only for R268. Casein hydrolysis was also observed only for the last strain. Sugars were utilized with formation of acid but no gas. Complex substrates like yeast extract and peptone supported a good growth. Gelatine does not support growth of the organisms. Strains R270^T and R273 were tolerant to 1% presence of NaCl in the medium and R268 tolerates the presence of 3% NaCl. The tree isolates grew in PY medium with 0.02% NaN_3. Indol was not produced, nitrate was not reduced, Voges–Proskauer reaction and Methyl-red test were negative. Like most thermophilic bacilli (Slepecky and Hemphill 1992), catalase reaction was positive for the tested strains.

The growth of strains R268, R270^T and R273 was inhibited by tetracycline, gentamycin, streptomycin, erythromycin, carbenicillin and chloramphenicol. They were resistant to ampicillin, oxacillin, penicillin and nalidixic acid. They differ in their insensitivity to penicillin and ampicillin from *A. amylolyticus* (Poli et al. 2006) and to ampicillin from *A. ayderensis* and *A. kestanbolensis* (Dulger et al. 2004).

Differentiating characteristics of the new type isolate compared with those of the closest neighbors are presented in Table 1. Between physiological properties, a lack of growth on galactose and lactose differed R270^T from the close relatives *A. voinovskiensis* (Yumoto et al. 2004) and *A. contaminans* (De Clerk et al. 2004). Unlike other strains, R270^T was nor able to reduce nitrate to nitrite. This strain also differed in its relation to oxygen-anaerobic growth was not observed in the conditions used.

Phylogenetic affiliation

The first 500 nucleotide bases were obtained from all three isolates and found to be 99.8% identical. The full sequences (1,517 nucleotides) were determined for strain R268 (ARB_6C2D05D2) and R270^T (AJ879076). The 16S rRNA gene-similarity among them was 99.9%. As the three strains have been found identical, further detailed study has been done on the strain R270^T deposited as a type strain. The phylogenetic tree in Fig. 1 shows that strain R270^T is positioned between *Geobacillus* species from one side and *Anoxybacillus* species from the other side. The closest sequence relatives found by BLAST search was ''*Anoxybacillus beppuensis*''AAB243446 (100% identical with the sequence of R270T, but submitted later than the sequence of this strain) and *Geobacillus tepidamans*^{T}, AY563003 (96.8% similarity), i.e., more than the level (3% distance) over which strains are generally attributed to separate taxa (Stackebrandt and Goebel 1994). Except for *G. tepidamans*, the similarity between R270^T and other species in the genus *Geobacillus* was less than 91%. According to Nazina et al. (2001) the observed levels of 16S rRNA gene sequence similarity in this genus are higher than 96.5%, and *Geobacillus tepidamans*^{T} is phylogenetically close to *Anoxybacillus* species (Schäffer et al. 2004). On the basis of phylogenetic similarity with *Anoxybacillus* species, a strain R270^T was related to genus *Anoxybacillus*.

G + C content

The G + C content of the genomic DNA for the strain R270^T was 41.7 mol%. The value obtained is significantly lower than those for the genus *Geobacillus* (48.2–58 mol%) (Nazina et al. 2001) and similar to the closest

Table 1 Comparative characteristics of a strain R270^T and the closest relatives

Characteristic	1	2	3	4	5
Cell length (μm)	3.3–7.0	2.0–2.5	1.5–5	4.0–10.0	3.9–4.7
Oxygen	Strict aerobe	Facultative anaerobe	Aerobe or facultative anaerobe	Facultative anaerobe	Strict aerobe
T_{opt} for growth (°C)	55	61	54	50	55
pH_{opt} for growth	6.0–6.5	5.6	7.0–8.0	7.0	7.0
Growth on					
Galactose	–	+	+	+	+
Sucrose	–	+	+	+	+
Starch	+	+	–	+	no data
Lactose	–	no data	+	+	no data
Nitrate reduction to nitrite	–	+	+	+	+
G + C Content (mol %)	41.7	43.5	43.9	44.4	42.4

1 R270^T; 2 *A. amylolyticus*^{T}, DSM15939T (Poli et al. 2006); 3 *A. voinovskiensis*^{T}, NCIMB13956T (Yumoto et al. 2004); 4 *A. contaminans* DSM15866T (De Clerck et al. 2004); 5 *Geobacillus tepidamans* GS5-97 (Schäffer et al. 2004)

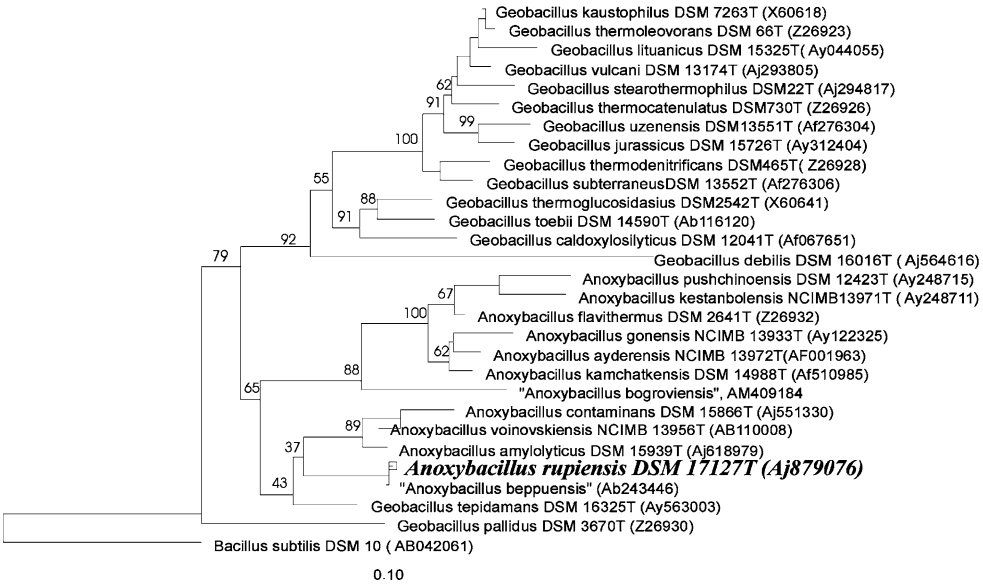

Fig. 1 16S rDNA sequence-based phylogenetic neighbour joining tree showing the phylogenetic relationship of strain R270 relative to the type strains of species in the genera *Geobacillus*, *Anoxybacillus* and sequences for the unpublished *Anoxybacillus* species ''*A. beppuensis*'' (identical with R270) and ''*A. bogroviensis*''. *B. subtilis* is included as outgroup. Bootstrap values (%) from 1,000 replicates are as shown. The tree topology by calculation by the FastML program was similar to the tree shown

Anoxybacillus relatives (*A. amylolyticus* 43.5 mol%; *A. voinovskiensis* 43.9 mol%; *A. contaminans* 44.3 mol%). G + C content of DNA was 43.2 mol% for *G. tepidamans*.

DNA–DNA homology

The results of experiments on the DNA–DNA homology between strain R270^T and its close relatives confirmed the phylogenetic differentiation of the strain as a new species. DNA–DNA homology with the closest validly published relative found by BLAST, *G. tepidamans*^{T} was 32.0%. This hybridization value is significantly less than the accepted 70% homology for species delineation (Stackebrandt et al. 2002) and clearly demonstrated its novelty at the species level.

Fatty acid profile

The fatty acid profile of strain R270^T was largely composed of branched saturated members and like other thermophilic bacilli contain anteiso-fatty acids as minor components (Table 2). Members of the genus *Anoxybacillus* contain iso-branched saturated fatty acids (iso-C15:0 and iso-C17:0) as major fatty acids. The major cellular fatty acids for R270^T were iso-C15:0 and iso-C17:0 (86.36% in total). Those values for other genus representatives were: 78.16% for *A. kestanbolensis* K_4^T (Dulger et al. 2004), 72.8% for *A. amylolyticus* (Poli et al. 2006), 68.59% for *A. contaminans* (De Clerck et al. 2004), and 65.7% for *A. voinovskiensis* (Yumoto et al. 2004). The iso-C16:0 (2.01%) value for R270^T was similar to that for other *Anoxybacillus* species and *G. tepidamans* and differed from published by Nazina et al. (2001) other *Geobacillus* species (*G. thermocatenulatus* 31.8%, *G. thermoleovorans* 21.0%; *G. thermoglucosidasius* 10.4%; *G. thermodenitrificans* 9.5%; *G. stearothermophilus* 6.2%).

Although the strain R270^T was consistently significantly similar to *G. tepidamans*^{T} (in their physiological properties, 16S rDNA, G + C value, low amount of iso-C16:0), it definitely differed from any other species in the genus *Geobacillus* in 16S rRNA gene similarity, G + C value, iso-C16:0 content. The similar physiological characteristics, G + C content of DNA, fatty acid profile and phylogenetic similarity (95.3–94.9% to the closest relatives) with representatives of the genus *Anoxybacillus* allow us to place the strain R270^T in the genus *Anoxybacillus*, as the type strain for the novel species, *A. rupiensis*.

Description of *Anoxybacillus rupiensis* sp. nov

Anoxybacillus rupiensis (N.L. masc. adj. rupiensis, originating from Rupi Basin, referring to the place of isolation of the type strain).

Cells of strain R270^T appeared as motile, large rods, approximately 3.3–7.0-μm long and 0.7–1.5-μm wide strain. Most cells occur in exponential growth phase singly or in chains. The cell wall structure is Gram positive. Terminal ellipsoidal or cylindrical endospores are observed. Whitish colonies of about 5 mm in diameter with irregular margin are formed on peptone-yeast extract plates. Obligate thermophiles growing between 35 and 67°C (optimum 55°C) and in a pH range from 5.5 to 8.5 (optimum 6.0–6.5). The strains are able to utilize a broad spectrum of carbohydrates such as sugars, polysaccharides and polyols in the presence of proteinaceous substrates or inorganic nitrogen. Growth on ribose, xylose, fructose, glucose and maltose occurs. It is not observed on galactose, L-rhamnose, raffinose, sucrose and lactose. Acid but no gas

Table 2 Cellular fatty acid composition (% w/w) of strain R270^T and some *Anoxybacillus* strains

Fatty acid	1	2	3	4	5	6
14:0 iso	–	–	1.3	–	–	0.6
14:0	0.30	–	1.3	2.91	7.3	4.1
15:0 iso	52.81	41.2	54.7	51.88	38.7	44.3
15:0 anteiso	1.64	2.13	8.0	7.52	2.0	6.6
15:0	0.31	0.1	–	–	0.9	–
16:0 iso	2.01	7.0	7.1	5.07	0.3	3.2
16:0 anteiso	–	0.12	–	–	–	–
16:0	5.44	6.3	1.9	11.32	14.5	15.1
17:0 iso	33.55	31.6	3.9	11.64	0.8	15.0
17:0 anteiso	3.94	0.7	–	7.02	0.1	6.1
17:0	–	–	–	–	0.5	–
18:0 iso	–	1.3	–	–	–	0.6
18:1	–	0.7	–	–	–	–
18:0	–	1.9	–	–	–	–

1 Strain R270^T; 2 *A. amylolyticus*^{T}, DSM15939T (Poli et al. 2006); 3 *A. voinovskiensis*^{T}, NCIMB13956T (Yumoto et al. 2004); 4 *A. contaminans* DSM15866T (De Clerck et al. 2004); 5 *A. pushchinoensis* DSM2641T (Pikuta et al. 2000, Pikuta et al. 2003) 6 *Geobacillus tepidamans* GS5-97 (Schäffer et al. 2004)

is produced by sugars. The organism degraded starch and xylan and not salicin, inulin and pectin. Growth is supported by manitol but not by ribitol, galactitol and sorbitol. Casein is hydrolysed, but not gelatin and olive oil. Citrate is not utilized. Phenyl-alanine is not deaminated, tyrosine is not degraded, nitrate is not reduced, indole is not produced, the Voges–Proskauer reaction is negative, catalase reaction is positive and methyl red test is negative. The major cellular fatty acids are iso-C15:0 and iso-C17:0. The DNA base composition of the type strain R270^T is 41.7%. The GenBank/EMBL accession number for the 16S rRNA gene sequence is AJ879076.

The type strain is R270^T, which was obtained from terrestrial hot spring at Rupi basin. It was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) under accession number 17127^T and in the National Bank for Industrial Microorganisms and Cell Cultures—Bulgaria (NBIMCC) under accession number 8387^T.

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