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ORIGINAL PAPER

Anoxybacillus thermarum sp. nov., a novel thermophilic bacterium isolated from thermal mud in Euganean hot springs, Abano Terme, Italy

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Abstract A novel aerobe thermophilic endospore-forming bacterium designated strain AF/04^T was isolated from thermal mud located in Euganean hot springs, Abano Terme, Padova, Italy. Strain AF/04^T was Gram-positive, motile, rod-shaped, occurring in pairs, or filamentous. The isolate grew between 55 and 67°C (optimum 65°C) and at pH 6.0–7.5 (optimum pH 7.2). The strain was aerobic and grew on maltose, trehalose, and sodium acetate as sole carbon sources. The G + C content of DNA was 53.5 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain AF/04^T falls within the genus *Anoxybacillus*. Levels of 16S rRNA gene sequence similarity between strain AF/04^T and the type strains of recognized *Anoxybacillus* species ranged from 95 to 99%. Chemotaxonomic data (major isoprenoid quinone–menaquinone-7; major fatty acid *iso*-C15:0 and *anteiso*-C17:0) supported the affiliation of strain AF/04^T to the genus *Anoxybacillus*. Based on phenotypic and chemotaxonomic characteristics, 16S rRNA gene sequence analysis and DNA–DNA hybridization data, it was proposed that strain AF/04^T (=DSM 17141^T = ATCC BAA 1156^T) should be placed in the genus *Anoxybacillus* as the type strain of a novel species, *Anoxybacillus thermarum* sp. nov.

Keywords *Anoxybacillus* · Taxonomy · Thermophilic · Lipid · DNA–DNA hybridisation · 16S rRNA analysis · Euganean hot spring

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The EMBL accession number for the 16S rRNA sequence of *Anoxybacillus thermarum* strain AF/04^T is AM402982.

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Introduction

The phylogenetic 16S rDNA sequence analysis revealed the isolate belongs to the genus *Anoxybacillus* (Pikuta et al. 2000). The first representative of this genus, *A. pushchinoensis* was described as strictly anaerobic and an emended description of the species was published later (Pikuta et al. 2003), according to which this species should be considered as aerotolerant anaerobe. At the time of writing, the genus *Anoxybacillus* contained eleven species: *Anoxybacillus pushchinoensis* and *Anoxybacillus flavithermus* (Pikuta et al. 2000), *Anoxybacillus gonensis* (Belduz et al. 2003), *Anoxybacillus contaminans* (De Clerck et al. 2004), *Anoxybacillus voinovskiensis* (Yumoto et al. 2004), *Anoxybacillus ayderensis* and *Anoxybacillus kestanbolensis* (Dulger et al. 2004), *Anoxybacillus kamchatkensis* (Kevbrin et al. 2005), *Anoxybacillus amylolyticus* (Poli et al. 2006a), *Anoxybacillus rupiensis* (Derekova et al. 2007) and *Anoxybacillus bogrovensis* (Atanassova et al. 2008). Moreover, recently a new subspecies of *Anoxybacillus kamchatkensis*, named *Anoxybacillus kamchatkensis* subsp. *asaccharedens*, has been isolated from a hot spring in Batman (Turkey) (Gul Guven et al. 2008).

The Abano–Montegrotto basin is a vast thermal area, close to the Euganean Hills (northeastern Italy). The heating of the waters is due to geothermal energy not linked to volcanic activity. This basin is fed by meteoric

water coming from distant areas and rising to the surface after being heated at a depth of 3,000 m. This zone is renowned for therapeutic applications made with the hot mud colonized with various microorganisms (Tolomio et al. 1999; Marcolongo et al. 2006).

The thermal mud is a hyperthermal poultice resulting from the mixture of a liquid component (high-temperature spring water containing sodium chloride, bromide, and iodide) and a solid component, mainly silt and clay taken from Lakes Arqua' and Lispida, also located in the Euganean district. A complex microbial community thrives in this site and is collectively indicated as "bioglea": an assemblage bacteria, algae, and protozoa. Thermal mud, for the therapeutic use, is pulled out from the bottom of the lakes, and taken to the thermal establishments. Here it is sieved and put in tanks, which are flooded with water which gushes from the spring shaft at a temperature ranging from 70 to 85°C.

The present paper describes the isolation, morphology, biochemical profile, 16S rRNA gene sequence, and results of DNA–DNA hybridization with close relatives of a novel thermophilic endospore-forming bacterium from thermal mud located in Euganean hot springs, Abano Terme, Padova, Italy.

On the basis of phenotypic features as well as DNA–DNA hybridization studies with the closest known species of *Anoxybacillus*, we propose to classify the new isolate AF/04^T as a new species, *Anoxybacillus thermarum* sp. nov.

Materials and methods

Sample source, enrichment, and isolation

Samples of mud were collected aseptically from tanks located in a hotel in Abano Terme (T 75–80°C; pH 7.5–8.0) at intervals up to 60 days of maturation (Galzigna et al. 1998) and were kept at +4°C until use. Sub-samples (1 ml) of the thermal mud were inoculated, at temperature range 60–80°C, into 10 ml of different enrichment media but the growth was observed in the medium A containing (% value are in w/v) peptone (Oxoid) 0.8%, yeast extract (Oxoid) 0.4% and NaCl 0.2%.

The isolation of pure cultures was carried out by serial dilution technique in liquid enrichment medium. The purity of the obtained isolates was confirmed microscopically and by colonies uniformity formed on solid medium plus 2% agar. Several strains were isolated and taxonomic properties of one of them, strain AF/04^T, will be presented in this note.

Anoxybacillus flavithermus DSM 2641^T and *Anoxybacillus pushchinoensis* DSM 12423^T were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Brunschweig, Germany (DSMZ) and were grown according to DSMZ catalog. *Anoxybacillus ayderensis* NCIMB 13972^T and *Anoxybacillus gonensis* NCIMB 13933^T were obtained from the National Collections of Industrial, Marine and Food Bacteria, Scotland, UK and were grown according to NCIMB catalog. *Anoxybacillus kamchatkensis* DSM 14988^T was kindly obtained from Vadim V. Kevbrin and grown as reported by Kevbrin et al. (2005).

Morphological, physiological, and biochemical tests

Cellular morphology and motility were determined by phase-contrast microscopy (Zeiss) and colony morphology was determined by Leica M8 stereomicroscope using cultures grown on media A agar plates for 24 h at the optimal temperature. Unless otherwise stated, the strain was characterized using the modified methods of Gordon and Pang (1973) and all growth tests were performed in liquid medium A. The pH dependence of growth was tested in the pH range 4.0–9.0. The temperature range was determined by incubation in liquid medium at temperatures between 30 and 80°C. Gram staining was performed according to Dussault (1955). KOH test was performed according to Halebian et al. (1981). Starch hydrolysis was tested by flooding cultures on solid medium A containing 0.2% (w/v) starch with Lugol's iodine. For casein hydrolysis, a solid medium A plus an equal quantity of skimmed milk was used. For gelatine hydrolysis and sensibility to lysozyme, enrichment medium A plus 1.0% (w/v) gelatine or 0.001% (w/v) lysozyme was used, respectively. For spore formation test, enrichment medium A plus 0.001% (w/v) $MnCl_2{\cdot}4H_2O$ was used. Urease activity, determined by rapid urease test, and utilization of Tween 60 for lipolytic activity was carried out according as described by Lanyi (1987). The motility of strain and other routine microbiological tests were performed as previously described (Poli et al. 2006a). Anaerobic growth test was performed as described by Gul Guven et al. (2008). Growth on carbon sources was tested on liquid mineral medium M162 (Poli et al. 2006b). The organic compounds tested (1%, w/v) were: D-glucose, D-lactose, D-maltose, D-fructose, D-cellobiose, D-galactose, D-mannose, D-ribose, D-trehalose, D-xylose, raffinose, glycerol, sucrose, sodium acetate, sodium citrate and sodium propionate. Moreover, ethanol (0.1%, v/v) and starch (0.2%, w/v) were also tested. All growth tests were done at 65°C and growth was scored positive if the A_{540} was greater than 0.300 after 3 days.

For the enzymatic assays, cells grown in medium A were collected during stationary growth phase by centrifugation at 9,000×*g* for 30 min. Wet cells (about 2.0 g) were suspended in 20 mM Tris–HCl at pH 7.8, lysed by ultrasonic

treatment (Heat System Instrument) for 4 min and by using lysozyme activity (3 mg of lysozyme for 1 g wet cells) and centrifuged at 15,000×*g* for 20 min. The supernatant (crude homogenate) was assayed for glycosil hydrolase activities, by incubating for 10 min at 65°C a reaction mixture containing in 1 ml final volume: 0.1 ml of the crude extract, 40 mM Tris–HCl pH 7.0 and 1 mM of the one of the following substrates: *p*-nitrophenyl-α-D-(glucopyranoside, galactopyranoside, maltoside); *p*-nitrophenyl-*β*-(glucopyranoside, xylopyranoside, lactopyranoside, maltoside); *p*-nitrophenyl *N*-acetyl-*β*-D-glucosamide (Poli et al. 2006b).

Antimicrobial susceptibility testing

Antibiotic sensitivity of strain was tested by using medium A with agar (1.8%, w/v) and Sensi discs (6 mm; Oxoid) (Romano et al. 1993). The following antibiotics (μg): neomycin (30), erythromycin (30), penicillin G (10U), chloramphenicol (50), kanamycin (30), nystatin (100), ampicillin (25), gentamicin (30), novobiocin (30), bacitracin (10 U), lincomycin (15), fusidic acid (10), vancomycin (30), streptomycin (25), and tetracycline (30), were used.

Chemosystematic characterization: lipid and fatty acid compositions

Lipid and fatty acid analysis were performed as reported by Nicolaus et al. (2001). Polar lipids were achieved from 3.0 g of freeze-dried cells grown in medium A at 65°C by the extraction with 65:25:4 $CHCl_3$/MeOH/H_2O. The lipid extract was analyzed by thin layer chromatography (TLC) on silica gel (0.25 mm, F_{254}, Merck) eluted with $CHCl_3$/MeOH/H_2O (65:25:4, by vol.). Lipids were detected by spraying the plates with 0.1% $Ce(SO_4)_2$ in 2 N H_2SO_4 followed by heating at 100°C for 5 min. Phospholipids and amino-lipids were detected on the plates upon spraying with the Dittmer–Lester and the ninhydrin reagents, respectively, and glycolipids were visualized with alpha-naphtol (Nicolaus et al. 2001). Authentic standards for comparing the R_f of appeared spot. Quinones were analyzed by LC/MS on a reverse-phase column, RP-18 Lichrospher (250 × 4 mm) column, eluted with *n*-hexane/ethylacetate (99:1 v/v) with a flow rate of 1.0 ml/min and identify by EI/MS and ^{1}H-NMR spectra. Fatty acid methyl esters (FAMEs) were obtained from complex lipids by acid methanolysis (Nicolaus et al. 2001) and analyzed using a Hewlett–Packard 5890A gas chromatograph fitted with a FID detector and equipped with an HP-V column with a flow rate of 45 ml/min using the temperature program of 120°C (1 min), from 120 to 250°C at 2°C/min. Identification of compounds was obtained with standards and by interpretation of mass spectra. NMR spectra, recorded at the NMR Service of Institute of Biomolecular Chemistry of CNR (Pozzuoli, Italy), were acquired on a Bruker DPX-300 operating at 300 MHz, using a dual probe.

16S rDNA sequencing and phylogenetic analysis

DNA was extracted and purified from bacterial cell cultures (about 250 mg of dry pellet) using the Genomic-DNA-Buffer Set and the Genomic-tip-100/G columns (QIAGEN SpA, Milano Italy), according to manufacturer's instructions. DNAs were dissolved in Tris 10 mM pH 8.0, 1 mM EDTA and were serial-diluted to a final concentration of 50 μg/ml as evaluated by UV-absorbance and fluorimetric assay (Quant-iT DNA, Invitrogen).

PCR mediated amplification of the 16S rRNA and purification of the PCR products were performed at the CRIBI Sequencing Service (Padova University, Italy). The 16S rRNA resulting sequence was manually aligned and was compared with representative sequences of organisms belonging to the *Firmicutes* group (Maidak et al. 1999). The 16S rRNA gene similarity values were calculated by pairwise comparison of the sequences within the alignment. For construction of the phylogenetic dendrogram, operations of the PHYLIP package were used (Felsenstein 2004); the pairwise evolutionary distances were computed from percent similarities by the correction of Jukes and Cantor (1969) and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987). *Brevibacillus centrosporus* was chosen as an outgroup organism.

The G + C mol% content was evaluated by modifying the procedure originally described by Gonzalez and Saiz-Jimenez (2002). Briefly, DNA samples were subjected to thermal denaturation in a reaction mixture containing a final volume of 25 μl: 10 mM Tris pH 8, 1 mM EDTA, 20 mM NaCl, 1× of the fluorescent DNA-intercalating dye EVA-green (Biotium) and 200 ng of DNA, by using an iQ5 (Bio-Rad) PCR-Real-Time apparatus. Determinations were performed in quadruplicate in a 96-well plate sealed by an optical tape (BioRad). Well factors were obtained from a replicate plate containing the mixture without DNA, while the experimental plate was inserted in the iQ5 apparatus during a hold step at 37°C. The incubation at 37°C was resumed for further 20 min, followed by a melting protocol from 50 to 100°C in step of 0.2°C, dwell time 15 s and acquisition of fluorescence data for each step. The fluorescence data were exported to the "Melting Profiler" version 0.7 software (Bio-Rad) for the evaluation of Tm. Bacterial DNA samples of known G + C content, ranging from 30 to 60%, were included for a standard curve G + C versus Tm design (linear correlation coefficient 0.999%) and the G + C content of the unknowns was extrapolated from the curve. DNA samples purification and DNA–DNA

percent homology evaluation by filter hybridization were performed as previously described (Romano et al. 2007).

Results

Morphological and biochemical analysis

Gram-positive rods microorganisms were isolated from samples of mud sediments collecting from the maturation tanks of a thermal establishment located in Euganean hot springs, Abano Terme, Padova, Italy. They were purified using serial repeated dilution technique followed by streaking on the solid enrichment medium A. The strain AF/04^T was the predominant organism in the enrichment medium A and it was the only colony forming organism at the highest dilutions. Cells of isolate AF/04^T were Gram-positive, motile rods, 3.0–3.5 μm long, and 0.3–0.5 μm wide with terminal, ellipsoidal to cylindrical endospores. Colonies were yellow, smooth, and circular. The isolate AF/04^T strain did not grow in anaerobic condition tested. It was aerobe. The temperature growth range was about 55–67°C (optimum 65°C). The pH growth range was from 6.0 to 7.5 (optimum pH 7.2). The isolate AF/04^T utilized maltose, trehalose, and sodium acetate as sole carbon sources and it was sensitive to neomycin (30 μg), erythromycin (30 μg), penicillin G (10 U), chloramphenicol (50 μg), kanamycin (30 μg) ampicillin (25 μg), gentamicin (30 μg), novobiocin (30 μg), bacitracin (10 U), lincomycin (15 μg), fusidic acid (10 μg), streptomycin (25 μg), and tetracycline (30 μg). The isolate was able to hydrolyse hippurate, to decompose tyrosine, catalase positive and it was sensitive to lysozyme (Table 1). Strain AF/04^T was negative for nitrate reduction, oxidase, urease, starch, casein, and gelatin hydrolysis, Tween 60 utilization, indole production, and phenylalanine deamination. It possessed an alpha and beta-glucosidase activity with an optimal pH value of 5.3 and 6.5, respectively.

Chemosystematic characterization: lipid and fatty acid compositions

The isolate AF/04^T strain possessed complex lipids based on fatty acids. The total lipid content was about 10% of the total dry weight of cells grown at 65°C in medium A. Under these conditions, three major phospholipids, 1,2 diacylglycero-3-phosphorylethanolamine (PEA), 1,2 dipalmitoyl-3-glycero-phosphatidic acid (AP) and cardiolipin (DPG), were identified.

FAMEs' composition, determined on cells grown under standard condition, was characterized by the abundance of branched acyl chains. The most abundant was *i*C15:0 (46.6%), other components were *i*C17:0 (6.6%), *ai*C17:0 (25.6%), *i*C16:0 (8.7%) and *ai*C15:0 (2.9%) (Table 2). Chromatographic analysis of quinones revealed the presence of one more abundant UV-absorbing band. ^{1}H-NMR spectrum showed that the quinone present is menaquinone (MK) type. LC/MS as well as EI/MS analyses of the quinone content of AF/04^T strain gave a molecular peak corresponding to MK7 as major compound (more than 90% of quinones).

Phylogenetic analysis and DNA–DNA hybridization studies

The DNA G + C content of strain AF/04^T was determined to be 53.5 mol%. Comparative 16S rRNA gene sequence analyses (EMBL nucleotide sequence accession number is AM402982) showed that strain AF/04^T is phylogenetically most closely affiliated to the genus *Anoxybacillus* and that the new isolate had a similarity level ranging 95.0–99.0% with respect to sequences of the type strains of recognized *Anoxybacillus* species. In particular, the closest phylogenetic neighbors were *A. flavithermus*, *A. kamchatkensis*, *A. ayderensis*, *A. gonensis* and *A. pushchinoensis* (sequence similarity correspondingly 99.4, 99.3, 99.0, 98.3 and 98.0%, respectively) and the more distant was *A. rupiensis* (95.0%). The results were presented by phylogenetic dendrogram (Fig. 1) and the strain AF/04^T fell within the radiation of the cluster comprising *Anoxybacillus* species.

The mean DNA–DNA reassociation values found between strain AF/04^T and the close *Anoxybacillus* species, showing high 16S rRNA gene sequence similarity, were in the range of 9.5–35.8%. In particular, the values found between strain AF/04^T and *A. flavithermus*, *A. kamchatkensis*, *A. ayderensis, A. gonensis* and *A. pushchinoensis* were 35.8, 9.5, 12.3, 8.8 and 9.2%, respectively. All these values were lower than the recommended threshold value of 70%, which is accepted as the definition of a genospecies (Wayne et al. 1987) and clearly demonstrated its novelty at species level.

Discussion

In Table 1 are reported the differential characteristics among strain AF/04^T and *Anoxybacillus* species. As regarding the relation to O_2, strain AF/04^T is aerobe microorganism. However, the term Anoxybacillus means "without oxygen Bacillus", and the first species of *Anoxybacillus* described was strictly anaerobic (*A. pushchinoensis*), some species described later as *A. rupiensis* grew well aerobically and most of the new *Anoxybacillus* species have been found to be aerobe/facultative anaerobes (*A. flavithermus*, *A. contaminans*, *A. amylolyticus*) or to be facultative anaerobe (*A. kamchatkensis*, *A. ayderensis*,

Table 1 Comparison of the phenotypic characteristics of strain AF/04^T and related species

Characteristics	1	2	3	4	5	6	7	8	9	10	11	12
Relation to O_2	Aerobe	Facultative anaerobe	Aerobe/ facultative anaerobe	Facultative anaerobe	Aerobe/ facultative anaerobe	Facultative aerobe	Aerobe/facultative anaerobe	Anaerobe	Aerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Temperature range (°C)	55–67	38–67	30–72	30–70	50–60	30–64	45–65	37–66	35–67	40–70	40–70	40–69
Optimum temperature (°C)	65	57–62	60–65	50	50	54	61	62	55	55–60	50–55	65
pH range	6.0–7.5	5.7–9.9	5.5–9.0	6.0–11.0	4.5–10.0	7.0–8.0	5.0–6.5	8.0–10.5	5.5–8.5	6.0–10.0	6.0–10.5	6.5–10.0
Optimum pH	7.2	6.8–8.5	7.0	7.5–8.5	7.0	7.0–8.0	5.6	9.5–9.7	6.0–6.5	7.5–8.0	7.5–8.5	8.0
Motility	+	+	+	+	+/−	−	+	−	+	+	+	−
NaCl (3%, w/v)	−	+[a]	−	−	+	−	−	+	−	+	+	−
Nitrate reduction	−	+[a]	+	+	+	+	−	+	−	+	+	−
Sensitive to Lysozyme	+	+[a]	ND	ND	ND	ND	+	ND	ND	ND	ND	ND
Gelatin hydrolysis	−	−	−	+	+	−	−	−	−	+	−	+
Starch hydrolysis	−	−	+	+	+	−	+		+	+	+	+
Casein hydrolysis	−	−[a]	+	ND	−	−	−	−	+	ND	ND	−
Oxidase	−	−	+	+	−	+	−	ND	ND	+	+	ND
Catalase	+	−	+	+	+	+	+	−	+	W	+	+
Utilization of												
Glucose	−	+	−	+	+	+	−	+	+	+	+	+
Galactose	−	+	ND	ND	+	+	+	ND	−	ND	ND	−
Lactose	−	−		−	+	+	−	ND	−	−	−	−
Xylose	−	−	−	+	+	+	−	+	+	+	−	−
Trehalose	+	+	ND	ND	+	−	+	+	ND	ND	ND	ND
G + C (mol %)	53.5	42.3	41.6	54	44.4	43.9	43.5	42.2	41.7	57	50	44.1

1 strain AF/04^T, *2 A. kamchatkensis* DSM 14988^T (Kevbrin et al. 2005), *3 A. flavithermus* DSM 2641^T (Pikuta et al. 2000), *4 A. ayderensis* NCIMB 13972^T (Dulger et al. 2004), *5 A. contaminans* DSM 15866^T (De Clerck et al. 2004), *6 A. voinovskiensis* NCIMB 13956^T (Yumoto et al. 2004), *7 A. amylolyticus* DSM 15939^T (Poli et al. 2006a), *8 A. pushchinoensis* DSM 12423^T (Pikuta et al. 2000), *9 A. rupiensis* DSM 17127^T (Derekova et al. 2007), *10 A. gonensis* NCIMB 13933^T (Belduz et al. 2003), *11 A. kestanbolensis* NCIMB 13971^T (Dulger et al. 2004), *12 A. bogrovensis* DSM 17956^T (Atanassova et al. 2008)

+ positive, − negative, *w* weak response, *ND* not determined

[a] Data obtained by present paper

Table 2 Fatty acid methyl ester profiles of strain AF/04^T and *Anoxybacillus* species

Fatty acids	1	2	3	4	5	6	7	8	9	10	11
*i*C14:0	–	0.9	–	–	–	1.3	–	–	–	1.2	0.9
C14:0	–	1.6	1.9	1.0	3.0	1.3	–	2.9	0.3	1.2	1.3
*i*C15:0	46.6	9.1	54.8	48.2	52.0	54.7	41.2	51.9	53.0	65.2	68.6
*ai*C15:0	2.9	7.7	4.0	3.6	7.0	8.0	2.1	7.5	1.6	2.6	3.5
C15:0	–	2.6	1.2	0.8	–	–	0.1	–	0.3	1.1	1.1
*i*C16:0	8.7	20.7	2.9	7.5	5.0	7.1	7.0	5.1	2.0	6.0	6.4
*ai*C16:0	–	0.4	–	–	–	–	0.1	–	–	–	–
C16:0	–	21.0	11.1	9.1	11.0	1.9	6.3	11.3	5.4	2.4	3.5
*i*C17:1ω5c	–	–	–	–	–	–	–	–	–	2.6	0.6
*i*C17:0	6.6	9.3	17.7	20.6	12.0	3.9	31.6	11.6	33.6	12.0	9.5
C17:0 anteiso A	–	–	–	–	–	–	–	–	–	0.8	–
*ai*C17:0	25.6	18.9	6.2	9.2	7.0	–	8.4	7.0	3.9	3.3	3.7
C17:0	–	2.6	–	–	–	–	0.7	–	–	–	
*ai*C17:1	–	–	–	–	–	7.1	–	–	–	–	
C17:1	–	–	–	–	–	2.6	–	–	–	–	–
*i*C18:0	–	2.0	–	–	–	–	0.1	–	–	–	–
*ai*C18:0	–	–	–	–	–	–	0.7	–	–	–	–
C18:0	–	3.0	–	–	–	–	1.9	–	–	–	–

1 strain AF/04^T, *2 A. kamchatkensis* DSM 14988^T (data obtained by present paper), *3 A. flavithermus* DSM 2641^T (Pikuta et al. 2000), *4 A. ayderensis* NCIMB 13972^T (Dulger et al. 2004), *5 A. contaminans* DSM 15866^T (De Clerck et al. 2004), *6 A. voinovskiensis* NCIMB 13956^T (Yumoto et al. 2004), *7 A. amylolyticus* DSM 15939^T (Poli et al. 2006a), *8 A. pushchinoensis* DSM 12423^T (Pikuta et al. 2003), *9 A. rupiensis* DSM 17127^T (Derekova et al. 2007), *10 A. gonensis* NCIMB 13933^T (Belduz et al. 2003), *11 A. kestanbolensis* NCIMB 13971^T (Dulger et al. 2004)

Values are shown as a percentage of the total fatty acid content for each strain

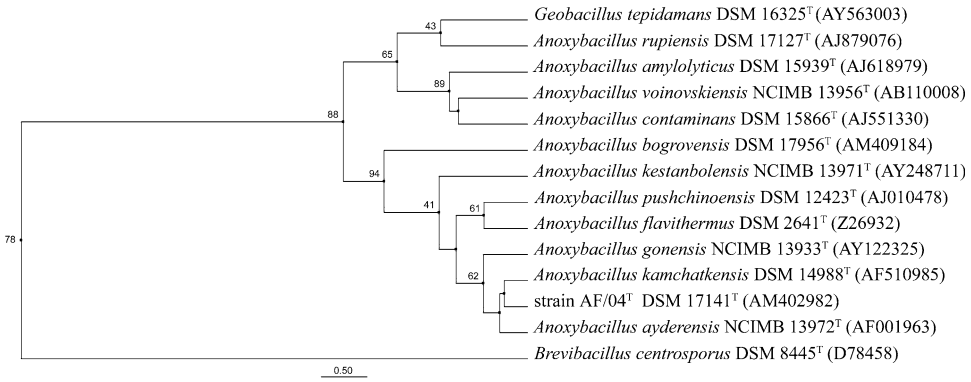

Fig. 1 *Phylogenetic dendrogram* indicating the position of the strain AF/04^T within the radiation of *Anoxybacillus*. The bootstrap values are given at the nods. *Scale bar* represents 0.50 substitutions per nucleotide position

A. gonensis, *A. kestanbolensis*, *A. bogrovensis*) or for some species anaerobic growth was registered only under certain conditions (*A. voinovskiensis*). Moreover, strain AF/04^T differentiates from the other related species in terms of the following phenotypic characteristics: optimal *T*°C, pH range for growth, lower NaCl tolerance (<3%, w/v), starch and casein hydrolysis, lack of oxidase activity. Regarding the nitrate reduction reaction, strain AF/04^T has been found negative unlike of the most *Anoxybacillus* species except for *A. amylolyticus*, *A. rupiensis* and *A. bogrovensis*. Another biochemical difference between the new isolate and closest relatives was the spectrum of carbohydrate using as sole carbon sources: among several carbon source tested, strain AF/04^T was able to grow only in the presence of maltose, trehalose, and sodium acetate. An α and β- glucosidase activity with an optimal pH value of 5.3 and 6.5, respectively, were found in the cytosol of the new species: these enzymatic activities have not been described in other *Anoxybacillus* species even if a constitutive amylase was observed for *A. amylolyticus* (Poli et al. 2006a) and *A. bogrovensis* (Atanassova et al. 2008) with an endo- and an exo-mechanism of enzyme action, respectively.

Strain AF/04^T although possesses the typical polar lipid pattern of *Anoxybacillus* genus (PEA, AP and DPG), shows a different fatty acid composition in term of percentage of the total fatty acid content (Table 2). Members of the genus *Anoxybacillus* contain iso-branched saturated fatty acids (*i*C15:0 and *i*C17:0) as major fatty acids. The major cellular fatty acids for strain AF/04^T were found *i*C15:0 (46.6%) and *ai*C17:0 (25.6%) even if the presence of *i*C17:0 was also observed (6.6%). To notice that *n*C16:0, reported for all *Anoxybacillus* species, was not detected for AF/04^T.

According to the low levels of DNA–DNA hybridisation between AF/04^T and the *Anoxybacillus* genus representative (9.5–35.8%) and on the basis of the above mentioned phenotypic and biochemical differences, we propose that strain AF/04^T should be placed in the genus *Anoxybacillus* as the type strain for the novel species *Anoxybacillus thermarum* sp. nov.

Description of *Anoxybacillus thermarum* sp. nov.

A. thermarum (ther.ma'rum. L. gen. pl. n. thermarum, of warm springs)

Cells are Gram-positive, motile rods, 3.0–3.5 μm long, and 0.3–0.5 μm wide with terminal, ellipsoidal to cylindrical endospores. Colonies are usually yellow, smooth, and circular. It is aerobic. Growth occurs in enrichment medium A (peptone Oxoid 0.8%, yeast extract Oxoid 0.4% and NaCl 0.2%), at temperature of 55–67°C (optimum 65°C) and at pH 6.0–7.5 (optimum pH 7.2). Catalase positive and oxidase negative. Hydrolyses hippurate, decomposes tyrosine, but not starch, casein, gelatin and Tween 60. Negative for nitrate reduction, indole production, and phenylalanine deaminase. Sensitive to lysozyme. It is able to grow on maltose, trehalose, and sodium acetate as sole carbon source. The following antibiotics inhibit the growth of the strain AF/04^T: neomycin (30 μg), erythromycin (30 μg), penicillin G (10 U), chloramphenicol (50 μg), kanamycin (30 μg), ampicillin (25 μg), gentamicin (30 μg), novobiocin (30 μg), bacitracin (10 U), lincomycin (15 μg), fusidic acid (10 μg), streptomycin (25 μg) and tetracycline (30 μg). Possesses α and β- glucosidase activity. The major menaquinone is MK7. The major fatty acids are iso-C15:0 (46.6%), anteiso-C17:0 (25.6%), iso-C16:0 (8.7%), iso-C17:0 (6.6%) and anteiso-C15:0 (2.9%). Major lipids are three phospholipids [1,2 diacylglycero-3-phosphorylethanolamine (PEA), 1,2 dipalmitoyl-3-glycero-phosphatidic acid (AP) and cardiolipin (DPG)]. The DNA G + C content of type strain AF/04^T is 53.5 mol%.

The type strain, AF/04^T (=DSM 17141^T = ATCC BAA 1156^T) was isolated from thermal mud located in Euganean hot springs, Abano Terme, Padova, Italy.

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