

Full Paper

***Geobacillus thermodenitrificans* subsp. *calidus*, subsp. nov., a thermophilic and α -glucosidase producing bacterium isolated from Kizilcahamam, Turkey**

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An α -glucosidase producing, thermophilic, facultatively anaerobic, and endospore-forming, motile, rod-shaped bacterial strain F84b^T was isolated from a high temperature well-pipeline sediment sample in Kizilcahamam, Turkey. The growth occurred at temperatures, pH and salinities ranging from 45 to 69°C (optimum 60°C), 7.0 to 8.5 (optimum 8.0) and 0 to 5% (w/v) (optimum 3.5%), respectively. Strain F84b^T was able to grow on a wide range of carbon sources. Starch and tyrosine utilization, amylase, catalase and oxidase activities, nitrate reduction, and gas production from nitrate were all positive. The G+C content of the genomic DNA was 49.6 mol%. The menaquinone content was MK-7. The dominant cellular fatty acids were iso-C17:0, iso-C15:0, and C16:0. In phylogenetic analysis of 16S rRNA gene sequence, strain F84b^T showed high sequence similarity to *Geobacillus thermodenitrificans* (99.8%) and to *Geobacillus subterraneus* (99.3%) with DNA hybridization values of 74.3% and 29.1%, respectively. In addition, the Rep-PCR and the intergenic 16S–23S rRNA gene fingerprinting profiles differentiated strain F84b^T from the *Geobacillus* species studied. The results obtained from the physiological and biochemical characters, the menaquinone contents, the borderline DNA–DNA hybridization homology, and the genomic fingerprinting patterns had allowed phenotypic, chemotaxonomic and genotypic differentiation of strain F84b^T from *G. thermodenitrificans*. Therefore, strain F84b^T is assigned to be a new subspecies of *G. thermodenitrificans*, for which the name *Geobacillus thermodenitrificans* subsp. *calidus*, subsp. nov. is proposed (The type strain F84b^T = DSM 22629^T = NCIMB 14582^T).

Key Words—DNA–DNA hybridization; genomic fingerprinting; *Geobacillus*; lipid; 16S rRNA gene; taxonomy; thermostable α -glucosidase

Introduction

The genus *Geobacillus* was first described by Nazina et al. (2001) with the type species of *Geobacillus stearothermophilus* DSM 22^T. The genus falls into the thermophilic bacilli genetic group 5 according to their 16S rRNA gene sequences and contains thermophilic, endospore-forming, aerobic members (Ash et al., 1991; Nazina et al., 2001). The members of the genus *Geoba-*

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The GenBank accession numbers for the 16S rRNA gene sequence of strain F84b^T, F84a and A333 are EU477773.2, EU477772 and EU326497, respectively.

cillus can exhibit growth at temperatures ranging from 35 to 78°C, and are widespread in various geographical locations. They have been defined as a phylogenetically coherent group of thermophilic bacilli displaying very high intragenetic similarity among their 16S rRNA gene sequences (96.5–99.2%) (Nazina et al., 2001, 2004). Since then, new representatives of the genus *Geobacillus* have been described and now the genus comprises seventeen validly published species. Examples include *Geobacillus kaustophilus* (Nazina et al. 2001; Priest et al. 1988), *Geobacillus subterraneus* (Nazina et al., 2001), *Geobacillus thermocatenulatus* (Golovacheva et al., 1975; Nazina et al., 2001), *Geobacillus thermoglucosidasius* (Nazina et al., 2001; Suzuki et al., 1983), *Geobacillus thermoleovorans* (Nazina et al., 2001; Zarilla and Perry, 1987), *Geobacillus thermodenitrificans* (Manachini et al., 2000; Nazina et al., 2001), and *Geobacillus uzenensis* (Nazina et al., 2001).

Isolation of novel thermophilic bacilli has received considerable attention and the interest in thermozymes is of importance for industrial applications (Zeikus et al., 1998). Turkey is rich in geothermal sources which vary in typical temperatures and pH values in a range from 25 to 100°C and 4.0 to 11.0. During our previous search, we isolated and screened thermophilic bacilli which were able to produce industrially important α -amylases and α -glucosidases from different geothermal regions of Turkey (Coleri et al., 2009). Such a thermophilic bacterium was isolated from a sediment sample of a high-temperature well-pipeline in the Middle Anatolian Region of Turkey. This strain, called F84b^T, was found to be unique in its ability to produce the highest amount of thermostable α -glucosidase per cell yield (77.18 U/g) after 15 h of growth at 60°C, when compared with other amylolytic and thermophilic isolates and α -glucosidase producing reference strains. The strain was capable of producing significant levels of α -glucosidase extracellularly, which might promise biotechnological importance. The current paper describes the taxonomy of this α -glucosidase-producing strain F84b^T with details of phenotypic, chemotaxonomic, and genetic characteristics. On the basis of the presented data, it is proposed that F84b^T should be classified as a novel subspecies, *Geobacillus thermodenitrificans* subsp. *calidus*, subsp. nov. (type strain = DSM 22629^T = NCIMB 14582^T).

Materials and Methods

Isolation and growth conditions. Strains F84b^T and F84a were obtained from sediment samples of a high-temperature well-pipeline in Kizilcahamam Province of Ankara. The water temperature and pH of this geothermal region were measured as 98°C and 7.0–7.5, respectively. Strain A333 was isolated from a soil sample of a high-temperature well-pipeline in Germencik Province of Aydin. Both of these mentioned high-temperature well-pipelines exist in the Middle Anatolian Region of Turkey. The samples (0.1 g) were incubated at 60°C with 250 rpm shaking for 24 h in 5 ml of the *G. thermoglucosidasius* medium (MI) containing 1% soluble starch (pH 7.0) to obtain the enrichment culture (Suzuki et al., 1976). The turbid enrichments were streaked on plates of MI containing 3% agar and incubated aerobically at 60°C for 24 h. Cultures were purified from the same medium by subculturing four times and the purity was confirmed microscopically. The first pure cultures of strains F84b^T and F84a were obtained on 8 September 2004, whereas strain A333 was isolated on 15 July 2004. Strains F84b^T, F84a and A333 were deposited in two culture collections with the deposition numbers of DSM 22629^T = NCIMB 14582^T, DSM 22628 = NCIMB 14581, and DSM 22625 = NCIMB 14578, respectively.

The following *Geobacillus* strains were used for references: *G. stearothermophilus* ATCC 12980^T, *G. subterraneus* DSM 13552^T, *G. thermodenitrificans* DSM 465^T, *G. thermoglucosidasius* DSM 2542^T, and *Geobacillus toebii* E134 DSM 22627.

Morphological and physiological characterizations. Cytology and colony morphology analyses were carried out using exponentially grown cultures on Nutrient Agar plates (containing 3% agar). The formation of spores was tested by using Nutrient Broth cultures of 18–24 h supplemented with 5 mg/L MnSO₄·4H₂O and observed using phase-contrast microscopy. Scanning Electron Microscopy (SEM) analysis used Nutrient Broth culture of F84b^T cells, grown at 60°C under shaking at 250 rpm for 18 h. The sample was prepared for electron microscopy using the procedure described by Hayat (1981), and examined in the Jeol JSM 6060 LV Scanning Electron Microscope. The temperature range was determined by incubating the strains in Nutrient Broth at temperatures from 30 to 80°C. The pH dependence of growth was tested in Nutrient Broth which was adjusted with either NaOH or HCl to various

pH values from 4.0 to 11.0. The effect of salinity was also checked in Nutrient Broth supplemented with 0 to 5% (w/v) concentrations of NaCl. The optimal growth at different temperatures, pH values and salinities were determined spectrophotometrically by measuring the optic density of the turbid cultures at 600 nm. Physiological characterization tests included: Gram staining, anaerobic growth, oxidase, catalase and amylase activity, casein, citrate, starch, tyrosine, gelatine and urea utilization, reduction of nitrate to nitrite, N₂ gas production from nitrate, acid production from sugars, methyl red test, Voges-Proskauer test, indole and H₂S production, susceptibility to lysozyme. These tests were carried out by the methods of Claus and Berkeley (1986). Test results were observed at days 1, 2 and 7. A disk diffusion test was performed by using Nutrient Agar plates for antimicrobial susceptibility testing. Plasmid DNA isolation was carried out by the procedure of Anderson and McKay (1983). All assays were performed with pure culture in triplicates.

Enzyme assays. Strain F84b^T was cultivated for 12 h in 1 L enzyme production medium MII (2% soluble starch from potato, 2% peptone, 0.2% yeast extract, 0.5% meat extract, 0.3% K₂HPO₄, and 0.1% KH₂PO₄) at 60°C, pH 8.0 under shaking at 250 rpm (Suzuki et al., 1976). Cell-free supernatant of F84b^T was separated from the medium by centrifugation. The centrifuged supernatant was fractionated with 40–65% ammonium sulfate and dialyzed overnight and the 310 ml dialyzate was concentrated 20-fold by centrifugation in a speed vacuum concentrator. Then it was applied to a cation-exchange chromatography column as described previously (Cihan et al., 2009). Protein concentration was determined by using Bradford reagent, and as the protein standard 0.1 to 10 mg/ml concentrations of BSA were used (Cihan et al., 2009). When determining the molecular weight of the enzyme, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the procedure of Laemmli (1970).

The α -glucosidase activity was determined spectrophotometrically by measuring the hydrolysis of *p*NPG (*para*-nitrophenol α -D-glucopyranoside, Sigma N1377) as described previously (Coleri et al., 2009). One unit of enzyme was defined as the amount of enzyme needed for hydrolysis of 1 μ mol *p*NPG per minute at 60°C, pH 6.8. The millimolar extinction coefficient of *p*NP (*para*-nitrophenol) at 400 nm, pH 6.8 and 60°C was measured as 19.1 mm⁻¹·cm⁻¹ and used to calcu-

late the product yield.

The effect of temperature on enzyme activity was determined between 35 to 95°C with 5°C intervals. The effect of pH on the enzyme activity was determined using 0.02 M sodium-citrate (pH 3.0–6.5), 0.1 M sodium phosphate (pH 7.0–8.5), 0.1 M sodium carbonate-bicarbonate (pH 9.0–9.5) and Ringer (pH 10.0–11.0) buffers at 60°C, all instead of potassium phosphate buffer (pH 6.8) in the standard assay mixture.

16S rRNA gene amplification and sequence analyses. The cultures growing on Nutrient Agar for 18 h at 60°C were used for genomic DNA extraction (Genomic DNA purification kit, Fermentas). The gene encoding 16S rRNA was amplified by Polymerase Chain Reaction (PCR) with the bacterial 16S-specific 27F forward primer and the 1492R reverse primer (Kuisiene et al., 2002). The sequence of the PCR-amplified 16S rRNA gene was determined by using an ABI 3100 gene sequencer with a Bigdye cycle sequencing kit. In the phylogenetic analysis, homology search was carried out using the basic BLASTN search program at the NCBI Web-site. A neighbor-joining phylogenetic tree (Saitou and Nei, 1987) was constructed with the MEGA package version 4 (Tamura et al., 2007) according to the Kimura 2-parameter method (Kimura, 1980).

Fatty acids and respiratory lipoquinone analyses. Fatty acids and respiratory lipoquinone analyses were performed by Dr. Brian Tindall (DSMZ Identification Service, Braunschweig, Germany) using minor modifications of the extraction method of Kuykendall et al. (1988), Miller (1982) and Tindall et al. (1990a, b). Fatty acids were further determined using Gas Chromatography (GC), and quinones were analyzed by HPLC.

PCR-based fingerprinting analyses of REP elements and intergenic 16S–23S rRNA gene. Rep-PCR genomic fingerprintings were performed with the (GTG)₅ and BOXA1R primers using the PCR conditions that were described by Versalovic et al. (1994). Primer sets S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 were used for the amplification of intergenic transcribed spacers (ITS) between 16S and 23S rRNA genes and PCR conditions were adjusted according to Daffonchio et al. (2003). The PCR products were electrophoresed in a 1.5% agarose gel, using 1 × TBE buffer at 120 V for 4 h. The resulting Rep-PCR fingerprintings were analyzed by the Bionumerics version 6.1 software packages (Applied Maths, Belgium). The DNA fragments that ranged from 320 to 5,300 bp, and 320 to 6,100 bp generated with BOXA1 and (GTG)₅-PCR

fingerprintings were included in the statistical analysis. Similarities of the digitized profiles were calculated using Pearson correlation and an average linkage (UP-GMA) dendrogram was obtained.

DNA base composition and DNA-DNA hybridization studies. DNA base composition and DNA-DNA hybridization analyses were carried out by the DSMZ Identification Service. DNA was extracted using the French press technique, and purified by the method of Cashion et al. (1977). In determining G+C base composition of the genomic DNA, the DNA was hydrolyzed with P1 nuclease and the nucleotides were dephosphorylized with bovine alkaline phosphatase (Mesbah et al., 1989). The resulting deoxyribonucleotides were analyzed using HPLC. The G+C content of the genomic DNA was calculated according to the method of Mesbah et al. (1989). Spectroscopic DNA-DNA hybridization was carried out at 67°C in $2 \times$ SSC as described by De Ley et al. (1970) with the modifications described by Huss et al. (1983).

Results and Discussion

Phenotypic characteristics of the strain

The morphological and physiological characteristics of strains F84b^T are given in the subspecies description. The cells of F84b^T are 0.6–0.9 µm wide and 1.6–2.9 µm long. The cell surface was observed to be smooth in SEM images. The phase-contrast and SEM micrographs of the isolate are shown in Fig. 1a and 1b, respectively. Microscopy observations revealed

that the cells of strains F84b^T, F84a and A333 were Gram-positive, motile and straight rods.

Strain F84b^T differed from closely related species *G. thermodenitrificans*, *G. subterraneus*, and *G. stearrowthermophilus* in some phenotypic characteristics such as cell size, spore shape, temperature, pH and NaCl requirements, casein, tyrosine, and gelatin utilization, reduction of nitrate to nitrite, gas production from nitrate, methyl red test, acid production from lactose, xylose, arabinose, raffinose, galactose and mannitol (Table 1). Beside these phenotypic differences, while strain A333 and *G. thermodenitrificans* were found to be susceptible to all of the antibiotics tested, a kanamycin resistance was observed in strains F84b^T and F84a. Furthermore, both strain F84b^T and F84a had plasmid DNA contents of 15.6 and 12.0 kb, but strain A333 differed from these strains by containing the plasmid bands of 19.0 and 17.7 kb.

α-Glucosidase activity

Strain F84b^T, F84a and A333 could produce starch-degrading enzymes with strong amylolytic and glycosidic activity, and α-glucosidase of strain A333 was characterized previously (Cihan et al., 2009). The α-glucosidase of strain A333 was found to be 45 kDa, having optimum activity at 60°C, and pH 6.8 in 16 h of cultivation. In the current study, fractions showing α-glucosidase activity from 2 to 7 of peak I was pooled for F84b^T anionic enzyme after cation-exchange chromatography. Strain F84b^T produced a significant amount of extracellular α-glucosidase with a specific

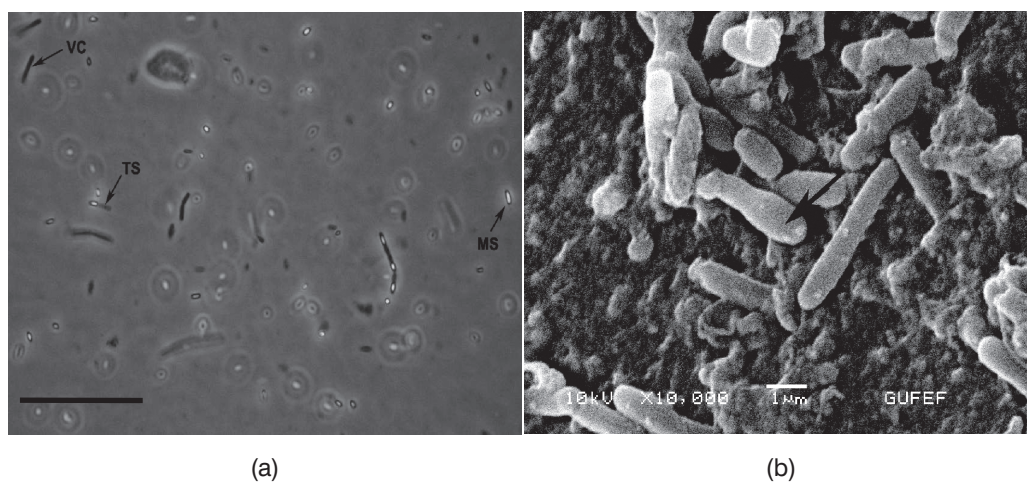


Fig. 1. (a) Phase-contrast (VC, vegetative cell; MS, mature spore; TS, terminal sporulation; bar, 10 µm), and (b) Scanning electron micrographs of strain F84b^T after 18 h of cultivation at 60°C in Nutrient Broth medium.

Table 1. Differentiating characteristics of strain F84b^T and the most phylogenetically related strains of *Geobacillus* species.

Characteristic	1	2	3	4	5	6
Cell width (μm)	0.6–0.9	0.7–0.9	0.6–0.8	0.5–1.0	0.8–1.2	0.6–1.0
Cell length (μm)	1.6–2.9	1.2–2.2	1.2–1.7	1.5–2.5	4.6–6.6	2.0–3.5
Spore shape	E	E	E	O	E	E
Growth conditions						
Temperature (°C)	45–69 (60)	45–69 (60)	45–69 (60)	45–70 (60)	45–65 (55)	37–65 (55)
pH	7.0–8.5 (8.0)	7.0–8.5 (8.0)	6.5–8.5 (7.5)	6.0–8.0 (7.7)	6.2–7.6 (7.0)	6.0–8.0 (7.0)
NaCl (%), (w/v)	0–5.0 (3.5)	0–4.5 (3.0)	0–4.5 (3.0)	0–3.0 (2.0)	0–3.0 (2.0)	0–5.0 (3.0)
Casein utilization	–	–	–	–	–	+
Tyrosine utilization	+	–	+	–	–	–
Gelatin utilization	–	–	–	–	–	+
Reduction of nitrate to nitrite	+	+	+	+	+	–
Gas from nitrate	+	–	–	+	–	–
Methyl red	–	–	–	–	+	+
Acid production from						
Lactose	–	–	–	+	–	–
D-(+)-Xylose	+	+	+	+	–	–
L-Arabinose	+	+	–	–	–	–
Raffinose	+	+	+	+	–	+
D-(+)-Galactose	+	+	+	+	+	–
D-(–)-Mannitol	+	+	+	+	–	–
Protease production	–	–	–	+	–	+
α-Glucosidase production	+	+	+	+	–	+

1, strain F84b^T; 2, strain F84a; 3, strain A333; 4, *G. thermodenitrificans* DSM 465^T; 5, *G. subterraneus* DSM 13552^T; 6, *G. stearothermophilus* DSM 22^T. Abbreviations: +, positive reaction; –, negative reaction; W+, weakly positive reaction; E, ellipsoidal; O, oval. The tests for the reference strains were also done in the present study.

activity (3.01 U/mg with *p*NPG substrate) higher than the strain A333 in about 12 h of cultivation at 60°C. The molecular weight of the enzyme was estimated as 43 kDa. Maximum activity of the α-glucosidase was expressed between the late exponential and stationary phases. The enzyme showed maximum activity at 60°C, which is its optimal temperature for growth. The F84b^T α-glucosidase also showed activity at a wide range of pH from 4.5 to 11.0, with an optimum pH value of 6.8.

Lipid and fatty acid composition

According to respiratory quinone analysis of strains F84b^T and F84a, the isoprenoidquinone content was determined as MK-7 (100%). The predominant menaquinone content of strain A333 and *G. thermodenitrificans* were found to be MK-7 (98%) and MK-6 (2%). The dominant cellular fatty acids were iso-C17:0, iso-C15:0, C16:0, anteiso-C17:0, and iso-C16:0 in strains F84b^T, F84a and A333. The iso-branched fatty acids (iso-C17:0, iso-C15:0 and iso-C16:0) were the major membranous lipids present with a percentage of 78 of

the total fatty acids, which was in the range described for genus *Geobacillus*. In fatty acid profiles, some slight differences were observed between strain F84b^T and the other phylogenetically related *Geobacillus* species. This can be seen in Table 2.

Phylogenetic position

The 16S rRNA gene sequence determined for the type strain F84b^T was 1,400 nucleotides long and the G+C content of 16S rRNA gene was found to be 59 mol%. The phylogenetic tree was constructed using the neighbor-joining method. It showed the position of strains F84b^T, F84a and A333 among the species of genus *Geobacillus* which fell into phylogenetic group 5 of endospore-forming bacteria (Fig. 2). As shown in Fig. 2, strains F84b^T, F84a, A333 and *G. thermodenitrificans* DSM 465^T were branched within the same cluster in the phylogenetic tree with a bootstrap value of 99% and the 16S rRNA gene sequence similarity values of 93.3% to 99.8% were obtained between strain F84b^T and the other members of the genus *Geobacillus*. According to the 16S rRNA gene se-

Table 2. Differences in cellular fatty acid methyl esters of strain F84b^T and the related *Geobacillus* species.

Fatty acid	1	2	3	4	5	6
C17:0 iso	37.09	36.72	35.88	34.23	21.52	18.50
C15:0 iso	34.51	33.96	32.34	35.64	33.46	37.80
C16:0	8.21	8.60	9.12	6.77	17.35	1.70
C17:0 anteiso	7.74	8.26	8.34	6.40	12.65	5.80
C16:0 iso	5.94	5.92	6.37	8.07	6.43	29.20
C15:0 anteiso	1.48	1.65	1.56	1.28	4.14	2.30
C15:0	1.20	1.17	1.16	1.79	—	1.40
C17:0	1.04	0.93	1.29	1.61	—	0.40
C19:0 iso	0.67	0.82	0.65	0.74	—	—
C18:0	0.62	0.70	0.83	0.55	—	—
C14:0	0.44	0.48	0.51	0.41	1.32	—
C18:0 iso	0.30	0.36	0.37	0.34	1.75	—
C14:0 iso	0.21	0.22	0.26	0.38	—	2.90
C17:1 ω5c iso	0.20	—	0.58	0.98	—	—
C19:0 anteiso	0.17	0.22	0.21	—	—	—
Others	0.18	0	0.53	0.81	1.38	0

Strains: 1, F84b^T; 2, F84a; 3, A333 (from this study); 4, *G. thermodenitrificans* DSM 465^T; 5, *G. stearothermophilus* DSM 22^T (Manachini et al., 2000); 6, *G. subterraneus* DSM 13552^T (Nazina et al., 2001). Values are percentages of total fatty acids. Major fatty acids are shown in bold.

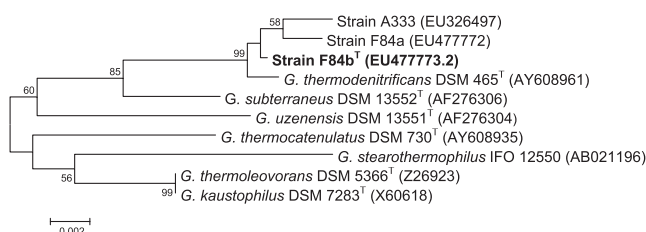


Fig. 2. Neighbor-joining evolutionary distance phylogenetic tree based on the 16S rRNA gene sequences of strain F84b^T and the representative members of genus *Geobacillus* of the family 'Bacillaceae.'

Bootstrap values (%) are based on 1,000 replicates and shown for branches with more than 50% bootstrap support. Bar indicates 0.002 substitutions per nucleotide position.

Table 3. DNA G+C content, 16S rRNA gene sequence similarity and DNA-DNA homology among the thermophilic isolates and the reference strains.

	G+C content (mol%)	a	F84b ^T b	c	a	F84a b	c	a	A333 b	c
F84b ^T	49.6	100								
F84a	49.0	100	95	3.7	100					
A333	45.1	99.9	77.4	4.1	99.9	78.3	3.5	100		
<i>G. thermodenitrificans</i> DSM 465 ^T	50.3 ^d	99.8	74.3	3.3	99.8	80.5	3.4	100	100	3.9
<i>G. subterraneus</i> DSM 13552 ^T	52.3 ^e	99.3	29.1	2.3	99.3	28.4	1.9	99.4	29.5	6.7

^a, 16S rRNA gene sequence similarity (%); ^b, Reassociation (%) with DNA from; ^c, deviation (+/–) of DNA homology; G+C content data were taken from ^d, Manachini et al., 2000; and ^e, Nazina et al., 2001.

quence similarity, F84b^T was found to be closely related to *G. thermodenitrificans* (99.8%), *G. subterraneus* (99.3%), *G. thermocatenulatus* (98.3%), *G. uzenensis* (98.1%), *G. kaustophilus* (98.1%), *G. thermoleovorans* (98.0%), and *G. stearothermophilus* (98.0%). Furthermore, strain F84b^T showed 100% and 99.9% 16S rRNA gene sequence similarity values with strains F84a and A333, as presented in Table 3.

The Rep-PCR and ITS fingerprintings

The Repetitive Extragenic Palindromic (REP) elements such as BOX and (GTG)₅ are known as evolutionary conserved sequences in prokaryotic genomes and can be used as oligonucleotide binding sites for PCR-mediated genomic fingerprinting (Rep-PCR). The Rep-PCR can be used to discriminate strains at the species and subspecies level (Versalovic et al., 1994). Rep-PCR has been used for the determination of the genetic diversity among the *Geobacillus* species (Meintanis et al., 2008). Beside these fingerprinting methods, the intergenic transcribed spacers (ITS) between the 16S and the 23S rRNA genetic loci are frequently used in PCR fingerprinting to discriminate at the species and intraspecies levels. The polymorphism in the ITS is due to the presence of tRNA genes that contain multiple ribosomal operons which differ in length and sequence (Daffonchio et al., 2003). This polymorphism was used for discriminating closely related *Geobacillus* species in previous studies of Daffonchio et al. (2003), Kuisiene et al. (2008) and Manachini et al. (2000).

Thus, strain F84a^T and its phylogenetically related *Geobacillus* species were subjected to Rep-PCR and ITS fingerprinting analyses. The (GTG)₅ and BOX-PCR fingerprinting patterns and their cluster analyses derived from strain F84b^T and its closely related *Geoba-*

cillus species are shown in Fig. 3a and 3b, respectively. The (GTG)₅ and BOX-PCR products generated a high number of bands giving discriminative information below species and subspecies level between these *Geobacillus* strains studied and as in the case of the phylogenetic tree obtained from the 16S rRNA gene, strain F84b^T, F84a, A333 and *G. thermodenitrificans* were branched within the same cluster on the dendrogram generated by the Rep-PCR patterns. Beside these results, the Rep-PCR fingerprinting patterns of F84b^T were the same as for strain F84a, whereas the banding patterns of strain A333 were very similar to those of *G. thermodenitrificans*. Therefore, the distinctive Rep-PCR bands were able to differentiate these strains genetically, and the *thermodenitrificans* cluster diverged into two groups, one of which was formed from strains F84b^T and F84a. The other group consisted of strain A333 and *G. thermodenitrificans*.

Not only the rep-PCR, but also the ITS fingerprinting patterns of these strains confirmed the genotypic di-

versity between these strains which is presented in Fig. 4. As in the case of rep-PCR analyses, while the ITS banding patterns of strain F84b^T and F84a (Fig. 4, Lanes 4 and 5) showed similar patterns, the fingerprinting profiles of strain A333 and *G. thermodenitrificans* (Fig. 4, Lanes 3 and 6) resembled each other. Both of these results derived from the Rep-PCR and ITS fingerprintings clearly showed the genetic divergence between strain F84b^T and *G. thermodenitrificans*, and thus on the basis of these fingerprint methods, it can be concluded that type strain F84b^T and strain F84a were genetically a distinct group, may not be assigned to the species *G. thermodenitrificans* and represent novel subspecies of *G. thermodenitrificans*, while strain A333 may belong to this species.

The G+C content and DNA-DNA hybridizations

The G+C content of the genomic DNA of strain F84b^T, F84a and A333 were 49.6, 49.0 and 45.1 mol%, which are in the range of *Geobacillus* species (Table

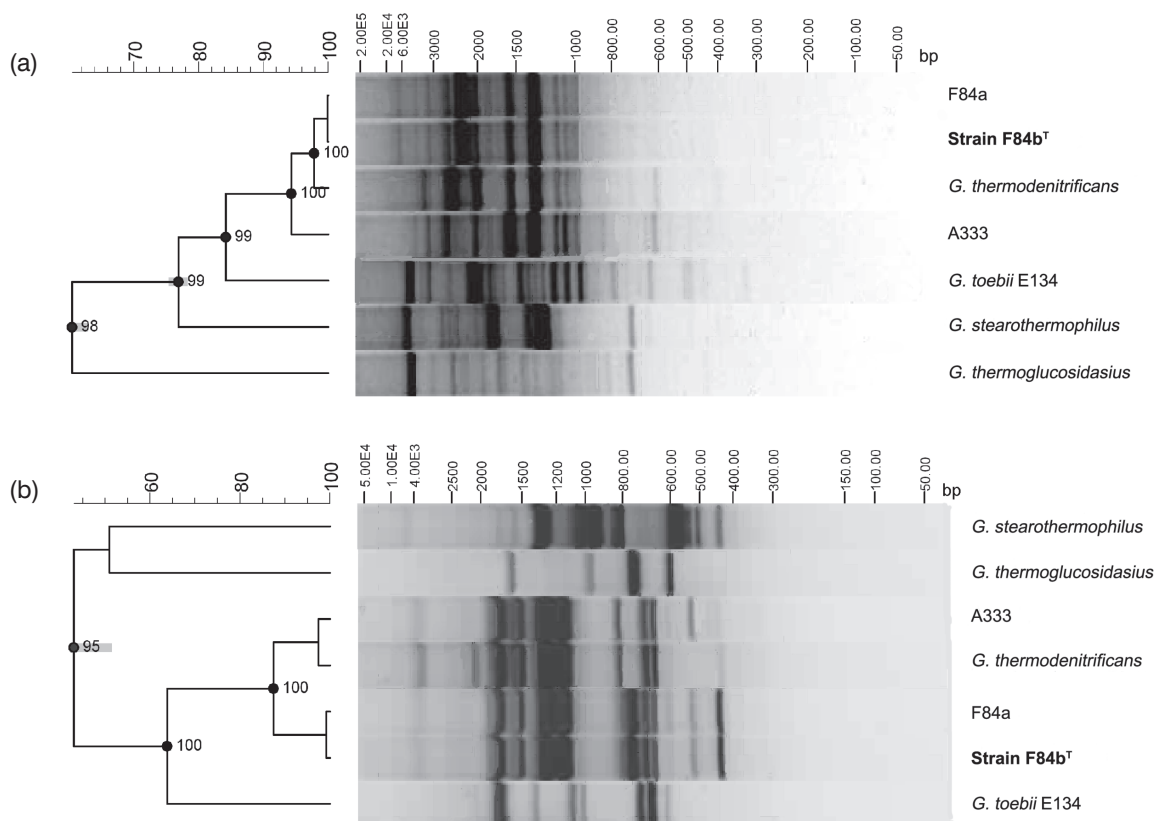


Fig. 3. Cluster analysis of digitized banding patterns, generated by rep-PCR using (a) the (GTG)₅ primer, and (b) the BOXA1R primer of strains from the genus *Geobacillus* (F84b^T, F84a, A333, *G. thermodenitrificans* DSM 22^T, *G. thermoglucosidasius* DSM 2542^T, *G. thermodenitrificans* DSM 465^T, *G. toebii* E134 DSM 22627). The dendrogram was constructed by using UPGMA, with correlation levels expressed as percentage values of the Pearson correlation coefficient.

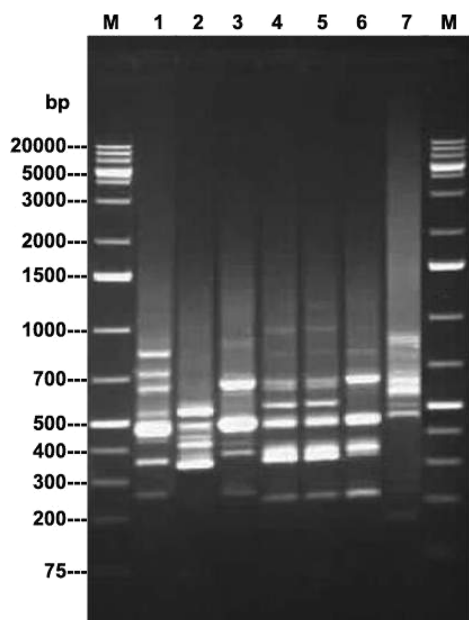


Fig. 4. ITS fingerprinting patterns of *Geobacillus* species, amplified by using the S-D-Bact-1494-a-S-20 and L-D-Bact-0035-a-A-15 primers (Lane M: 1 kb plus DNA ladder (SM1343, Fermentas), Lane 1: *G. stearothermophilus* DSM 22^T, Lane 2: *G. thermoglucosidasius* DSM 2542^T, Lane 3: *G. thermodenitrificans* DSM 465^T, Lane 4: F84b^T, Lane 5: F84a, Lane 6: A333 and Lane 7: *G. toebii* E134 DSM 22627).

3).

In order to verify the taxonomic positions of these strains, they were subjected to DNA-DNA hybridization analyses with each other and with their closely related species: *G. thermodenitrificans* and *G. subterraneus*. The DNA-DNA homology data are given in Table 3. Strain A333 had DNA-DNA hybridization values of 100% with *G. thermodenitrificans* and 29.5% with *G. subterraneus*. In addition, 77.4% and 78.3% DNA reassociation values were found between strain A333 and strains F84b^T, F84a, respectively. Thus, strain A333 is assigned to be a species of *G. thermodenitrificans* as a result of the data obtained from both phenotypic and genotypic characters and also from the complete DNA homology with this mentioned type strain.

On the other hand, strains F84b^T and F84a were found to be 74.3% and 80.5% similar to *G. thermodenitrificans* and 29.1% and 28.4% to *G. subterraneus* according to the DNA-DNA hybridization results, respectively. They also shared a DNA homology of 95% with each other. The ad hoc committee recommends a threshold value of 70% DNA-DNA similarity for the definition of a novel bacterial species (Stackebrandt et

al., 2002). Nevertheless similarity values can be within an accuracy of about 10%, so values in close vicinity have to be regarded as critical. The critical DNA-DNA hybridization values between strains F84b^T, F84a and *G. thermodenitrificans* could not differentiate F84b^T and F84a from all the other species of genus *Geobacillus* into a separate species. But, when the physiologic, phylogenetic, genetic and chemotaxonomic data are considered, in addition to the borderline DNA homology, a novel subspecies of *Geobacillus thermodenitrificans* is proposed for which the name *Geobacillus thermodenitrificans* subsp. *calidus*, subsp. nov. is recommended; the type strain is F84b^T. A description of the new subspecies is given below.

Emendation of *Geobacillus thermodenitrificans* (Manachini et al., 2000) Nazina et al. (2001)

On the basis of the description of *Bacillus thermodenitrificans* given by Manachini et al. (2000), the following points are modified: Endospores are oval or ellipsoidal. Nitrate is reduced to nitrite. Gas from nitrate is variable. Protease production is variable.

Description of *Geobacillus thermodenitrificans* subsp. *calidus*, subsp. nov.

Geobacillus thermodenitrificans subsp. *calidus* (Etymologies: *ca' li. dus*, L. Masc. Adj., referring to [habitats] hot).

Cells are motile, spore-forming, straight rods with approximate sizes of $0.6\text{--}0.9 \times 1.2\text{--}2.9 \mu\text{m}$. They exist singly. Terminal, ellipsoidal endospores occur in non swollen sporangia. Colonies are cream, opaque, circular, and flat with lobate edges, with a dry-powdery surface. Catalase activity is positive. Grows well aerobically but is facultatively anaerobic. Gram-positive and thermophilic. Growth occurs at 45–69°C (optimum 60°C) and pH 7.0–8.5 (optimum 8.0). Growth range is within 0% to 4.5–5% (w/v) concentrations of NaCl, optimum being at 3.0–3.5% (w/v). Starch utilization, amylase and reduction of nitrate to nitrite are also positive. Citrate, gelatin and urea can not be utilized. Protease activity is negative. Methyl red and Voges-Proskauer tests are negative. Indole and H₂S are not produced. Acid is produced from maltose, D-fructose, D-glucose, galactose, sucrose, D-xylose, D-mannose, L-arabinose, raffinose and D-mannitol, but not from lactose or D-sorbitol. Produces a high amount of thermostable α -glucosidase extracellularly and the amount of α -glucosidase production per cell yield for the strains

F84a and F84b^T differs from 36.6 U/g to 77.2 U/g, respectively. It is sensitive to lysozyme. The following antibiotics: vancomycin (30 µg); bacitracin (10 µg); chloramphenicol (30 µg); rifampicin (30 µg); tetracycline (30 µg); penicillin G (10 µg); neomycin (30 µg); novobiocin (30 µg), and azithromycin (15 µg) inhibit the growth, but it is resistant to kanamycin (30 µg). The menaquinone content is MK-7 (100%). The dominant cellular fatty acids are iso-C17:0, iso-C15:0, C16:0, anteiso-C17:0, and iso-C16:0. The strain contains two plasmid bands of 12.0 and 15.6 kb. The G+C content of the genomic DNA is 49.0–49.6 mol%.

The type strain is F84b^T (=DSM 22629^T = NCIMB 14582^T). It was isolated from a sediment sample of a high-temperature well-pipeline in Kizilcahamam Province of Ankara, Turkey.

Geobacillus thermodenitrificans subsp. *thermodenitrificans* (Manachini et al., 2000) Nazina et al. 2001 is automatically created on the basis of the description of *Bacillus thermodenitrificans* given by Manachini et al. (2000).

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