Bacillus marmarensis sp. nov., an alkaliphilic, protease-producing bacterium isolated from mushroom compost

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A Gram-stain-positive, obligately alkaliphilic bacterium designated strain GMBE 72^T was isolated from mushroom compost from Yalova, located in the Marmara region of Turkey. Cells were aerobic, straight rods and they formed subterminal to terminal ellipsoidal endospores. The isolate was catalase-positive, oxidase-negative and motile and contained a type A1γ peptidoglycan based on *meso*-diaminopimelic acid. The strain grew at pH 8.0–12.5. The major cellular fatty acid was anteiso-$C_{15:0}$. The genomic DNA G+C content was 40.2 mol%. Phylogenetic analyses based on 16S rRNA gene sequencing showed that strain GMBE 72^T belonged to the genus *Bacillus* and exhibited 98.2 % sequence similarity to *Bacillus pseudofirmus* DSM 8715^T. DNA–DNA reassociation was 56 % between GMBE 72^T and *B. pseudofirmus* DSM 8715^T. According to our polyphasic characterization, strain GMBE 72^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus marmarensis* sp. nov. is proposed. The type strain is GMBE 72^T (=DSM 21297^T =JCM 15719^T).

Many alkaliphilic *Bacillus* species belong to the sixth rRNA group in the genus *Bacillus* (Nielsen *et al.*, 1994) and have been isolated from different habitats, as described by Agnew *et al.* (1995), Nielsen *et al.* (1995) and Lim *et al.* (2006a, b). They have been studied extensively because of their taxonomic and ecological significance and also for their use for biotechnological and industrial purposes (Fritze *et al.*, 1990; Horikoshi, 1996; Kumar & Takagi, 1999; Denizci *et al.*, 2004; Nogi *et al.*, 2005). Alkaliphilic bacteria have been isolated in order to investigate their diversity in terms of physiological adaptation to high pH and the industrial applications of their enzymes such as alkaline protease (Horikoshi, 1996, 1999; Kumar & Takagi, 1999; Yumoto *et al.*, 1998; Jasvir *et al.*, 1999; Takami *et al.*, 1999; Takami & Horikoshi, 2000; Takami & Krulwich, 2000; Gupta *et al.*, 2002; Saeki *et al.*, 2002). At the time of writing, 26 alkaliphilic and alkalitolerant *Bacillus* species have been identified (Vedder, 1934; Spanka & Fritze, 1993; Nielsen *et al.*, 1995; Agnew *et al.*, 1995; Fritze, 1996; Switzer Blum *et al.*, 1998; Yumoto *et al.*, 1998, 2003; Olivera *et al.*, 2005; Nogi *et al.*, 2005; Ghosh *et al.*, 2007; Lee *et al.*, 2008; Borsodi *et al.*, 2008).

In this study, we describe the results of a polyphasic study aimed at the characterization of strain GMBE 72^T, which was isolated from mushroom compost from the Marmara region of Turkey during screening for alkaliphilic bacteria (Oner *et al.*, 2006). Compost samples were diluted serially with 0.9 % (w/v) saline solution and spread on agar containing 0.1 % glucose, 0.2 % peptone, 0.5 % yeast extract, 0.1 % K_2HPO_4, 0.02 % $MgSO_4.7H_2O$ and 0.5 % skimmed milk (skimmed milk was sterilized separately). The medium was adjusted to pH 10.0 by addition of 10 % Na_2CO_3 solution after sterilization and plates were incubated for 72 h at 30 °C. A clear zone around colonies indicated protease-producing micro-organisms; isolate GMBE 72^T was selected as an alkaline protease producer on the basis of the diameter of the clear zone around its colonies. Subculturing was performed on DSMZ 31 medium (http://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium31.pdf) for 24 h at 30 °C and the isolate was maintained as glycerol stocks at −70 °C.

Colony morphology was examined by growing strains on DSMZ 31 medium for 24 h at 30 °C. Cell morphology was

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GMBE 72^T is EU621902.

Micrographs of strain GMBE 72^T and a maximum-parsimony 16S rRNA gene sequence-based tree are available as supplementary material with the online version of this paper.

investigated with light microscopy (Leica DM 4000) at ×1000 magnification. Gram staining was determined with the Sigma Gram stain kit according to the manufacturer's instructions. Motility was observed after 12 and 24 h in semi-solid DSMZ 31 medium. Growth was tested in DSMZ 31 medium at 5–65 °C. The NaCl requirement for growth was studied in DSMZ 31 medium supplemented with 0–20 % (w/v) NaCl. Growth at different pH values was tested according to Nielsen *et al.* (1995). All physiological and biochemical tests were performed using the methods of Gordon *et al.* (1973). The medium was adjusted to pH 10 as described by Nielsen *et al.* (1995). Carbon source utilization tests were performed using API 50 CH galleries according to Nielsen *et al.* (1995). Tests were performed twice and unclear results are not given.

The cell-wall diamino acid type of strain GMBE 72^T was determined from whole-cell hydrolysates as described by Hasegawa *et al.* (1983). For cellular fatty acid analyses, the strain was grown on TSA at 28 °C for 24 h and fatty acid methyl ester analysis was performed according to the instructions of the Sherlock Microbial Identification System (MIDI; Microbial ID). Determination of the DNA G+C content and DNA–DNA hybridization were performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Genomic DNA was isolated using DNAzol (Molecular Research Center, Inc.) according to the manufacturer's instructions. PCR amplification of the 16S rRNA gene was performed with universal primers 27f (5′-AGAGTTTGATCCTCAG-3′) and 1385r (5′-CGGTGTGTACAAGGCCC-3′). An amplicon of approximately 1.4 kb was purified by using the EZ-10 Spin Column PCR purification kit (Bio Basic Inc.) according to the manufacturer's instructions. DNA sequencing using four primers, namely 27f, 357f, 907r, 1385r, was performed by the dideoxy chain-termination method with an ABI Prism 3100 DNA analyser using an ABI Prism Big Dye

Table 1. Phenotypic characteristics that serve to differentiate strain GMBE 72^T from type strains of phylogenetically related *Bacillus* species

Strains: 1, *Bacillus marmarensis* sp. nov. GMBE 72^T; 2, *B. pseudofirmus* DSM 8715^T (data from this study); 3, *B. wakoensis* N-1^T (data from Nogi *et al.*, 2005); 4, *B. krulwichiae* JCM 1169^T (Yumoto *et al.*, 2003). ND, No data available; V, variable.

Characteristic	1	2	3	4
O_2 requirement	Aerobic	Strictly aerobic	ND	Aerobic/anaerobic
Colony morphology				
Pigmentation	Cream–yellowish	Yellow	Yellowish	Colourless
Margin	Entire	Irregular	ND	ND
Cell size (μm)	0.8–1.1 × 2–2.5	0.6–0.8 × 2.0–4.0	0.5–0.8 × 1.5–2.0	0.5–0.7 × 1.5–2.6
Growth with/at:				
12 % NaCl	+	+	−	+
14 % NaCl	−	+	−	+
pH 11	+	−	−	−
10 °C	−	+	+	−
45 °C	+	+	+	−
Hydrolysis of:				
Hippurate	−	+	ND	+
Starch	−	+	+	+
Gelatin	+	+	−	V
Casein	+	+	−	V
Tween 20	−	−	−	+
Tweens 40 and 60	−	+	−	+
Tween 80	+	−	ND	+
Reduction of nitrate	−	−	+	+
Growth on:				
D-Mannose	+	+	+	−
D-Xylose	−	+	+	+
D-Ribose	+	+	−	+
Cellobiose	+	+	+	−
D-Sorbitol	−	−	+	−
D-Galactose	−	−	−	+
DNA G+C content (mol%)	40.2	39	38.1	41

Terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's protocol. 16S rRNA gene sequences of close relatives of strain GMBE 72^T with validly published names were retrieved from the GenBank database using BLASTN (Altschul *et al.*, 1997). A phylogenetic tree was constructed by using the software package MEGA version 4 (Kumar *et al.*, 2004) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (distance options according to Kimura's two-parameter model; Kimura, 1980, 1983) and clustering were based on the neighbour-joining and maximum-parsimony methods. The tree topology was examined by the bootstrap method of resampling (Felsenstein, 1985) using 1000 bootstraps.

Strain GMBE 72^T formed cream–yellowish-coloured, circular, entire and convex colonies on DSMZ 31 medium. Cells were Gram-stain-positive, aerobic, motile, straight rods (0.8–1.1 × 2.0–2.5 μm). Cells were mainly single or occasionally in pairs or in short chains, with subterminal to terminal endospores (Supplementary Fig. S1, available in IJSEM Online). The strain was catalase-positive and oxidase-negative and did not reduce nitrate to nitrite. Growth was observed at 15–45 °C. The strain grew at pH 8.0–12.5; no growth was observed at pH 7.0. The strain grew at salt concentrations in the range 0–12 % (w/v) NaCl. The growth and phenotypic characteristics that serve to distinguish strain GMBE 72^T from the type strains of closely related *Bacillus* species are shown in Table 1.

The cell wall contained *meso*-diaminopimelic acid in the peptidoglycan, suggesting the occurrence of peptidoglycan type A1γ (as in the case of the great majority of members of the genus *Bacillus*; Claus & Berkeley, 1986).

The cellular fatty acid composition of GMBE 72^T was dominated by anteiso-$C_{15:0}$ (41.1 %). The type strain of the most closely related species, *Bacillus pseudofirmus* DSM 8715^T, also exhibited anteiso-$C_{15:0}$ as the predominant fatty acid; iso-$C_{15:0}$ was the second most abundant fatty acid in both strains (Table 2). This fatty acid profile corresponded well with the profile of the type species of the genus, *Bacillus subtilis* (Kämpfer, 1994), including the major acids anteiso-$C_{15:0}$ and iso-$C_{15:0}$.

Comparative 16S rRNA gene sequencing analyses showed that strain GMBE 72^T was closely related to *B. pseudofirmus* DSM 8715^T (98.2 %), *Bacillus wakoensis* N-1^T (93.4 %), *Bacillus krulwichiae* AM31D^T (93.2 %) and *B. subtilis* 168 (92.4 %). The phylogenetic tree reconstructed using the neighbour-joining method showed that strain GMBE 72^T is a member of group 6 (Nielsen *et al.*, 1994) of the genus *Bacillus*. Strain GMBE 72^T formed a clade with *B. pseudofirmus* DSM 8715^T with a bootstrap value of 100 % (Fig. 1). The maximum-parsimony phylogenetic tree showed essentially the same position for strain GMBE 72^T (Supplementary Fig. S2). The DNA G+C content of strain GMBE 72^T was 40.2 mol%.

Table 2. Fatty acid profiles of strain GMBE 72^T and *B. pseudofirmus* DSM 8715^T

Values are percentages of total fatty acid. Data were determined in this study; only fatty acids accounting for at least 1 % of the total fatty acids are listed. −, Not detected (<1.0 %).

Fatty acid	GMBE 72^T	*B. pseudofirmus* DSM 8715^T
iso-$C_{10:0}$	−	1.0
iso-$C_{14:0}$	5.0	3.6
$C_{14:0}$	1.7	1.6
iso-$C_{15:0}$	24.2	16.5
anteiso-$C_{15:0}$	41.1	50.8
$C_{16:1}\omega7c$ alcohol	6.9	5.1
iso-$C_{16:0}$	2.7	2.5
$C_{16:1}\omega11c$	4.5	4.7
$C_{16:0}$	2.0	2.4
anteiso-$C_{17:0}$	4.5	4.7
iso-$C_{17:0}$	1.0	−
$C_{18:1}\omega9c$	1.1	−
Summed feature*	3.7	3.1

*Summed feature containing iso-$C_{17:1}$ I and/or anteiso-$C_{17:1}$ B.

The mean value for DNA–DNA hybridization between strain GMBE 72^T and *B. pseudofirmus* DSM 8715^T was 56 % (individual values 54.4 and 57.6 %). This value for hybridization is lower than the recommended threshold value accepted for defining a novel species (Wayne *et al.*, 1987), supporting the distinct position of strain GMBE 72^T within the genus *Bacillus*. Phenotypic properties of strain GMBE 72^T, such as the ability to hydrolyse Tween 80 and

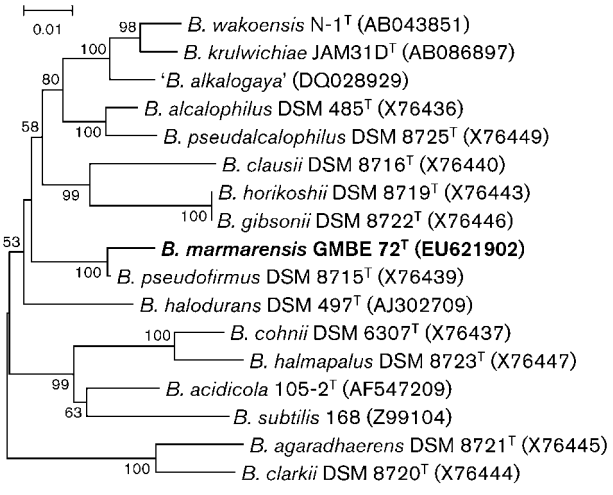

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the position of strain GMBE 72^T. Bootstrap values are shown as percentages of 1000 replicates; only values above 50 % are shown. Bar, 0.01 substitutions per nucleotide position.

the inability to hydrolyse starch and to grow in 14 % NaCl, also supported the view that the isolate was distinguishable from closely related *Bacillus* species (Table 1). Therefore, on the basis of physiological, biochemical and phylogenetic properties, strain GMBE 72^T represents a novel species within the genus *Bacillus*, for which the name *Bacillus marmarensis* sp. nov. is proposed.

Description of *Bacillus marmarensis* sp. nov.

Bacillus marmarensis (mar.ma.ren′sis. N.L. masc. adj. *marmarensis* pertaining to the region of Marmara, where the type strain was isolated).

Cells are aerobic, Gram-stain-positive, motile rods (0.8–1.1 × 2.0–2.5 μm) that produce ellipsoidal spores that are located subterminally to terminally. Colonies are cream–yellowish, circular, entire and convex. Catalase-positive and oxidase-negative. No growth is detected at pH 7.0–7.5. Growth occurs at pH 8–12.5 and 10–45 °C. The organism is able to grow at 12 % NaCl. It hydrolyses casein, gelatin and Tween 80, but does not hydrolyse urea, starch or Tweens 20, 40 or 60. Does not reduce nitrate to nitrite and does not utilize Simmons' citrate. Heterotrophic, utilizing D- and L-arabinose, ribose, glucose, fructose, mannose, methyl α-D-mannoside, methyl α-D-glucoside, *N*-acetylglucosamine, salicin, maltose, cellobiose, trehalose, sucrose, glycogen, xylitol, gentiobiose, turanose, D-arabitol and 2-ketogluconate, but not utilizing glycerol, erythritol, D- or L-xylose, adonitol, methyl β-D-xyloside, galactose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, amygdalin, arbutin, aesculin, lactose, melibiose, inulin, melezitose, raffinose, D-lyxose, D-tagatose, D- or L-fucose, L-arabitol, gluconate or 5-ketogluconate. Major fatty acids are anteiso-$C_{15:0}$ and iso-$C_{15:0}$. The detailed fatty acid profile is listed in Table 2. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The DNA G+C content of the type strain is 40.2 mol% (determined by HPLC).

The type strain, GMBE 72^T (=DSM 21297^T =JCM 15719^T), was isolated from mushroom compost from Yalova, Turkey.

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