Bacillus alveayuensis sp. nov., a thermophilic bacterium isolated from deep-sea sediments of the Ayu Trough

Seung Seob Bae, Jung-Hyun Lee and Sang-Jin Kim

Correspondence
Sang-Jin Kim
s-jkim@kordi.re.kr

Microbiology Laboratory, Korea Ocean Research and Development Institute, PO Box 29, Ansan, 425-600, Republic of Korea

Two thermophilic, spore-forming strains, TM1^T and TM5, were isolated from deep-sea sediment (4000 m below sea level) of the Ayu Trough in the western Pacific Ocean. Cells of the two strains were Gram-positive, motile and rod-shaped. Their spores were ellipsoidal, subterminal to terminal and occurred in swollen sporangia. The two strains grew at temperatures up to 65 °C and in the pH range 6.5–9.0. The NaCl concentration for optimal growth was 3.0% (w/v) and growth was inhibited by 5% (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strains TM1^T and TM5 belonged to the genus *Bacillus*, and that strain TM1^T was most closely related to *Bacillus aeolius* DSM 15084^T (96.7%), *Bacillus smithii* DSM 4216^T (96.1%), *Bacillus methanolicus* NCIMB 13113^T (95.8%) and *Bacillus pallidus* DSM 3670^T (95.7%). Between the 16S rRNA gene sequences of strains TM1^T and TM5 there were only three nucleotide differences, implying that the two strains were of the same species. The cellular fatty acid profiles of the two strains were also very similar, with iso-C_{15:0}, iso-C_{16:0}, C_{16:0}, iso-C_{17:0} and anteiso-C_{17:0} as the major components. The G + C content of strain TM1^T was 38.7%. On the basis of phenotypic and molecular data, strains TM1^T and TM5 represent a novel species of the genus *Bacillus*, for which the name *Bacillus alveayuensis* sp. nov. is proposed. The type strain is TM1^T (=KCTC 10634^T=JCM 12523^T).

Thermophilic bacilli – aerobic or facultatively anaerobic, endospore-forming organisms – that grow optimally over the temperature range 45 to <70 °C have been isolated from both thermophilic and mesophilic environments (Sharp *et al.*, 1992). Several thermophilic bacilli have been isolated from ocean-bottom mud samples (Egorova, 1938), deep ocean-basin cores (Bartholomew & Paik, 1966), shallow marine hot springs (Hjörleifsdóttir *et al.*, 1989) and deep-sea hydrothermal vents (Marteinsson *et al.*, 1995).

Currently, thermophilic bacilli are classified into seven genera: *Bacillus*, *Brevibacillus* (Sneath, 1986), *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Aneurinibacillus* (Heyndrickx *et al.*, 1997), *Geobacillus* (Nazina *et al.*, 2001), *Sulfobacillus* (Dufresne *et al.*, 1996) and *Thermobacillus* (Touzel *et al.*, 2000). Molecular taxonomy shows that most of the thermophilic bacilli described to date belong to either

Bacillus rRNA group 1 or *Bacillus* rRNA group 5 (Ash *et al.*, 1991; Rainey *et al.*, 1994). *Bacillus* rRNA group 1 contains the thermophilic species *Bacillus smithii* and *Bacillus coagulans*, as well as their mesophilic relatives (Ash *et al.*, 1991). *Bacillus* rRNA group 5 includes the genus *Geobacillus* and an aerobic, Gram-positive coccus, *Saccharococcus thermophilus* (Ash *et al.*, 1991; Rainey *et al.*, 1994; Nazina *et al.*, 2001).

Gram characterization of the strains was done by using the Bio-Rad Gram staining kit according to the manufacturer's instructions. Morphologies of cells and spores were observed under a phase-contrast microscope (Zeiss Axioplan) at 1000× magnification. To enhance sporulation, cells were grown in marine broth 2216 (MB; Difco) containing 5 mg MnSO₄ l⁻¹ at 55 °C for 1 day. Catalase and oxidase activities were determined by bubble production in a 3% hydrogen peroxide solution and by oxidation of 1% *p*-aminodimethylaniline oxalate, respectively. Acid production from carbohydrates and other physiological tests were determined with the API 50CHB and API 20NE systems (bioMérieux), respectively, according to the manufacturer's instructions, with the exception that concentration of NaCl in the saline solution was 2.0% (w/v) instead of 0.5%. To determine the optimal growth temperature, cells were tested at 30–70 °C in MB; to test the optimal

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains TM1^T and TM5 are AY605232 and AY606801, respectively.

A table giving details of the cellular fatty acid compositions of strains TM1^T and TM5 and related thermophilic *Bacillus* species is available as supplementary material in IJSEM Online.

growth pH, the distilled water in MB was replaced by the following buffers (Sigma) at a concentration of 20 mM: for pH 4, 5 and 5.5, MES; for pH 6 and 6.5, PIPES; for pH 7 and 7.5, HEPES; for pH 8, 9 and 10, AMPSO. The requirement of NaCl was tested using modified MB (per litre distilled water: 5 g bactopectone, 1 g yeast extract, 0.01 g $\text{FePO}_4 \cdot 4\text{H}_2\text{O}$) supplemented with different concentrations of NaCl. Turbidity was monitored automatically by a temperature-gradient incubator (TVS126MA; Advantec) and the optimal temperature, pH and salinity were determined by calculating the growth rate in the exponential phase. Hydrolysis of casein, starch and tributyrin was tested as described by Marteinsson *et al.* (1996) and Scholz *et al.* (1987). To confirm anaerobic growth, cells were inoculated into MB medium in a serum vial capped with an aluminium seal and cultivated at 55 °C for 7 days.

Genomic DNA was extracted from 1 ml aliquots of cells of TM1^T and TM5 cultured in MB medium by using the Wizard genomic DNA purification kit (Promega). The 16S rRNA gene was amplified by PCR and purified as described by Sohn *et al.* (2004). The PCR products were sequenced by using a BigDye terminator cycle sequencing kit (Applied Biosystems) and an ABI PRISM 3100 Genetic Analyser (Applied Biosystems). The nearly complete (1500 nt) 16S rRNA gene sequences of the strains were used for phylogenetic analysis. Alignment gaps and unidentified base positions were not taken into consideration for the analysis. The 16S rRNA gene sequences of strains TM1^T and TM5 were compared with those in the GenBank database by using the BLAST algorithm (Altschul *et al.*, 1990). Related sequences and the novel sequences were aligned by using CLUSTAL_X (Thompson *et al.*, 1997), and the alignment was refined using PHYDIT (Chun, 1995) and manual comparison considering the secondary structures. The phylogenetic analysis was performed by using the computer packages PHYLIP (Felsenstein, 1993) and PAUP* 4.0 (Swofford, 1998). Phylogenetic trees were inferred using the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), maximum-parsimony (Fitch, 1972) and neighbour-joining algorithms (Saitou & Nei, 1987). Distance matrices for the neighbour-joining and Fitch–Margoliash methods were generated according to the model of Jukes & Cantor (1969). The robustness of the topology in the phylogenetic trees was evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

Total lipids were extracted from cells that had been incubated at 55 °C for 1 day by using the method of Folch *et al.* (1957). For analysis of the fatty acid composition, total lipids were converted to fatty acid methyl esters (FAMES) by serial addition of 1.5% NaOH and 5% HCl in methanol; both reactions were repeated at 65 °C for 20 min. FAMES were analysed as described by Sohn *et al.* (2004). The G+C content (mol%) of strain TM1^T was determined by using the melting temperature method as

described by Mandel *et al.* (1970) and Marmur & Doty (1962). The melting temperature of purified chromosomal DNA extracted from *Escherichia coli* K-12 (KCTC 2443) was also determined, to serve as a control.

Strains TM1^T and TM5 had similar characteristics with respect to their cellular and colonial morphologies. Cells of both strains were Gram-positive, motile, rod-shaped (0.5–1.0 µm wide and 2.5–5 µm long) and occurred singularly or in chains. They produced ellipsoidal endospores that lay in terminal or subterminal positions and usually caused the sporangia to swell. Colonies that formed after 1 day incubation on marine agar 2216 (Difco) at 55 °C were circular, opaque, cream and convex with entire margins. They did not grow under anaerobic conditions. Both strains grew between 40 and 65 °C, with optimum growth at 55 °C. The strains grew in the pH range 6.5–9.0; the optimal growth pH was between pH 7.0 and 7.5. The NaCl concentration range for growth of strains TM1^T and TM5 was 0–4.0% (w/v), with the optimal concentration at 3.0 and 2.0% (w/v), respectively. From tests done using the API 20NE system (bioMérieux), both strains gave positive results for aesculin hydrolysis (β -glucosidase), acid production from glucose and assimilation of glucose, mannose and maltose, and negative results for oxidase, gelatin hydrolysis, reduction of nitrate to nitrite, reduction of nitrates to nitrogen, indole production, arginine dihydrolase, urease, β -galactosidase, utilization of citrate and assimilation of arabinose, mannitol, *N*-acetylglucosamine, gluconate, caprate, adipate, malate, citrate and phenyl acetate. From tests done using the API 50CHB system (bioMérieux), acid production by both strains was positive for D-fructose, D-glucose, D-mannose, D-trehalose, maltose, sucrose, aesculin, 5-ketogluconate and glycerol, and negative for other substrates tested. Neither strain hydrolysed starch or tributyrin, but casein was hydrolysed by both strains. Physiological characteristics useful for differentiating between strains TM1^T and TM5 and related thermophilic *Bacillus* species are summarized in Table 1.

The 16S rRNA gene sequences of strains TM1^T and TM5 were continuous stretches of 1532 and 1530 nt, respectively. The level of 16S rRNA gene sequence similarity between the two strains was 99.8%; hence, both strains should be assigned to a single taxon (Fig. 1). Based on 16S rRNA gene sequence similarity data, it was found that the closest relatives of strain TM1^T were *Bacillus aeolius* DSM 15084^T (96.7%), *B. smithii* DSM 4216^T (96.1%), *Bacillus methanolicus* NCIMB 13113^T (95.8%) and *Bacillus pallidus* DSM 3670^T (95.7%). Sequence similarity of strain TM1^T with other *Bacillus* species with validly published names was less than 97%. There are widely accepted criteria for delineating species in current bacteriology: these state that strains with DNA relatedness values of less than 70% or with more than 3% difference in their 16S rRNA gene sequences are considered to represent different species (Wayne *et al.*, 1987).

The cellular fatty acids of strains TM1^T and TM5 ranged

Table 1. Physiological characteristics of strains TM1^T and TM5 that differentiate them from related thermophilic *Bacillus* type strains

Strains: 1, TM1^T; 2, TM5; 3, *B. aeolius* DSM 15084^T; 4, *B. smithii* DSM 4216^T; 5, *B. methanolicus* NCIMB 13113^T; 6, *B. pallidus* DSM 3670^T. +, Positive; –, negative; d, variable; w, weak reaction; ND, not determined. All strains were positive for acid production from D-glucose, and negative for reduction of nitrate to nitrite and acid production from lactose.

Characteristic	1	2	3*	4†	5‡	6§
Growth at:						
70 °C	–	–	–	–	–	+
65 °C	+	+	+	+	–	+
25 °C	–	–	–	+	–	–
pH 8.0	+	+	+	–	–	+
Growth in the presence of:						
3 % NaCl	+	+	+	–	–	+
10 % NaCl	–	–	–	–	–	+
Catalase	+	+	+	+	+	+
Oxidase	–	–	+	+	+	+
Hydrolysis of:						
Aesculin	+	+	–	d	ND	d
Casein	+	+	+	–	–	–
Starch	–	–	+	w	–	w
Tributyrin	–	–	ND	ND	ND	+
Utilization of citrate	–	–	+	d	ND	–
Anaerobic growth	–	–	–	+	–	–
Acid from:						
D-Fructose	+	+	+	+	–	+
D-Galactose	–	–	–	d	–	–
D-Mannose	+	+	+	d	ND	–
D-Ribose	–	–	+	d	+	–
D-Trehalose	+	+	+	+	–	d
D-Xylose	–	–	+	d	–	–
Cellobiose	–	–	+	d	–	–
Maltose	+	+	+	d	+	d
Rhamnose	–	–	–	d	ND	–
Raffinose	–	–	+	–	+	ND
Sucrose	+	+	+	–	–	+
Glycogen	–	–	–	d	–	–
Glycerol	+	w	+	d	–	ND
Mannitol	–	–	+	d	+	–
Sorbitol	–	–	–	d	+	ND
5-Ketogluconate	+	+	+	–	ND	ND
DNA G + C content (mol%)	38.7	ND	40.8	38.2–41.7	48–50	39–41

*Data from Gugliandolo *et al.* (2003).

†Data from Nakamura *et al.* (1988).

‡Data from Arfman *et al.* (1992).

§Data from Scholz *et al.* (1987).

from C₁₂ to C₁₈ and included saturated, monoenoic and iso-branched components. In both strains, iso-C_{15:0}, iso-C_{16:0}, C_{16:0}, iso-C_{17:0} and anteiso-C_{17:0} were the major fatty acids. A comparison of cellular fatty acid compositions of strains TM1^T and TM5 and related thermophilic *Bacillus* species is given in a Supplementary Table in IJSEM Online.

The DNA G + C content of strain TM1^T was 38.7 mol%. On the basis of the above-mentioned phenotypic and genetic characteristics of strains TM1^T and TM5, we consider the two strains to be distinct from other *Bacillus* species and propose that they are assigned to a novel species, *Bacillus alveayuensis* sp. nov.

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences, showing relationships between strains TM1^T and TM5 and some closely related taxa of the family *Bacillaceae*. Bootstrap percentage values based on neighbour-joining analyses of 1000 replications have been included at nodes, and solid circles indicate that the corresponding nodes (groupings) are also recovered in Fitch–Margoliash, maximum-parsimony and maximum-likelihood trees. Bar, 0.1 nucleotide substitution per position.

Description of *Bacillus alveayuensis* sp. nov.

Bacillus alveayuensis [al.ve.a.yu.en'sis. L. n. *alveus* trough; N.L. masc. adj. *ayuensis* pertaining to Ayu (as a locality); N.L. masc. adj. *alveayuensis* pertaining to the Ayu Trough in the Pacific Ocean].

Cells are Gram-positive, rod-shaped (0.5–1.0 µm wide and 2.5–5 µm long), motile and occur singularly or in chains. They produce ellipsoidal endospores that lie in terminal or subterminal positions and usually cause the sporangia to swell. Colonies that form after 1 day incubation on marine agar 2216 at 55 °C are circular, opaque and cream. Obligate aerobe. Grows optimally at 55 °C, pH 7.0–7.5 and 3.0 % NaCl. Does not grow in marine broth 2216 below 40 °C or higher than 65 °C and 4.0 % NaCl. Data on the utilization of carbon sources and on the hydrolysis of chromogenic substrates are shown in Table 1. The major fatty acids are 13-methyl tetradecanoic acid (iso-C_{15:0}), 14-methyl pentadecanoic acid (iso-C_{16:0}), hexadecanoic

acid (C_{16:0}), 15-methyl hexadecanoic acid (iso-C_{17:0}) and 14-methyl hexadecanoic acid (anteiso-C_{17:0}).

The type strain (TM1^T=KCTC 10634^T=JCM 12523^T) and a reference strain (TM5) were isolated from deep-sea sediment of the Ayu Trough (4000 m below sea level) in the western Pacific Ocean. The DNA G + C content of the type strain is 38.7 mol%.

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