The Journal of Microbiology (2010) Vol. 48, No. 6, pp. 867-871
DOI 10.1007/s12275-010-0148-0

NOTE

Bacillus gaemokensis sp. nov., Isolated from Foreshore Tidal Flat Sediment from the Yellow Sea

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(Received April 16, 2010 / Accepted August 16, 2010)

A Gram-positive, rod-shaped, endospore-forming organism, strain BL3-6^T, was isolated from tidal flat sediments of the Yellow Sea in the region of Tae-An. A 16S rRNA gene sequence analysis demonstrated that this isolate belongs to the *Bacillus cereus* group, and is closely related to *Bacillus mycoides* (99.0% similarity), *Bacillus thuringiensis* (99.0%), *Bacillus weihenstephanensis* (99.0%), *Bacillus cereus* (98.9%), *Bacillus anthracis* (98.8%), and *Bacillus pseudomycoides* (98.1%). The phylogenetic distance from any validly described *Bacillus* species outside the *Bacillus cereus* group was less than 95.6%. The DNA G+C content of the strain was 39.4 mol% and the major respiratory quinone was menaquinone-7. The major cellular fatty acids were iso-$C_{14:0}$ (17.8%), iso-$C_{16:0}$ (15.8%), and iso-$C_{12:0}$ (11.3%). The diagnostic amino acid of the cell wall was *meso*-diaminopimelic acid and the major cell wall sugar was galactose. The results of DNA-DNA hybridization (<55.6%) and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain BL3-6^T from the published *Bacillus* species. BL3-6^T therefore represents a new species, for which the name *Bacillus gaemokensis* sp. nov. is proposed, with the type strain BL3-6^T (=KCTC 13318^T =JCM 15801^T).

***Keywords*:** *B. cereus*, *B. gaemokensis* sp. nov., phylogenetic, new species

Bacillus cereus group comprises the following six recognized species: *B. cereus*, *B. anthracis*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, and *B. weihenstephanensis* (Ash *et al*., 1991; Drobniewski, 1993; Lechner *et al.*, 1998). These are Gram-positive, spore-forming, rod-shaped bacteria that appear in diverse habitats and as part of the intestinal flora of various animals (Drobniewski, 1993; Lechner *et al.*, 1998; von Stetten *et al.*, 1999; Stenfors Arnesen *et al.*, 2008). Sequence comparison of the 16S rRNA gene from 4 strains within the *B. cereus* group - *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. mycoides* have shown that they are very closely related (Seki *et al.*, 1978; Nakamura, 1998; Kim *et al.*, 2008). DNA-DNA hybridization has been applied in the classification of species in this group, but it is not easy to differentiate the taxa due to their high genetic homology (Kaneko *et al.*, 1978; Nakamura and Jackson, 1995). The members of the *B. cereus* group have been traditionally classified as different species based on their distinct virulence and morphological characteristics: *B. mycoides* and *B. pseudomycoides* are distinguished from *B. cereus* by rhizoidal colony shape and fatty acid composition (Nakamura, 1998). *B. weihenstephanensis* is physiologically separated by its unique phychrotolerant characteristic (Lechner *et al.*, 1998). *B. cereus* is widely present in food production environments and causes food poisoning and other infections (Drobniewski, 1993; Granum and Lund, 1997). *B. anthracis* and *B. thuringiensis* are mammalian and insect pathogens that cause acute and often lethal disease (Jackson *et al.*, 1997; Cardazzo *et al.*, 2008). In this study, we explore and survey microbial diversity from foreshore tidal flat sediment of the Yellow Sea, and have taxanomically described a new *Bacillus* species.

The samples of tidal flat sediment (20-25 cm depth) were collected from the oil-contaminated foreshore of the Gaemok Harbor in Tae-An, a coastal region of the Yellow Sea in the Republic of Korea. Bacterial strains were isolated by the dilution-plating technique on a tryptic soy broth medium (pH 7.3, TSB, Difco, USA) solidified with 15.0 g agar per liter (TSA), and incubated at 30°C. Isolates were subcultured several times to obtain a purified culture, and were then further characterized. The reference strains used in the study were *B. anthracis* ATCC 14185 (the genomic DNA was granted by courtesy of Dr. Kim Wonyong, College of Medicine, Chung-Ang University), *B. cereus* KCTC 3624^T, *B. mycoides* KCTC 3453^T, *B. pseudomycoides* KCTC 3862^T, *B. thuringiensis* KCTC 3452^T, and *B. weihenstephanensis* KCTC 3975^T. Strains were cultured routinely on TSA medium and

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stored at -80°C.

Strains were subjected to biochemical, physiological, and morphological analyses to determine the differential phenotypic properties of the novel isolates as described previously (Chang *et al.*, 2002, 2008). The morphology of bacterial cells grown on TSB medium was examined by bright field microscopy (Nikon Optiphot-2, Japan), phase-contrast microscopy (Nikon 80i), and electron microscopy. For the electron microscopy analysis, cells were fixed in a 2.5% paraformaldehyde/glutaraldehyde mixture, coated with gold in a sputter coater (SC502, Polaron), and observed using a scanning electron microscope (S4300N, Hitachi). Cells were negatively stained with 1% (w/v) uranyl acetate and the flagella type was observed using a model CM-20 transmission electron microscope (Philips; Chang *et al.*, 2002). The temperature (5-60°C) and NaCl ranges (0-7%, w/v) for growth were evaluated over a period of 7 days of incubation in TSB medium. Optical density was monitored at 600 nm (Bio-Rad, USA) in order to assess growth. Anaerobic growth was examined under anaerobic conditions (Forma Anaerobic System, USA) using a gas phase of $H_2/CO_2/N_2$ (7:5:88%, v/v). The pH range (pH 4.0-10.0 in 1.0-unit increments) for growth was determined in buffered medium in a 500 ml flask (working volume of 350 ml) at 30°C. The TSB medium was buffered by three different solutions, 50 mM succinic acid/NaOH (pH 4-6), 100 mM Na_2HPO_4/NaH_2PO_4 (pH 6-8), or 50 mM 2-amino-2-methyl-1,3-propanediol/HCl (pH 8-10). Motility was tested in a semi-solid TSA medium containing 0.4% agar. Spore and Gram staining and activity for catalase and oxidase were assessed using standard procedures and previously described methods (Smibert and Krieg, 1994; Chang *et al.*, 2008). Fingerprinting of various biochemical characteristics was performed using API 20E and 50CH galleries (bioMérieux, France) according to the manufacturers' instructions.

Respiratory quinones were examined as described previously (Komagata and Suzuki, 1987) using TLC and HPLC. To analyze cellular fatty acids, the strains were grown on a TSA medium at 28℃ for 48 h and determined according to a standard protocol (Sherlock Microbial Identification System; MIDI, USA). The extracts of fatty acids were analyzed using an automated GC system (model 6890N and 7683 auto-sampler; Agilent) with the associated software package. Analysis of the cell wall peptidoglycan was performed as described previously (Schleifer and Kandler, 1972) with the modification that TLC on cellulose was applied instead of paper chromatography. The cell wall sugar patterns in whole-cell wall hydrolysates were obtained as described previously (Staneck and Roberts, 1974), using TLC on cellulose plates with the solvent system.

DNA-DNA hybridization was determined by the microplate method, as previously described (Ezaki *et al*., 1989) with three replications for each sample. The plate was prehybridized for 30 min and then hybridized with photobiotin-labelled probes in the presence of 2× SSC and 50% formamide at 45°C for 4 h. The fluorescence intensity was measured by a Flouroskan Ascent Fluorescent plate reader (Thermo Life Science, UK). The highest and lowest values were excluded from each sample, and the means of the remaining three values are quoted as the DNA relatedness value. The DNA G+C content (mol%) of the genomic DNA was analyzed by real-time thermocycling (Thermocycler, Bio-Rad) with SYBR Green I, using a fluorimetric method, as described previously (Gonzalez and Saiz-Jimenez, 2002). The relative G+C content was calculated using *E. coli* KCTC 2441^T DNA (50.8 mol%, Tm) as a standard.

Nearly complete 16S rRNA gene sequences for strain BL3-6^T were determined according by Chang *et al*. (2008). The sequence (1,351 bp) of the strain was aligned manually against sequences retrieved from the GenBank database. Phylogenetic trees were constructed using the neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981), and maximum-parsimony (Fitch, 1971) methods. Evolutionary distance matrices were generated according to the model of Jukes and Cantor (1969) by using the PHYLIP software package (Felsenstein, 1993). The stability of the resultant trees was assessed by bootstrap analyses (Felsenstein, 1985) of the neighbor-joining method based on 1,000 resamplings.

Strain BL3-6^T was positive for catalase activity, but negative for oxidase activity. This strain grew in both aerobic and anaerobic conditions. Growth occurred at 15-40°C (optimum, 30°C) and at pH values of 5.0-9.0 (optimum, pH 7.0). The strain grew in the presence of NaCl concentrations of 0-6.0% (w/v) but not concentrations above 7%. The detailed phenotypic characteristics of the novel isolate are summarized in Table 1 and in the species description.

A preliminary analysis of the 16S rRNA gene sequence for strain BL3-6^T was performed by an initial BLAST search against the GenBank database. The results demonstrated that the isolate belongs to the *B. cereus* group and is closely related to *B. mycoides* ATCC 6462^T and *B. thuringiensis* ATCC 10792^T. Strain BL3-6^T and the members of *B. cereus* group were presented in a single clade sharing common ancestry with the genus *Bacillus*. The clade was confirmed by other treeing algorithms and supported by a 100% bootstrap value. The results of our comprehensive phylogenetic analyses are shown in Fig. 1. The closest phylogenetic relatives to strain BL3-6^T were *B. mycoides* (99.0% similarity) followed by *B. thuringiensis* (99.0%), *B. weihenstephanensis* (99.0%), *B. cereus* (98.9%), *B. anthracis* (98.8%), and *B. pseudomycoides* (98.1%). Similarities between the isolate and the members of the *B. cereus* group were greater than 98% (98.1-99.0%), whereas their similarity to other *Bacillus* species was below 95.6%. Previous studies also demonstrated a high degree of sequence similarity between species of the *B. cereus* group (Ash *et al*., 1991; Henderson *et al.*, 1994; Lechner *et al*., 1998; Nakamura, 1998; Kim *et al*., 2008). A lower sequence variation was observed in sequence comparisons within the group and additional DNA-DNA hybridization was subsequently carried out to differentiate these closely related species more accurately.

DNA-DNA hybridization values between strain BL3-6^T and *B. anthracis* ATCC 14185, *B. weihenstephanensis* KCTC 3975^T, *B. mycoides* KCTC 3453^T, *B. cereus* KCTC 3624^T, *B. thuringiensis* KCTC 3452^T, and *B. pseudomycoides* KCTC 3862^T were 56.6 (±4.2), 53.2 (±3.7), 42.1 (±2.5), 40.0 (±3.5), 35.8 (±4.3), and 29.7 (±2.2)%, respectively. The values of intraspecies of the *B. cereus* group range from 29.7% to 56.6%, whereas their 16S rRNA sequence similarities were 98.1-99.0%. Considering that the *B. cereus* group showed a high degree of genetic homogeneity between species group (Ash *et al*., 1991; Henderson *et al.*, 1994; Nakamura, 1998), these lower

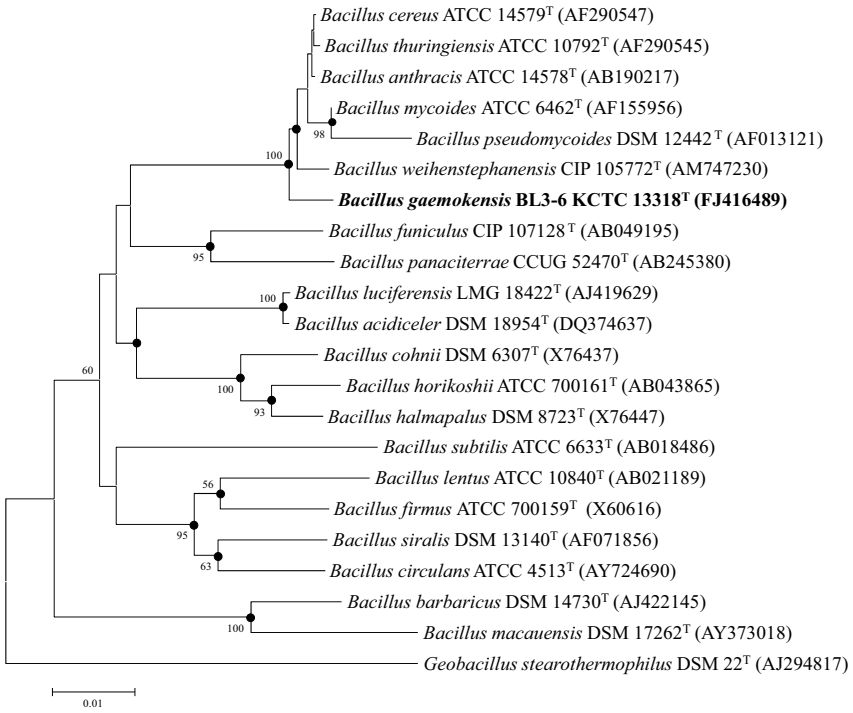

Fig. 1. Phylogenetic tree inferred by neighbour-joining method based on 16S rRNA gene sequences (1,351 bp). It shows relationships of strain BL3-6^T within the genus *Bacillus*. Bootstrap values are calculated from 1,000 replications and values >50% are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the tree generated with the maximum-parsimony algorithm. Bar, 0.01 changes per nucleotide position.

Table 1. Differential biochemical characteristics of strain BL3-6^T and closely related species
Strains: 1, strain BL3-6^T; 2, *B. mycoides* KCTC 3453^T; 3, *B. thuringiensis* KCTC 3452^T; 4, *B. weihenstephanensis* KCTC 3975^T; 5, *B. cereus* KCTC 3624^T; 6, *B. pseudomycoides* KCTC 3862^T; 7, *B. anthracis* (data from Claus and Berkeley, 1986). Data from this study except for *B. anthracis*. All strains are positive for catalase, gelatinase, and glucose. Symbols: +, positive; -, negative; ND, not determined; w, weakly positive.

Characteristics	1	2	3	4	5	6	7
Oxidase	-	-	-	-	v	-	-
Nitrate reduction	+	-	+	+	+	+	-
Anaerobic growth	+	+	+	-	+	+	+
Growth temperature	15-40	7-30	10-43	5-43	10-50	15-40	ND
NaCl 10%	-	-	+	+	-	-	-
Arginine dihydrolase	+	-	+	-	+	+	-
Acetoin production (VP)	+	+	+	+	+	+	-
Citrate utilization	-	-	+	-	+	-	+
Acid production from:							
Ribose	+	-	+	-	w	+	-
Glucose	+	+	+	+	+	+	-
Fructose	+	+	+	+	+	+	-
Mannose	+	-	+	-	-	-	-
N-acetyl-glucosamine	-	-	+	+	+	+	-
Arbutin	+	-	-	+	+	-	-
Esculin	-	w	+	+	+	-	-
Salicin	+	-	+	+	+	-	-
Cellobiose	-	-	-	-	+	-	-
Maltose	+	+	+	+	+	+	-
Sucrose	+	+	+	-	+	-	-
Trehalose	+	+	+	+	+	+	-
Starch	+	-	+	-	+	+	-
Glycogen	+	-	+	-	+	+	+
Turanose	+	-	+	-	-	-	-

Table 2. Cellular fatty acid compositions of strain BL3-6^T and related *Bacillus* species. Strains: 1, strain BL3-6^T; 2, *B. mycoides* KCTC 3453^T; 3, *B. thuringiensis* KCTC 3452^T; 4, *B. weihenstephanensis* KCTC 3975^T; 5, *B. cereus* KCTC 3624^T; 6, *B. pseudomycoides* KCTC 3862^T. All data from this study. Values are percentages of total fatty acid detected. t, trace amount (<1.0%); -, not detected. Summed feature 2 contains iso-$C_{16:0}$ I/14:0 3OH and/or $C_{14:0}$ 3OH/ iso-$C_{16:1}$. Summed feature 3 contains-$C_{16:1}$ ω7c/ iso-$C_{15:0}$ 2OH, and/or iso-$C_{15:0}$ 2OH/$C_{16:1}$ ω7c.

Fatty acid	1	2	3	4	5	6
12:0 iso	11.3	t	t	1.6	t	6.4
12:0	1.6	t	t	t	t	3.5
13:0 iso	5.0	11.8	11.2	16.6	13.9	10.6
13:0 anteiso	6.0	t	1.1	1.7	1.7	4.9
14:0 iso	17.8	2.8	5.1	4.7	4.3	2.4
14:0	4.3	3.2	3.6	4.5	3.1	3.4
15:0 iso	5.1	33.5	31.3	29.2	33.2	33.2
15:0 anteiso	6.1	2.1	3.4	2.7	4	1.9
15:0	1.7	-	t	-	-	1.1
16:1 ω7c alcohol	-	2.1	t	1.3	t	-
16:0 iso	15.8	5.5	4.5	5.8	4.2	6.8
16:1 ω11c	-	2.9	-	1.2	-	
16:0	4.2	5.3	3.1	7.7	3.1	8.2
15:0 2OH	-	t	t	-	1.1	-
iso 17:1 ω10c	-	10.6	2	5.7	2.6	-
iso 17:1 ω5c	-	2	4.2	2.1	5.3	-
17:1 anteiso A	t	-	-	-	t	-
17:0 iso	2.0	6.8	13.7	7.6	5.9	8.9
17:0 anteiso	3.3	t	-	1	1.5	t
18:1 iso H	1.3	-	-	-	-	-
18:0	-	-	-	-	-	2.6
Summed Feature 2	4.8	t	3.5	-	2.9	-
Summed Feature 3	7.6	6.8	9.7	5.4	9.1	3.8

values strongly supported that strain BL3-6^T can be differentiated from members of this group. Furthermore, the values were much lower than the suggested threshold value for species delineation (Wayne *et al.*, 1987), indicating that strain BL3-6^T represents a novel species distinct from members of the genus *Bacillus* (Stackebrandt and Goebel, 1994).

The DNA G+C content of strain BL3-6^T was 39.4 mol% (mean), which is consistent with the range of values for the *B. cereus* group (31.7-40.1 mol%; Priest *et al.*, 1988) within the genus *Bacillus* (32-69%; Claus and Berkeley, 1986). The major menaquinone MK-7 of strain BL3-6^T corresponded with that found in members of the genus *Bacillus,* and strain BL3-6^T shared this characteristic with this genus (Claus and Berkeley, 1986; Ahmed *et al.*, 2007). In the analysis of complete cell-wall hydrolysates, the *meso*-diaminopimelic acid in strain BL3-6^T was determined to be a diagnostic amino acid, indicating that the peptidoglycan type is A1γ (Schleifer and Kandler, 1972). The type species of the genus *Bacillus*, *B. subtilis*, produced results similar to strain BL3-6^T, which also supports assignment of the strain to the genus *Bacillus* (Drobniewski, 1993; Ahmed *et al.*, 2007). Additionally, galactose was determined to be the major cell- wall sugar in strain BL3-6^T.

The major cellular fatty acids of strain SL3-6^T were iso-$C_{14:0}$ (17.8%), iso-$C_{16:0}$ (15.8%), and iso-$C_{12:0}$ (11.3%). The fatty acid profile of strain was remarkably different from those of the *B. cereus* group in the content of iso-$C_{13:0}$, iso-$C_{15:0}$, and $C_{16:0}$ (Table 2). In the fatty acid analysis, all the members of the *B. cereus* group showed similar profiles with the largest amounts iso-$C_{15:0}$ (29.1-33.5%). The major cellular fatty acids of this group were iso-$C_{13:0}$ and iso-$C_{15:0}$ (Nakamura, 1998; Venkateswaran *et al.*, 2003). The results of the fatty acid analysis clearly segregated strain SL3-6^T from the closely related species. According to the polyphasic taxonomic data presented in this study, the isolated strain BL3-6^T can be differentiated from members of the genus *Bacillus* and assigned to a novel species by the name of *Bacillus gaemokensis* sp. nov.

Description of *Bacillus gaemokensis* sp. nov.

Bacillus gaemokensis: ga.e.mo.ken'sis. N.L. masc. adj. gaemokensis, pertaining to the Gaemok, Republic of Korea, the geographical origin of the type strain of the species.

Cells grow as Gram-positive, straight rod (1.5-2.0×6.0-7.0 μm), and round-ended bacteria and occur singly and in pairs. Cells are motile with peritrichous flagella and contain terminal ellipsoidal endospores. Colonies grown on TSA medium for 48 h are round, entire, convex, and grayish-white (2.2-3.4 mm in diameter). Cells are catalase-positive and oxidase-negative. Cells grow at 15-40°C; optimum growth occurs at 30°C. Growth occurs at pH 5.0-9.0; optimum growth occurs at pH 7.0. Cells grow in 5% NaCl but not above 7%. Cells grow under an anaerobic condition. Positive for nitrate reduction, starch, casein, and gelatin hydrolysis. Acid production from arbutin, glycerol, ribose, glucose, fructose, mannose, maltose, trehalose, sucrose, salicin, turanose, and glycogen, but not from cellobiose, esculin, xylose, adonitol, galactose, sorbose, rhamnose, inositol, mannitol, sorbitol, lactose, raffinose, fucose, *N*-acetyl-glucosamine or arabinose. Positive for production of acetoin, gelatinase, and arginine dihydrolase, but not production of indole, urease, H_2S, nitrogen, or citrate utilization. The diagnostic amino acid is *meso*-diaminopimelic acid and the major cell-wall sugar is galactose. Predominant cellular fatty acids were iso-$C_{12:0}$, iso-$C_{14:0}$, and iso-$C_{16:0}$. The major menaquinone is MK-7. The DNA G+C content is 39.4 mol%.

The type strain, BL3-6^T (=KCTC 13318^T =JCM 15801^T), was isolated from oil-contaminated tidal flat sediment collected from Gaemock Harbor in the Yellow Sea coastal region of Tae-An, Republic of Korea.

We are grateful to Dr. Jean. P. Euzeby (Society for Systematic and Veterinary Bacteriology, France) for his advice on nomenclatural queries. This work was supported by grants NMC0300938, KOSEF (no. M10508040002-07N0804-00216) and the KRIBB Research Initiative Program funded by the Ministry of Education, Science and Technology, Republic of Korea.

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