

NOTE

***Bacillus manliponensis* sp. nov., a New Member of the *Bacillus cereus* Group Isolated from Foreshore Tidal Flat Sediment<sup>§</sup>**

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A Gram-positive, endospore-forming, new *Bacillus* species, strain BL4-6<sup>T</sup>, was isolated from tidal flat sediment of the Yellow Sea. Strain BL4-6<sup>T</sup> is a straight rod, with motility by peritrichate flagella. The cell wall contains *meso*-diaminopimelic acid, and the major respiratory quinone is menaquinone-7. The major fatty acids are iso-C<sub>15:0</sub> and summed feature 3 (containing C<sub>16:1</sub> ω7c/ iso-C<sub>15:0</sub> 2OH, and/or iso-C<sub>15:0</sub> 2OH/C<sub>16:1</sub> ω7c). Cells are catalase-positive and oxidase-negative. The G+C content of the genomic DNA is 38.0 mol%. Based on a comparative 16S rRNA gene sequence analysis, the isolate belongs to the genus *Bacillus*, forms a clade with the *Bacillus cereus* group, and is closely related to *Bacillus mycoides* (98.5%), *Bacillus cereus* (98.5%), *Bacillus anthracis* (98.4%), *Bacillus thuringiensis* (98.4%), *Bacillus weihenstephanensis* (98.1%), and *Bacillus pseudomycooides* (97.5%). The isolate showed less than 85% similarity of the *gyrA* gene sequence and below 95% similarity of the *rpoB* gene sequence to the members of this group. DNA-DNA relatedness between strain BL4-6<sup>T</sup> and *B. cereus* group was found to be in a range of 22.8–42.3%, and thus BL4-6<sup>T</sup> represents a unique species. On the basis of these studies, strain BL4-6<sup>T</sup> (=KCTC 13319<sup>T</sup> =JCM 15802<sup>T</sup>) is proposed to represent the type strain of a novel species, *Bacillus manliponensis* sp. nov.

**Keywords:** *Bacillus cereus* group, *Bacillus manliponensis* sp. nov., phylogenetic, new species

*Bacillus anthracis*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycooides*, and *Bacillus weihenstephanensis* are members of the *B. cereus* group (Ash *et al.*, 1991; Drobniowski, 1993; Lechner *et al.*, 1998). Most members of this group have been classified as different species based on their distinct virulence and physiological characteristics (Claus and Berkeley, 1986; Priest *et al.*, 1988; Drobniowski, 1993; Nakamura and Jackson, 1995). However, sequence comparison of the 16S rRNA gene within this group has shown that they are very closely related and it is not easy to differentiate the taxa due to their high genetic homology (Kaneko *et al.*, 1978; Ash *et al.*, 1991; Henderson *et al.*, 1994; Nakamura, 1998; Kim *et al.*, 2008). There have been reports documenting that the only established difference between *B. cereus* and *B. thuringiensis* strains is the presence of genes coding for insecticidal toxins, usually present in plasmids (Thorne, 1993; Helgason *et al.*, 2000). *B. thuringiensis* can no longer be dis-

tinguished from *B. cereus* when these plasmids are lost (Thorne, 1993). Helgason *et al.* (2000) contended that *B. anthracis*, *B. thuringiensis*, and *B. cereus* should be considered as belonging to the same species due to the close similarity of the genomes. There have been no new species added to this group during the past decade. Recently, we reported a new *Bacillus* species within the *B. cereus* group (Jung *et al.*, 2010). In this study, we report another novel species of this group obtained from foreshore tidal flat sediment of the Yellow Sea.

For isolation of the strain, about 50 g of tidal flat sediment (25 cm depth) was collected from the oil-contaminated foreshore of Malipo beach in Tae-An, a coastal region of the Yellow Sea in the Republic of Korea. Collected samples were placed individually into sterile tubes (50 ml, Corning, USA) and pooled into an anaerobic pouch (Gas Pak, Becton Dickinson Microbiology Systems, USA) in an icebox, and then transported to the laboratory and processed immediately. Each of the samples was serially diluted in saline solution (0.85% NaCl, w/v), spread onto a tryptic soy broth medium (pH 7.3, TSB, Difco, USA) solidified with 15.0 g agar per liter

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**Table 1.** Differential physiological characteristics of strain BL4-6<sup>T</sup> and closely related *Bacillus* species

Characteristic	1	2	3	4	5	6	7
Anaerobic growth	+	+	+	+	-	+	+
Growth temperature	15-40	15-40	7-30	10-40	5-40	10-50	15-40
Growth in NaCl 10%	-	-	-	+	+	-	-
Arginine dihydrolase	-	+	-	+	-	+	+
Oxidase	-	-	-	-	-	v	-
Acetoin production (VP)	-	+	+	+	+	+	+
Citrate utilization	-	-	-	+	-	+	-
Nitrate reduction	+	+	-	+	+	+	+
Hydrolysis:							
Starch	-	+	+	+	+	+	-
Casein	-	-	+	+	+	+	+
Acid production from:							
Ribose	+	+	-	+	-	w	+
Fructose	-	+	+	+	+	+	+
Mannose	-	+	-	+	-	-	-
<i>N</i> -Acetyl-glucosamine	+	-	-	+	+	+	+
Arbutin	-	+	-	-	+	+	-
Esculin	+	-	w	+	+	+	-
Salicin	-	+	-	+	+	+	-
Cellobiose	-	-	-	-	-	+	-
Sucrose	-	+	+	+	-	+	-
Trehalose	-	+	+	+	+	+	+
Starch	-	+	-	+	-	+	+
Glycogen	-	+	-	+	-	+	+
Turanose	-	+	-	+	-	-	-

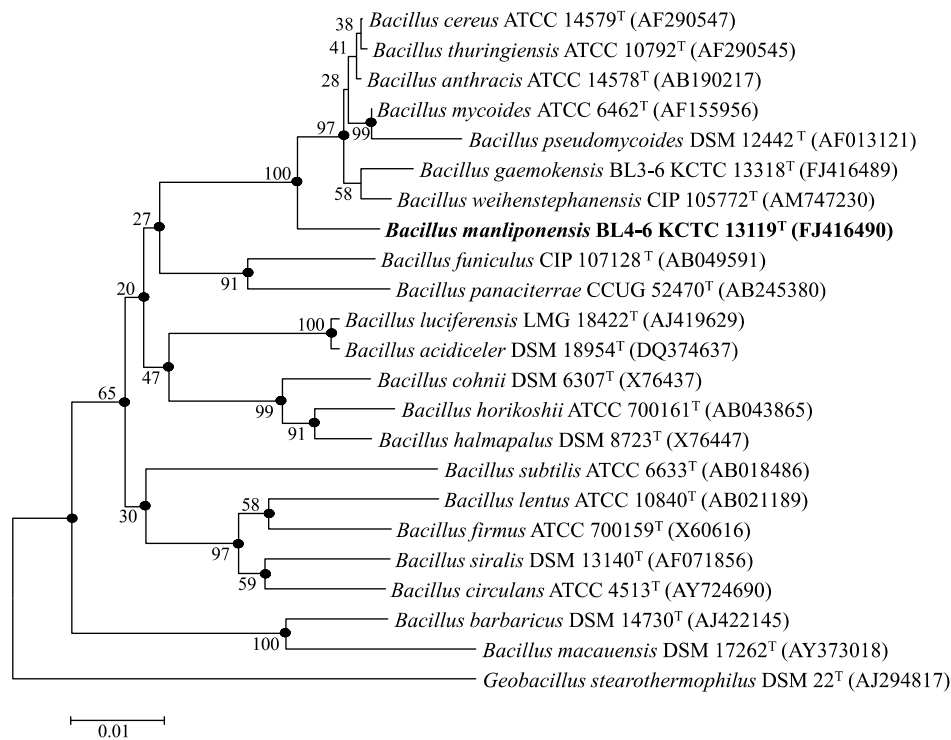
Taxa are indicated as: 1, strain BL4-6<sup>T</sup>; 2, *B. gaemokensis* KCTC 13318<sup>T</sup>; 3, *B. mycoides* KCTC 3453<sup>T</sup>; 4, *B. thuringiensis* KCTC 3452<sup>T</sup>; 5, *B. weihenstephanensis* KCTC 3975<sup>T</sup>; 6, *B. cereus* KCTC 3624<sup>T</sup>; 7, *B. pseudomycooides* KCTC 3862<sup>T</sup>. All data are taken from this study and Jung *et al.* (2010). All taxa are positive for catalase, gelatinase, glucose, and maltose. DNase reaction and hydrolysis of Tween 40 and 60, but negative for hydrolysis of Tween 20 and 80. +, positive; -, negative; v, variable; w, weakly positive.

(TSA), and incubated at 30°C for 48 h. The isolated strains were subcultured several times to obtain a purified culture, and were then further characterized. The reference strains used in this study were *B. cereus* KCTC 3624<sup>T</sup>, *B. mycoides* KCTC 3453<sup>T</sup>, *B. pseudomycooides* KCTC 3862<sup>T</sup>, *B. thuringiensis* KCTC 3452<sup>T</sup>, *B. weihenstephanensis* KCTC 3975<sup>T</sup>, and *B. gaemokensis* BL3-6<sup>T</sup> (KCTC 13318<sup>T</sup>). Strains were cultured routinely on TSA media under identical conditions and stored in a deep freezer (-80°C) as skim milk (Difco) suspensions (10%, w/v).

To determine the differential phenotypic properties of the novel isolate, strains were subjected to physiological, biochemical, and morphological analyses (Smibert and Krieg, 1994; Chang *et al.*, 2002). The morphology of the bacterial cells was examined by phase-contrast microscopy (Nikon 80i, Japan) and electron microscopy. For the electron microscope analysis, cells were fixed in a 2.5% paraformaldehyde/glutaraldehyde mixture, coated with gold in a Sputter Coater (SC502, Polaron), and observed with a scanning electron microscope (S4300N, Hitachi, Japan). Cells were negatively stained with 1% (w/v) uranyl acetate and the flagella type was observed using a model CM-20 Philips transmission electron microscope (Chang *et al.*, 2002). The temperature (5-60°C) and NaCl range (0-10%, w/v) for growth were determined over a period of 3-7 days of incubation in TSB medium. The pH range (pH 4.0-10.0) for growth was determined in 250 ml of buffered TSB me-

dium at 30°C in a 500 ml flask (Corning). The medium was buffered by three different solutions, 50 mM succinic acid/NaOH (pH 4-6), 100 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> (pH 6-8), or 50 mM 2-amino-2-methyl-1,3-propanediol/HCl (pH 8-10). Optical density was monitored at 600 nm (Bio-Rad, USA) in order to assess growth. The physiological and biochemical characterizations were assessed using standard procedures and previously described methods (Smibert and Krieg, 1994; Chang *et al.*, 2002). All tests were performed under identical conditions of growth temperature and culture medium for up to 7 days incubation. Motility test, Gram staining, spore test, indole production, oxidase, catalase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease, DNase activity, citrate utilization, nitrate reduction, hydrolysis of casein, starch, gelatin, Tween 20, 40, 60, and 80, and amino acids, the Voges-Proskauer test and acid production from various carbohydrates were assessed. Anaerobic growth was determined by monitoring for growth in Hungate tubes (Bellco, USA) under anaerobic conditions (gas phase of N<sub>2</sub> 88: H<sub>2</sub> 7: CO<sub>2</sub> 5, v/v; Forma anaerobic system, USA). The anaerobic bacteria *Clostridium pasteurianum* KCTC 1674<sup>T</sup> was concurrently cultured as a standard (Chang *et al.*, 2008).

Strain BL4-6<sup>T</sup> is Gram-positive, motile, spore-forming, and rod-shaped. Cells tested positive for catalase activity, but negative for oxidase activity. In contrast to its closest relatives, strain BL4-6<sup>T</sup> was negative for acetoin production and acid



**Fig. 1.** Phylogenetic tree inferred by neighbour-joining method based on 16S rRNA gene sequences (1,400 bp). It shows relationships of strain BL4-6<sup>T</sup> within the genus *Bacillus*. Bootstrap values are calculated from 1,000 replications. 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.

production from fructose and trehalose. Detailed phenotypic characteristics of the strain BL4-6<sup>T</sup> are provided in Table 1 and in the species description.

A nearly complete 16S rRNA sequence (1,400 bp) for strain BL4-6<sup>T</sup> was analyzed by Chang *et al.* (2008). Strain BL4-6<sup>T</sup> showed the highest similarity of the 16S rRNA gene sequence to *B. mycooides* (98.5% similarity) and members of the *B. cereus* group. For improvement of the phylogenetic analysis, additional analyses of the *gyrA* and *rpoB* gene sequences were performed to corroborate new species status as described previously (Vogler *et al.*, 2002; Antwerpen *et al.*, 2007). Phylogenetic trees were inferred by using the maximum-parsimony (Fitch, 1971), maximum-likelihood (Felsenstein, 1981), and neighbour-joining (Saitou and Nei, 1987) methods. The PHYLIP software package (Felsenstein, 1993) was used for all phylogenetic analyses. The reliability of the resultant trees inferred by the neighbour-joining method was evaluated by bootstrap analyses (Felsenstein, 1985) based on 1,000 resamplings. A comparison of the 16S rRNA gene sequence for strain BL4-6<sup>T</sup> was performed by an initial BLAST search against sequences obtained from the GenBank database. The analysis demonstrated that the isolate belongs to the *B. cereus* group and is closely related to *B. mycooides* ATCC 6462<sup>T</sup> and *B. cereus* ATCC 14579<sup>T</sup>. Strain BL4-6<sup>T</sup> and the members of the *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 (Supplementary data Figs. 1 and 2). The results of our comprehensive phylogenetic analyses are shown in Fig. 1. The

closest phylogenetic relatives to strain BL4-6<sup>T</sup> were *B. mycooides* (98.5% similarity), followed by *B. cereus* ATCC 14579<sup>T</sup> (98.5%), *B. anthracis* ATCC 14578<sup>T</sup> (98.4%), *B. thuringiensis* ATCC 10792<sup>T</sup> (98.4%), *B. weihenstephanensis* CIP 105772<sup>T</sup> (98.1%), *B. gaemokensis* KCTC 13318<sup>T</sup> (97.8%), and *B. pseudomycooides* DSM 12442<sup>T</sup> (97.5%). Similarities between the strain BL4-6<sup>T</sup> and the members of the *B. cereus* group were greater than 97% (97.5–98.5%), whereas their similarity to other *Bacillus* species was below 95.9%. Previous studies also demonstrated a high degree of sequence similarity between species of the *B. cereus* group (Kaneko *et al.*, 1978; Ash *et al.*, 1991; Henderson *et al.*, 1994). The analysis based on the *gyrA* and *rpoB* gene sequence demonstrated that the isolate belongs to the *B. cereus* group, which corresponded to those of the 16S rRNA gene analysis (Supplementary data Figs. 3 and 4). The *gyrA* and *rpoB* trees also supported that strain BL4-6<sup>T</sup> formed an independent phylogenetic line in this group. Based on the *gyrA* gene sequences, the closest relative to strain BL4-6<sup>T</sup> was *B. cereus* (84.6%) but their similarity was less than 85%. For the *rpoB* gene sequence, their similarity was less than 95% (the closest neighbor *B. cereus* 94.6%; Ko *et al.*, 2003).

A lower sequence variation was observed in sequence comparisons within the group, and additional DNA-DNA hybridization was subsequently performed to differentiate these closely related species more accurately (Stackebrandt and Ebers, 2006). DNA-DNA hybridization was determined by the microplate method, as previously described (Ezaki *et al.*, 1989; Chang *et al.*, 2008), with three replications for each

**Table 2.** Cellular fatty acid compositions of strain BL4-6<sup>T</sup> and closely related *Bacillus* species. Taxa are indicated as: 1, strain BL4-6<sup>T</sup>; 2, *B. gaemokensis* KCTC 13318<sup>T</sup>; 3, *B. mycoides* KCTC 3453<sup>T</sup>; 4, *B. thuringiensis* KCTC 3452<sup>T</sup>; 5, *B. weihenstephanensis* KCTC 3975<sup>T</sup>; 6, *B. cereus* KCTC 3624<sup>T</sup>; 7, *B. pseudomycooides* KCTC 3862<sup>T</sup>. All data are taken from this study and Jung *et al.* (2010a). Values are percentages of total fatty acid detected. t, trace amount (<1.0 %); -, not detected.

Fatty acid	1	2	3	4	5	6	7
12:0 iso	1.2	11.3	t	t	1.6	t	6.4
12:0	t	1.6	t	t	t	t	3.5
13:0 iso	8.2	5.0	11.8	11.2	16.6	13.9	10.6
13:0 anteiso	1.4	6.0	t	1.1	1.7	1.7	4.9
14:0 iso	4.7	17.8	2.8	5.1	4.7	4.3	2.4
14:0	3.7	4.3	3.2	3.6	4.5	3.1	3.4
15:0 iso	28.8	5.1	33.5	31.3	29.2	33.2	33.2
15:0 anteiso	3.7	6.1	2.1	3.4	2.7	4	1.9
15:0	t	1.7	-	t	-	-	1.1
16:1 ω7c alcohol	-	-	2.1	t	1.3	t	-
16:0 iso	3.3	15.8	5.5	4.5	5.8	4.2	6.8
16:1 ω11c	-	-	2.9	-	1.2	-	-
16:0	2.2	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	7.5	-	2	4.2	2.1	5.3	-
17:1 anteiso A	1.5	t	-	-	-	t	-
17:0 iso	7.5	2.0	6.8	13.7	7.6	5.9	8.9
17:0 anteiso	5.5	3.3	t	-	1	1.5	t
18:1 iso H	-	1.3	-	-	-	-	-
18:0	-	-	-	-	-	-	2.6
Summed Feature 2 <sup>a</sup>	6.0	4.8	t	3.5	-	2.9	-
Summed Feature 3 <sup>a</sup>	12.6	7.6	6.8	9.7	5.4	9.1	3.8

<sup>a</sup> Fatty acids that could not be separated by GC using the Microbial Identification System (Microbial ID) software were considered summed features. Summed feature 2 contains iso-C<sub>16:0</sub> 1/14:0 3OH and/or C<sub>14:0</sub> 3OH/ iso-C<sub>16:1</sub>. Summed feature 3 contains C<sub>16:1</sub> ω7c/ iso-C<sub>15:0</sub> 2OH, and/or iso-C<sub>15:0</sub> 2OH/C<sub>16:1</sub> ω7c.

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 Fluoroskan Ascent Fluorescent plate reader (Thermo Life Science, UK). The highest and lowest values were excluded for each sample, and the means of the remaining three values are cited as the DNA relatedness value. DNA-DNA hybridization values between strain BL4-6<sup>T</sup> and the members of the *B. cereus* group showed a low degree of genetic relatedness. The values among species of the *B. cereus* group ranged from 22.8% to 42.3%, whereas these species exhibited high 16S rRNA sequence similarities, ranging from 97.5% to 98.5%. Based on the high degree of genetic homogeneity between species of the *B. cereus* group (Kaneko *et al.*, 1978; Ash *et al.*, 1991; Henderson *et al.*, 1994), these low DNA-DNA hybridization values strongly support that strain BL4-6<sup>T</sup> 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 BL4-6<sup>T</sup> represents a novel species distinct from validly described species.

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<sup>T</sup> DNA (50.8 mol%, Tm) as a standard (Jung *et al.*, 2010b). The DNA

G+C content of strain BL4-6<sup>T</sup> was 38.0 mol% (mean). This is consistent with the range of values (31.7-40.1 mol%) for the *B. cereus* group (Priest *et al.*, 1988), and its taxonomic position corresponds with members of this group and the genus *Bacillus* (32-69%; Claus and Berkeley, 1986).

Cellular fatty acid profiles of the isolate and relatives grown on TSA medium at 28°C for 48 h were determined by extracting and analyzing the fatty acid methyl esters (FAMES) according to a standard protocol (Sherlock Microbial Identification System; MIDI, USA; Sasser, 1990; Chang *et al.*, 2002). The FAMES were separated by an automated GC system (model 6890N and 7683 autosampler; Agilent) and identified using the associated software package (MIDI, USA). The predominant fatty acids of strain BL4-6<sup>T</sup> (Table 2) were iso-C<sub>15:0</sub> (28.8%), Summed Feature 3 (12.6%; comprising the unknown fatty acid C<sub>16:1</sub> ω7c/ iso-C<sub>15:0</sub> 2OH, and/or iso-C<sub>15:0</sub> 2OH/C<sub>16:1</sub> ω7c), and iso-C<sub>13:0</sub> (8.2%). All the members of the *B. cereus* group except *B. gaemokensis* KCTC 13318<sup>T</sup> showed similar profiles with the largest amounts iso-C<sub>15:0</sub> (28.8-33.5%). Variation in the relative amounts of Summed Feature 3 for strain BL4-6<sup>T</sup> was found, and the data can be employed to differentiate our isolate from closely related species (Kämpfer *et al.*, 1994; Ahmed *et al.*, 2007).

For the analyses of quinine and cell wall type, the isolate and relatives were grown on TSA medium at 30°C for 48 h. Analysis of the cell wall peptidoglycan was performed as described previously (Schleifer, 1985; Jung *et al.*, 2009) with the

modification that TLC on cellulose was applied instead of paper chromatography. Respiratory quinones were examined as described previously (Komagata and Suzuki, 1987; Chang *et al.*, 2002) using TLC and HPLC. In the analysis of complete cell-wall hydrolysates, the *meso*-diaminopimelic acid in strain BL4-6<sup>T</sup> was determined to be a diagnostic amino acid. The data represented peptidoglycan type A1 $\gamma$ , sharing this characteristic with the genus *Bacillus* (Ahmed *et al.*, 2007). The major quinone of strain BL4-6<sup>T</sup> was MK-7, which corresponded with that found in members of the genus *Bacillus* (Claus and Berkeley, 1986; Ahmed *et al.*, 2007).

On the basis of its cell wall peptidoglycan type, major quinone MK-7, fatty acid pattern, DNA G+C content (38.0 mol%), DNA-DNA hybridization values (<42.3%), 16S rRNA gene (<98.5% similarity), and the *gyrA* gene (<85%) and the *rpoB* gene (<95%) sequence analyses, strain BL4-6<sup>T</sup> can be differentiated from members of the genus *Bacillus* as a novel species, for which the name *Bacillus manliponensis* sp. nov. is proposed.

#### Description of *Bacillus manliponensis* sp. nov.

*Bacillus manliponensis*: man.li.po.nen'sis. N.L. masc. adj. manliponensis, pertaining to the Malipo, Republic of Korea, geographical origin of the type strain of the species).

Cells in TSA medium culture are Gram-positive, straight rod-shaped (1.5-2.0  $\times$  6.0-7.8  $\mu$ m) occurring singly or in pairs, motile with peritrichous flagella. They produce ellipsoidal endospores that lay in terminal position, with no swelling of sporangia. Cell walls contain *meso*-diaminopimelic acid. Surface colonies on TSA agar plates are 2.0-3.2 mm in diameter, circular, convex, translucent to semi-opaque, white gray, and shiny with smooth or erose margin. Growth occurred at 15-40°C (optimum, 30°C) and at pH values of 5.0-9.0 (optimum, pH 7.0). Cells are facultatively anaerobic. Growth fails at 7.0% NaCl. Cells are catalase-positive and oxidase-negative. Positive reactions for nitrate reduction, gelatinase, and hydrolysis of DNase, Tween 40 and 60; negative for production of acetoin, indole, H<sub>2</sub>S, nitrogen, urease,  $\beta$ -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophane desaminase, hydrolysis of casein, starch, Tween 20 and 80, and for citrate utilization. Positive reactions for ribose, glucose, N-acetyl-glucosamine, esculin and maltose; negative for fructose, mannose, arbutin, salicin, cellobiose, sucrose, trehalose, starch, glycogen, turanose, and the others. Cellular fatty acids include mainly iso-C<sub>15:0</sub> and summed feature 3 (containing C<sub>16:1</sub>  $\omega$ 7c/ iso-C<sub>15:0</sub> 2OH, and/or iso-C<sub>15:0</sub> 2OH/ C<sub>16:1</sub>  $\omega$ 7c). The major menaquinone is MK-7. The DNA G+C content is 38.0 mol%. The type strain, BL4-6<sup>T</sup> (=KCTC 13319<sup>T</sup> =JCM 15802<sup>T</sup>), was isolated from oil-contaminated tidal flat sediment collected from Malipo in the Yellow Sea coastal region of Tae-An, Republic of Korea.

The 16S rRNA gene sequences of strain BL4-6<sup>T</sup> (=KCTC 13319<sup>T</sup> =JCM 15802<sup>T</sup>) are available in the DDBJ/EMBL/GenBank database under the accession number FJ416490.

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