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Bacillus hunanensis sp. nov., a slightly halophilic bacterium isolated from non-saline forest soil

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Abstract A novel Gram-stain-positive, slightly halophilic, catalase- and oxidase-positive, endospore-forming, motile, aerobic, rod-shaped bacterium, designated strain JSM 081003^T, was isolated from non-saline forest soil in Hunan Province, China. Growth occurred with 0.5–15% (w/v) NaCl (optimum 2–4%) at pH 6.5–10.5 (optimum pH 7.5–8.5) and at 5–40°C (optimum 30°C). *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The major cellular fatty acids were iso-C15:0, anteiso-C15:0 and iso-C14:0. Strain JSM 081003^T contained MK-7 as the predominant respiratory quinone, and diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol as the major polar lipids. The genomic DNA G + C content of strain JSM 081003^T was 40.9 mol%. A phylogenetic analysis based on 16S rRNA gene sequence comparisons revealed that strain JSM 081003^T should be assigned to the genus *Bacillus*, and was related most closely to the type strains of *Bacillus lehensis* (sequence similarity 99.6%), *Bacillus oshimensis* (99.4%) and *Bacillus patagoniensis* (96.6%); lower than 96.0% sequence similarity was observed with other *Bacillus* species. The combination of phylogenetic analysis, DNA–DNA relatedness

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSM 081003^T is HM054473.

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values, phenotypic characteristics and chemotaxonomic data supports the view that strain JSM 081003^T represents a new species of the genus *Bacillus*, for which the name *Bacillus hunanensis* sp. nov. is proposed. The type strain is JSM 081003^T (= DSM 23008^T = KCTC 13711^T).

Keywords *Bacillus hunanensis* sp. nov. · Halophilic · Non-saline soil

Introduction

Halophilic, halotolerant, alkaliphilic and/or alkalitolerant bacilli species are not only widely distributed throughout various types of saline environments (Ash et al. 1991; Nielsen et al. 1994; Ventosa et al. 1998; Arahal and Ventosa 2002; Romano et al. 2005; Lim et al. 2006a, b; Yumoto 2007; Chen et al. 2009a, b) but also were isolated from non-saline environments (Nielsen et al. 1995; Echigo et al. 2005, 2007; Usami et al. 2007). Those bacteria attracted increasing interest, attributable to their ability to grow under extreme conditions as well as to the potential use of their enzymes in biotechnological applications (Horikoshi 1999; Margesin and Schinner 2001; Nogi et al. 2005; Krulwich et al. 2007). During an investigation of the diversity of halophilic and halotolerant bacteria in Xiaoxi National Natural Reserve (28°42′15″–28°53′15″N 110°6′50″–110°21′35″E), China, a slightly halophilic, endospore-forming, Gram-stain-positive bacterium, designated strain JSM 081003^T, was isolated from a non-saline forest soil sample. Based on the results of a polyphasic taxonomic study, this strain is considered to represent a novel species of the genus *Bacillus*.

Materials and methods

Strains and culture conditions

Strain JSM 081003^T was isolated from a non-saline forest soil sample using the dilution plating technique on marine agar 2216 (MA; Difco) supplemented with 10% (w/v) NaCl and cultivated at 28°C for 2 weeks. After primary isolation, the strain was purified by repeated streaking and subculturing on MA plates (4–5 times) and examining by light microscopy. The isolate was maintained as serial transfers on MA slants, otherwise lyophilized cultures at 4°C and also deep-frozen at −80°C in 20% (v/v) glycerol. For comparison, two type strains, *Bacillus lehensis* DSM 19099^T and *Bacillus oshimensis* DSM 18940^T, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). Unless indicated otherwise, morphological, physiological, molecular and chemotaxonomic studies were performed with cells grown on MA (pH 8.0) at 30°C.

Phenotypic characterization

In order to compare strain JSM 081003^T and strains *B. lehensis* DSM 19099^T and *B. oshimensis* DSM 18940^T under the same laboratory conditions, a phenotypic characterization of these three strains was carried out following the recommendations of the proposed minimal standards for describing new taxa of aerobic, endospore-forming bacteria (Logan et al. 2009). Cell morphology was examined by using phase-contrast microscopy (DM3000, 100×HCX PL Fluotar oil immersion objective, Ph3; Leica) with cells grown on MA plus 10 mg $MnSO_4$ for 1–3 days at 30°C and 4–7 days at room temperature (18–20°C). The Gram staining and the KOH lysis test were carried out according to Smibert and Krieg (1994) and Gregersen (1978), respectively. Flagella were examined according to the methods described by Smibert and Krieg (1994). Growth in the absence of NaCl was investigated on nutrient agar (NA) and in nutrient broth (NB) prepared according to the formula of Atlas (1993) except the addition of NaCl. Tolerance of NaCl was tested on NA as well as in NB at different NaCl concentrations [0.1 and 0.5% (w/v), and 1–30% (w/v) in increments of 1%]. Growth was tested at various temperatures (4, 5–55°C, in increments of 5°C) and at different pH (5.0–11.0, in increments of 0.5 pH units) on MA as well as in NB supplemented with 2.5% (w/v) NaCl. The buffer solutions described by Chen et al. (2007) were used for pH experiments. Methyl red and Voges–Proskauer tests and determination of H_2S production from L-cysteine, hydrolysis of aesculin, indole production, nitrate and nitrite reduction and activities of arginine dihydrolase, lysine and ornithine decarboxylase, phenylalanine deaminase and urease were performed as described by Smibert and Krieg (1994). Hydrolysis of casein, cellulose, DNA, gelatin, starch, Tween 20, 40, 60 and 80 was determined as

described by Cowan and Steel (1965). Growth under anaerobic conditions was determined on MA supplemented with 0.5% (w/v) glucose and with or without 0.1% (w/v) nitrate using the GasPak Anaerobic Systems (BBL) according to the manufacturer's instructions. Determination of acid production from carbohydrates and utilization of carbon and nitrogen sources was performed as described by Ventosa et al. (1982). Observation of motility and tests of catalase and oxidase activities were detected as described previously (Chen et al. 2007). Other enzymic activities were assayed using API ZYM strips (bioMérieux) according to the manufacturer's instructions with 3% (w/v) NaCl. All the physiological and biochemical tests were repeated for three times.

Determination of 16S rRNA gene sequence, phylogenetic analysis and DNA–DNA hybridization

The 16S rRNA gene sequence was amplified by PCR and sequenced as described by Cui et al. (2001). Pairwise sequence similarities were calculated using a global alignment algorithm, implemented at the EzTaxon server (Chun et al. 2007). Phylogenetic analysis was performed using the software package MEGA version 4.1 (Tamura et al. 2007) after multiple alignment of sequence data by CLUSTAL_X (Thompson et al. 1997). Distances were calculated using distance options according to Kimura's two-parameter model (Kimura 1980) and clustering was performed with the neighbour-joining method (Saitou and Nei 1987). Maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Kluge and Farris 1969) trees were generated using the tree making algorithms contained in the PHYLIP package (Felsenstein 2002). Bootstrap analysis was used to evaluate the tree topology by means of 1000 resamplings (Felsenstein 1985). DNA–DNA hybridization experiments were performed using the optical renaturation method (De Ley et al. 1970; Huß et al. 1983; Jahnke 1992). Every hybridization experiment was performed with three replications and the relatedness value was expressed as the mean of the three values.

Chemotaxonomic characterization

Diamino acid in cell wall was analysed as described by Hasegawa et al. (1983). Isoprenoid quinones were analysed by HPLC as described by Groth et al. (1996). Polar lipids were extracted according to the method of Minnikin et al. (1979) and were identified by two-dimensional TLC and spraying with appropriate detection reagents (Collins and Jones 1980). Fatty acids were determined according to Sasser (1990) using the Microbial Identification System (Microbial ID) with cells grown in marine broth 2216 (Difco) in flasks on a rotary shaker (with shaking at 200 rpm) at 30°C for 2 days. Genomic DNA was isolated according to Hopwood et al. (1985) and the G + C content was determined using the HPLC method (Mesbah et al. 1989).

Results and discussion

Phenotypic characteristics

Strain JSM 081003^T was slightly halophilic and strictly aerobic and the cells were motile, Gram-stain-positive rods, forming ellipsoidal endospores that lay in central unswollen sporangia. Colonies were yellow-pigmented, somewhat convex and opaque with smooth, glistening surfaces and circular margins and 2–3 mm diameter after incubation for 3–5 days at 30°C on MA. The strain grew optimally in the presence of 2–4% (w/v) NaCl, at pH 7.5–8.5 and at 30°C. Detailed phenotypic properties that differentiate strain JSM 081003^T from related *Bacillus* species are summarized in Table 1 and also mentioned in the species description below.

Phylogenetic analysis based on 16S rRNA gene sequence comparison and DNA–DNA relatedness

The almost-complete 16S rRNA gene sequence (1427 bp) of the organism was determined (GenBank/EMBL/DDBJ accession number HM054473). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JSM 081003^T should be assigned to the genus *Bacillus*, and was related most closely to the type strains of *B. lehensis* (16S rRNA gene sequence similarity 99.6%; Ghosh et al. 2007), *B. oshimensis* (99.4%; Yumoto et al. 2005) and *B. patagoniensis* (96.6%; Olivera et al. 2005); lower than 96.0% sequence similarity was observed with other *Bacillus* species. In the neighbour-joining phylogenetic tree, strain JSM 081003^T formed a robust

Table 1 Characteristics used to distinguish strain JSM 081003^T from the type strains of phylogenetically related *Bacillus* species

Characteristic	1	2	3
Colony pigmentation	Yellow	Creamy yellow	Creamy
Spore position	Central	Subterminal	Terminal
Motility	+	+	−
Urease	−	+	+
Hydrolysis of			
DNA	−	−	+
Aesculin	+	+	–
Tween 80	−	−	+
Growth condition			
NaCl range (%, w/v)	0.5–15	0.5–15	0–18
NaCl optimum (%, w/v)	2–4	5–8	2–4
pH range	6.5–10.5	6.5–10.5	7–11.0
pH optimum	7.5–8.5	8.0	8.5
Temperature range (°C)	5–40	10–40	10–40
Temperature optimum (°C)	30	30	25–30
Acid production from			
Amygdalin	+	−	−
D-Fructose	−	+	−
Glycogen	−	+	−
Lactose	−	+	−
D-Mannitol	+	−	−
D-Mannose	−	−	+
Melibiose	+	−	+
D-Ribose	−	−	+
D-Sorbitol	−	−	+
Trehalose	−	+	+
D-Xylose	−	+	+
Menaquinone content (%):			
MK-6	4.2	2.5	1.2
MK-7	93.5	97.5	98.8
MK-8	2.3	0	0

Strains *1 B. hunanensis* sp. nov. JSM 081003^T, *2 B. lehensis* DSM 19099^T, *3 B. oshimensis* DSM 18940^T

"+" Positive, "−" Negative

All strains are aerobic, Gram-stain-positive rods, with growth in the presence of 0.5–15% (w/v) at pH 6.5–10.5 and at 10–40°C. All strains produce ellipsoidal endospores that lie in unswollen sporangia. All strains are positive for catalase and oxidase activities and hydrolysis of casein, gelatin, starch, Tween 20, 40 and 60, but negative for hydrolysis of cellulose, nitrate and nitrite reduction, H_2S and indole production, methyl red and Voges–Proskauer test and activities of arginine dihydrolase, lysine and ornithine decarboxylase and phenylalanine deaminase. All strains produce acid from D-glucose, glycerol, maltose and sucrose, but not from *N*-acetylglucosamine, adonitol, L-arabinose, cellobiose, dulcitol, D-galactose, *myo*-inositol, melezitose, raffinose, L-rhamnose or D-salicin. All data were obtained from this study

lineage with the type strains of *B. lehensis* and *B. oshimensis* supported by a significant bootstrap resampling value (100%) (Fig. 1). However, JSM 081003^T occupied a distinct lineage in the phylogenetic trees constructed by using maximum-likelihood and maximum-parsimony methods (Supplementary Fig. S1). Levels of DNA–DNA relatedness between strain JSM 081003^T and strains *B. lehensis* DSM 19099^T and *B. oshimensis* DSM 18940^T were 41% (SD, 2.5%) and 43% (SD, 2.6%), respectively, values

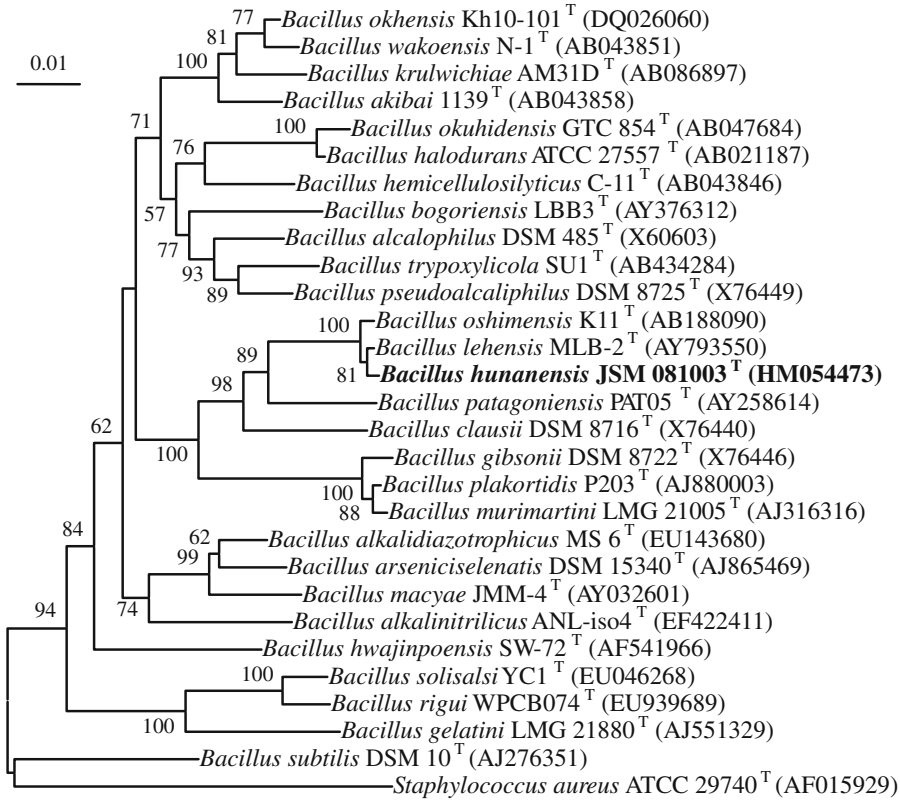

Fig. 1 Phylogenetic tree showing the phylogenetic positions of strain JSM 081003^T and related taxa based on 16S rRNA gene sequence analysis constructed using the neighbour-joining method. *Numbers* at nodes are bootstrap percentages (>50%) based on a neighbour-joining analysis of 1000 resampled datasets. *Bar* 1 substitutions per 100 nucleotides

that are well below the threshold value (70%) recommended by Wayne et al. (1987) for the definition of members of a species. Therefore, it would appear that, on the basis of the phylogenetic and DNA–DNA hybridization data, strain JSM 081003^T represents a new species of the genus *Bacillus* according to accepted criteria (Wayne et al. 1987; Stackebrandt and Goebel 1994).

Chemotaxonomic characteristics and DNA base composition

Chemotaxonomic data for strain JSM 081003^T were consistent with the assignment of the strain to the genus *Bacillus*. The strain possessed a cell-wall type based on *meso*-diaminopimelic acid as the diagnostic diamino acid. Strain JSM 081003 ^{T} contained MK-7 (93.5%) as the predominant menaquinone, with MK-6 (4.2%) and MK-8 (2.3%) present in minor amounts. The polar lipids of this strain consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and two unknown phospholipids. The fatty acid profile of strain JSM 081003^T was similar to those of the type strains of the two phylogenetically related *Bacillus* species, although there were differences in the proportions of some components (Table 2). The fatty acid profile of JSM 081003^T contained the major compounds iso-C15:0 (43.6%), anteiso-C15:0 (23.4%) and iso-C14:0 (10.2%), which are characteristic of numerous members within the genus *Bacillus* (Kämpfer 1994). The DNA G + C content of strain JSM 081003^T was 40.9 mol%.

Taxonomic conclusion

The results of the phylogenetic analysis and of morphological and chemotaxonomic investigations supported the affiliation of strain JSM 081003^T to the genus *Bacillus*. However, the strain could be clearly distinguished from its phylogenetic relatives by several phenotypic differences, such as the yellow-pigmentation, the positive results of acid production from amygdalin and D-mannitol and the negative results of urease activity and acid production from trehalose and D-xylose (Table 1). Together with some chemotaxonomic differences (Tables 1, 2) and low

Table 2 Fatty acid profiles of strain JSM 081003^T and related *Bacillus* species

Fatty acid[a]	1	2	3
Straight-chain			
C14:0	0.9	1.2	0.8
C16:0	3.0	3.4	1.9
C18:0	0.1	1.3	0.3
Unsaturated			
C16:1ω7*c* alcohol	3.7	0.6	0.3
C16:1ω11*c*	0.6	–	0.2
C18:1ω9*c*	0.8	0.5	0.4
Branched			
iso-C14:0	10.2	6.7	6.6
iso-C15:0	43.6	63.9	64.0
anteiso-C15:0	23.4	13.3	16.8
iso-C16:0	5.2	3.4	2.8
iso-C17:0	6.0	3.9	3.8
anteiso-C17:0	1.8	1.4	1.4
Summed feature 3	0.6	–	0.1

Strains *1 B. hunanensis* sp. nov. JSM 081003^T, *2 B. lehensis* DSM 19099^T, *3 B. oshimensis* DSM 18940^T

Values are percentages of total fatty acid content. "–" Not detected. All data were obtained from this study

[a] Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 3 comprises C16:1 ω7*c* and/or C16:1 ω6*c*

levels of DNA–DNA relatedness between strain JSM 081003^T and the type strains of *B. lehensis* and *B. oshimensis*, the results of the polyphasic taxonamic study presented here allow us to assign the isolate to a novel species, for which we propose the name *Bacillus hunanensis* sp. nov.

Description of *B. hunanensis*

B. hunanensis (hu.nan.en'sis. N.L. masc. adj. *hunanensis* pertaining to Hunan Province, China, the source of the sample from which the type strain was isolated).

Cells are Gram-stain-positive, catalase- and oxidase-positive, motile, aerobic, slightly halophilic, straight rods, approximately 0.6–0.8 μm wide and 1.5–3.5 μm long, occurring singly, as pairs or as short chains, producing ellipsoidal endospores that lie in central unswollen sporangia. Colonies are yellow-pigmented, somewhat convex and opaque, have smooth, glistening surfaces and circular margins and are 2–3 mm in diameter on MA. No diffusible pigments are produced. Growth occurs with 0.5–15% (w/v) NaCl (optimum 2–4%) and at pH 6.5–10.5 (optimum pH 7.5–8.5) and at 5–40°C (optimum 30°C). No growth occurs in the absence of salts. Nitrate and nitrite is not reduced. Negative for tests of methyl red, Voges–Proskauer, H_2S and indole production. Aesculin, casein, gelatin, starch, Tween 20, 40 and 60 are hydrolyzed, but cellulose, DNA and Tween 80 are not. Acids are produced from amygdalin, D-glucose, glycerol, maltose, D-mannitol, melibiose and sucrose, but not from *N*-acetylglucosamine, adonitol, L-arabinose, cellobiose, dulcitol, D-fructose, D-galactose, glycogen, *myo*-inositol, lactose, D-mannose, melezitose, raffinose, L-rhamnose, D-ribose, D-salicin, D-sorbitol, trehalose or D-xylose. The following compounds are utilized as sole sources of carbon and energy or sole sources of carbon, nitrogen and energy: *N*-acetylglucosamine, dextrin, D-fructose, D-glucose, maltose, D-mannose, sucrose, D-mannitol, citrate and L-asparagine; the following are not utilized: L-arabinose, cellobiose, D-galactose, lactose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, trehalose, D-xylose, adonitol, D-arabitol, glycerol, *myo*-inositol, D-sorbitol, acetate, butyrate, gluconate, propionate, succinate, L-alanine, L-arginine, L-glutamic acid, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine and L-valine. Constitutive enzymes expressed are acid and alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), -glucosidase, lipase (C14) and α-mannosidase; arginine dihydrolase, cystine arylamidase, α-fucosidase, α- and β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, β-glucuronidase, leucine arylamidase, lysine decarboxylase, naphthol-AS-BI-phosphohydrolase, ornithine decarboxylase, phenylalanine deaminase, trypsin, urease and valine arylamidase are not observed. *meso*-Diaminopimelic acid is present in the cell-wall peptidoglycan as the diagnostic diamino acid. Possesses MK-7 as the predominant menaquinone, and diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylglycerol as the major polar lipids. Major fatty acids are iso-C15:0, anteiso-C15:0 and iso-C14:0. The DNA G + C content of the type strain is 40.9 mol% (HPLC method).

The type strain is JSM 081003^T (= DSM 23008^T = KCTC 13711^T), which is the isolate on which the species description is based. It was isolated from non-saline forest soil in Hunan Province, China.

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