Bacillus neizhouensis sp. nov., a halophilic marine bacterium isolated from a sea anemone

Yi-Guang Chen,^{1,2} Yu-Qin Zhang,³ Yong-Xia Wang,² Zhi-Xiong Liu,¹ Hans-Peter Klenk,⁴ Huai-Dong Xiao,¹ Shu-Kun Tang,² Xiao-Long Cui² and Wen-Jun Li^{2,5}

Correspondence

Wen-Jun Li
wjli@ynu.edu.cn
Yi-Guang Chen
mchenjsu@yahoo.com.cn

¹College of Biology and Environmental Sciences, College of Chemistry and Chemical Engineering, Jishou University, Jishou 416000, PR China

²The Key Laboratory for Microbial Resources of the Ministry of Education, PR China, and Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming 650091, PR China

³Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100050, PR China

⁴Deutsche Sammlung von Mikroorganismen und Zellkulturen, D-38124 Braunschweig, Germany

⁵Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, PR China

A novel Gram-stain-positive, slightly halophilic, facultatively alkaliphilic, non-motile, catalase- and oxidase-positive, endospore-forming, rod-shaped, aerobic bacterium, strain JSM 071004^T, was isolated from a sea anemone collected from Neizhou Bay in the South China Sea. Growth occurred with 0.5–10% (w/v) total salts (optimum 2–4%) and at pH 6.5–10.0 (optimum pH 8.5) and 4–30 °C (optimum 25 °C). *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The predominant respiratory quinone was menaquinone 7 (MK-7) and the polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The major cellular fatty acids were anteiso-C_{15:0} and iso-C_{15:0}. The genomic DNA G + C content was 39.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain JSM 071004^T belongs to the genus *Bacillus*, being related most closely to the type strain of *Bacillus agaradhaerens* (sequence similarity 97.3%), followed by the type strains of *Bacillus cellulosilyticus* (96.2%), *Bacillus clarkii* (96.1%) and *Bacillus polygoni* (96.0%). The combination of phylogenetic analysis, DNA–DNA hybridization, phenotypic characteristics and chemotaxonomic data support the proposal that strain JSM 071004^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus neizhouensis* sp. nov. is proposed, with JSM 071004^T (=CCTCC AB 207161^T =DSM 19794^T =KCTC 13187^T) as the type strain.

There has been increasing interest in halophilic, halo-tolerant, alkaliphilic and/or alkalitolerant *Bacillus* species, attributable to their ability to grow under extreme conditions as well as to the use of their enzymes in biotechnological applications (Ash *et al.*, 1991; Nielsen *et al.*, 1994, 1995; Horikoshi, 1999; Margesin & Schinner, 2001; Arahal & Ventosa, 2002; Yoon *et al.*, 2004; Nogi *et al.*, 2005; Romano *et al.*, 2005; Lim *et al.*, 2006a, b; Carrasco *et al.*, 2007; Krulwich *et al.*, 2007; Yumoto, 2007; Aino *et al.*, 2008). During investigation of the diversity of the microbial population of invertebrates inhabiting the South China Sea (Chen *et al.*, 2009a, b; Xiao *et al.*,

2009), a slightly halophilic, facultatively alkaliphilic, endospore-forming, Gram-stain-positive bacterium, designated JSM 071004^T, was isolated from a sea anemone collected from the Neizhou Bay, China. Based on the results of a polyphasic taxonomic study, this strain is considered to represent a novel species of the genus *Bacillus*.

Strain JSM 071004^T was isolated from homogenates of a sea anemone by plating 1:10 serial dilutions of the sample on marine agar 2216 (MA; Difco) cultivated at 25 °C for 2 weeks. After primary isolation and purification, the isolate was preserved both on MA slants at 4 °C and in marine broth 2216 (MB; Difco) supplemented with 20% (v/v) glycerol at –80 °C. Unless otherwise indicated,

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morphological and physiological studies were performed with cells grown on MA at pH 8.5 and 25 °C. Cell morphology was examined by light microscopy (model BH 2; Olympus). Gram staining was carried out by using the standard Gram reaction combined with the KOH lysis method (Gregersen, 1978). Flagella and endospores were examined according to the methods of Leifson and Schaeffer–Fulton, respectively (Smibert & Krieg, 1994). Growth was tested at various temperatures (0, 4 and 5–45 °C, in increments of 5 °C) and at different pH (5.0–11.0, in increments of 0.5 pH units) on MA and in MB. For pH experiments, the buffer solutions described by Chen *et al.* (2007) were used. Growth at different total-salt concentrations (0, 0.1, 0.5 and 1–15 %, w/v, in increments of 1 %) was determined on MA prepared according to the formula of Atlas (1993). The concentration of all salts contained in MA was changed (keeping proportions the same) in the salt-response experiment. Methyl red and Voges–Proskauer tests, H₂S production from L-cysteine, hydrolysis of aesculin, indole production and nitrate and nitrite reduction were tested as recommended by Smibert & Krieg (1994). Hydrolysis of casein, cellulose, DNA, gelatin, starch, Tweens 20, 40, 60 and 80 and urea was determined as described by Cowan & Steel (1965). Determination of acid production from carbohydrates, as well as utilization of carbon and nitrogen sources, was performed as recommended by Ventosa *et al.* (1982). Motility, growth under anaerobic conditions, antibiotic susceptibility and catalase and oxidase activities were detected as described previously (Chen *et al.*, 2007). Other enzymic activities were tested by using API ZYM systems (bioMérieux) according to the manufacturer's instructions with 3 % (w/v) NaCl.

Strain JSM 071004^T was slightly halophilic and facultatively alkaliphilic, with optimum growth occurring at 2–4 % (w/v) total salts and at pH 8.5. It was able to grow at 4–30 °C, with optimum growth at 25 °C. Colonies were pale-yellow pigmented, low convex and translucent with circular/slightly irregular margins and 1–2 mm diameter after incubation for 3–5 days at 25 °C on MA (pH 8.5). Cells were Gram-stain-positive, endospore-forming, catalase- and oxidase-positive, aerobic, straight rods. The results of other phenotypic tests are summarized in the species description and in Table 1.

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). The 16S rRNA gene was amplified and sequenced as described previously (Cui *et al.*, 2001). Pairwise sequence similarities were calculated using a global alignment algorithm, which was implemented at the EzTaxon server (Chun *et al.*, 2007). Phylogenetic analysis was performed by 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 by the neighbour-joining method

Table 1. Characteristics used to distinguish strain JSM 071004^T from the type strains of phylogenetically related *Bacillus* species

Strains: 1, *Bacillus neizhouensis* sp. nov. JSM 071004^T (data from this study); 2, *B. agaradhaerens* DSM 8721^T (Nielsen *et al.*, 1995; Lim *et al.*, 2006a, b); 3, *B. cellulosilyticus* N-4^T (Nogi *et al.*, 2005); 4, *B. clarkii* DSM 8720^T (Nielsen *et al.*, 1995; Lim *et al.*, 2006a, b); 5, *Bacillus polygoni* YN-1^T (Aino *et al.*, 2008); 6, *B. vedderi* DSM 9768^T (Nielsen *et al.*, 1995; Nogi *et al.*, 2005; Lim *et al.*, 2006b); 7, *B. saliphilus* 6AG^T (Romano *et al.*, 2005); 8, *B. chagannorensis* CG-15^T (Carrasco *et al.*, 2007). +, Positive; –, negative; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8
Oxidase	+	–	–	–	–	+	+	–
Facultatively anaerobic	–	–	ND	–	–	+	ND	+
Motility	–	+	+	ND	–	+	–	+
Hydrolysis of:								
Casein	–	+	–	+	+	–	–	–
Gelatin	+	–	–	+	–	+	+	–
Starch	–	+	+	–	–	–	–	–
Tween 20	–	–	+	–	+	–	ND	ND
Tweens 40 and 60	–	+	+	+	+	+	ND	ND
Growth at:								
15 % (w/v) NaCl	–	+	–	+	–	–	+	+
4 °C	+	–	–	–	–	–	–	–
35 °C	–	+	+	+	+	+	+	+
pH 6.5	+	–	–	–	–	–	–	+
pH 11	–	+	–	+	+	–	–	+
DNA G+C content (mol%)	39.8	39.5	39.6	42.4	42.9	38.3	48.4	53.8

(Saitou & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and parsimony (Kluge & Farris, 1969) trees (not shown) were generated by using the treeing algorithms contained in the PHYLIP package (Felsenstein, 2002). Bootstrap analysis was used to evaluate the tree topology of the neighbour-joining data by means of 1000 resamplings (Felsenstein, 1985). DNA–DNA hybridization was carried out at 37 °C by using photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989).

The DNA G+C content of strain JSM 071004^T was 39.8 mol%. The almost-complete 16S rRNA gene sequence (1479 bp) was determined. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain JSM 071004^T belongs to the genus *Bacillus*, being related most closely to the type strain of *Bacillus agaradhaerens* (sequence similarity 97.3 %; Nielsen *et al.*, 1995), followed by the type strains of *Bacillus cellulosilyticus* (96.2 %; Nogi *et al.*, 2005), *Bacillus clarkii* (96.1 %; Nielsen *et al.*, 1995) and *Bacillus polygoni* (96.0 %; Aino *et al.*, 2008). Sequence similarities between strain JSM 071004^T and other *Bacillus* species were below 96.0 %. In the phylogenetic tree, the novel isolate formed a distinct subline with the type strain of *B. agaradhaerens* (three methods; Fig. 1). To establish

Fig. 1. Phylogenetic tree showing the positions of strain JSM 071004^T and related taxa, based on 16S rRNA gene sequence analysis and reconstructed using the neighbour-joining method. 'm' or 'p' labels indicate branches that were also found with the maximum-likelihood (Felsenstein, 1981) or parsimony (Kluge & Farris 1969) algorithms, respectively; asterisks indicate branches that were recovered with all three methods. Numbers at nodes indicate bootstrap values (>50%) based on neighbour-joining analysis of 1000 resampled data-sets. Bar, 1 substitution per 100 nucleotides.

the precise taxonomic position of strain JSM 071004^T, DNA–DNA hybridizations were performed between the novel isolate and *B. agaradhaerens* DSM 8721^T, which revealed the level of DNA–DNA relatedness between them to be 13.4%, far below the threshold value of 70% recommended by Wayne *et al.* (1987) for assigning strains to the same species. It is therefore evident, based on the results of phylogenetic analysis and DNA–DNA hybridization, that strain JSM 071004^T represents a previously unknown species of the genus *Bacillus* (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Amino acids of whole-cell hydrolysates were 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 & Jones, 1980). Fatty acids were determined for the new isolate as well as for the reference strain *B. agaradhaerens* DSM 8721^T as described by Sasser (1990) using the Microbial Identification System (MIDI; Microbial ID) with cells grown in MB (pH 8.5) in flasks on a rotary shaker at 200 r.p.m. at 25 °C for 3 days. Chemotaxonomic data for strain JSM 071004^T were consistent with its assignment to the genus *Bacillus*. The strain possessed a cell-wall type based on *meso*-diaminopimelic acid and contained MK-7 (99.6%) as the predominant menaquinone, with MK-6 (0.4%) present in trace amounts. The polar lipids of this strain consisted of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The fatty acid profile of strain JSM 071004^T was similar to that of the type strain of *B. agaradhaerens* (Table 2). The major fatty acids of strain JSM 071004^T were anteiso-C_{15:0} (50.4%) and iso-C_{15:0} (12.1%).

The results of phylogenetic analysis and morphological and chemotaxonomic studies supported the view that strain JSM 071004^T should be assigned to the genus *Bacillus*. However, the comparatively low growth temperatures (4–30 °C), as well as its inability to hydrolyse Tweens 40 and 60, differentiated the new isolate markedly from phylogenetically related *Bacillus* species (Table 1). Strain JSM 071004^T could also be clearly distinguished from its phylogenetically closest neighbour, *B. agaradhaerens*, by some other discriminative taxonomic markers, such as its oxidase activity, its ability to hydrolyse gelatin and to grow at pH 6.5 (Table 1) and the low level of DNA–DNA relatedness between them. Overall, the results presented here allowed us to assign the novel isolate to a novel species, for which we propose the name *Bacillus neizhouensis* sp. nov.

Description of *Bacillus neizhouensis* sp. nov.

Bacillus neizhouensis (nei.zhou.en'sis. N.L. masc. adj. *neizhouensis* pertaining to Neizhou Bay, from which the sample that yielded the type strain was collected).

Cells are Gram-stain-positive, non-motile, catalase- and oxidase-positive, aerobic, straight rods, approximately 0.5–0.6 µm wide and 3.0–5.0 µm long, occurring singly, as pairs or as short chains, producing ellipsoidal endospores that lie in subterminal swollen sporangia. Colonies are pale-yellow pigmented, low convex and translucent with circular/slightly irregular margins, 1–2 mm in diameter after incubation for 3–5 days at 25 °C on marine agar 2216 (Difco). Slightly halophilic and facultatively alkaliphilic; growth occurs with 0.5–10% (w/v) total salts (optimum 2–4%) and at pH 6.5–10.0 (optimum pH 8.5). Growth occurs at 4–30 °C, with an optimum of 25 °C. Nitrate is reduced but nitrite is not. Negative for tests of methyl red,

Table 2. Fatty acid composition of strain JSM 071004^T and *Bacillus agaradhaerens* DSM 8721^T

Data are percentages of the total fatty acids, those representing <1.0% in both strains being omitted. –, Not detected.

Fatty acid	<i>B. neizhouensis</i> JSM 071004 ^T	<i>B. agaradhaerens</i> DSM 8721 ^T
iso-C _{14:0}	5.1	1.0
iso-C _{15:0}	12.1	18.5
anteiso-C _{15:0}	50.4	45.6
iso-C _{16:0}	3.5	3.0
C _{16:1} ω7c alcohol	7.4	3.4
C _{16:1} ω11c	5.8	1.5
C _{16:0}	–	3.5
iso-C _{17:0}	4.0	3.8
anteiso-C _{17:0}	6.1	9.5
anteiso-C _{17:1} ω10c	2.8	5.5
C _{18:1} ω9c	–	2.7

Voges–Proskauer, H₂S and indole production. Aesculin and gelatin are hydrolysed, but casein, cellulose, DNA, starch, Tweens 20, 40, 60 and 80 and urea are not. Acids are produced from D-fructose, D-glucose, maltose, sucrose and trehalose, but not from adonitol, L-arabinose, cellobiose, dulcitol, D-galactose, glycerol, *myo*-inositol, lactose, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, starch, D-sorbitol or D-xylose. The following compounds are utilized as sole sources of carbon and energy or sole sources of carbon, nitrogen and energy: D-glucose, maltose, trehalose and L-leucine; the following substances are not utilized: L-arabinose, cellobiose, dextrin, D-fructose, D-galactose, D-lactose, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, D-salicin, sucrose, D-xylose, adonitol, D-arabitol, glycerol, *myo*-inositol, D-mannitol, D-sorbitol, acetate, butyrate, citrate, gluconate, propionate, succinate, L-alanine, L-arginine, L-asparagine, L-glutamic acid, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-methionine, L-proline, L-serine and L-valine. Constitutive enzymes expressed are alkaline phosphatase, β-glucosidase, β-glucuronidase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase; acid phosphatase, α-chymotrypsin, cystine arylamidase, α-fucosidase, α-galactosidase, β-galactosidase, N-acetyl-β-glucosaminidase, α-glucosidase, esterase (C4), esterase lipase (C8), lipase (C14), α-mannosidase, trypsin and valine arylamidase are not observed. Susceptible to (μg per disc) ampicillin (30), carbenicillin (30), chloramphenicol (30), gentamicin (10), kanamycin (30), nitrofurantoin (30), novobiocin (30), polymyxin B (30), rifampicin (5) and streptomycin (10), but not to lincomycin (2), nalidixic acid (20), tetracycline (30) or tobramycin (10). *meso*-Diaminopimelic acid is present in the cell-wall peptidoglycan as the diagnostic diamino acid. Possesses MK-7 as the predominant menaquinone and diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine as polar lipids. The major fatty acids are

anteiso-C_{15:0} and iso-C_{15:0}. The DNA G + C content of the type strain is 39.8 mol% (HPLC method).

The type strain, JSM 071004^T (=CCTCC AB 207161^T =DSM 19794^T =KCTC 13187^T), was isolated from homogenates of a sea anemone collected from Neizhou Bay in the South China Sea.

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