Bacillus hemicentroti sp. nov., a moderate halophile isolated from a sea urchin

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A novel Gram-staining-positive, moderately halophilic, facultatively alkaliphilic, non-motile, catalase-positive, oxidase-negative, endospore-forming, facultatively anaerobic rod, designated JSM 076093^T, was isolated from a sea urchin (*Hemicentrotus pulcherrimus*) collected from Naozhou Island in the South China Sea. Growth occurred with 0.5–25 % (w/v) NaCl (optimum 5–8 %) and at pH 6.0–10.5 (optimum pH 8.0) and 5–40 °C (optimum 30–35 °C). *meso*-Diaminopimelic acid was present in the cell-wall peptidoglycan. The predominant respiratory quinone was menaquinone 7 and the polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and one unidentified phospholipid. The major cellular fatty acids (>10 % of the total) were anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and iso-C_{14:0}. The genomic DNA G+C content was 38.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain JSM 076093^T belonged to the genus *Bacillus* and was related most closely to *Bacillus hwajinpoensis* SW-72^T (99.1 % 16S rRNA gene sequence similarity) and *Bacillus algicola* KMM 3737^T (97.3 %). The combination of results from the phylogenetic analysis, DNA–DNA hybridization and phenotypic and chemotaxonomic characterization supported the conclusion that strain JSM 076093^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus hemicentroti* sp. nov. is proposed, with JSM 076093^T (=DSM 23007^T=KCTC 13710^T) as the type strain.

The genus *Bacillus*, in the phylum *Firmicutes*, is a large collection of aerobic or facultatively anaerobic, rod-shaped, endospore-forming bacteria and is widely distributed in the environment. The genus is heterogeneous and contains several phylogenetic groups on the basis of 16S rRNA gene sequence analysis (Ash *et al.*, 1991; Stackebrandt & Liesack, 1993; Nielsen *et al.*, 1994; Ventosa *et al.*, 1998). The genus is attracting interest because its members have great biotechnological potential for the production of compatible solutes or hydrolytic enzymes (Horikoshi, 1999; Margesin & Schinner, 2001; Arahall & Ventosa, 2002; Nogi *et al.*, 2005;

Krulwich *et al.*, 2007). At the time of writing, the genus *Bacillus* consisted of more than 200 species with validly published names (Euzéby, 2010), including the recently described *Bacillus chungangensis* (Cho *et al.*, 2010), *B. galliciensis* (Balcázar *et al.*, 2010), *B. halochares* (Pappa *et al.*, 2010), *B. horneckiae* (Vaishampayan *et al.*, 2010), *B. marmarensis* (Denizci *et al.*, 2010), *B. methylotrophicus* (Madhaiyan *et al.*, 2010), *B. oceanisediminis* (Zhang *et al.*, 2010), *B. rigui* (Baik *et al.*, 2010), *B. siamensis* (Sumpavapol *et al.*, 2010) and *B. trypoxylicola* (Aizawa *et al.*, 2010). During an investigation of the diversity of the microbial population of invertebrates inhabiting the South China Sea (Chen *et al.*, 2009a, b, c, 2010; Huang *et al.*, 2009; Xiao *et al.*, 2009), a moderately halophilic, facultatively alkaliphilic, endospore-forming, Gram-staining-positive bacterium, designated strain JSM 076093^T, was isolated from a sea urchin

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain JSM 076093^T is HM460885.

Two supplementary figures are available with the online version of this paper.

(*Hemicentrotus pulcherrimus*) collected from Naozhou Island (20° 52–56' N 110° 33–38' E), China.

For isolation, serial dilutions (1:10) of sea urchin homogenate were plated on marine agar 2216 (MA; Difco) supplemented with 0–20% (w/v) NaCl and incubated at 28 °C for 7–14 days. A yellow colony on MA supplemented with 10% (w/v) NaCl was picked and purified. Strain JSM 076093^T was maintained on slants of MA supplemented with 3% (w/v) NaCl (MA3; pH 8.0), as lyophilized cultures at 4 °C and in 20% (v/v) glycerol at –80 °C. For comparison, *Bacillus hwajinpoensis* DSM 16206^T and *Bacillus algicola* KCTC 13005^T were obtained from the DSMZ and the KCTC, respectively. Unless indicated otherwise, morphological, physiological, molecular and chemotaxonomic studies were performed with cells grown on MA3 at 30 °C.

Strain JSM 076093^T, *B. hwajinpoensis* DSM 16206^T and *B. algicola* KCTC 13005^T were phenotypically characterized in this study according to 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 phase-contrast microscopy (DM3000, ×100 HCX PL Fluotar oil immersion objective, Ph3; Leica) with cells grown on MA3 supplemented with 10 mg MnSO₄ for 1–7 days at 30 °C. The Gram-staining and KOH lysis tests were carried out according to Smibert & Krieg (1994) and Gregersen (1978), respectively. Flagella and endospores were examined according to the methods of Leifson and Schaeffer-Fulton, respectively (Smibert & 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) without the addition of NaCl. Growth with 0.1, 0.5 and 1–30% (w/v) NaCl (in increments of 1% NaCl) was tested on NA and in NB. Growth at 4 and 5–55 °C (in increments of 5 °C) and at pH 5.0–11.0 (in increments of 0.5 pH units) was tested in NB, using the buffer solutions described by Chen *et al.* (2007) to adjust the pH. The methyl red and Voges–Proskauer tests and determination of H₂S production from L-cysteine, aesculin hydrolysis, indole production, nitrate and nitrite reduction, glucose oxidation/fermentation and phenylalanine deaminase and urease activities were performed as described by Smibert & Krieg (1994). Hydrolysis of casein, cellulose, DNA, gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80 and xanthine was determined as described by Cowan & 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 System (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 for catalase and oxidase activities were detected as described previously (Chen *et al.*, 2007). Other enzyme activities were assayed using API ZYM strips (bioMérieux), according to the

manufacturer's instructions except for the preparation of inocula with 5% (w/v) NaCl. All physiological and biochemical tests were repeated three times.

Cells of strain JSM 076093^T were Gram-staining-positive, endospore-forming, non-motile, moderately halophilic, facultatively alkaliphilic rods, with optimum growth occurring with 5–8% (w/v) NaCl and at pH 8.0 and 30–35 °C. Colonies were yellow, flat and opaque with circular margins and were 3–4 mm in diameter after 3–4 days. The phenotypic properties that differentiate strain JSM 076093^T from its closest phylogenetic neighbours are given in Table 1.

Table 1. Characteristics used to distinguish strain JSM 076093^T from its closest phylogenetic neighbours

Strains: 1, JSM 076093^T; 2, *B. hwajinpoensis* DSM 16206^T; 3, *B. algicola* KCTC 13005^T. All data were obtained from this study unless indicated otherwise. All strains are endospore-forming, Gram-staining-positive rods and positive for catalase, nitrate reduction and hydrolysis of gelatin, starch and Tween 20. All strains are negative for indole and H₂S production, nitrite reduction, phenylalanine deaminase, Voges–Proskauer test and hydrolysis of cellulose, DNA, hypoxanthine and xanthine. C, Central; CR, creamy; LY, light yellow; s, swollen; ss, slightly swollen; ST, subterminal; U, unswollen; Y, yellow; +, positive; –, negative.

Characteristic	1	2	3
Colony colour	Y	LY	CR
Spore position	ST	C to ST	ST
Sporangium	U or ss	ss	s
NaCl (% w/v)			
Range	0.5–25	0.5–20	0–4
Optimum	5–8	2–4	0
pH			
Range	6.0–10.5	6.0–10.0	6.5–10.5
Optimum	8.0	7.5–8.0	7.5–8.5
Temperature (°C)			
Range	5–40	10–40	10–45
Optimum	30–35	30–35	30
Oxidase	–	+	–
Urease	+	–	+
Facultatively anaerobic	+	–	–
Glucose fermentation	+	–	–
Methyl red test	+	–	–
Hydrolysis of:			
Aesculin	+	+	–
Casein	+	+	–
Tween 40	+	+	–
Tween 60	+	+	–
Tween 80	–	+	–
Source*	Sea urchin	Seawater	Brown alga
DNA G + C content (mol%)*	38.8	40.9	37.4

*Data for columns 2 and 3 were obtained from Yoon *et al.* (2004) and Ivanova *et al.* (2004), respectively.

Genomic DNA was isolated according to Hopwood *et al.* (1985) and the G + C content was determined using HPLC (Mesbah *et al.*, 1989). The 16S rRNA gene sequence was amplified by PCR and sequenced as described by Cui *et al.* (2001). Primers A 8–27f (5'-CCGTCGACGAGCTCAGAGTTTGATCCTGGCTCAG-3') and B 1523–1504r (5'-CCCGGGTACCAAGCTTAAGGAGGTGATCCAGCCGCA-3') were used. Pairwise sequence similarities were calculated using a global alignment algorithm implemented at the EzTaxon server (Chun *et al.*, 2007). After multiple sequence alignment using CLUSTAL_X (Thompson *et al.*, 1997), phylogenetic analysis was performed using MEGA version 3.1 (Kumar *et al.*, 2004). 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 & Nei, 1987). Maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) trees were generated using the algorithms contained in the PHYLIP package (Felsenstein, 2002). Bootstrap analysis by means of 1000 resamplings was used to evaluate tree topologies (Felsenstein, 1985). DNA–DNA hybridization was performed using the optical renaturation method (De Ley *et al.*, 1970; Huß *et al.*, 1983; Jahnke, 1992) with three replications.

The DNA G + C content of strain JSM 076093^T was 38.8 mol%. An almost-complete 16S rRNA gene sequence (1482 bp) was determined. The phylogenetic analysis revealed that strain JSM 076093^T should be assigned to the genus *Bacillus* and that it was most closely related to *B. hwajinpoensis* SW-72^T (99.1% 16S rRNA gene sequence similarity; Yoon *et al.*, 2004) and *B. algicola* KMM 3737^T (97.3%; Ivanova *et al.*, 2004). Less than 95.0% sequence similarity was observed with members of other species of the genus *Bacillus*. The neighbour-joining tree confirmed that strain JSM 076093^T was phylogenetically closely related to the genus *Bacillus*. Strain JSM 076093^T formed a robust lineage (100% bootstrap support) with *B. hwajinpoensis* SW-72^T and *B. algicola* KMM 3737^T (Fig. 1). Similar topologies were obtained in trees constructed using the maximum-likelihood and maximum-parsimony methods (Supplementary Fig. S1, available in IJSEM Online). DNA–DNA

relatedness between strain JSM 076093^T and *B. hwajinpoensis* DSM 16206^T and *B. algicola* KCTC 13005^T was (mean ± SD) 30.7 ± 3.2 and 10.5 ± 1.5%, respectively, which is well below the 70% threshold value recommended by Wayne *et al.* (1987) for the definition of species. Therefore, on the basis of phylogenetic analysis and DNA–DNA relatedness and in accordance with accepted criteria (Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994), it would appear that strain JSM 076093^T represents a novel species of the genus *Bacillus*.

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 identified by two-dimensional TLC and spraying with appropriate detection reagents (Collins & Jones, 1980). Fatty acids were determined for strain JSM 076093^T and the reference strains according to Sasser (1990), using the Microbial Identification System (Microbial ID) with cells grown in marine broth 2216 (Difco) in a rotary shaker (200 r.p.m.) at 30 °C for 2 days.

The chemotaxonomic data for strain JSM 076093^T were consistent with the assignment of strain JSM 076093^T to the genus *Bacillus*. Strain JSM 076093^T possessed a cell-wall type based on *meso*-diaminopimelic acid as the diagnostic diamino acid. The isolate contained MK-7 (95.8%) as the predominant menaquinone, with MK-6 (1.5%) and MK-8 (2.7%) present in minor amounts. The polar lipids consisted of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and one unidentified phospholipid (Supplementary Fig. S2). The fatty acid profile of strain JSM 076093^T was similar to those of the reference strains, although there were differences in the proportions of some components (Table 2). The major fatty acids were anteiso-C_{15:0} (47.5%), anteiso-C_{17:0} (12.9%), iso-C_{16:0} (12.8%) and iso-C_{14:0} (9.0%), which are characteristic of many members within the genus *Bacillus* (Kämpfer, 1994).

The results of the phylogenetic analysis and the morphological and chemotaxonomic investigations supported the affiliation of strain JSM 076093^T to the genus *Bacillus*. However, the abilities to grow under anaerobic conditions,

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain JSM 076093^T and closely related members of the genus *Bacillus*. Bootstrap values (>50%) based on 1000 resampled datasets are shown at branch nodes. Bar, 1 substitution per 100 nucleotides.

Table 2. Fatty acid compositions of strain JSM 076093^T and related species of the genus *Bacillus*

Strains: 1, JSM 076093^T; 2, *B. hwajinpoensis* DSM 16206^T; 3, *B. algicola* KCTC 13005^T. All data were taken from this study.

Fatty acid (%)	1	2	3
Saturated			
C _{14:0}	0.6	0.2	0.4
C _{16:0}	6.0	0.5	1.0
C _{18:0}	0.9	0.1	0.2
Unsaturated			
C _{16:1} ω7c alcohol	2.8	10.0	3.3
C _{16:1} ω11c	1.2	0.4	0.2
C _{18:1} ω9c	0.6	0.2	0.3
Branched			
anteiso-C _{13:0}	0.2	0.2	—
iso-C _{13:0}	—	—	0.7
iso-C _{14:0}	9.0	31.0	7.6
iso-C _{15:0}	3.5	6.8	11.7
anteiso-C _{15:0}	47.5	30.3	50.2
iso-C _{16:0}	12.8	16.8	14.0
iso-C _{17:0}	0.9	0.6	2.3
anteiso-C _{17:0}	12.9	2.5	6.1
Summed features*			
3	0.4	0.1	0.2
4	0.3	0.3	0.3

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C_{16:1}ω7c and/or C_{16:1}ω6c. Summed feature 4 consisted of iso-C_{17:1} I and/or anteiso-C_{17:1} B.

ferment glucose and grow with up to 25 % (w/v) NaCl, as well as the colony pigmentation, the positive result for the methyl red test and several other phenotypic characteristics, clearly differentiated the isolate from its closest phylogenetic relatives. In conclusion, strain JSM 076093^T represents a novel species of the genus *Bacillus*, for which we propose the name *Bacillus hemicentroti* sp. nov.

Description of *Bacillus hemicentroti* sp. nov.

Bacillus hemicentroti [he.mi.cen.tro'ti. N.L. gen. n. *hemicentroti* of *Hemicentrotus* (*Hemicentrotus pulcherrimus*, a sea urchin), the source of isolation of the organism].

Cells are Gram-staining-positive, catalase-positive, oxidase-negative, non-motile, facultatively anaerobic, straight rods, approximately 0.6–0.9 µm wide and 2.5–3.5 µm long, occurring singly, as pairs or as short chains and producing ellipsoidal endospores that lie in subterminal unswollen or slightly swollen sporangia. Colonies are yellow, flat and opaque, have smooth surfaces and circular margins and are 3–4 mm in diameter on MA supplemented with 3 % (w/v) NaCl. No diffusible pigments are produced. Moderately halophilic and facultatively alkaliphilic; growth occurs with 0.5–25 % (w/v) NaCl (optimum 5–8 %), at pH 6.0–10.5 (optimum pH 8.0) and at 5–40 °C (optimum 30–35 °C).

Nitrate is reduced to nitrite, but nitrite is not reduced further. Positive for glucose fermentation, methyl red test and urease, but negative for phenylalanine deaminase, H₂S and indole production and the Voges–Proskauer test. Aesculin, casein, gelatin, starch and Tweens 20, 40 and 60 are hydrolysed, but cellulose, DNA, hypoxanthine, Tween 80 and xanthine are not. Acids are produced from L-arabinose, D-fructose, D-glucose, glycogen, maltose, D-mannose, melibiose, raffinose, starch, sucrose, D-mannitol and D-sorbitol, but not from cellobiose, D-galactose, lactose, melezitose, L-rhamnose, D-ribose, trehalose, D-xylose, amygdalin, D-salicin, adonitol, dulcitol, glycerol, *myo*-inositol or *N*-acetylglucosamine. As sole sources of carbon or carbon and nitrogen, utilizes L-arabinose, dextrin, D-fructose, D-glucose, maltose, melibiose, D-ribose, sucrose, D-mannitol, gluconate, L-alanine and L-asparagine, but not cellobiose, D-galactose, glycogen, lactose, D-mannose, melezitose, raffinose, L-rhamnose, trehalose, D-xylose, amygdalin, D-salicin, adonitol, D-arabitol, glycerol, *myo*-inositol, D-sorbitol, acetate, butyrate, citrate, propionate, succinate, *N*-acetylglucosamine, L-arginine, L-glutamic acid, glycine, L-histidine, hydroxy-L-proline, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine or L-valine. Constitutive enzymes are acid and alkaline phosphatases, α-chymotrypsin, esterase (C4), esterase lipase (C8), β-galactosidase, α-glucosidase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase, but not cystine arylamidase, α-fucosidase, α-galactosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, β-glucuronidase, lipase (C14), α-mannosidase, trypsin or valine arylamidase. *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, phosphatidylglycerol and one unidentified phospholipid as the polar lipids. The major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0}, iso-C_{16:0} and iso-C_{14:0}.

The type strain, JSM 076093^T (=DSM 23007^T=KCTC 13710^T), was isolated from a homogenate of a sea urchin (*Hemicentrotus pulcherrimus*) collected from Naozhou Island in the South China Sea. The DNA G+C content of the type strain is 38.8 mol% (HPLC).

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