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

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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 DSM 76093T, was isolated from a sea urchn (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-C15:0, anteiso-C17:0, iso-C16:0 and iso-C14:0. The genomic DNA G+C content was 38.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that strain DSM 76093T belonged to the genus Bacillus and was related most closely to Bacillus hwajinpoensis SW-72T (99.1 % 16S rRNA gene sequence similarity) and Bacillus algicola KMM 3737T (97.3 %). The combination of results from the phylogenetic analysis, DNA–DNA hybridization and phenotypic and chemotaxonomic characterization supported the conclusion that strain DSM 76093T represents a novel species of the genus Bacillus, for which the name Bacillus hemicentroti sp. nov. is proposed, with DSM 76093T (=DSM 23007T=KCTC 13710T) 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; Arahal & Ventosa, 2001; Nogri 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 changangensis (Cho et al., 2010), B. gallicensis (Balcázar et al., 2010), B. halochares (Pappa et al., 2010), B. hornneckiae (Vaishampayan et al., 2010), B. marmarensis (Denizci et al., 2010), B. methylotrophicus (Madhavian 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 DSM 76093T, was isolated from a sea urchin...
(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, Tween 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 without or with 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 described as 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 alkalophilic 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 pheno-type 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

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

*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’-CCGTCGAGCCCAGAGGTTGATCCTGGCCTCAG-3’) and B 1523–1504r (5’-CCCGGGTACCAAGGATGGTATCCACGCGCA-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 (Klug & 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, 1970; Huß et al., 1983; Jahnke, 1992) with three replications.

The DNA G+C content of strain JSM 076093T was 38.8 mol%. An almost-complete 16S rRNA gene sequence (1482 bp) was determined. The phylogenetic analysis revealed that strain JSM 076093T should be assigned to the genus Bacillus and that it was most closely related to B. hwajinpoensis SW-72T (99.1% 16S rRNA gene sequence similarity; Yoon et al., 2004) and B. algicola KMM 3737T (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 076093T was phylogenetically closely related to the genus Bacillus. Strain JSM 076093T formed a robust lineage (100% bootstrap support) with B. hwajinpoensis SW-72T and B. algicola KMM 3737T (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 076093T and B. hwajinpoensis DSM 16206T and B. algicola KCTC 13005T 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 the 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 076093T 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 076093T 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 076093T were consistent with the assignment of strain JSM 076093T to the genus Bacillus. Strain JSM 076093T 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 076093T 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-C15:0 (47.5%), anteiso-C17:0 (12.9%),iso-C16:0 (12.8%) and iso-C14:0 (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 076093T to the genus Bacillus. However, the abilities to grow under anaerobic conditions,
Table 2. Fatty acid compositions of strain JSM 076093T and
related species of the genus Bacillus

<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>1</th>
<th>2</th>
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<tbody>
<tr>
<td>Saturated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C14:0</td>
<td>0.6</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>C16:0</td>
<td>6.0</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>C18:0</td>
<td>0.9</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Unsaturated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:1ω7c alcohol</td>
<td>2.8</td>
<td>10.0</td>
<td>3.3</td>
</tr>
<tr>
<td>C16:1ω11c</td>
<td>1.2</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>C18:1ω9c</td>
<td>0.6</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Branched</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anteiso-C13:0</td>
<td>0.2</td>
<td>0.2</td>
<td>–</td>
</tr>
<tr>
<td>iso-C13:0</td>
<td>–</td>
<td>–</td>
<td>0.7</td>
</tr>
<tr>
<td>iso-C14:0</td>
<td>9.0</td>
<td>31.0</td>
<td>7.6</td>
</tr>
<tr>
<td>iso-C15:0</td>
<td>3.5</td>
<td>6.8</td>
<td>11.7</td>
</tr>
<tr>
<td>anteiso-C15:0</td>
<td>47.5</td>
<td>30.3</td>
<td>50.2</td>
</tr>
<tr>
<td>iso-C16:0</td>
<td>12.8</td>
<td>16.8</td>
<td>14.0</td>
</tr>
<tr>
<td>iso-C17:0</td>
<td>0.9</td>
<td>0.6</td>
<td>2.3</td>
</tr>
<tr>
<td>anteiso-C17:0</td>
<td>12.9</td>
<td>2.5</td>
<td>6.1</td>
</tr>
<tr>
<td>Summed features*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.4</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Summed features represent two or three fatty acids that cannot be separated by the Microbial Identification System. Summed feature 3 consisted of C16:1ω7c and/or C16:1ω6c. Summed feature 4 consisted of iso-C17:1ω1 and/or anteiso-C17:1ω1 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 076093T 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, HisS and indole production and the Voges–Proskauer test. Aesculin, casein, gelatin, starch and Tween 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-xyllose, amygdalin, D-salicin, adonitol, dulcitol, glycerol, myo-inositol or N-acetylglycosamidase. As sole sources of carbon or carbon and nitrogen, utilizes D-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-xyllose, amygdalin, D-salicin, adonitol, D-arabitol, glycerol, myo-inositol, D-sorbitol, acetate, butyrate, citrate, propionate, succinate, N-acetylglycosamidase, 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, z-chymotrypsin, esterase (C4), esterase lipase (C8), β-galactosidase, α-glucosidase, leucine arylamidase and naphthol-AS-Bl-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-C15:0, anteiso-C17:0, iso-C16:0 and iso-C14:0.

The type strain, JSM 076093T (=DSM 23007T =KCTC 13710T), 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).

Acknowledgements

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