Bacillus oceanisediminis sp. nov., isolated from marine sediment

Jianli Zhang,1 Jiewei Wang,1 Caiyuan Fang,1 Fei Song,1 Yuhua Xin,2 Lei Qu1 and Kai Ding1

1School of Life Science and Technology, Beijing Institute of Technology, Beijing 100081, PR China
2China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China

A Gram-stain-positive, spore-forming, rod-shaped and aerobic bacterium was isolated from a sediment sample from the South Sea in China. The isolate, designated H2T, grew at 4–45 ºC (optimum 37 ºC) and pH 6–10 (optimum pH 7.0). The cell-wall peptidoglycan contained meso-diaminopimelic acid. The major isoprenoid quinone was MK-7 and the polar lipids consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unknown aminophospholipid. The major fatty acid was iso-C15 : 0. The genomic DNA G+C content of strain H2T was 44.8 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate formed a monophyletic clade with Bacillus firmus IAM 12464T. DNA–DNA relatedness between the isolate and B. firmus ATCC 14575T was low (27.5 %). Strain H2T also had a phenotypic profile that readily distinguished it from its closest phylogenetic neighbours. It is evident from the combination of genotypic and phenotypic data that the organism should be classified in a novel species of the genus Bacillus, for which the name Bacillus oceanisediminis sp. nov. is proposed. The type strain is H2T (=CGMCC 1.10115T =JCM 16506T).

The application of chemotaxonomic, numerical phenetic and molecular systematic methods has led to an improved description and a division of the genus Bacillus. Some species have been reclassified in novel genera or transferred to other genera (Ash et al., 1993; Stackebrandt & Liesack, 1993; Shida et al., 1996; Waino et al., 1999; Nazina et al., 2001; Yoon et al., 2001; Albert et al., 2007; Zhou et al., 2009). Many of these reclassifications are well supported by relatively conserved phenotypic traits such as the quinone system, peptidoglycan composition and polar lipid and cellular fatty acid profiles (Albert et al., 2007). Falsibacillus is a newly described genus, which currently comprises a single species, Falsibacillus pallidus comb. nov., and is represented by the type strain, CW 7T. The taxon F. pallidus comb. nov. is linked to the illegitimately named taxon Bacillus pallidus Zhou et al. 2008 and the creation of the genus Falsibacillus was primarily based on significant chemotaxonomic differences in comparison with the type species of the genus Bacillus. Bacillus subtulis (Zhou et al., 2008, 2009). The genus Bacillus currently encompasses more than 150 species with validly published names and contains at least six phylogenetically distinct groups on the basis of molecular analyses of 16S rRNA gene sequences (Ash et al., 1991; Nielsen et al., 1994; Schlesner et al., 2001; Yoon et al., 2004; Carrasco et al., 2007). Members of the genus Bacillus have been isolated from a wide variety of aquatic and terrestrial environments. A Gram-stain-positive bacterium, strain H2T, was isolated during an ecological survey of marine organisms in China and found to have morphological properties consistent with the genus Bacillus.

Strain H2T was isolated from a marine sediment collected at a depth of 823 m in the South Sea, China. A standard dilution-plating technique was used to isolate the strain from marine agar 2216 (MA; Difco) at 37 ºC for 3 days. Strain H2T was maintained on LB agar (10 g tryptone, 5 g yeast extract, 10 g NaCl, 1 l distilled water; pH 7.0) at 4 ºC and stored in 20 % (v/v) glycerol suspensions at −20 ºC.

To characterize strain H2T phenotypically, standard phenotypic tests were performed according to procedures described elsewhere (Gordon et al., 1973, 1977; Smibert & Krieg, 1994; Zhang et al., 2008, 2009). Cellular morphology was examined by light microscopy (BH-2; Olympus). Gram staining was performed as described by Gerhardt et al. (1981). The morphology, size and pigmentation of colonies were observed on LB agar, MA and nutrient agar after 1 day at 37 ºC. Catalase activity was determined on freshly growing colonies using 3 % (v/v) hydrogen peroxide solution. The oxidase reaction was performed on filter paper moistened with a 1 % (w/v) aqueous solution of N,N,N’,N’-tetramethyl-p-phenylenediamine. Growth under

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain H2T is GQ292772.

A comparison of the fatty acid profiles of strain H2T and its closest phylogenetic neighbours is available with the online version of this paper.

Correspondence
Jianli Zhang
zhangjianli@bit.edu.cn

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various pH, temperature and sodium chloride regimes was determined on LB agar after incubation for up to 7 days. Acid production from carbohydrates was determined using methods described by Gordon et al. (1974) and utilization of sole carbon sources was determined according to Gordon & Mihm (1957). Resistance to antibiotics was examined using impregnated filter-paper discs (Goodfellow & Orchard, 1974) on LB agar and incubation at 37 °C for up to 7 days. Resistance to lysozyme was determined by the method of Gordon et al. (1973). Strain H2T was Gram-stain-positive and aerobic. The cells were spore-forming rods (0.6–0.8 by 2.0–3.0 μm). After 1 day at 37 °C on LB agar, colonies were circular, creamy white and 1–3 mm in diameter. Strain H2T grew at 4–45 °C (optimum 37 °C) and pH 6–10 (optimum pH 7.0). A range of phenotypic properties separated strain H2T from its closest phylogenetic neighbours (Table 1). In particular, the isolate could be differentiated from Bacillus firmus ATCC 14575T in that it was positive for utilization of D-galactose, inulin, myo-inositol, lactose, D-sorbitol and xylitol. Additional phenotypic properties are presented in the species description and Table 1.

Biomass for most of the chemotaxonomic and molecular systematic studies was prepared as described by Zhang et al. (2002), with the modification of using shake flasks of LB broth. Preparation of the cell wall and determination of the peptidoglycan composition were performed using the methods described by Schleifer (1985), with the modification that cellulose sheets instead of paper were used for TLC. Isoprenoid quinones were extracted and purified according to Collins et al. (1987) and purified menaquinones were determined by reversed-phase HPLC (Wu et al., 1989). Polar lipids were examined by two-dimensional TLC and identified using the method of Minnikin et al. (1984). The cellular fatty acid profile was determined for cells cultured on tryptic soy broth agar at 28 °C for 24 h. Cells were saponified and the cellular fatty acids were extracted, purified, methylated and quantified by GC using the standard Microbial Identification System (MIDI; Sasser, 1990; Kämpfer & Kroppenstedt, 1996). The G+C content of the DNA was determined using the thermal denaturation method (Marmur & Doty, 1962) with Escherichia coli AS 1.365 as a reference.

**Table 1.** Comparison of properties of strain H2T with the three most closely related type strains of species in the genus Bacillus and the type strain of Falsibacillus pallidus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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<tbody>
<tr>
<td>Isolation source</td>
<td>Marine sediment</td>
<td>Soil</td>
<td>Patient with neonatal sepsis</td>
<td>Spacecraft-assembly facility</td>
<td>Forest soil</td>
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<tr>
<td>Reduction of nitrate</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
<td>–</td>
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<td>Oxidase</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Arginine dihydrolase</td>
<td>+</td>
<td>–</td>
<td>ND</td>
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<td>–</td>
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<tr>
<td>Growth at/with:</td>
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<tr>
<td>Temperature range (°C)</td>
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<td>10–40</td>
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<td>25–60</td>
<td>15–42</td>
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<td>Temperature optimum (°C)</td>
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<tr>
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<tr>
<td>Gelatin</td>
<td>+</td>
<td>–</td>
<td>ND</td>
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<td>+</td>
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<tr>
<td>Starch</td>
<td>+</td>
<td>+</td>
<td>ND</td>
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<td>Sole carbon sources</td>
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<tr>
<td>D-Galactose</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<td>–</td>
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<tr>
<td>Glycerol</td>
<td>W</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Inulin</td>
<td>+</td>
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<tr>
<td>myo-Inositol</td>
<td>+</td>
<td>–</td>
<td>+</td>
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<tr>
<td>Lactose</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>–</td>
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<tr>
<td>Maltose</td>
<td>W</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<tr>
<td>D-Mannitol</td>
<td>+</td>
<td>V</td>
<td>+</td>
<td>–</td>
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<tr>
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<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
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<tr>
<td>D-Sorbitol</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Trehalose</td>
<td>W</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<tr>
<td>Xylitol</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Acid production from D-mannitol</td>
<td>–</td>
<td>+</td>
<td>ND</td>
<td>+</td>
<td>–</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>44.8</td>
<td>43.7</td>
<td>40.8</td>
<td>ND</td>
<td>42.3</td>
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</table>

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Analysis of the cell-wall peptidoglycan showed that strain H2\textsuperscript{T} possessed the A1\textsubscript{y} type, with meso-diaminopimelic acid as the diagnostic diamino acid, which is in common with a large majority of members of the genus Bacillus (Priest et al., 1988). Strain H2\textsuperscript{T} contained MK-7 as the major menaquinone. The polar lipids detected were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unknown aminophospholipid. The cellular fatty acid profile of strain H2\textsuperscript{T} contained iso-C\textsubscript{15}:0 (35.65 %), C\textsubscript{16:0} 97c alcohol (9.97 %), iso-C\textsubscript{14}:0 (9.71 %), anteiso-C\textsubscript{15}:0 (9.61 %), iso-C\textsubscript{16}:0 (9.21 %) and summed feature 4 (iso-C\textsubscript{17}:1 I and/or anteiso-C\textsubscript{17}:1 B; 6.73 %) as the major fatty acids (>5 %) (Supplementary Table S1, available in IJSEM Online). These iso- and anteiso-branched fatty acids of the 14–17-carbon series are typical of those found in the cell membranes of members of the genus Bacillus (Kämpfer, 1994; Albert et al., 2005). Thus, these chemotaxonomic features of strain H2\textsuperscript{T} were typical of those for the genus Bacillus (Priest et al., 1988; Heyrman et al., 2004; Albert et al., 2005, 2007; Wieser et al., 2005; Lim et al., 2006a, b; Xue et al., 2008). In addition, the DNA G+C content of strain H2\textsuperscript{T} was 44.8 mol%, which is within the range for the genus Bacillus (Nielsen et al., 1995; Priest et al., 1988).

Chromosomal DNA preparation from strain H2\textsuperscript{T}, PCR-mediated amplification of the 16S rRNA gene and purification of the amplification product were carried out according to Rainey et al. (1996) and the amplification product was sequenced directly using the method of Lu et al. (2001). Nucleotide sequences were sequenced automatically using an Applied Biosystems DNA sequencer (model 377) and software provided by the manufacturer. The resultant 16S rRNA gene sequence was compared with those available from the GenBank database using the BLAST program to determine an approximate phylogenetic affiliation for strain H2\textsuperscript{T}. Multiple alignment with closely related sequences was performed using CLUSTAL X version 1.8 (Thompson et al., 1997). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), minimum-evolution and maximum-parsimony algorithms in MEGA version 3.1 (Kumar et al., 2004). Evolutionary distance matrices were generated according to Kimura (1980). Topologies of the resultant unrooted trees were evaluated by bootstrap analysis (Felsenstein, 1985) using 1000 resamplings of the neighbour-joining dataset.

An almost-complete 16S rRNA gene sequence (1393 nt) was obtained for strain H2\textsuperscript{T}. Phylogenetic analysis showed that the isolate belonged in the phyletic group classically defined as the genus Bacillus. The type strains of species of the genus Bacillus with the greatest pairwised similarity to strain H2\textsuperscript{T} were B. firmus IAM 12464\textsuperscript{T} (98.5 % 16S rRNA gene sequence similarity), B. infantis SMC 4352-1\textsuperscript{T} (98.3 %) and B. nealsornii FO-92\textsuperscript{T} (97.7 %). The sequence similarities between strain H2\textsuperscript{T} and other members of the genus Bacillus were below 97.0 %. The initial alignment included 16S rRNA gene sequences from members of nearly all of the species of the genus Bacillus with validly published names. Of these, 25 sequences were found to have a close relationship with strain H2\textsuperscript{T} and were selected for further analysis. It is apparent from the neighbour-joining phylogenetic tree (Fig. 1) that strain H2\textsuperscript{T} forms a coherent cluster with B. firmus IAM 12464\textsuperscript{T} and B. infantis SMC 4352-1\textsuperscript{T} within the genus Bacillus and is distantly related to other members of the genus. The same results were obtained with the other methods of phylogenetic reconstruction used in the study, minimum evolution and maximum parsimony (data not shown).

There are widely accepted criteria for delineating species in current bacteriology, stating that strains with <70 % DNA–DNA relatedness or with <97 % 16S rRNA gene sequence similarity are considered to represent different species (Wayne et al., 1987; Stackebrandt & Goebel, 1994; Stackebrandt et al., 2002). Many members of the genus Bacillus with >98.5 % 16S rRNA gene sequence similarity are considered to belong to different species. For example, the 16S rRNA gene sequences of the type strains of Bacillus bataviensis, B. soli, B. drentensis, B. novalis and B. vireti show 98.7–99.6 % pairwise similarity (Ko et al., 2006). DNA–DNA relatedness provides a reliable way of distinguishing between representatives of species that share high 16S rRNA gene sequence similarity (Goodfellow et al., 1998). In the present study, DNA–DNA relatedness studies were carried out between the isolate and its closest phylogenetic neighbours, B. firmus ATCC 14575\textsuperscript{T}, B. infantis SMC 4352-1\textsuperscript{T} and B. nealsornii ATCC BAA-519\textsuperscript{T}, using the liquid renaturation method (De Ley et al., 1970; Huß et al., 1983; Jahnke, 1992) using a model Lambda 35 UV/VIS spectrometer equipped with a temperature program controller (Perkin–Elmer) and the results were expressed as the mean of three determinations. Strain H2\textsuperscript{T} showed low levels of DNA–DNA relatedness with B. firmus ATCC 14575\textsuperscript{T} (27.5 %), B. infantis SMC 4352-1\textsuperscript{T} (23.1 %) and B. nealsornii ATCC BAA-519\textsuperscript{T} (20.7 %); all of these values are well below the 70 % cut-off point.

Although strain H2\textsuperscript{T} showed a higher 16S rRNA gene sequence similarity with F. pallidus CW 7\textsuperscript{T} (97 %) than with the type strain of the type species of the genus Bacillus, B. subtilis NCDO 1769\textsuperscript{T} (96 %), the isolate did not belong to the genus Falsibacillus. Chemotaxonomic data showed some significant differences in comparison with F. pallidus CW 7\textsuperscript{T} (Supplementary Table S1). Polar lipid profiles have proved to be a most useful tool for the classification and determination of members of the family Bacillaceae in recent years (Zhou et al., 2009). The polar lipid profile of B. subtilis has been shown to be characteristic of the genus Bacillus and should form part of the genus description (Kämpfer et al., 2006). The polar lipid profile of B. subtilis comprises diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, an unknown aminophospholipid and β-gentibiosyldiacylglycerol (Kämpfer et al., 2006; Albert et al., 2007), while the polar lipids of F. pallidus comprise diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two aminophosphoglycolipids, five unknown...
phospholipids, two phosphoglycolipids and one glycolipid (Zhou et al., 2009). The polar lipid profile of strain H2T was similar to those for the genus Bacillus. The significant differences in the cellular fatty acid profile and phenotypic properties (Table 1 and Supplementary Table S1) can be used to distinguish strain H2T from F. pallidus CW 7T and, therefore, it is concluded that the isolate should be placed in the genus Bacillus.

On the basis of the taxonomic data described above, strain H2T represents a novel species of the genus Bacillus, for which the name Bacillus oceanisediminis sp. nov. is proposed.

**Description of Bacillus oceanisediminis sp. nov.**

*Bacillus oceanisediminis* (o.ce.a.ni.se.di’ni.isi. L. n. oceanus ocean; L. n. sedimen -inis sediment; N.L. gen. n. oceanisediminis of/from sediment of the ocean).

Cells are 0.6–0.8 μm in width and 2–3 μm in length, Gram-stain-positive, aerobic, round-ended rods that occur singly or in pairs and occasionally in short chains. Ellipsoidal endospores are formed subterminally, paracentrally or centrally. After 1 day at 37°C on LB agar, colonies are creamy white, slightly convex in the centre, smooth, round and 1–3 mm in diameter; edges are usually irregular with pointed projections that may spread and become rhizoid in old cultures. Diffusible pigments are not formed. Grows at 4–45°C (optimum 37°C), at pH 6–10 (optimum pH 7.0) and with 0–13% (w/v) NaCl. Positive for catalase, oxidase, β-glucosidase and arginine dihydrolase, but negative for lipase, phenylalanine deaminase and urease. Nitrate is reduced. Indole and methyl red tests are positive, but Voges–Proskauer and egg-yolk reactions are negative. Hydrogen sulphide is not produced. Adenine, casein, gelatin, starch and tyrosine (weak) are hydrolysed; aesculin and arbutin are not hydrolysed. Acid is formed from D-glucose, inulin, glycogen, raffinose and sucrose but not from L-arabinose, cellobiose, D-fructose, D-galactose, glycerol, myo-inositol, lactose, maltose, D-mannitol, melezitose, melibiose, x-L-rhamnose, D-sorbitol, trehalose, D-xylitol or D-xylene. As sole carbon and energy sources, utilizes cellobiose, meso-erythritol, D-glucose, glycogen, melezitose, raffinose, x-L-rhamnose, sucrose, acetate, fumarate, lactic acid, malate, pyruvic acid, sorbic acid, succinate and tartrate (weak), but not L-arabinose, D-fructose, melibiose, D-ribose, L-sorbitase, D-xylene, benzoate, citrate, malonate, propionate or oxalate. Resistant to (μg per disc unless otherwise stated) aztreonam (30), but susceptible to lysozyme (0.005%, w/v), amikacin (30), amoxicillin plus clavulanic acid (10), ampicillin (10), cefalotaxime (30), chloramphenicol (30), ciprofloxacin (5), cindamycin hydrochloride (2), erythromycin (15), gentamicin sulphonate (10), kanamycin sulphonate (30), mezlocillin (75), oxolinic (5), penicillin G (10 U), rifampicin (5), streptomycin sulphonate (10), tetracycline hydrochloride (30) and tobramycin sulphonate (10). Additional phenotypic properties are shown in Table 1. Chemotaxonomic properties are typical of the genus Bacillus: the cell-wall peptidoglycan contains meso-diaminopimelic acid, the major isoprenoid quinone type is MK-7 and the polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and an unknown aminophospholipid. The fatty acid profile (≥2%) is composed of iso-C15:0, C16:1ω7c alcohol, iso-C14:0 anteiso-C15:0, iso-C16:0, summed feature 4 (iso-C17:1ω1 and/or anteiso-C17:1ω2, B), iso-C16:1ω6c, iso-C17:1ω7c, anteiso-C17:1ω9c, anteiso-C17:0, iso-C17:0 and C16:1ω11c. The genomic DNA G+C content of the type strain is 44.8 mol%.

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The type strain, H2T (=CGMCC 1.10115T = JCM 16506T), was isolated from a sediment sample from the South Sea, China.

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