Oceanobacillus arenosus sp. nov., a moderately halophilic bacterium isolated from marine sand

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A Gram-stain-positive, spore-forming, rod-shaped, motile, strictly aerobic bacterium, designated CAU 1183^T, was isolated from marine sand and its taxonomic position was investigated by using a polyphasic approach. The bacterium grew optimally at 30 °C, at pH 8.5 and in the presence of 2 % (w/v) NaCl. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain CAU 1183^T formed a distinct lineage within the genus *Oceanobacillus* and exhibited the highest similarity to *Oceanobacillus chungangensis* CAU 1051^T (97.6 %). The strain contained MK-7 as the predominant isoprenoid quinone and anteiso-$C_{15:0}$ was the major cellular fatty acid. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid. The polar lipid pattern of strain CAU 1183^T consisted of diphosphatidylglycerol, phosphatidylglycerol and unidentified lipids, including two phospholipids, two glycolipids, a phosphoglycolipid and two lipids. The G+C content of the genomic DNA was 37.5 mol%. On the basis of phenotypic, chemotaxonomic and phylogenetic data, strain CAU 1183^T should be assigned to a novel species in the genus *Oceanobacillus*, for which the name *Oceanobacillus arenosus* sp. nov. is proposed. The type strain is CAU 1183^T (=KCTC 33037^T=CECT 8560^T).

The genus *Oceanobacillus*, a member of the family *Bacillaceae*, was proposed by Lu *et al.* (2001) with the description of *Oceanobacillus iheyensis* as the type species. The genus *Oceanobacillus* comprises aerobic, Gram-stain-positive, motile, rod-shaped bacteria that are characterized chemotaxonomically by the presence of menaquinone 7 (MK-7) as the major isoprenoid quinone and anteiso-$C_{15:0}$ as the predominant cellular fatty acid (Namwong *et al.*, 2009; Lee *et al.*, 2010; Whon *et al.*, 2010). At the time of writing, the genus comprised 17 recognized species with validly published names: *O. iheyensis* (Lu *et al.*, 2001), *O. picturae* (Heyrman *et al.*, 2003), *O. oncorhynchi* (Yumoto *et al.*, 2005), *O. chironomi* (Raats & Halpern, 2007), *O. profundus* (Kim *et al.*, 2007), *O. caeni* (Nam *et al.*, 2008), *O. kapialis* (Namwong *et al.*, 2009), *O. sojae* (Tominaga *et al.*, 2009), *O. neutriphilus* (Yang *et al.*, 2010), *O. locisalsi* (Lee *et al.*, 2010), *O. kimchii* (Whon *et al.*, 2010), *O. chungangensis* (Lee *et al.*, 2013), *O. indicireducens* (Hirota *et al.*, 2013a), *O. polygoni* (Hirota *et al.*, 2013b), *O. pacificus* (Yu *et al.*, 2014), *O. limi* (Amoozegar *et al.*, 2014) and *O. luteolus* (Wu *et al.*, 2014) (http://www.bacterio.net/oceanobacillus.html). Members of the genus *Oceanobacillus* have been isolated from various habitats such as marine environments (deep-sea sediment, marine solar salterns and salt lakes), mural paintings, freshwater fish, insects, activated sludge, food, soy sauce production equipment and soil. In the course of the screening of bacteria from marine environments, a bacterial strain, designated CAU 1183^T, was isolated from a sand sample collected on Jeju island (33°23′39.3″N 126°14′22.9″E) in the Republic of Korea. The purpose of the present study was to establish the taxonomic position of this bacterial strain by using a polyphasic approach that included the determination of phenotypic, chemotaxonomic and genotypic properties.

Strain CAU 1183^T was isolated from a marine sand sample according to Gordon & Mihm (1962) using marine agar 2216 (MA; Difco). The sand sample was crushed and diluted with sterilized saline solution so that appropriate dilutions could be spread on MA plates. The plates were incubated under aerobic conditions at 30 °C for 7 days. A pure single colony was purified by subculturing and preserved at −80 °C in marine broth 2216 (MB; Difco) supplemented with 25 % (w/v) glycerol for taxonomic

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 1183^T is JX233495.

Two supplementary figures are available with the online Supplementary Material.

analysis. The type strains of four closely related species and the type strain of the type species of the genus, *O. iheyensis* KCTC 3954^T, were used as reference strains in most analyses. *O. iheyensis* KCTC 3954^T, *O. chungangensis* KCTC 33035^T, *O. profundus* KCTC 13625^T and *O. caeni* KCTC 13061^T were obtained from the Korean Collection for Type Cultures (Taejon, Korea).

Genomic DNA of strain CAU 1183^T was extracted using the method of Marmur (1961). PCR amplification of the 16S rRNA gene was performed following established procedures (Cho *et al.*, 2008). The amplified 16S rRNA gene was sequenced directly using a BigDye terminator version 3.1 cycle sequencing kit and an automatic 3730 DNA sequencer (Applied Biosystems). Multiple alignments and calculation of 16S rRNA gene sequence similarity between strain CAU 1183^T and a broad selection of closely related strains were carried out by using EzTaxon (http://www.ezbiocloud.net/eztaxon) and CLUSTAL_X 2.1 (Larkin *et al.*, 2007). Evolutionary distance matrices were created by the neighbour-joining method defined by Jukes & Cantor (1969). Phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987), least-squares (Fitch & Margoliash, 1967) and maximum-likelihood (Felsenstein, 1981) algorithms in the PHYLIP package (Felsenstein, 1989). Branch support in the neighbour-joining tree was evaluated by the bootstrap resampling method

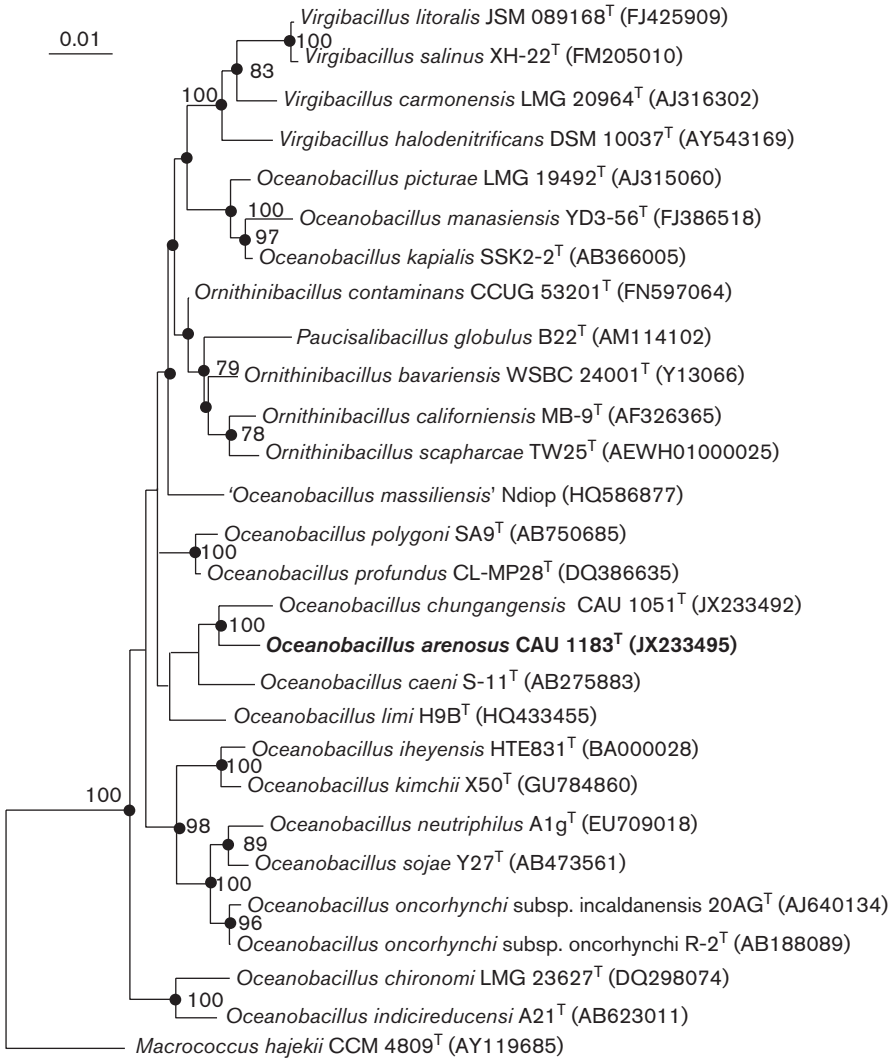

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 1183^T and the type strains of species of *Oceanobacillus* and related genera. Filled circles indicate that the corresponding nodes were also recovered in trees generated with the maximum-likelihood and least-squares algorithms. Numbers at nodes indicate percentage levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >70 % are given. Bar, 0.01 substitutions per nucleotide position. *Macrococcus hajekii* CCM 4809^T was used as the outgroup organism.

based on 1000 replicates (Felsenstein, 1985). The extent of DNA–DNA relatedness between CAU 1183^T and the most closely related phylogenetic neighbour, *O. chungangensis* KCTC 33035^T, was determined using the fluorometric microplate method (Ezaki *et al.*, 1989), as modified by Goris *et al.* (1998). The G+C content of the genomic DNA was examined by HPLC, according to the method of Tamaoka & Komagata (1984).

Strain CAU 1183^T was cultivated on MA at 30 °C for the investigation of morphological, physiological and biochemical characteristics, except for spore formation, which was assessed on nutrient sporulation medium (Nicholson & Setlow, 1990). Cell morphology was examined under a DM 1000 light microscope (Leica Microsystems) using cells from an exponentially growing culture. The Gram reaction was assessed using the bioMérieux Gram-staining kit. Motility was assessed with an MB culture using the hanging-drop method (Bowman, 2000). After 5 days of growth, spore formation was determined by staining with malachite green as described by Conn *et al.* (1957). Growth on MA at 4, 10, 20, 30, 37, 45 and 55 °C was evaluated in an aerobic incubator (model MIR-253; Sanyo Electric) and an anaerobic chamber (model Bactron; Sheldon Manufacturing) by measuring the turbidity of the broth after 72 h. The optimal growth pH was investigated by culturing at 30 °C in MB that was adjusted to pH 4.5–10.0 (at intervals of 0.5 pH units) using sodium acetate/acetic acid and Na_2CO_3 buffers, and the pH was confirmed after autoclaving. Growth in the absence of NaCl and in the presence of up to 15.0 % (w/v) NaCl was explored by culturing in MB prepared according to the Difco formula except that NaCl was excluded and that 0.45 % (w/v) $MaCl_2$. $6H_2O$ and 0.06 % (w/v) KCl were added.

Oxidase activity was assessed from the oxidation of 0.1 % (w/v) tetramethyl *p*-phenylenediamine (Cappuccino & Sherman, 2002). Catalase activity was tested by bubble production in 3 % (v/v) H_2O_2 solution. Hydrolysis of casein, starch and urea was evaluated according to the methods of Lányi (1987) and Smibert & Krieg (1994). Acid production from carbohydrates, enzyme activities and other physiological and biochemical features were tested using the API 50CH and API 20E systems (bioMérieux) at 30 °C. API 20E strips were read after 24 h and API 50CH strips after 24 and 48 h.

For fatty acid analysis, cell mass of strain CAU 1183^T, *O. iheyensis* KCTC 3954^T, *O. chungangensis* KCTC 33035^T, *O. profundus* KCTC 13625^T and *O. caeni* KCTC 13061^T was harvested from TSA (Difco) plates after cultivation for 3 days at 30 °C. The physiological age of the biomass was standardized by observing growth development during incubation of the different cultures and choosing the moment of harvesting according to the standard MIDI method (Sherlock Microbial Identification System version 6.1). Cellular fatty acid methyl esters were obtained according to the method of Minnikin *et al.* (1980) and separated with an Agilent 6890N gas chromatograph fitted with an Agilent 7683 autosampler. Peaks were identified using the Microbial Identification software package (Moore library version 5.0; MIDI database TSBA6). Peptidoglycan was analysed as described by Schleifer & Seidl (1985). The polar lipids of strain CAU 1183^T were identified using two-dimensional TLC by the method of Minnikin *et al.* (1984). The plate was sprayed with 10 % ethanolic molybdatophosphoric acid (for total lipids), molybdenum blue (for phospholipids), ninhydrin (for aminolipids) and α-naphthol/sulfuric acid reagent (for glycolipids). Menaquinones were determined by HPLC using an isocratic solvent system [methanol/isopropyl ether (3 : 1, v/v)] and a solvent flow rate of 1 ml min^{-1} (Hiraishi *et al.*, 1984).

The nearly complete 16S rRNA gene sequence of strain CAU 1183^T (1547 bp) was determined and compared to reference sequences for other bacterial species in public databases. Phylogenetic analysis indicated that the novel isolate fell into the genus *Oceanobacillus*. The neighbour-joining tree is shown in Fig. 1. Pairwise analysis indicated that the most closely related strains were *O. chungangensis* CAU 1051^T (97.6 % 16S rRNA gene sequence similarity), *O. profundus* CL-MP28^T (96.5 %), *O. polygoni* SA9^T (96.3 %) and *O. caeni* S-11^T (95.7 %). The DNA–DNA relatedness between CAU 1183^T and the most closely related strain, *O. chungangensis* KCTC 33035^T, was 44.2 % when CAU 1183^T was used as the probe and 43.6 % when *O. chungangensis* KCTC 33035^T was used as the probe. These values are well below the 70 % cut-off point recommended by Wayne *et al.* (1987) for the delineation of genomic species, supporting the proposal that strain CAU 1183^T represents a separate species. The DNA G+C content of strain CAU 1183^T was 37.5 mol%, which is in accordance with previously reported values for the genus *Oceanobacillus*, ranging from 33.6 mol% (*O. caeni* S-11^T; Nam *et al.*, 2008) to 40.6 mol% (*O. polygoni* SA9^T; Hirota *et al.*, 2013b).

The detailed phenotypic characteristics of strain CAU 1183^T are presented in Table 1 and the species description. Cells of strain CAU 1183^T were Gram-stain-positive, strictly aerobic and motile, forming ovoid spores in a central position (Fig. S1, available in the online Supplementary Material). Colonies on MA were cream coloured, entire, circular and convex, 1.2–2.0 mm in diameter after 3 days of cultivation at 30 °C. No colonies were formed under anaerobic growth conditions. Cell were rod-shaped, approximately 0.5–1.0 μm in diameter and 1.5–4.3 μm long. Growth occurred at 20–37 °C (optimum, 30 °C), at pH 5.5–9.0 (optimum, pH 8.5) and in the presence of 0–8 % (w/v) NaCl (optimum, 2 %). The phenotypic characteristics of strain CAU 1183^T differed from those of its closest relative, *O. chungangensis* KCTC 33035^T (Lee *et al.*, 2013), and other members of the genus *Oceanobacillus* (Table 1). The growth temperature of CAU 1183^T could be distinguished from those of *O. profundus* (15–42 °C), *O. polygoni* (5–48 °C) and *O. iheyensis* (15–42 °C). In addition, the ranges of pH and NaCl concentration for growth of strain CAU 1183^T differed from those of

O. polygoni (pH 7.0–12.0 and 3–12 % NaCl) and *O. iheyensis* (pH 6.5–10.0 and 0–21 % NaCl). Strain CAU 1183^T differed from its closest relatives, *O. profundus* CL-MP28^T (Kim *et al.*, 2007), *O. polygoni* SA9^T (Hirota *et al.*, 2013b) and *O. caeni* S-11^T (Nam *et al.*, 2008), and the type species *O. iheyensis* (Lu et al., 2001) by its acid production from starch and glycogen. In addition, strain CAU 1183^T differed from *O. iheyensis* (Lu *et al.*, 2001) by its acid production from trehalose.

Overall, the chemotaxonomic characteristics of strain CAU 1183^T were in agreement with previously published data for the genus *Oceanobacillus*. The predominant isoprenoid quinone detected in strain CAU 1183^T was MK-7, in line with all members of the genus *Oceanobacillus* and consistent with data for the type species of the genus, *O. iheyensis* (Lu *et al.*, 2001). The strain contained saturated and branched-chain fatty acids (Table 2). The peptidoglycan of strain CAU 1183^T contained *meso*-diaminopimelic acid as the diagnostic cell-wall diamino acid, which was present in strains of other recognized species of the genus *Oceanobacillus*. The major fatty acids (>10 %) were anteiso-$C_{15:0}$ (50.4 %), $C_{16:0}$ (11.5 %), anteiso-$C_{17:0}$ (10.3 %) and iso-$C_{15:0}$ (10.3 %). The following fatty acids were also present (>1 %): $C_{16:0}$ (8.5 %), iso-$C_{14:0}$ (5.0 %), iso-$C_{17:0}$ (2.4 %) and $C_{14:0}$ (1.4 %) (Table 2). This fatty acid profile was almost the same as those of its closest relatives, although there were differences in the amounts and presence of some fatty acids. anteiso-$C_{15:0}$ was the major compound in strain CAU 1183^T and the type strains of other closely related species of the genus *Oceanobacillus*, *O. chungangensis* KCTC 33035^T, *O. profundus* KCTC 13625^T, *O. caeni* KCTC 13061^T and *O. polygoni* SA9^T, but not in *O. iheyensis* KCTC 3954^T. Strain CAU 1183^T contained diphosphatidylglycerol and phosphatidylglycerol as the major polar lipids, as in *O. iheyensis* KCTC 3954^T. In addition, cells of the novel isolate contained unidentified lipids: two phospholipids, two glycolipids, one phosphoglycolipid and two lipids (Fig. S2). Diphosphatidylglycerol and phosphatidylglycerol

Table 1. Phenotypic properties of strain CAU 1183^T and the type strains of closely related species of the genus *Oceanobacillus*

Strains: 1, CAU 1183^T; 2, *O. chungangensis* KCTC 33035^T; 3, *O. profundus* KCTC 13625^T; 4, *O. caeni* KCTC 13061^T; 5, *O. polygoni* SA9^T (Hirota *et al.*, 2013b); 6, *O. iheyensis* KCTC 3954^T. Data were obtained in this study unless indicated. All strains produced ellipsoidal spores.

Characteristic	1	2	3	4	5	6
Spore position*	C	T	T[b]†	C[c]	C[d]	T/ST[e]
Temperature for growth (°C)						
Range	20–37	20–37[a]	15–42[b]	20–45[c]	5–48[d]	15–42[e]
Optimum	30	30[a]	35[b]	35[c]	35[d]	30[e]
pH for growth						
Range	5.5–9.0	4.5–10.0[a]	6.5–9.5[b]	6.0–9.0[c]	7.0–12.0[d]	6.5–10.0[e]
Optimum	8.5	5.0[a]	8.0[b]	7.0[c]	9.0[d]	8.3[e]
NaCl concentration for growth (%, w/v)						
Range	0–8	0–10[a]	0–14[b]	0–10[c]	3–12[d]	0–21[e]
Optimum	2	0[a]	2[b]	3.5[c]	3[d]	3[e]
Hydrolysis of:						
Casein	−	−	+	−	+	+
Gelatin	+	−	+	−	−	+
Aesculin	+	+	+	−	+	+
Acid production from:						
Amygdalin	+	+	+	+	−	+
Glycerol	−	−	+	+	+	+
D-Galactose	+	+	+	−	−	−
D-Mannose	−	+	+	+	+	+
Melezitose	−	−	+	+	+	+
Melibiose	+	−	−	−	−	−
Raffinose	+	−	+	−	−	−
D-Ribose	−	+	+	+	+	+
D-Sorbitol	+	+	−	−	−	−
Trehalose	+	+	+	+	+	−
L-Arabinose	−	+	−	−	−	+
L-Rhamnose	−	−	−	+	−	−
DNA G+C content (mol%)	37.5	36.3[a]	40.2[b]	33.6[c]	40.6[d]	35.8[e]

*C, Central; ST, subterminal; T, terminal.

†Data taken from: *a*, Lee *et al.* (2013); *b*, Kim *et al.* (2007); *c*, Nam *et al.* (2008); *d*, Lu *et al.* (2001).

Table 2. Cellular fatty acid compositions of strain CAU 1183^T and the type strains of closely related species of the genus *Oceanobacillus*

Strains: 1, CAU 1183^T; 2, *O. chungangensis* KCTC 33035^T; 3, *O. profundus* KCTC 13625^T; 4, *O. caeni* KCTC 13061^T; 5, *O. polygoni* SA9^T (data from Hirota *et al.*, 2013b); 6, *O. iheyensis* KCTC 3954^T. All data were from this study unless indicated otherwise. Values are percentages of total fatty acids. Only fatty acids amounting to >1.0 % in all strains are shown. −, Not detected.

Fatty acid	1	2	3	4	5	6
Saturated						
$C_{14:0}$	1.4	1.5	−	−	2.0	4.1
$C_{16:0}$	11.5	16.4	4.9	6.7	9.0	9.9
Branched-chain						
anteiso-$C_{15:0}$	50.4	44.2	53.3	56.7	60.3	32.0
anteiso-$C_{17:0}$	10.3	13.3	18.0	16.9	8.6	4.2
iso-$C_{14:0}$	5.0	4.8	3.7	2.6	3.5	6.8
iso-$C_{15:0}$	10.3	9.6	8.5	8.0	8.3	33.5
iso-$C_{16:0}$	8.5	6.6	8.6	5.4	3.5	6.1
iso-$C_{17:0}$	2.4	2.5	1.9	1.8	1.5	2.3

were also detected as the major polar lipids in *O. luteolus* WM-1^T, *O. limi* H9B^T, *O. chungangensis* CAU 1051^T, *O. sojae* JCM 15792^T and *O. kapialis* SSK2-2^T. Phosphatidylethanolamine was detected in *O. pacificus* XH204^T, *O. polygoni* SA9^T and *O. indicireducens* A21^T, but this polar lipid was not detected in strain CAU 1183^T.

In conclusion, it is evident from the phenotypic, chemotaxonomic and genotypic data that strain CAU 1183^T represents a novel species of the genus *Oceanobacillus*, for which the name *Oceanobacillus arenosus* sp. nov. is proposed.

Description of *Oceanobacillus arenosus* sp. nov.

Oceanobacillus arenosus (a.re.no′sus. L. masc. adj. *arenosus* sandy).

Gram-stain-positive, obligately aerobic, motile rods with optimal growth at 30 °C (range 20–37 °C), pH 8.5 (range pH 5.5–9.0) and 2 % (w/v) NaCl (range 0–8 %). Forms endospores in a central position. Catalase and oxidase tests are positive. Aesculin and gelatin are hydrolysed. Starch, urea and citrate are not hydrolysed. Acid is produced from starch, amygdalin, D-fructose, D-galactose, D-glucose, maltose, D-mannitol, melibiose, lactose, raffinose, D-sorbitol, trehalose, D-xylose, glycogen and salicin. The major isoprenoid quinone is MK-7. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The polar lipid pattern consists of diphosphatidylglycerol, phosphatidylglycerol, two unidentified phospholipids, two unidentified glycolipids, one unidentified phosphoglycolipid and two unidentified lipids. The major cellular fatty acid is anteiso-$C_{15:0}$.

The type strain, CAU 1183^T (=KCTC 33037^T=CECT 8560^T), was isolated from marine sand collected from Jeju Island in the Republic of Korea. The DNA G+C content of the type strain is 37.5 mol%.

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