Oceanobacillus chungangensis sp. nov., isolated from a sand dune

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A Gram-stain-positive, spore-forming, rod-shaped, motile, strictly aerobic bacterial strain, designated CAU 1051^T, was isolated from a sand dune and its taxonomic position was investigated using a polyphasic approach. Strain CAU 1051^T grew optimally at pH 5.0 and 30 °C. NaCl was not required for growth but up to 10.0 % (w/v) NaCl was tolerated. Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain CAU 1051^T formed a distinct lineage within the genus *Oceanobacillus* and was most closely related to *Oceanobacillus profundus* CL-MP28^T, *Oceanobacillus caeni* S-11^T, and *Oceanobacillus picturae* LMG 19492^T (96.8 %, 95.6 % and 95.3 % similarity, respectively). DNA–DNA reassociation analysis showed that strain CAU 1051^T displayed 28.2 ± 0.7 % relatedness to *O. profundus* KCTC 13625^T. Strain CAU 1051^T contained MK-7 as the only isoprenoid quinone and anteiso-$C_{15:0}$ as the major fatty acid. The cell wall peptidoglycan of strain CAU 1051^T contained *meso*-diaminopimelic acid. The polar lipids were composed of diphosphatidylglycerol, phosphatidylglycerol, six unidentified phospholipids, an unidentified glycolipid, and six unidentified polar lipids. The major whole-cell sugars were glucose and ribose. The DNA G+C content was 36.3 mol%. On the basis of phenotypic data and phylogenetic inference, strain CAU 1051^T represents a novel species of the genus *Oceanobacillus* for which the name *Oceanobacillus chungangensis* sp. nov. is proposed. The type strain is CAU 1051^T (=KCTC 33035^T=CCUG 63270^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-positive, motile, rod-shaped bacteria that are characterized chemotaxonomically by the presence of 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 harbours 11 characterized 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) and *O. kimchii* (Whon *et al.*, 2010) (http://www.bacterio.net/). Members of the genus *Oceanobacillus* have been isolated from various habitats such as deep-sea sediment, mural paintings, freshwater fish, insects, activated sludge, food, soy sauce production equipment, and a marine solar saltern. At the time of writing, the description of the genus *Oceanobacillus* has been emended twice (Yumoto *et al.*, 2005; Lee *et al.*, 2006). In the course of the screening of bacteria with biotechnological potential from marine environmental samples, a bacterial strain, designated CAU 1051^T, was isolated from a sand dune sample collected on Jeju Island (33° 21′ 25.03″ N 126° 29′ 59.25″ E and 33° 22′ 06.16″ N 126° 33′ 11.11″ 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 describing chemotaxonomic, phenotypic and genotypic properties.

Isolation was performed according to Gordon & Mihm (1962) using marine agar 2216 (MA; Difco), supplemented with cycloheximide (50 mg^{-1}) and nalidixic acid (20 mg^{-1}). The sample was crushed with a mortar in 10 ml saline solution. Two dilution series were made of the

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

sample; one series was plated directly on MA and the other was plated after heating for 15 min at 80 °C to select for endospores. The agar plates were incubated under aerobic conditions at 30 °C for 14 days. Single colonies on the plates were purified by subculturing. Strain CAU 1051^T was one of the isolates that appeared by direct plating on MA. Pure cultures were preserved at −70 °C in marine broth (MB; Difco) supplemented with 25 % (v/v) glycerol. The type strains of six closely related species including the type strain of the type species of the genus, *O. iheyensis*, were used as reference strains in most analyses. *O. iheyensis* KCTC 3954^T, *O. profundus* KCTC 13625^T, *O. caeni* KCTC 13061^T, *O. picturae* KCTC 3821^T, *O. kapialis* KCTC 13177^T and *O. chironomi* KCTC 13626^T were obtained from the Korean Collection for Type Cultures (KCTC; Taejon, Korea).

Genomic DNA of strain CAU 1051^T was isolated by the method of Marmur (1961). The 16S rRNA gene was amplified by PCR following established procedures (Nam *et al.*, 2004). The amplified 16S rRNA gene was sequenced directly using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and an automated DNA sequencer (model 3730; Applied Biosystems). The nearly complete 16S rRNA gene sequence of strain CAU 1051^T (1533 bp) was determined and compared with the corresponding sequences of other bacterial strains in the GenBank database. Multiple alignments with sequences of a broad selection of members of the genus *Oceanobacillus* and calculation of sequence similarity levels were carried out by using CLUSTAL X (Thompson *et al.*, 1997) and the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim *et al.*, 2012). Evolutionary distance matrices were generated by the neighbour-joining method described 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 software package (Felsenstein, 1989), and tree topology was evaluated by the bootstrap resampling method (Felsenstein, 1985) with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package. Phylogenetic analysis indicated that strain CAU 1051^T fell in the genus *Oceanobacillus*. The neighbour-joining tree is shown in Fig. 1. The trees obtained with the two other treeing methods used showed essentially the same topology (Fig. S1, available in IJSEM Online). Pairwise analysis showed that the most closely related species were *O. profundus* CL-MP28^T (96.8 % 16S rRNA gene sequence similarity), *O. caeni* S-11^T (95.6 %), *O. picturae* LMG 19492^T (95.3 %), *O. kapialis* SSK2-2^T (94.9 %), *O. chironomi* LMG 23627^T (94.9 %), and *O. iheyensis* HTE831^T (94.6 %).

Strain CAU 1051^T was cultivated routinely on MA at 30 °C to investigate all morphological, physiological and biochemical characteristics, except for spore formation that was assessed on nutrient sporulation medium (Nicholson & Setlow, 1990). Cell morphology was examined by light microscopy (DM 1000; Leica) and transmission electron microscopy (JEM 1010; JEOL) using cells from an exponentially growing culture. For transmission electron microscopy, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and the grids were examined after being air-dried. Gram staining was carried out using the bioMérieux Gram staining kit according to the manufacturer's instructions. Motility was assessed using the hanging-drop method. After 5 days of growth, spore formation was determined by staining with malachite green as described previously by Conn *et al.* (1957). Cells were motile and ovoid to rod-shaped, 0.5–0.7 × 1.7–3.3 mm in size. In addition, cells possessed two flagella at polar and subpolar positions (Fig. S2).

Growth on MA at 4, 10, 20, 30, 37, 45 and 55 °C in an aerobic incubator (MIR-253; Sanyo) and in an anaerobic chamber (Bactron; Sheldon) was evaluated by measuring the turbidity of the broth after 72 h. Anaerobic medium was degassed by bubbling with a stream of N_2 for 20 min; pH adjustment with concentrated HCl or NaOH (10 %) and dispensation into serum bottles was carried out in an anaerobic chamber. Growth was tested at 30 °C in MB adjusted to pH 4.5–10.0 at increments of 0.5 pH units by using sodium acetate/acetic acid and sodium carbonate buffers. Growth in the absence of NaCl and in the presence of 0–15.0 % (w/v) NaCl at 1 % intervals was investigated at 30 °C in MB prepared according to the formula of the Difco medium except that NaCl was excluded and that 0.45 % (w/v) $MgCl_2 . 6H_2O$ and 0.06 % (w/v) KCl were added. Catalase activity was determined by gas production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was determined from the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Cappuccino & Sherman, 2002). Hydrolysis of casein, gelatin and aesculin, and nitrate reduction were determined according to Lányí (1987) and Smibert & Krieg (1994). Acid production from carbohydrates, enzyme activity, 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 h and 48 h.

The morphological, cultural, physiological and biochemical characteristics of strain CAU 1051^T are given in Table 1 and in the species description. Overall, the results obtained in this study are in agreement with previously published data for the 11 recognized species of the genus *Oceanobacillus* (Lu *et al.*, 2001; Heyrman *et al.*, 2003; Lee *et al.*, 2006, 2010; Kim *et al.*, 2007; Raats and Halpern 2007; Nam *et al.*, 2008; Namwong *et al.*, 2009; Tominaga *et al.*, 2009; Yang *et al.*, 2010; Whon *et al.*, 2010). However, strain CAU 1051^T differed from its closest relatives, *O. profundus* KCTC 13625^T, *O. caeni* KCTC 13061^T, *O. picturae* KCTC 3821^T, *O. kapialis* KCTC 13177^T and *O. chironomi* KCTC 13626^T, and from the type strain of the type species of the genus *Oceanobacillus*, *O. iheyensis* KCTC 3954^T (Lu *et al.*, 2001), by its ability to produce acid from D-ribose and D-sorbitol and by its optimum pH for growth and optimum NaCl tolerance.

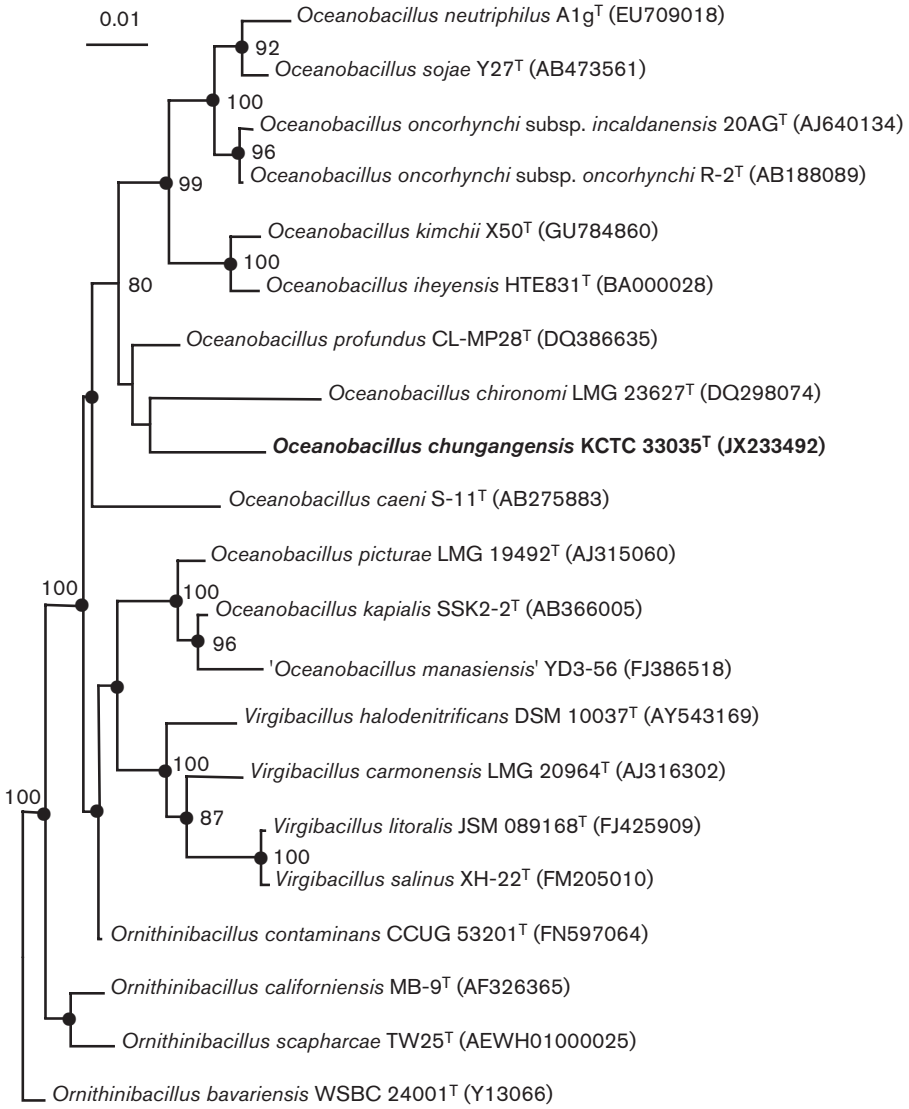

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 1051^T and the type strains of recognized species of the genus *Oceanobacillus*. Filled circles indicate that the corresponding nodes were also recovered in the trees generated with the maximum-likelihood and least-squares algorithms. Numbers at nodes indicate levels of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values >70 % are shown. *Ornithinibacillus bavariensis* WSBC 24001^T (GenBank accession no. Y13066) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

For determination of cellular fatty acids, the cell mass of strain CAU 1051^T, *O. profundus* KCTC 13625^T, *O. caeni* KCTC 13061^T, *O. picturae* KCTC 3821T^T, *O. kapialis* KCTC 13177^T, *O. chironomi* KCTC 13626^T and *O. iheyensis* KCTC 3954^T was harvested from tryptic soy agar (TSA; Difco) after cultivation for 3 days at 30 °C. The physiological age of biomass of all the strains for fatty acid analysis was standardized by observing growth profiles of cultures and harvesting according to a standard MIDI protocol (Sherlock Microbial Identification System, version 6.1). Cellular fatty acid methyl esters (FAMEs) were obtained as described by Minnikin *et al.* (1980), and separated by an automated gas chromatography system (model 6890N and 7683 autosampler; Agilent). Peaks were identified by using the Microbial Identification software package (MOORE library ver. 5.0; MIDI database TSBA6). The polar lipids of strain CAU 1051^T were identified using two-dimensional TLC by the method of Minnikin *et al.* (1984). The plates were sprayed with 10 % ethanolic molybdatophosphoric acid (for total lipids), molybdenum blue (for phospholipids), ninhydrin (for aminolipids) and α-naphthol/sulphuric acid reagent (for glycolipids) (Sigma-Aldrich). The following analyses were performed on strain CAU 1051^T only: menaquinones were analysed as described

Table 1. Differential properties of strain CAU 1051^T and the type strains of the most closely related species of the genus *Oceanobacillus*

Strains: 1, CAU 1051^T (data from this study); 2, *O. profundus* KCTC 13625^T (this study and Kim *et al.*, 2007); 3, *O. caeni* KCTC 13061^T (this study and Nam *et al.*, 2008); 4, *O. picturae* KCTC 3821^T (this study and Lee *et al.*, 2006); 5, *O. kapialis* KCTC 13177^T (this study and Namwong *et al.*, 2009); 6, *O. chironomi* KCTC 13626^T (this study and Raats & Halpern, 2007); 7, *O. iheyensis* KCTC 3954^T (this study and Lu *et al.*, 2001); +, Positive; −, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6	7
Growth temperature (°C)							
Range	20–37	15–42	20–45	5–40	8–43	12–46	15–42
Optimum	30	35	35	30	37	37	30
pH for growth							
Range	4.5–10.0	6.5–9.5	6.0–9.0	9.0–10.0	6.0–9.0	6.5–10.0	6.5–10.0
Optimum	5.0	8.0	7.0	9.5	8.0	8.5	8.3
NaCl tolerance (%)							
Range	0–10.0	0–14	0–10	0–10	0.5–24	0–11	0–21
Optimum	0	2.0	3.5	7.0	10.0	2.0	3.0
Nitrate reduction	+	+	+	+	+	+	−
Hydrolysis of:							
Casein	−	+	−	w	+	−	+
Gelatin	−	+	−	w	+	+	+
Aesculin	+	+	−	w	−	+	+
Acid production from:							
Glycerol	−	w	w	+	−	w	w
L-Arabinose	w	−	−	−	+	w	w
D-Ribose	+	w	w	−	−	w	w
D-Xylose	w	w	w	−	−	w	w
D-Galactose	w	w	−	−	−	w	−
D-Glucose	w	w	w	+	+	w	w
D-Fructose	+	w	w	−	+	w	w
D-Mannose	+	w	w	+	+	−	w
L-Rhamnose	−	−	w	−	−	−	−
D-Mannitol	w	w	w	−	+	−	w
D-Sorbitol	w	−	−	−	−	−	−
Amygdalin	w	w	w	−	−	−	w
Salicin	+	w	w	−	−	−	w
Maltose	w	w	w	−	+	w	w
Melibiose	−	−	−	−	−	w	−
Trehalose	w	w	w	−	−	−	−
Melezitose	−	w	w	−	−	−	w
Raffinose	−	w	−	−	−	−	−
DNA G+C content (mol%)	36.3	40.2	33.6	39.5	38.5	38.1	35.8

previously (Komagata & Suzuki, 1987) using reversed-phase HPLC with the solvent methanol/isopropyl ether (3 : 1) and a flow rate of 1 ml min^{-1}; whole cell sugars were analysed by TLC according to the method of Komagata & Suzuki (1987); peptidoglycan was analysed as described by Schleifer & Seidl (1985), with the modification that a cellulose sheet was substituted to chromatography paper. The extent of DNA–DNA relatedness between strain CAU 1051^T and the nearest phylogenetic neighbour were estimated using the fluorometric microplate method (Ezaki *et al.*, 1989) as modified by Goris *et al.* (1998). The G+C content of the genomic DNA of strain CAU 1051^T was determined using HPLC by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC.

The peptidoglycan of strain CAU 1051^T contained *meso*-diaminopimelic acid as the diagnostic cell-wall diamino acid, which was also present in *O. picturae* (Lee *et al.*, 2006), *O. kapialis* (Namwong *et al.*, 2009), *O. locisalsi* (Lee *et al.*, 2010) and *O. neutriphilus* (Yang *et al.*, 2010). However, this was significantly different from that of another closely related genus, *Ornithinibacillus*, which contained L-Orn–D-Asp (Kämpfer *et al.*, 2010). Menaquinone 7 (MK-7) was the major respiratory quinone. These characteristics are in agreement with those of numerous species of the genus *Oceanobacillus*, including the type species, *O. iheyensis* (Lu *et al.*, 2001). Diphosphatidylglycerol and phosphatidylglycerol

were the only polar lipids identified in strain CAU 1051^T. The other unidentified polar lipids were six phospholipids, one glycolipid and six polar lipids (Fig. S3). TLC analysis of the murein structure of strain CAU 1051^T revealed the presence of glucose and ribose.

The cellular fatty acid profile of the novel isolate contained saturated and branched-chain fatty acids (Table 2). The fatty acids present were anteiso-$C_{15:0}$, $C_{16:0}$, anteiso-$C_{17:0}$, iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{14:0}$, iso-$C_{17:0}$ and $C_{14:0}$. The major compound was anteiso-$C_{15:0}$ (44.2 %) which is characteristic of numerous taxa within the genus *Oceanobacillus*. The fatty acid profile of strain CAU 1051^T was almost the same as that of its closest relatives; however, the amounts and presence of $C_{14:0}$ were different from those observed in the type strains of most species of the genus *Oceanobacillus*. The mean DNA–DNA relatedness value determined between strain CAU 1051^T and *O. profundus* KCTC 13625^T was 28.2±0.7 %. This value is 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 1051^T represents a separate species. The genomic DNA of strain CAU 1051^T had a G+C content of 36.3 mol%.

These data provide sufficient evidence to recognize strain CAU 1051^T as a representative of a novel species of the genus *Oceanobacillus*, for which the name *Oceanobacillus chungangensis* sp. nov. is proposed.

Description of *Oceanobacillus chungangensis* sp. nov.

Oceanobacillus chungangensis (chung.ang.en′sis. N.L. masc. adj. *chungangensis* belonging to Chung-Ang University where the taxonomic studies on this species were performed).

Cells are Gram-stain-positive rods, measuring approximately 0.5–0.7 μm in diameter and 1.7–3.3 μm in length, and are obligately aerobic, ellipsoidal-spore-formers that are motile by means of polar and subpolar flagella. Colonies on MA are cream, circular, convex with entire margins after 3 days of incubation at 30 °C. Growth occurs at 20–37 °C (optimum, 30 °C) and at pH 4.5–10.0 (optimum, pH 5.0). NaCl is not required for growth but up to 10.0 % (w/v) NaCl is tolerated. Catalase- and oxidase-positive. Aesculin is hydrolysed. Acid is produced from D-ribose, D-fructose, D-mannose and salicin. The cell wall peptidoglycan contains *meso*-diaminopimelic acid. The major isoprenoid quinone is MK-7. The polar lipid pattern consists of diphosphatidylglycerol, phosphatidylglycerol, six unidentified phospholipids, one unidentified glycolipid and six unidentified polar lipids. The major cellular fatty acids (>10 % of the total fatty acids) are anteiso-$C_{15:0}$, $C_{16:0}$ and anteiso-$C_{17:0}$.

The type strain CAU 1051^T (=KCTC 33035^T=CCUG 63270^T), was isolated from a sand dune sample collected from Jeju Island in the Republic of Korea. The DNA G+C content of the type strain is 36.3 mol%.

Table 2. Cellular fatty acid compositions (%) of strain CAU 1051^T and the type strains of the most closely related species of the genus *Oceanobacillus*

Strains: 1, CAU 1051^T; 2, *O. profundus* KCTC 13625^T; 3, *O. caeni* KCTC 13061^T; 4, *O. picturae* KCTC 3821^T; 5, *O. kapialis* KCTC 13177^T; 6, *O. chironomi* KCTC 13626^T; 7, *O. iheyensis* KCTC 3954^T, All data are from this study. Only those fatty acids amounting to >1.0 % in all strains are shown, −, Not detected.

Fatty acid	1	2	3	4	5	6	7
Saturated							
$C_{14:0}$	1.5	−	−	−	−	1.4	4.1
$C_{16:0}$	16.4	4.9	6.7	5.8	3.9	17.4	9.9
Branched-chain							
anteiso-$C_{15:0}$	44.2	53.3	56.7	52.2	47.2	50.3	32.0
anteiso-$C_{17:0}$	13.3	18.0	16.9	14.1	21.5	21.3	4.2
iso-$C_{14:0}$	4.8	3.7	2.6	4.0	3.6	1.1	6.8
iso-$C_{15:0}$	9.6	8.5	8.0	2.3	7.4	4.3	33.5
iso-$C_{16:0}$	6.6	8.6	5.4	7.2	8.3	2.5	6.1
iso-$C_{17:0}$	2.5	1.9	1.8	3.8	4.0	1.2	2.3

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