Bacillus chungangensis sp. nov., a halophilic species isolated from sea sand

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The taxonomic position of a Gram-stain-positive, endospore-forming, halophilic strain, designated CAU 348^T, isolated from sea sand was investigated using a polyphasic approach. Colony morphology, biochemical tests and chemotaxonomic investigations revealed that strain CAU 348^T had the characteristics of the genus *Bacillus*. Comparative 16S rRNA gene sequence analysis showed that the organism formed a hitherto unknown subline within the genus *Bacillus*. Sequence divergence values of more than 4.3 % from other described *Bacillus* species, together with phenotypic differences, showed that the unidentified bacterium represents a previously unrecognized member of this genus. The genotypic and phenotypic data indicated that strain CAU 348^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus chungangensis* sp. nov. is proposed. The type strain is CAU 348^T (=KCTC 13566^T =CCUG 57835^T).

Aerobic, endospore-forming, halophilic Gram-positive rods are taxonomically very diverse and have been isolated from different saline habitats such as salterns, estuarine water, salt lakes, salty foods, sea ice and deep-sea hydrothermal vents (Agnew *et al.*, 1995; Arahall *et al.*, 1999; Nielsen *et al.*, 1994; Ventosa *et al.*, 1989; Yoon *et al.*, 2004). Recently, novel approaches in the study of bacterial systematics have aided the reclassification of many species assigned to the genus *Bacillus* (Ash *et al.*, 1993; Heyndrickx *et al.*, 1998; Shida *et al.*, 1996; Wisotzkey *et al.*, 1992). In particular, comparative 16S rRNA gene sequence analysis has shown that the genus *Bacillus* contains six phylogenetically distinct groups and that many alkaliphilic/halophilic bacilli belong to rRNA *Bacillus* group 6 (Ash *et al.*, 1991; Nielsen *et al.*, 1994).

In the course of screening micro-organisms from marine habitats in Jeju island, an aerobic, Gram-stain-positive, halophilic bacterium, designated CAU 348^T, was isolated and characterized. The genotypic and phenotypic data obtained in this study suggest that the strain CAU 348^T represents a novel species of the genus *Bacillus*.

The procedure for isolation of strain CAU 348^T followed that of Gordon & Mihm (1962) by using glucose-yeast extract agar (GYEA) (1⁻¹: 10 g yeast extract, 10 g glucose, 15 g agar) supplemented with 50 mg cycloheximide and 20 mg nalidixic acid 1⁻¹. A sand sample was diluted with

sterilized distilled water, spread onto the GYEA medium and incubated aerobically for 3 days at 30 °C.

The pure culture of CAU 348^T was preserved in 25 % (v/v) glycerol at –70 °C. Cell morphology was examined by light microscopy (model DM 1000; Leica). Flagellum type was examined using cells from exponentially growing cultures. The cells were negatively stained with 1 % (w/v) phosphotungstic acid and, after air drying, the grids were examined using a TEM (model CM-20; Philips). Catalase activity was determined by bubble production in a 3 % (v/v) hydrogen peroxide solution. Oxidase activity was tested by means of the oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck). Hydrolysis of casein, starch and urea was determined on GYEA according to the methods described by Cowan & Steel (1965), Lányi (1987) and Smibert & Krieg (1994). Acid production from carbohydrates was tested as described by Leifson (1963) and using the API 50CH system (bioMérieux), according to the manufacturer's instructions, with incubation for up to 3 days at 30 °C. Growth at various NaCl concentrations (0–15 %) at 30 °C was investigated on GYEA or glucose-yeast extract broth (GYEB). Growth at various temperatures and pH values was investigated on GYEA between 4 and 45 °C.

Cellular fatty acid methyl esters were extracted after incubation for 3 days on tryptic soy agar (TSA; Difco) by acid methanolysis (Minnikin *et al.*, 1980) and analysed using a Hewlett Packard series II gas chromatograph model 5890A equipped with a 5 % phenyl methyl silicon-fused silica capillary column (HP 19091B-102). Preparation of cell walls and analysis of peptidoglycan structures were carried out using the methods described by Schleifer

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CAU 348^T is FJ514932.

An electron micrograph showing a cell of strain CAU 348^T is available as supplementary material with the online version of this paper.

(1985), with the modification that TLC on cellulose sheets was performed instead of paper chromatography. Polar lipids were extracted from freeze-dried cell material (100 mg) by using a two-stage method and separated by two-dimensional silica-gel TLC as described by Tindall (1990a, b). Menaquinones were analysed as described by Komagata & Suzuki (1987) using reversed-phase HPLC.

Genomic DNA of strain CAU 348^T was isolated and purified by the method of Marmur (1961). The G+C content of the genomic DNA was determined using SYBR Green I with a real-time thermocycler (model 7300; Applied Biosystems) and the fluorimetric method of Gonzalez & Saiz-Jimenez (2002). PCR amplification and sequencing of the 16S rRNA gene were carried out following established procedures (Nam *et al.*, 2004). The PCR amplification products were sequenced directly using a BigDye Terminator cycle sequencing kit and an automatic DNA sequencer (model 3730; Applied Biosystems). Multiple alignments with sequences from a broad selection of *Bacillus* species and calculations of sequence similarity were carried out by using CLUSTAL X (Thompson *et al.*, 1997) and the EzTaxon server (Chun *et al.*, 2007). A phylogenetic tree was constructed using the neighbour-joining algorithm (Saitou & Nei, 1987) from the PHYLIP suite of programs (Felsenstein, 1989). Evolutionary distance matrices were generated by the neighbour-joining method described by Jukes & Cantor (1969) and tree topology was evaluated by bootstrap resampling (Felsenstein, 1985) with 1000 replicates of the neighbour-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package.

Colonies of strain CAU 348^T were 0.2–0.3 mm wide and 0.8–1.0 mm long. Cells of the isolate were short rods, approximately $0.8 \times 2.5 \mu\text{m}$ after 3 days of cultivation at 30 °C on GYEA, and were strictly aerobic. Strain CAU 348^T was motile by means of peritrichous flagella (Supplementary Fig. S1, available in IJSEM Online). Strain CAU 348^T grew with 0–15 % (w/v) NaCl [optimum 5 % (w/v) NaCl], at 4–45 °C (optimum 30 °C) and at pH 4.5–9.0 (optimum pH 7.0).

Strain CAU 348^T contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. The predominant isoprenoid quinone in strain CAU 348^T was unsaturated menaquinone with seven isoprene units (MK-7). The major polar lipids of strain 348^T were phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. TLC analysis of whole-cell hydrolysates revealed mainly ribose and glucose. The cellular fatty acid profile of the strain grown on TSBA was characterized by saturated branched fatty acids such as iso-C_{13:0} (2.5 %), iso-C_{14:0} (1.1 %), iso-C_{15:0} (68.8 %), anteiso-C_{15:0} (17.4 %), iso-C_{16:0} (1.3 %), C_{16:0} (2.3 %), iso-C_{17:0} (2.1 %), and anteiso-C_{17:0} (3.2 %). Cells were positive for catalase, oxidase and urease but negative for starch hydrolysis. API 50CHB test strips provided adequate phenotypic data to describe the new isolate, and a detailed

description of the key phenotypic characteristics of the isolate is given in Table 1 and the species description. Acid was produced from fructose, glucose, rhamnose, ribose, salicin, sucrose, D-tagatose, arbutin and aesculin, but not from glycerol, D-arabinose, galactose, mannitol, mannose, raffinose or D-xylose.

The DNA G+C content of strain CAU 348^T was 35.0 mol%. The nearly complete 16S rRNA gene sequence of strain CAU 348^T (1468 bp) was determined and compared with the corresponding sequences of other bacterial strains in the GenBank database. A phylogenetic tree based on 16S rRNA gene sequence data from strain CAU 348^T and corresponding sequences from the type strains of recognized *Bacillus* species as well as *Aneurinibacillus migulanus* DSM 2895^T was constructed according to the neighbour-joining algorithm (Fig. 1). The genus *Bacillus* embraces a very diverse range of organisms. It is recognized, however, that the genus is not monophyletic and comprises several distinct 16S rRNA gene sequence lineages. The tree topology, supported by high bootstrap values, clearly separated strain CAU 348^T within the genus *Bacillus* lineage from *Aneurinibacillus migulanus* DSM 2895^T, which formed an outlying single-membered cluster. It is evident from our 16S rRNA gene sequence

Table 1. Differential phenotypic properties between strain CAU 348^T and the type strains of closely related *Bacillus* species

Strains: 1, *Bacillus chungangensis* sp. nov. CAU 348^T; 2, *B. carboniphilus* Kasumi 6^T (data from Fujita *et al.*, 1996); 3, *B. sporothermodurans* M215^T (Pettersson *et al.*, 1996); 4, *B. shackletonii* SSI024^T (Logan *et al.*, 2004); 5, *B. acidicola* 105-2^T (Albert *et al.*, 2005). +, Positive; w, weakly positive; –, negative.

Characteristic	1	2	3	4	5
Hydrolysis of:					
Casein	+	+	–	w	–
Starch	–	+	–	–	–
Acid from:					
D-Arabinose	–	–	–	+	–
Fructose	+	–	w	+	–
Glucose	+	–	–	+	–
Mannose	–	–	–	+	–
Rhamnose	+	–	–	–	–
Ribose	+	–	w	–	–
Salicin	+	–	–	+	–
Sucrose	+	–	–	–	–
D-Tagatose	+	w	–	+	–
D-Xylose	–	+	–	–	–
Mannitol	–	–	–	w	–
Arbutin	+	–	–	+	–
Aesculin	+	+	–	+	–
Cell-wall sugar(s)*	Rib, Glc	Rib, Xyl	Rib, Glc	Rib	Rib, Glc, Gal

*Gal, Galactose; Glc, glucose; Rib, ribose; Xyl, xylose.

Fig. 1. Neighbour-joining phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relationships between strain CAU 348^T and members of the genus *Bacillus*. Bootstrap values (>50%) based on 1000 resamplings are shown at branch nodes. Bar, 0.01 substitutions per nucleotide position.

study that strain CAU 348^T forms a distinct subclade with *Bacillus carboniphilus* JCM 9731^T, *B. sporothermodurans* M215^T, *B. shackletonii* LMG 18435^T, *B. acidicola* 105-2^T, *B. seohaeanensis* BH724^T, *B. oleronius* ATCC 700005^T, *B. isabelliae* CVS-8^T and *B. ginsengihumi* Gsoil 114^T. The pairwise similarity between strain CAU 348^T and *B. carboniphilus* JCM 9731^T was 95.6% and similarities with other described members of this genus were lower, indicating clearly that strain CAU 348^T represents a novel species belonging to the genus *Bacillus* (Stackebrandt & Goebel, 1994).

These phenotypic and chemotaxonomic data, together with the 16S rRNA gene sequence analysis, provide sufficient evidence to support the proposal that strain CAU 348^T, isolated from sea sand, represents a hitherto unrecognized species within the genus *Bacillus*. The name *Bacillus chungangensis* sp. nov. is proposed for this novel taxon.

Description of *Bacillus chungangensis* sp. nov.

Bacillus chungangensis (chung.an.gen'sis. N.L. masc. adj. *chungangensis* named after Chung-Ang University, where taxonomic studies on the type strain were performed).

Cells are strictly aerobic, Gram-stain-positive, motile, spore-forming short rods, 0.8 × 2.5 µm. Colonies are creamy, smooth and circular on GYE media agar after 72 h incubation at 30 °C. Growth occurs at 4–45 °C (optimum 30 °C), pH 4.5–9.0 (optimum pH 7.0) and 0–15%

(w/v) NaCl (optimum 5%). Catalase- and oxidase-positive. Acid production occurs from ribose, glucose, fructose, rhamnose, arbutin, aesculin, salicin, sucrose and D-tagatose but not from glycerol, D-arabinose, D-xylose, galactose, mannose, mannitol or raffinose. The cell wall contains meso-diaminopimelic acid. The major isoprenoid quinone is MK-7. The major polar lipids are phosphatidylglycerol, diphosphatidylglycerol and phosphatidylethanolamine. Whole-cell hydrolysates contain mainly ribose and glucose. The predominant cellular fatty acids (>10%) are iso-C_{15:0} and anteiso-C_{15:0}. The DNA G+C content of the type strain is about 35.0 mol%.

The type strain, CAU 348^T (=KCTC 13566^T = CCUG 57835^T), was isolated from sea sand from Jeju island, Republic of Korea.

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