**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 348T, isolated from sea sand was investigated using a polyphasic approach. Colony morphology, biochemical tests and chemotaxonomic investigations revealed that strain CAU 348T 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 348T represents a novel species of the genus *Bacillus*, for which the name *Bacillus chungangensis* sp. nov. is proposed. The type strain is CAU 348T (=KCTC 13566T = CCUG 57835T).

Aerobic, endospore-forming, halophilic Gram-positive rods are taxonomically very diverse and have been isolated from different saline habitats such as salters, estuarine water, salt lakes, salty foods, sea ice and deep-sea hydrothermal vents (Agnew et al., 1995; Arahal 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 348T, was isolated and characterized. The genotypic and phenotypic data obtained in this study suggest that the strain CAU 348T represents a novel species of the genus *Bacillus*.

The procedure for isolation of strain CAU 348T followed that of Gordon & Mihm (1962) by using glucose-yeast extract agar (GYEA) (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 348T 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 GYE agar (model DM 1000; Leica). Flagellum type was examined using cells from exponentially growing cultures. 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 GYE or glucose-yeast extract broth (GYEB). Growth at various temperatures and pH values was investigated on GYE 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...
Genomic DNA of strain CAU 348T 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 with 1000 replicates of the neighbour-joining algorithm (Fig. 1). It is evident from our 16S rRNA gene sequence comparison 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 348T and corresponding sequences from the type strains of recognized Bacillus species as well as Aneurinibacillus migulanus DSM 2895T 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 348T within the genus Bacillus lineage from Aneurinibacillus migulanus DSM 2895T, which formed an outlying single-membered cluster. It is evident from our 16S rRNA gene sequence

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

<table>
<thead>
<tr>
<th>Characteristic</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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<tr>
<td>Casein</td>
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<td>Starch</td>
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<td><strong>Acid from:</strong></td>
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<tr>
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<td>w</td>
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<td>Aesculin</td>
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<td>–</td>
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<td>Rib, Glc</td>
<td>Rib, Xyl</td>
<td>Rib, Glc</td>
<td>Rib, Glc, Gal</td>
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</table>

*Gal, Galactose; Glc, glucose; Rib, ribose; Xyl, xylose.
study that strain CAU 348 T forms a distinct subclade with Bacillus carboniphilus JCM 9731T, B. sporthoderomurans M215T, B. shackletonii LMG 18435T, B. acidicola 105–2T, B. seohaeanensis BH724T, B. oleronius ATCC 700005T, B. isabeliae CVS-8T and B. ginsengihumi Gsoll 114T. The pairwise similarity between strain CAU 348 T and B. carboniphilus JCM 9731T 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, ascinulin, salicin, sucrose and D-tagatose but not from glycerol, D-arabinose, D-xylose, galactose, mannose, mannotol 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 contains mainly ribose and glucose. The predominant cellular fatty acids (＞10 %) are iso-C15:0 and anteiso-C15:0. The DNA G+C content of the type strain is about 35.0 mol%.

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

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References


