Pontibacillus marinus sp. nov., a moderately halophilic bacterium from a solar saltern, and emended description of the genus *Pontibacillus*

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A moderately halophilic, Gram-positive, rod-shaped bacterium (BH030004^T) was isolated from a solar saltern in Korea. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain BH030004^T belonged to the genus *Pontibacillus*. Chemotaxonomic data (DNA G+C content, 42 mol%; major isoprenoid quinone, MK-7; cell-wall type, A1 γ -type meso-diaminopimelic acid; major fatty acids, iso-C_{15:0} and anteiso-C_{15:0}) also supported the affiliation of the isolate to the genus *Pontibacillus*. Although the 16S rRNA gene sequence similarity between strain BH030004^T and *Pontibacillus chungwhensis* DSM 16287^T was relatively high (99.1%), physiological properties and DNA–DNA hybridization (about 7% DNA–DNA relatedness) allowed genotypic and phenotypic differentiation of strain BH030004^T from the type strain of *P. chungwhensis*. Therefore, strain BH030004^T represents a novel species of the genus *Pontibacillus*, for which the name *Pontibacillus marinus* sp. nov. is proposed. The type strain is BH030004^T (=KCTC 3917^T=DSM 16465^T).

Aerobic, spore-forming, moderately halophilic, Gram-positive rods constitute a taxonomically diverse group and have been isolated from marine environments and related habitats. They were originally assigned to the genus *Bacillus*, but molecular and chemical analyses have shown that they form several phylogenetically distinct lineages within the group classically defined as the genus *Bacillus* (Ash *et al.*, 1991; Stackebrandt & Liesack, 1993; Nielsen *et al.*, 1994). Some of these distinct lineages have been described as the novel genera *Halobacillus* (Spring *et al.*, 1996; Yoon *et al.*, 2003), *Gracilibacillus* (Wainø *et al.*, 1999), *Virgibacillus* (Heyndrickx *et al.*, 1998; Heyrman *et al.*, 2003), *Filobacillus* (Schlesner *et al.*, 2001) and *Lentibacillus* (Yoon *et al.*, 2002). Recently, Lim *et al.* (2005) reported the species *Pontibacillus chungwhensis*, which is the only species described in the genus *Pontibacillus* to date. In this study, another novel Gram-positive bacterium belonging to the genus *Pontibacillus* was isolated from a solar saltern in Korea and its taxonomic status and phenotypic properties are described.

Strain BH030004^T was isolated from a solar saltern of the Yellow Sea in Korea, using marine agar 2216 (MA) (Difco) supplemented with 5% (w/v) NaCl and 3 days incubation at 35 °C. Requirements for, and tolerance of, NaCl were determined in nutrient broth (Difco) supplemented with modified artificial sea water [containing (l⁻¹) 0–30% (w/v) NaCl, 5.94 g MgSO₄·7H₂O, 4.53 g MgCl₂·6H₂O, 0.64 g KCl and 1.3 g CaCl₂]. The isolates were routinely grown aerobically on MA for 2 days at 30 °C unless otherwise indicated. Anaerobic growth was determined by incubation in an anaerobic chamber for 5 days at 30 °C on MA. Growth was tested at different temperatures (4–55 °C) on MA and at different pH values (5.0–10.0) in nutrient broth supplemented with artificial sea water containing 2.36% (w/v) NaCl. The cellular morphology of strain BH030004^T was examined using light microscopy and transmission electron microscopy. Motility was observed at 12 and 36 h in wet mounts by means of a light microscope (E600 apparatus; Nikon). For visualization of flagella, cells were mounted on Formvar-coated copper grids (Electron Microscopy Science), negatively stained with 2% (w/v) uranyl acetate for 15 s and then subjected to transmission electron microscopy (JEM-1010 apparatus; JEOL). Gram staining was determined using the bioMérieux Gram stain kit, according to the manufacturer's instructions. Oxidase activity was tested using a Bactident Oxidase strip (Merck) and

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BH030004^T is AY603977.

Fatty acid profiles of strain BH030004^T and *P. chungwhensis* DSM 16287^T are available as supplementary material in IJSEM Online.

catalase activity was determined by bubble production in 3 % (v/v) hydrogen peroxide solution. Nitrate reduction and hydrolysis of aesculin, casein, starch, Tween 80, urea, hypoxanthine, tyrosine, gelatin and xanthine were determined on MA as described by Lanyi (1987). Acid production from carbohydrates was tested as described by Leifson (1963); all suspension media were supplemented with artificial sea water containing 2.36 % (w/v) NaCl.

The whole-cell fatty acid profile of strain BH030004^T was determined by using GC/MS according to the instructions of the Microbial Identification System (MIDI; Microbial ID) after cultivation of the micro-organism for 2 days at 30 °C on either MA or MA supplemented with 3 % (w/v) NaCl. The peptidoglycan and isoprenoid quinones of the strain were analysed as described by Komagata & Suzuki (1987). The DNA G + C content of strain BH030004^T was determined by reverse-phase HPLC using the method of Tamaoka & Komagata (1984). DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness between strain BH030004^T and *P. chungwhensis* KCTC 3890^T. Genomic DNA from the isolate and from *P. chungwhensis* KCTC 3890^T was isolated and purified according to the method described by Yoon *et al.* (1996). The isolated DNA samples were serially diluted from 200 to 0.5 ng and then denatured by boiling for 10 min. The series of dilutions was applied to positively charged nylon membranes with a slot device (Schleicher & Schuell) under a slight vacuum. Random-primed DNA labelling with digoxigenin-dUTP and hybridization were performed using a DIG High Prime DNA labelling kit and hybridization solutions (Roche Applied Science) according to the manufacturer's instructions. Detection of hybrids on the nylon membrane was performed using an enzyme immunoassay detection kit (Roche Applied Science). The membranes were incubated in 5 ml freshly prepared Coloursubstrate solution (Roche Applied Science) for 16 h in the dark. The membranes were washed with distilled water and then the colour intensities of the series of dilutions were quantified using Bio-Rad GelDoc scanning software. The signals produced by self-hybridization were inferred as 100 %, and relatedness values (percentages) were calculated from duplicate samples.

The 16S rRNA gene sequence of strain BH030004^T was analysed as described previously (DeLong, 1992). The 16S rRNA gene sequence was aligned together with those of representative members of selected genera by using the CLUSTAL W program (Thompson *et al.*, 1994). Sequence similarity values were computed using Similarity Matrix version 1.1 (Ribosomal Database Project II; <http://rdp.cme.msu.edu/index.jsp>; Cole *et al.*, 2003). Gaps at the 5' and 3' ends of the alignment were omitted for further analyses. Phylogenetic trees were constructed using three different methods, the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms, available in PHYLIP software, version 3.6 (Felsenstein, 2002). Evolutionary distance matrices were calculated according to the

algorithm of the Kimura two-parameter model for the neighbour-joining method. A bootstrap analysis (1000 replications) was performed, using the neighbour-joining method in the PHYLIP package, to evaluate the stability of the phylogenetic tree.

On MA, strain BH030004^T formed creamy, smooth and circular/slightly irregular colonies. It grew optimally on media containing 2–5 % (w/v) NaCl, but did not grow without NaCl or in the presence of more than 10 % (w/v) NaCl. The organism grew in nutrient agar (Difco) supplemented with artificial sea water, but not in nutrient agar with just NaCl, i.e. the strain requires other salts in addition to NaCl for growth. Anaerobic growth was not observed in an anaerobic chamber. Growth occurred from pH 6.0 to 9.0 (optimum, pH 7.0–7.5) and at temperatures between 15 and 40 °C (optimum, 30 °C). Strain BH030004^T showed obvious Gram-, catalase- and oxidase-positive reactions and reduced nitrate to nitrite. The cells were rods, 0.4–0.9 µm wide and 3.3–4.0 µm long, and were motile by means of peritrichous flagella after 2 days incubation at 35 °C on MA. Spherical endospores were formed in a terminal position in swollen sporangia. Analysis of the cell-wall peptidoglycan of the isolate showed that the diagnostic diamino acid was *meso*-diaminopimelic acid (A1γ-type). The major isoprenoid quinone was MK-7. The genomic DNA G + C content was about 42.0 mol%.

An almost-complete 16S rRNA gene sequence of strain BH030004^T was obtained and used for initial BLAST searches (<http://www.ncbi.nlm.nih.gov/blast/>) in GenBank and the phylogenetic analyses. The tree constructed by the neighbour-joining method showed that strain BH030004^T formed a phyletic line (with a 100 % bootstrap value) distinct from the most closely related species, *P. chungwhensis* (Fig. 1). The topologies of phylogenetic trees constructed using the maximum-likelihood and maximum-parsimony algorithms were similar to that of the tree constructed using neighbour-joining analysis (data not shown). The strain was most closely related to *P. chungwhensis* KCTC 3890^T (99.1 % 16S rRNA gene sequence similarity) and it was confirmed that differences between the two sequences were mainly localized in the second variable region of the 16S RNA molecules. Levels of 16S rRNA gene sequence similarity between strain BH030004^T and other related taxa were less than 95.1 %. The predominant cellular fatty acids of strain BH030004^T were iso-C_{15:0} (~65.1 %), anteiso-C_{15:0} (~10.2 %), C_{16:1ω7c} alcohol (~8.8 %) and iso-C_{14:0} (~4.0 %) on MA (details available in a supplementary table in IJSEM Online). No significant differences in fatty acid profiles were found between cells grown on MA and those grown on MA supplemented with 3 % (w/v) NaCl. Two major cellular fatty acids, iso-C_{15:0} and anteiso-C_{15:0}, of the test strain composed about 65 and 10 % of the total fatty acid content, respectively, whereas the corresponding fatty acids in *P. chungwhensis* KCTC 3890^T accounted for about 28 and 31 % of the total fatty acid content, respectively. Although

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic relationships of strain BH030004^T and other related taxa of low-G+C Gram-positive bacteria. Bootstrap values are shown as percentages of 1000 replicates. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 0.01 changes per nucleotide position.

Table 1. Characteristics of strain BH030004^T and some other related species

Strains: 1, strain BH030004^T; 2, *P. chungwhensis* DSM 16287^T; 3, *Lentibacillus salicampi* KCCM 41560^T; 4, *Halobacillus salinus* KCCM 41590^T; 5, *Gracilibacillus halotolerans* DSM 11805^T. Data from Lim *et al.* (2005), Yoon *et al.* (2002, 2003), Wainø *et al.* (1999) and this study. Symbols: +, positive; –, negative; w, weak; v, variable; NA, not available.

Characteristic	1	2	3	4	5
Cell size (μm)	0.4–0.9 × 3.3–4.0	0.6–0.9 × 2.3–3.0	0.4–0.7 × 2.0–4.0	0.7–1.1 × 1.5–4.0	0.4–0.6 × 2.0–5.0
Pigmentation	Cream	Yellow	Cream	Pale orange–yellow	Creamy white
Flagellation	Peritrichous	Peritrichous	One flagellum	Peritrichous	Peritrichous
Gram reaction	+	+	+/–	+	+
Spore shape*	S	S	S/O	E	E
Spore position†	T	T	T	C/ST	C/T
NaCl range (% w/v)	1–9	1–15	2–23	0–23	0–20
Oxidase	+	–	+	+	+
Nitrate reduction	+	–	NA	NA	+
Hydrolysis of:					
Aesculin	+	–	–	+	+
Casein	–	+	+	+	–
Gelatin	–	–	NA	+	+
Starch	–	+	–	–	+
Urea	–	–	–	–	+
Acid production from:					
D-Glucose	–	+	NA	+	+
D-Fructose	+	–	–	+	NA
D-Mannitol	–	–	–	+	+
D-Ribose	+	+	–	–	NA
D-Xylose	–	–	–	–	+
Maltose	+	+	–	+	NA
D-Trehalose	+	+	–	+	NA
Major fatty acids	iso-C _{15:0} , anteiso-C _{15:0} , C _{16:1} ω7c alcohol	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0}	anteiso-C _{15:0} , iso-C _{16:0} , anteiso-C _{17:0}	iso-C _{15:0} , anteiso-C _{15:0} , iso-C _{16:0}	anteiso-C _{15:0} , C _{16:0} , anteiso-C _{17:0}
Cell-wall type‡	<i>m</i> -DAP (A1γ)	<i>m</i> -DAP (A1γ)	<i>m</i> -DAP (A1γ)	L-Orn–D-Asp (A4β)	<i>m</i> -DAP (A1γ)
DNA G+C content (mol%)	42	41	44	45	38

*E, Ellipsoidal; O, oval; S, spherical.

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

‡*m*-DAP, *meso*-Diaminopimelic acid.

strain BH030004^T and *P. chungwhensis* DSM 16287^T had different fatty acid ratios, the profiles were similar. Therefore, comparative 16S rRNA gene sequence analysis clearly indicated that the novel strain was a member of the genus *Pontibacillus*. In addition to the results of phylogenetic analysis, many chemotaxonomic properties also supported the affiliation of the isolate to the genus *Pontibacillus* (Table 1).

The DNA–DNA relatedness between strain BH030004^T and *P. chungwhensis* KCTC 3890^T was about 7%, which is sufficient for classification of the novel strain as a different species (Stackebrandt *et al.*, 2002). Phenotypic properties of strain BH030004^T, including pigmentation, NaCl range, oxidase, nitrate reduction, hydrolyses and acid production, also supported its distinction from closely related taxa (Table 1).

On the basis of the phenotypic and phylogenetic properties, it is proposed that strain BH030004^T represents a novel species of the genus *Pontibacillus*, for which the name *Pontibacillus marinus* sp. nov. is proposed.

Description of *Pontibacillus marinus* sp. nov.

Pontibacillus marinus (ma'rin.us. L. masc. adj. *marinus* of the sea).

Cells are rods, approximately 0.4–0.9 µm wide and 3.3–4.0 µm long, motile by means of peritrichous flagella. Spherical endospores are formed terminally in swollen sporangia. Strictly aerobic. Catalase- and oxidase-positive. Nitrate is reduced to nitrite. Colonies are cream in colour, flat, smooth and circular/slightly irregular on MA. Growth occurs at 15–40 °C (optimum, 30 °C), pH 6.0–9.0 (optimum, pH 7.0–7.5) and 1–9% (w/v) NaCl (optimum, 2–5%). Tween 80 and aesculin are hydrolysed. Hydrolysis of casein, starch, gelatin, L-tyrosine, hypoxanthine, xanthine and urea is not observed. Acids are produced from sucrose, D-melibiose, D-trehalose, D-raffinose, D-fructose, D-ribose and maltose, but not from D-glucose, glycerol, D-xylose, L-arabinose, L-rhamnose, α-D-lactose, adonitol, D-mannitol, inositol or D-mannose. Major cellular fatty acids on MA are iso-C_{15:0} (65.12%), anteiso-C_{15:0} (10.15%), C_{16:1ω7c} alcohol (8.82%) and iso-C_{14:0} (4.0%). DNA G + C content is 42.0 mol% (HPLC).

The type strain is BH030004^T (=KCTC 3917^T=DSM 16465^T), isolated from a solar saltern of the Yellow Sea in Korea.

Emended description of the genus *Pontibacillus* Lim *et al.* 2005

The description is as given by Lim *et al.* (2005) with the following amendments. DNA G + C content is 40.8–42.0 mol% (HPLC). On MA, predominant cellular fatty acids are anteiso-C_{15:0} and iso-C_{15:0}.

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