Bacillus seohaeanensis sp. nov., a halotolerant bacterium that contains L-lysine in its cell wall

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A halotolerant, round-endospore-forming, aerobic, Gram-positive bacterium, designated BH724^T, was isolated from a solar saltern at Taeon in Korea. Cells of this strain were rod-shaped and found to be non-motile. Strain BH724^T grew at salinities of 0–10 % (w/v) NaCl with an optimum of 3 % (w/v) NaCl and at temperatures of 15–50 °C with an optimum of 40 °C. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain BH724^T belonged to the genus *Bacillus* and that *Bacillus aquimaris* TF-12^T, *Bacillus marisflavi* TF-11^T and *Bacillus vietnamensis* JCM 11124^T were its closest neighbours, sharing 97.3, 97.2 and 97.0 % 16S rRNA gene sequence similarity, respectively. The genomic DNA G + C content was 39 mol% and the predominant menaquinone was MK-7. Its major cellular fatty acids were anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{16:0} and iso-C_{14:0}. The peptidoglycan type was A1 α , linked directly through L-lysine. On the basis of morphological, chemotaxonomic, physiological and phylogenetic properties, strain BH724^T represents a novel species of the genus *Bacillus*, for which the name *Bacillus seohaeanensis* sp. nov. is proposed. The type strain is BH724^T (=KCTC 3913^T = DSM 16464^T).

The presence of a considerable diversity of organisms, constituting both moderately halophilic as well as halotolerant bacteria, has been revealed from extreme environments such as hypersaline environments (Ventosa *et al.*, 1998; Oren, 1999; Yoon *et al.*, 2001a, 2003). Aerobic, rod-shaped, endospore-forming, halophilic and/or halotolerant Gram-positive bacteria were originally classified in the genus *Bacillus*, but recently they have been reclassified as members of new genera or transferred to other genera by the application of molecular methods and improved phenotypic approaches (Arahal *et al.*, 2000; Ash *et al.*, 1993; Heyndrickx *et al.*, 1998; Jeon *et al.*, 2005; Schlesner *et al.*, 2001; Shida *et al.*, 1996; Wainø *et al.*, 1999; Yoon *et al.*, 2001b). In the course of screening halophilic bacteria, we isolated a halotolerant bacterium belonging to the genus *Bacillus*. The results of a polyphasic characterization of this *Bacillus* strain, BH724^T, are presented here. Our results suggest that the strain represents a novel species of the genus *Bacillus*.

Strain BH724^T was isolated from a solar saltern at Taeon on the west coast of Korea. For isolation, soil samples were diluted serially with 1 % (w/v) saline solution, spread on

marine agar 2216 (MA; Difco) with a final concentration of 11.4 % (w/v) total salts and incubated for 2 days at 35 °C. Isolate BH724^T was routinely grown aerobically on MA for 2 days at 35 °C except where indicated otherwise. Requirements for, and tolerance of, NaCl were determined in nutrient broth supplemented with modified artificial seawater 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 CaCl₂·2H₂O (Levring, 1946). Growth was tested at different temperatures (4–55 °C) and pH values (5.0–10.0) in marine broth (MB; Difco). To test for growth on single carbon sources at a concentration of 10 g l⁻¹, yeast extract and peptone were omitted from the MB. Cell growth was monitored by measurement of optical density at 600 nm. The cellular biomass of strain BH724^T was obtained from MB cultures at 35 °C.

Cell morphology was studied using light microscopy and transmission electron microscopy. Motility was observed at 12 and 36 h in wet mounts under a light microscope (Nikon E600). The flagellum type was examined by transmission electron microscopy using cells from the exponential growth phase. Cells were mounted on Formvar-coated copper grids and negatively stained with 1 % potassium phosphotungstate (pH 7.0). Grids were examined in a Phillips 201

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BH724^T is AY667495.

transmission electron microscope operating at 80 kV. Endospores were stained according to the method of Schaeffer–Fulton (Smibert & Krieg, 1981).

Gram staining was determined using the bioMérieux Gram Stain kit according to the manufacturer's instruction. Catalase activity was determined by bubble production in 3% (v/v) aqueous hydrogen peroxide solution. Oxidase activity was tested by oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine using a Bactident Oxidase strip (Merck). Nitrate reduction and hydrolysis of aesculin, casein, starch, Tween 80, urea, hypoxanthine, tyrosine, gelatin and xanthine were determined on MA according to the methods described by Cowan & Steel (1965), Lanyi (1987) and Smibert & Krieg (1994). Acid production from carbohydrates was tested as described by Leifson (1963). Growth under anaerobic conditions was determined in an anaerobic

chamber (5% H₂, 10% CO₂, 85% N₂; Mart Microbiology) after 5 days' incubation at 35 °C on MA.

For quantitative analysis of whole-cell fatty acids, strain BH724^T was cultivated on MA for 2 days at 30 °C. Fatty acid methyl esters were analysed by GC/MS according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Isoprenoid quinones were analysed as described by Komagata & Suzuki (1987) using HPLC apparatus fitted with a reversed-phase column (GROM-SIL 100 ODS-2FE; Grom). Methanol/2-propanol (2:1, v/v) was used as the mobile phase and quinone was detected at 270 nm. The peptidoglycan structure was elucidated by the DSMZ Identification Service. Qualitative analyses of amino acids and peptides in peptidoglycan hydrolysates were carried out as described by Schleifer (1985) and Schleifer & Kandler (1972) with paper chromatography (Rhuland *et al.*,

Table 1. Phenotypic characteristics that differentiate strain BH724^T from related members of the genus *Bacillus*

Data from Noguchi *et al.* (2004), Yoon *et al.* (2003) and this study. +, Positive; –, negative; W, weakly positive; V, variable; ND, no data available; DAP, diaminopimelic acid. All of the micro-organisms were positive for the hydrolysis of casein.

Characteristic	BH724 ^T	<i>B. marisflavi</i>	<i>B. aquimaris</i>	<i>B. vietnamensis</i>
Gram staining	+	+	V	+
Colony colour	Cream	Pale yellow	Pale orange/yellow	ND
Cell size (µm)	0.5–0.6 × 1.3–1.8	0.6–0.8 × 1.5–3.5	0.5–0.7 × 1.2–3.5	0.5–1.7 × 2.0–3.0
Spore position	Central	Subterminal or central	Central	Central
Spore shape	Sphere	Ellipsoid	Ellipsoid	Ellipsoid
Motility	–	+	+	+
Growth in/at				
10 °C	–	+	+	+
50 °C	+	–	–	–
pH 4.5	–	+	–	–
pH 9.0	–	+	–	+
0% NaCl	+	+	W	+
15% (w/v) NaCl	–	+	+	+
Oxidase	+	–	–	+
Hydrolysis of:				
Aesculin	+	+	–	+
Tween 80	–	–	+	ND
Starch	–	–	+	+
Urea	+	–	–	ND
Acid production from:				
Aesculin	+	+	–	+
Arbutin	+	+	–	–
Glycerol	+	+	–	+
D-Mannitol	+	+	–	+
D-Mannose	W	+	–	–
Melibiose	–	+	–	–
D-Raffinose	–	W	–	–
Salicin	–	+	–	–
Starch	–	–	+	+
D-Xylose	+	–	–	–
Cell wall type	A1α (L-Lys direct)	meso-DAP	meso-DAP	meso-DAP
DNA G+C content (mol%)	39	49	38	43

1955). The quantitative analysis of amino acids in the total hydrolysate was performed by gas chromatography and GC/MS as described by MacKenzie (1987) and Hasegawa *et al.* (1983). The N terminus of the interpeptide bridge was determined by dinitrophenylation according to Schleifer (1985). The G+C content (mol%) was determined by reversed-phase HPLC using the method of Tamaoka & Komagata (1984).

DNA–DNA hybridization was carried out to evaluate the genomic DNA relatedness between strain BH724^T and closely related type strains of the genus *Bacillus*. Strains for comparative analysis were grown aerobically on MA for 2 days at 35 °C and chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996). DNA–DNA hybridization was determined based on the filter-hybridization method described by Seldin & Dubnau (1985). Isolated DNA samples were serially diluted from 200 to 0.5 ng and denatured by boiling for 10 min. The series of dilutions was applied to positively charged nylon membranes (Hybond-N⁺; Amersham) 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 the enzyme immunoassay Detection Starter kit II (Roche Applied Science). Membranes were incubated in 5 ml freshly prepared Colorsubstrate solution (Roche Applied Science) for 16 h in the dark. The membranes were washed with distilled water and the colour intensities of the series of dilutions were quantified using Bio-Rad GelDoc scanning software. The signals produced by self-hybridization were taken as 100 % and relatedness values (percentages) were calculated from duplicate samples.

The 16S rRNA gene was amplified by PCR using primers Eubac 27F and 1492R (DeLong, 1992) and the PCR products were purified using the QIAquick PCR Purification kit (Qiagen). Sequencing of the purified 16S rRNA gene was performed using an ABI PRISM BigDye Terminator Cycle Sequencing kit (Applied Biosystems), as recommended by the manufacturer, and five primers (337F, 785F, 1225F, 518R and 1100R). The purified sequencing reaction mixtures were electrophoresed automatically using an Applied Biosystems model 377 automatic DNA sequencer. The 16S rRNA gene sequence of strain BA724^T was aligned together with those of other *Bacillus* species using CLUSTAL W (Thompson *et al.*, 1994). Sequence similarity values were computed using Similarity Matrix version 1.1 (Ribosomal Database Project II; <http://rdp.cme.msu.edu/>) (Cole *et al.*, 2003). Gaps at the 5' and 3' ends of the alignment were omitted in further analyses. Phylogenetic trees were inferred using three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Kluge & Farris, 1969) methods available in the PHYLIP software package version 3.6

(Felsenstein, 2002). Evolutionary distance matrices for the neighbour-joining method were calculated using the algorithm of the Kimura two-parameter model (Kimura, 1980) with the DNADIST program. To evaluate the stability of the phylogenetic tree, a bootstrap analysis (1000 replications) was performed with the SEQBOOT, DNADIST, NEIGHBOR and CONSENSE programs in the PHYLIP package.

Strain BH724^T on MA medium formed creamy, smooth and circular/slightly irregular colonies after incubation at 35 °C for 2 days. Growth was not observed in the absence of salts. The strain grew at salt concentrations in the range of 0–10 % (w/v) NaCl, with optimum growth occurring at 3–5 % (w/v) NaCl in the presence of artificial seawater. The strain did not grow in the presence of more than 12 % (w/v) NaCl. Growth occurred from pH 5.0 to 8.0 (optimum, pH 7.5) in marine broth. Growth was observed at temperatures between 15 and 50 °C, with an optimum growth temperature of 35–40 °C, and growth was not observed at 10 °C.

Table 2. Cellular fatty acid compositions of strain BH724^T and related species on MA supplemented with 3 % (w/v) NaCl

Data are expressed as percentages of total fatty acids. Fatty acids representing less than 0.5 % in all strains are not included. ND, Not detected. Data for reference strains were taken from Yoon *et al.* (2003).

Fatty acid	BH724 ^T	<i>B. marisflavi</i>	<i>B. aquimaris</i>
Straight-chain fatty acids			
C _{14:0}	0.4	1.7	0.9
C _{15:0}	0.7	1.1	0.5
C _{16:0}	1.3	1.0	0.5
Branched fatty acids			
iso-C _{14:0}	8.2	9.1	6.5
iso-C _{15:0}	20.3	22.9	46.9
iso-C _{16:0}	14.3	7.7	4.2
iso-C _{17:0}	1.6	1.2	0.8
anteiso-C _{15:0}	36.7	27.7	22.2
anteiso-C _{17:0}	4.8	7.1	2.3
Unsaturated fatty acids			
iso-C _{15:1}	ND	ND	0.7
C _{16:1} ω7c alcohol	4.0	2.5	8.4
C _{16:1} ω11c	0.6	ND	1.3
iso-C _{17:1} ω10c	0.4	ND	1.8
Hydroxy fatty acids			
C _{15:0} 2-OH	1.3	5.5	ND
C _{17:0} 2-OH	ND	1.2	ND
iso-C _{14:0} 3-OH	1.1	2.0	ND
iso-C _{15:0} 3-OH	1.6	6.1	ND
iso-C _{16:0} 3-OH	1.2	1.5	ND
Summed feature 4*	0.9	1.8	2.9

*Summed features represent groups of two or three fatty acids that can not be separated by GLC with the MIDI system. Summed feature 4 contains iso-C_{17:1} I and/or anteiso-C_{17:1} B.

Fig. 1. Neighbour-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship of strain BH724^T to other related taxa. Numbers at branching nodes are bootstrap values (percentages of 1000 replications); only values greater than 50% are indicated. Bar, 0.01 substitutions per nucleotide position.

Strain BH724^T was a Gram-positive, strictly aerobic rod, 0.5–0.6 µm wide and 1.3–1.8 µm long. It formed spherical endospores at central positions and cells were non-motile and did not possess flagella. The phenotypic characteristics of strain BH724^T are compared with those of type strains of closely related *Bacillus* species in Table 1.

The predominant isoprenoid quinone was MK-7. The cellular fatty acid profile of strain BH724^T was characterized by the presence of branched fatty acids such as anteiso-C_{15:0} (36.7%), iso-C_{15:0} (20.3%) and iso-C_{16:0} (14.3%) as the major fatty acids on MA and its composition was slightly different from that of the other closely related type strains (Table 2). The DNA G + C content of strain BH724^T was 39 mol%. The major fatty acid profile, major isoprenoid quinone and DNA G + C content are typical of the group classically defined as the genus *Bacillus* (Table 1). However, the peptidoglycan of strain BH724^T contained the amino acids lysine, alanine and glutamic acid in a molar ratio of 0.6:1.8:1.0. Dinitrophenylation revealed lysine as the only N-terminal amino acid. The peptides L-Ala–D-Glu and L-Lys–D-Ala were detected by two-dimensional TLC in the partial hydrolysate. From these data, it was concluded that the peptidoglycan type is A1α (L-Lys direct) (Schleifer & Kandler, 1972) (murein type A11 according to the DSMZ catalogue of strains, 2001). The majority of endospore-forming, Gram-positive, rod-shaped bacteria possess meso-diaminopimelic acid as the diagnostic diamino acid of the cell wall peptidoglycan. Therefore, strain BH724^T could be distinguished from closely related species within the genus *Bacillus* based on cellular fatty acid profiles and cell wall peptidoglycan type.

The almost complete 16S rRNA gene sequence (1401 nt) of strain BH724^T was obtained and used for an initial BLAST search in GenBank and for phylogenetic analysis. The similarity values of strain BH724^T to closely related species with validly published names were *Bacillus aquimaris* JCM 11545^T (97.3%), *Bacillus marisflavi* JCM 11544^T (97.2%) and *Bacillus vietnamensis* JCM 11124^T (97.0%). Similarity values to other type strains of *Bacillus* species were 95.1–96.9%. A tree constructed by neighbour-joining analysis showed that strain BH724^T formed a distinct phylogenetic line with a bootstrap value of 82.3% compared with the closely related type strains *B. aquimaris* JCM 11545^T, *B. marisflavi* JCM 11544^T and *B. vietnamensis* JCM 11124^T (Fig. 1). The topologies of phylogenetic trees built using the maximum-likelihood and maximum-parsimony algorithms were similar to that of the tree constructed by neighbour-joining analysis (data not shown).

DNA–DNA relatedness between strain BH724^T and *B. aquimaris* JCM 11545^T, *B. marisflavi* JCM 11544^T and *B. vietnamensis* JCM 11124^T was about 50, 40 and 30%, respectively, and the values were low enough to allocate strain BH724^T to a novel species of the genus *Bacillus* (Wayne, 1994). On the basis of the phenotypic and multiple molecular analyses, we propose that strain BH724^T should be assigned to the genus *Bacillus* within a novel species for which the name *Bacillus seohaeanensis* sp. nov. is proposed.

Description of *Bacillus seohaeanensis* sp. nov.

Bacillus seohaeanensis (seo.hae.an.en'sis. N.L. masc. adj. *seohaeanensis* of Seohaean, the Korean name for the west coast of Korea, where the type strain was isolated).

Cells are aerobic, Gram-positive, endospore-forming (spherical, central) rods, $0.5\text{--}0.6 \times 1.3\text{--}1.8\text{ }\mu\text{m}$. Colonies are creamy, smooth and circular to slightly irregular. Cells are non-motile and do not possess flagella. Grows at salinities of 0–10 % (w/v) NaCl. Optimal growth at 3–5 % (w/v) NaCl. No growth occurs in the presence of more than 12 % (w/v) NaCl. Grows between 15 and 50 °C (optimum 35–40 °C) and from pH 5.0 to 8.0 (optimum pH 7.5) in marine broth. Glucose, fructose, maltose, glycerol, yeast extract and peptone support growth as the single carbon and energy source. Growth does not occur under anaerobic conditions on MA. Oxidase- and catalase-positive. Nitrate is not reduced to nitrite. Aesculin, casein and urea are hydrolysed. Gelatin, starch, L-tyrosine, Tween 80, xanthine and hypoxanthine are not hydrolysed. Acid is produced from D-glucose, maltose, D-trehalose, D-xylose, D-ribose, glycerol, D-mannitol, D-fructose, arbutin and D-mannose, but not from L-arabinose, D-rhamnose, lactose, adonitol, D-raffinose, D-salicin and D-melibiose. The cell-wall peptidoglycan type is A1 α (L-Lys direct). The predominant isoprenoid quinone is MK-7. The major fatty acids are anteiso-C_{15:0}, iso-C_{15:0} and iso-C_{16:0}. The G + C content of the type strain is 39 mol%.

The type strain is strain BH724^T (= KCTC 3913^T = DSM 16464^T), isolated from a solar saltern at Taean on the west coast of Korea.

Acknowledgements

This work was supported by the 21C Frontier Microbial Genomics and Application Centre Programme of the Ministry of Science and Technology, Republic of Korea.

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