Halobacillus yeomjeoni sp. nov., isolated from a marine solar saltern in Korea

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A Gram-positive or Gram-variable, motile, endospore-forming, halophilic bacterial strain, MSS-402^T, was isolated from a marine solar saltern in Korea, and subjected to a polyphasic taxonomic study. Some cells of strain MSS-402^T were long filamentous rods. The isolate grew optimally at 37 °C and in the presence of 3–5 % (w/v) NaCl. Strain MSS-402^T had cell-wall peptidoglycan based on L-Orn–D-Asp, MK-7 as the predominant menaquinone and anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} as major fatty acids. The DNA G + C content was 42.9 mol%. Phylogenetic analyses based on 16S rRNA gene sequences showed that strain MSS-402^T falls within the evolutionary radiation of species of the genus *Halobacillus*. Levels of 16S rRNA gene sequence similarity between strain MSS-402^T and the type strains of recognized *Halobacillus* species ranged from 98.0 % (with *Halobacillus halophilus*) to 99.2 % (with *Halobacillus litoralis* and *Halobacillus trueperi*). Levels of DNA–DNA binding indicated that strain MSS-402^T represents a genomic species that is distinct from recognized *Halobacillus* species. Strain MSS-402^T was differentiated from *Halobacillus* species by means of several phenotypic characteristics. On the basis of its phenotypic properties and phylogenetic and genetic distinctiveness, strain MSS-402^T (= KCTC 3957^T = DSM 17110^T) should be classified as the type strain of a novel *Halobacillus* species, for which the name *Halobacillus yeomjeoni* sp. nov. is proposed.

The genus *Halobacillus* was first described by Spring *et al.* (1996); at present, it comprises six species with validly published names, *Halobacillus halophilus*, *Halobacillus litoralis* and *Halobacillus trueperi* (Spring *et al.*, 1996), *Halobacillus salinus* (Yoon *et al.*, 2003), *Halobacillus karajensis* (Amoozegar *et al.*, 2003) and *Halobacillus locisalis* (Yoon *et al.*, 2004).

The genus *Halobacillus* is differentiated from the genus *Bacillus* and other related genera as having a cell-wall peptidoglycan type based on L-Orn–D-Asp (Spring *et al.*, 1996; Shida *et al.*, 1997; Yoon *et al.*, 2001, 2002; Lim *et al.*, 2005). Recently, in the course of screening for novel microorganisms present in marine solar salterns of the west coast of the Korean peninsula, a *Halobacillus*-like bacterial strain, MSS-402^T, was isolated and characterized taxonomically. In this study, we focused on strain MSS-402^T because it was found to have an interesting morphological property, i.e. some cells are crooked, long, filamentous rods. The aim of the present work was to investigate the exact taxonomic

status of strain MSS-402^T by using polyphasic taxonomic characterization.

Water collected from a marine solar saltern at Seosan, Korea, provided the source for isolation of the bacterial strains. A standard dilution plating technique was used to isolate strain MSS-402^T at 30 °C on marine agar 2216 (MA; Difco) supplemented with 8 % (w/v) NaCl. *H. halophilus* KCTC 3685^T, *H. litoralis* KCTC 3687^T and *H. trueperi* KCTC 3686^T were obtained from the Korean Collection for Type Cultures, Taejeon, Korea. *H. karajensis* DSM 14948^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *H. salinus* HSL-3^T and *H. locisalis* MSS-155^T were obtained from the studies of Yoon *et al.* (2003, 2004). To investigate its morphological, physiological and biochemical characteristics, strain MSS-402^T was routinely cultivated on MA supplemented with 3 % (w/v) NaCl at 30 °C. The cell morphology was examined by light microscopy (E600; Nikon) and transmission electron microscopy. For the latter, the cells were negatively stained with 1 % (w/v) phosphotungstic acid and, after air-drying, the grids were examined by using a model CM-20 transmission electron microscope (Philips). The presence of flagella was examined by transmission electron microscopy using cells from

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exponentially growing cultures. The Gram-reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on MA supplemented with 3 % (w/v) NaCl and on MA supplemented with 3 % (w/v) NaCl and nitrate, both of which had been prepared anaerobically using nitrogen. The optimal pH for growth was determined in trypticase soy broth (Difco), supplemented with 3 % (w/v) NaCl and 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1 distilled water)⁻¹, that was adjusted to various pH values (pH 4.0 initially, rising to pH 10.5 at intervals of 0.5 pH units). The pH was adjusted prior to sterilization to various levels by the addition of HCl or Na_2CO_3 . Growth in the absence of NaCl was investigated in trypticase soy broth without NaCl, supplemented with 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1 distilled water)⁻¹. The requirement for ions for growth was investigated in trypticase soy broth supplemented with 3 % (w/v) NaCl using $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KCl or CaCl_2 at the concentrations indicated by Bruns *et al.* (2001). Growth at various NaCl concentrations was investigated in marine broth 2216 (Difco) (more than 2 %, w/v, NaCl at intervals of 1 %) and in trypticase soy broth supplemented with 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (1 distilled water)⁻¹ (less than 2 %, w/v, NaCl at intervals of 0.5 %). Growth at various temperatures (4–50 °C) was measured on MA supplemented with 3 % (w/v) NaCl. Oxidase and catalase activities and hydrolysis of casein and starch were determined as described by Cowan & Steel (1965). Hydrolysis of Tweens 20, 40, 60 and 80 was determined as described by Cowan & Steel (1965), with the modification that artificial sea water was used instead of distilled water. Hydrolysis of aesculin, gelatin and urea and nitrate reduction were determined as described by Lanyi (1987), with the modification that artificial sea water was used for preparation of media. The artificial sea water contained the following (1 distilled water)⁻¹: 23.6 g NaCl, 0.64 g KCl, 4.53 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.94 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 1.3 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Bruns *et al.*, 2001). H_2S production was tested as described by Bruns *et al.* (2001). Hydrolysis of hypoxanthine, tyrosine and xanthine was performed on MA supplemented with 3 % (w/v) NaCl, using the substrate concentrations indicated by Cowan & Steel (1965). Acid production from carbohydrates was determined as described by Leifson (1963). Enzyme activities and other physiological properties were determined using the API ZYM and API 20E systems (bioMérieux); the cell suspension used to inoculate the systems was prepared by using artificial sea water (Bruns *et al.*, 2001).

Cell mass for analyses of the cell wall and menaquinones and for DNA extraction was produced in marine broth 2216 supplemented with 3 % (w/v) NaCl at 37 °C. Preparation of cell-wall peptidoglycan was carried out using the method described by Schleifer & Kandler (1972), and the peptidoglycan structure was determined using an automated amino acid analyser (model L-8500A; Hitachi). Menaquinones were analysed as described by Komagata & Suzuki (1987), using reverse-phase HPLC. Chromosomal

DNA was isolated and purified according to the method described previously (Yoon *et al.*, 1996), with the exception that RNase T1 was used in combination with RNase A. For fatty acid methyl ester analysis, cell mass of strain MSS-402^T was harvested from agar plates after cultivation for 2 days at 37 °C on MA supplemented with 3 % (w/v) NaCl. The fatty acid methyl esters were extracted and prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984), with the modification that the DNA was hydrolysed and the resultant nucleotides were analysed by reverse-phase HPLC. The 16S rRNA gene was amplified by using a PCR with two universal primers, as described previously (Yoon *et al.*, 1998). Sequencing of the 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded, and the means of the remaining three values were quoted as the DNA–DNA binding values.

The morphological, cultural, physiological and biochemical characteristics of strain MSS-402^T are shown in Table 1 or are given in the species description below. Cells of strain MSS-402^T were Gram-variable rods or long filamentous rods, measuring 0.4–0.6 × 2.0–100 µm after incubation on MA supplemented with 3 % (w/v) NaCl at 37 °C; a few cells greater than 100 µm in length were also observed (Fig. 1). This cellular morphological property was not found in other *Halobacillus* species (Spring *et al.*, 1996; Amoozegar *et al.*, 2003; Yoon *et al.*, 2003, 2004). Strain MSS-402^T required Na^+ and Mg^{2+} ions for growth, but did not require K^+ or Ca^{2+} . The 16S rRNA gene sequence of strain MSS-402^T determined in this study comprised 1522 nt, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. Comparative 16S rRNA gene sequence analyses showed that strain MSS-402^T is phylogenetically most closely affiliated to the genus *Halobacillus* (Fig. 2). In the phylogenetic tree based on the neighbour-joining algorithm, strain MSS-402^T fell within the radiation of the cluster comprising *Halobacillus* species (Fig. 2). The same tree topology was found in trees generated with the maximum-likelihood and maximum-parsimony algorithms (data not shown). The 16S rRNA gene sequence of strain MSS-402^T had similarity levels of 98.0–99.2 % with respect to sequences of the type strains of recognized *Halobacillus* species (Fig. 2). Sequence similarities with respect to all other species included in the phylogenetic analysis were lower than 94.8 % (Fig. 2).

The chemotaxonomic properties of strain MSS-402^T support the result of monothetic phylogenetic classification, namely that the isolate might be a member of the genus *Halobacillus*. Cell-wall analysis showed that strain MSS-402^T

Table 1. Differential phenotypic properties of *Halobacillus* species

Species: 1, *H. yeomjeoni* sp. nov.; 2, *H. halophilus* (data from Claus *et al.*, 1983; Spring *et al.*, 1996); 3, *H. trueperi* (Spring *et al.*, 1996); 4, *H. litoralis* (Spring *et al.*, 1996); 5, *H. salinus* (Yoon *et al.*, 2003); 6, *H. karajensis* (Amoozegar *et al.*, 2003); 7, *H. locisalis* (Yoon *et al.*, 2004). All species are positive for catalase and oxidase. All species are negative for nitrate reduction (not determined for *H. karajensis*), urease, anaerobic growth and hydrolysis of tyrosine (not determined for *H. karajensis*). Symbols: +, positive; –, negative; w, weakly positive; v, variable; ND, not determined.

Characteristic	1	2	3	4	5	6	7
Cell morphology	Rods or long filamentous rods	Cocci or oval	Rods	Rods	Rods	Rods	Rods
Flagellation	Single	Single or peritrichous	Peritrichous	Peritrichous	Peritrichous	Absent	Single
Gram staining	+	+	+	+	+	+	+
Spore shape	Ellipsoidal	Spherical	Ellipsoidal or spherical	Ellipsoidal or spherical	Ellipsoidal	Ellipsoidal or spherical	Ellipsoidal
Spore position	Central or subterminal	Central or lateral	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal	Central or subterminal
Colony colour	Light yellow	Orange	Orange	Orange	Pale orange–yellow	Cream or white	Light orange–yellow
Maximum temperature for growth (°C)	48	37	44	43	45	49	42
Growth at:							
pH 5.0	–	–	–	–	+	–	+
pH 5.5	–	–	–	–	+	–	+
Growth with:							
0.5 % NaCl	+	–	+	+	+	–	–
25 % NaCl	–	–	+	+	–	–	–
Hydrolysis of:							
Aesculin	–	–	–	–	+	+	+
Casein	+	+	–	–	+	+	–
Gelatin	+	+	+	+	+	+	–
Starch	–	+	–	–	–	+	+
Tween 80	+	–	–	–	+	–	+
Acid production from:							
D-Fructose	–	–	+	+	+	+	+
D-Galactose	–	–	+	–	w	–	–
Maltose	+	–	+	+	+	+	–
Sucrose	+	–	+	+	+	–	+
D-Xylose	–	–	–	+	–	–	–
D-Glucose	+	–	+	+	+	+	+
D-Mannitol	+	–	–	+	+	+	–
D-Trehalose	+	–	+	+	+	ND	+
DNA G+C content (mol%)	42.9	40.1–40.9	43	42	45	41.3	44.0

*Positive when Mg²⁺ ion is added.

Fig. 1. Micrographs of strain MSS-402^T after cultivation for 3 days at 37 °C on MA supplemented with 3% (w/v) NaCl. (a) Transmission electron micrograph. Bar, 1 µm. (b) Light micrograph. Bar, 10 µm.

had the peptidoglycan type A4β, based on L-Orn–D-Asp, as described by Schleifer & Kandler (1972). This cell-wall peptidoglycan type is a key marker that differentiates strain MSS-402^T and the genus *Halobacillus* from other aerobic or facultatively anaerobic, endospore-forming, rod-shaped genera (Spring *et al.*, 1996; Shida *et al.*, 1997; Wainø *et al.*, 1999; Yoon *et al.*, 2001). The predominant isoprenoid quinone found in strain MSS-402^T was unsaturated

menaquinone with seven isoprene units (MK-7). The fatty acids detected in strain MSS-402^T were anteiso-C_{15:0} (40.4%), anteiso-C_{17:0} (23.0%), iso-C_{16:0} (19.3%), iso-C_{15:0} (8.8%), iso-C_{17:0} (3.5%), iso-C_{14:0} (3.4%) and C_{16:0} (1.6%). This fatty acid profile was similar to those of the type strains of *Halobacillus* species, although there were differences in the proportions of some fatty acids (Yoon *et al.*, 2003, 2004). The DNA G + C content of strain MSS-402^T was 42.9 mol%.

There were some differences between strain MSS-402^T and recognized *Halobacillus* species in terms of phenotypic characteristics (Table 1). DNA–DNA binding data were sufficient to categorize strain MSS-402^T as representing a species that is distinct from recognized *Halobacillus* species (Wayne *et al.*, 1987). The mean DNA–DNA relatedness levels between strain MSS-402^T and the type strains of six recognized *Halobacillus* species were in the range 13–26%. Therefore, on the basis of the data presented, strain MSS-402^T should be placed in the genus *Halobacillus* as a member of a novel species, for which the name *Halobacillus yeomjeoni* sp. nov. is proposed.

Description of *Halobacillus yeomjeoni* sp. nov.

Halobacillus yeomjeoni (yeom.jeo'ni. N.L. gen. n. *yeomjeoni* of yeomjeon, the Korean name for a marine solar saltern).

Cells are rods or long filamentous rods, measuring 0.4–0.6 × 2.0–100 µm, on MA supplemented with 3% (w/v) NaCl at 37 °C; a few cells in old cultures are greater than 100 µm in length (Fig. 1). Gram-positive, but Gram-variable in old cultures. Central or subterminal ellipsoidal endospores are observed in swollen sporangia. Colonies are circular, slightly raised, glistening, light yellow in colour and 1–2 mm in size after 2 days incubation at 30–37 °C on MA supplemented with 3% (w/v) NaCl. Na⁺ and Mg²⁺ ions are required for growth. The optimal growth temperature is 37 °C; growth occurs at 15 and 48 °C, but not at 10 or 49 °C. The optimal pH for growth is 7.0–8.0; growth occurs at pH 6.0, but not at pH 5.5. Optimal growth occurs in the presence of 3–5% (w/v) NaCl; growth occurs in the presence of 0.5% (w/v) and 21% (w/v) NaCl, but not without NaCl

Fig. 2. Neighbour-joining tree, based on 16S rRNA gene sequence data, showing the phylogenetic positions of strain MSS-402^T, *Halobacillus* species and some other related taxa. Bootstrap values (1000 replications) are shown as percentages at each node only if they are 50% or greater. Bar, 0.01 substitutions per nucleotide position.

or in the presence of > 22 % (w/v) NaCl. Tweens 20, 40 and 60 are hydrolysed. Hypoxanthine and xanthine are not hydrolysed. The Voges-Proskauer test is negative. Indole and H₂S are not produced. Arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase are absent. In assays with the API ZYM system, alkaline phosphatase, esterase (C4), esterase lipase (C8), naphthol-AS-BI-phosphohydrolase and β -galactosidase are present, but lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, acid phosphatase, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase are absent. Acid is produced from D-cellobiose, D-ribose and D-mannose. Acid is not produced from L-arabinose, lactose, D-melezitose, melibiose, D-raffinose, L-rhamnose, *myo*-inositol or D-sorbitol. The cell wall contains peptidoglycan based on L-Orn-D-Asp. The predominant menaquinone is MK-7. The major fatty acids are anteiso-C_{15:0} (40.4 %), anteiso-C_{17:0} (23.0 %) and iso-C_{16:0} (19.3 %). The DNA G + C content is 42.9 mol%.

The type strain, MSS-402^T (= KCTC 3957^T = DSM 17110^T), was isolated from a marine solar saltern of the Yellow Sea in Korea.

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