Halobacillus karajensis sp. nov., a novel moderate halophile

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A moderately halophilic, Gram-positive, spore-forming bacterium was isolated from surface saline soil of the Karaj region, Iran. The strain, designated MA-2^T, was strictly aerobic with rod-shaped cells that occurred singly, in pairs or short chains. It contained L-Orn-D-Asp-type peptidoglycan and the major respiratory lipoquinone was MK-7. It was non-motile and had an ellipsoidal endospore located centrally or subterminally. Growth occurred at 10–49 °C and in the pH range 6.0–9.6. Strain MA-2^T grew at salinities of 1–24 % (w/v) NaCl, showing optimal growth at 10 % (w/v). The DNA G+C content was 41.3 mol%. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain MA-2^T was associated with *Bacillus* rRNA group 1. The micro-organisms showing the closest phylogenetic relationship to strain MA-2^T were *Halobacillus litoralis* and *Halobacillus trueperi*. On the basis of phenotypic and chemotaxonomic characteristics, 16S rRNA gene sequence analysis and DNA–DNA similarity data, it is proposed that strain MA-2^T (=DSM 14948^T=LMG 21515^T) should be placed in the genus *Halobacillus* as the type strain of a novel species, *Halobacillus karajensis* sp. nov.

Moderately halophilic bacteria include a heterogeneous group of micro-organisms consisting of different genera and species that grow in various salty environments (Ventosa *et al.*, 1998). As described by Kaurichev (1980), soils containing more than 0.2 % (w/v) soluble salt are considered to be saline soils and such soils are scattered all over the world.

The microbiota of hypersaline soils are more similar to those of non-saline soils than the microbiota from hypersaline waters. This suggests that general features of the environment are more important in determining the microbiota in a particular habitat than are individual factors such as high salinity (Quesada *et al.*, 1983). Moderately halophilic spore-forming bacteria, which are an important group, were formerly placed in the genus *Bacillus* (Claus & Berkeley, 1986; Slepecky & Hemphill, 1991), but now are placed in separate genera. The spore-forming moderate halophiles are found mostly in marine or hypersaline environments. Strain MA-2^T was isolated from moderately saline soil of the Karaj region, Iran, and its phenotypic and chemotaxonomic characteristics, cell wall composition, DNA G+C content, DNA–DNA relatedness to other species and 16S rRNA gene sequences have been determined. The data obtained strongly support the suggestion

that this strain represents a novel species of the genus *Halobacillus*, and it has therefore been classified as *Halobacillus karajensis* sp. nov.

Soil samples were obtained from the geographical area of Karaj in Iran. The salinity of the samples was determined to be about 5 % (w/v, as total salt). Aliquots of the soil were added to a broth medium containing (g l⁻¹): NaCl, 81; MgCl₂·6H₂O, 7; MgSO₄·7H₂O, 9.7; CaCl₂, 0.36; KCl, 2; NaHCO₃, 0.06; NaBr, 0.026 (total salts, 100 g l⁻¹). The pH was adjusted to 7.2–7.4 with 1 M KOH prior to autoclaving. Incubation was carried out at 34 °C under aerobic conditions. After a few days of incubation, agar plates were streaked, resulting mostly in white opaque colonies. Pure cultures were isolated after repeated re-streaking.

A pure culture of strain MA-2^T (=DSM 14948^T=LMG 21515^T) was grown and maintained in a complex medium containing (g l⁻¹): NaCl, 100; MgSO₄·7H₂O, 5; casein peptone, 5; yeast extract, 3 (as recommended by Spring *et al.*, 1996). A sporulation medium (Atlas, 1997) plus 5 % (w/v) NaCl was used for sporulation. Incubation was carried out on a shaker at 150 r.p.m. and 34 °C. Morphological and physiological characteristics of the isolate were studied on nutrient agar or nutrient broth plus 10 % (w/v) NaCl. Reference strains *Halobacillus trueperi* DSM 10404^T, *Halobacillus litoralis* DSM 10405^T and *Halobacillus halophilus* DSM 2266^T were obtained from the DSMZ.

The EMBL accession number for the 16S rDNA sequence of strain MA-2^T (=DSM 14948^T) is AJ486874.

SEM was used for morphological examinations. Strain MA-2^T was prepared for SEM according to the method of Bozzola & Russell (1999) and specimens were observed on a Zeiss DSM 960 EM. Gram staining (Burke method) was performed and the result was confirmed by the KOH test (Baron & Finegold, 1990). Motility was analysed by the wet-mount method (Murray *et al.*, 1994). Catalase, oxidase and urease activity, nitrate reduction, hydrolysis of aesculin, methyl red, Voges–Proskauer and indole production were checked as recommended by Smibert & Krieg (1994). Hydrolysis of Tween 80 was examined as described by Harrigan & McCance (1976). Utilization of citrate and propionate was determined as recommended by Claus & Berkeley (1986). Utilization of various carbohydrates and the production of acid were respectively determined as described by Atlas (1997) and Parry *et al.* (1988). All the above tests were performed using medium containing 10 % (w/v) NaCl.

Antibiotic sensitivity tests were performed on Mueller–Hinton agar plus 10 % (w/v) NaCl seeded with a bacterial suspension containing 1.5×10^6 c.f.u. ml⁻¹ using discs (bioMérieux) impregnated with various antibiotics. The plates were incubated at 34 °C for 48 h and the inhibition zone was interpreted according to the manufacturer's manual. The specific growth rate (μ) was calculated as 0.693 h⁻¹ using a spectroscopic method (Shimadzu model UV-160 A) at 620 nm. Growth at various temperatures (5, 10, 15, 20, 25, 30, 35, 38, 40, 45, 49, 50 and 55 °C) was determined. The pH range for growth was deduced and the final pH was adjusted to between 5 and 10. At pH values greater than 6, Tris buffer was used, and, at pH values below 6, sodium acetate buffer was used. Other physiological and biochemical tests were performed as described previously (Quesada *et al.*, 1984; Ventosa *et al.*, 1982).

The interpeptide bridge in the cell-wall peptidoglycans was analysed using the method described by Schleifer & Kandler (1972). Cell-wall hydrolysates were separated by one- or two-dimensional chromatography on cellulose thin-layer plates (Merck).

Fatty acid methyl esters were obtained from 40 mg (wet wt) cells by saponification, methylation and extraction as described previously (Kämpfer & Kroppenstedt, 1996; Kroppenstedt, 1985; Miller, 1982). The fatty acid methyl ester mixtures were separated by an automated GC system (model 5890 series II and 7673 autosampler; Agilent) controlled by MIS software (Microbial ID). Peaks were integrated automatically and fatty acid names and percentages were determined using the Microbial Identification standard software package (Sasser, 1990).

For determination of DNA base composition and DNA–DNA hybridization, DNA was isolated using a French pressure cell and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). The G + C content was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). DNA–DNA

hybridization studies were carried out according to the method of De Ley *et al.* (1970) with the modification described by Huß *et al.* (1983), using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were computed with the program TRANSFER.BAS (Jahnke, 1992).

Genomic DNA extraction, PCR-mediated amplification of the 16S rDNA, purification of PCR products and electrophoresis of sequencing products were done as described previously (Rainey *et al.*, 1996). The 16S rDNA sequence was aligned manually with published sequences from the *Bacillus/Clostridium* group contained in the DSMZ database of 16S rDNA sequences. The ae2 editor (Maidak *et al.*, 1999) was used to align the 16S rDNA sequences of strain MA-2^T against sequences of *Halobacillus* type strains available from public databases. Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). The least-squares method (De Soete, 1983) was used to construct a phylogenetic dendrogram from distance matrices.

Cells of the isolate were rod-shaped (0.8–0.9 × 2.5–4.0 µm), Gram-positive and strictly aerobic and occurred singly, in pairs or in short chains (Fig. 1). Under non-optimal conditions for growth, particularly at pH 9.0–9.5, a filamentous form appeared. The isolate was non-motile in media containing the various salt concentrations and pH values used in this study; this characteristic differentiated strain MA-2^T from all known Gram-positive, spore-forming, moderately halophilic bacteria (Arahal *et al.*, 1999; Garabito *et al.*, 1997; Spring *et al.*, 1996; Schlesner *et al.*, 2001; Ventosa *et al.*, 1989). MA-2^T formed ellipsoidal or spherical spores after growing on sporulation agar for 4 days. Spores were central or subterminal in position. Colonies were smooth, circular, white, entire, opaque and approx. 2 mm in diameter after 2 days at 34 °C on nutrient agar plus 10 % (w/v) NaCl. Other characteristics that differentiate strain MA-2^T

Fig. 1. Scanning electron micrograph of cells of *Halobacillus karajensis* sp. nov. MA-2^T from an exponentially growing culture. Bar, 1 µm.

Table 1. Useful characteristics that distinguish *Halobacillus karajensis* sp. nov. from other related Gram-positive endospore-forming rods

Species: 1, *H. karajensis* sp. nov.; 2, *H. halophilus*; 3, *H. litoralis*; 4, *H. trueperi*; 5, *Bacillus halophilus*; 6, *Salibacillus salexigens*; 7, *Salibacillus marismortui*. Data from Arahal *et al.* (1999), Garabito *et al.* (1997), Spring *et al.* (1996), Ventosa *et al.* (1989) and this study. Abbreviations: E, ellipsoidal; S, spherical; C, central; ST, subterminal; T, terminal; *m*-Dpm, *meso*-diaminopimelic acid; ND, not determined.

Character	1	2	3	4	5	6	7
Morphology	Rods, single or in chains	Coccoid	Rods	Rods	Rods	Rods	Rods
Pigmentation	White	Orange	Orange	Orange	None	None	Cream
Spore shape	S/E	S	E/S	E/S	E	E	E
Spore position	C/ST	C/ST	C/ST	C/ST	C	C/ST	T/ST
Sporangium swollen	+	—	ND	ND	—	+	+
Motility	—	+	+	+	+	+	+
NaCl concentration for growth (% w/v):							
Range	1–24	2–20	0.5–25.0	0.5–30.0	3–30	7–20	5–25
Optimum	10	10	10	10	15	10	10
Temperature range for growth (°C)	10–49	15–40	10–43	10–44	15–50	15–45	15–50
pH range for growth	6.0–9.6	7–9	6.0–9.5	6.0–9.5	6–8	6–11	6–9
Hydrolysis of:							
Gelatin	+	+	+	+	—	+	+
Casein	+	+	—	—	—	+	+
Aesculin	+	—	—	—	+	+	ND
Starch	+	+	—	—	—	—	—
Urea	—	—	—	—	+	—	+
Acid production from:							
D-Galactose	—	—	—	+	—	—	—
Glucose	+	—	+	+	+	+	+
Maltose	+	—	+	+	—	+	+
D-Xylose	—	—	+	—	+	—	—
Cell-wall type	Orn–D–Asp	Orn–D–Asp	Orn–D–Asp	Orn–D–Asp	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm
G + C content (mol%)	41.3	40.1–40.9	42.0	43.0	51.5	39.5	40.7

from related species are either shown in Table 1 or are included in the species description. MA-2^T did not contain diaminopimelic acid in the cell-wall peptidoglycan, but possessed Orn–D–Asp.

Whole-cell fatty acid compositions of various *Halobacillus* species are shown in Table 2. In general, the fatty acid patterns are very similar among species of this genus and differences are due mainly to varying quantities of some fatty acids. Branched fatty acids of the iso- and anteiso-types with chain lengths of 15:0, 16:0 and 17:0 are clearly dominant, as observed in many other species of aerobic, spore-forming bacilli. In contrast, the abundance of the unsaturated fatty acids 16:1 ω 7c alcohol and 16:1 ω 11c seems to be a characteristic of the genus *Halobacillus*.

The G + C content of strain MA-2^T was 41.3 mol%. DNA–DNA hybridization experiments revealed low levels of similarity between strain MA-2^T and *H. trueperi* DSM 10404^T (32.4%), *H. litoralis* DSM 10405^T (22.7%) and *H. halophilus* DSM 2266^T (16.9%).

The almost-complete 16S rDNA sequence consisting of 1529 nt was compared with sequences from members of the *Bacillaceae*. Members of the genus *Halobacillus* were

the closest phylogenetic neighbours. Binary similarity values ranged from 99.3% (*H. litoralis* DSM 10405^T, *H. trueperi* DSM 10404^T) to 97.5% (*H. halophilus* NCIMB 2269^T). Similarly high values separated the type strains of *H. litoralis* and *H. trueperi* (99.5%). Phylogenetic trees were obtained using different distance-matrix-based clustering algorithms such as neighbour-joining and maximum-likelihood, as compiled in the PHYLIP package (Felsenstein, 1993). The different algorithms gave consistent results, placing strain MA-2^T in a cluster together with *H. litoralis* and *H. trueperi* (Fig. 2).

Considering the phylogenetic, phenotypic and chemotaxonomic characteristics of the isolate, it is concluded that strain MA-2^T is part of rRNA group 1 of *Bacillus* (Ash *et al.*, 1991) and belongs to the genus *Halobacillus*. The data presented in this paper indicate that strain MA-2^T is somewhat related to *H. trueperi*. However, our strain exhibited differences from this and other known species of this genus. In contrast to the *Halobacillus* species described so far, strain MA-2^T was non-motile. It formed white or cream-coloured colonies, whereas other species of the genus produce orange-pigmented colonies.

Strain MA-2^T grew well at pH 6.0–9.6, whereas *H. halophilus*

Table 2. Cellular fatty acid composition of type strains of *Halobacillus* species

Strains: 1, *H. halophilus* DSM 2266^T; 2, *H. trueperi* DSM 10404^T; 3, *H. litoralis* DSM 10405^T; 4, *H. karajensis* sp. nov. DSM 14948^T. Strains were grown on MB medium at 28 °C for 48 h prior to analysis. Values are percentages of total fatty acids. The position of the double bond in unsaturated fatty acids is located by counting from the methyl (ω) end of the carbon chain; *cis* and *trans* isomers are respectively indicated by the suffixes *c* and *t*. Summed features represent groups of two or three fatty acids that could not be separated by GLC with the MIDI system. Summed feature 4 contained one or more of iso-C17:1 I and/or anteiso-C17:1 B. ECL, Equivalent chain length; —, not detected.

ECL	Fatty acid	1	2	3	4
13:618	iso-14:0	10.6	23.2	—	2.0
14:623	iso-15:0	7.4	6.6	16.3	11.3
14:715	anteiso-15:0	42.1	19.2	45.6	42.4
15:000	15:0	0.8	—	—	0.3
15:387	16:1 ω 7 <i>c</i> alcohol	8.8	12.2	2.6	9.7
15:627	iso-16:0	14.2	28.0	1.2	6.9
15:756	16:1 ω 11 <i>c</i>	0.6	0.7	1.3	0.9
15:998	16:0	1.0	1.0	0.9	1.1
16:388	iso-17:1 ω 10 <i>c</i>	—	—	2.5	1.1
16:478	Summed feature 4	0.9	1.2	6.8	3.3
16:631	iso-17:0	1.7	2.8	7.8	5.0
16:724	anteiso-17:0	11.6	5.1	15.0	16.0

does not grow below pH 7 or above pH 9. It formed filaments at higher pH values (9.0–9.5) and shorter and thicker bacillus cells at lower pH values. This is in contrast to the situation observed in *H. litoralis* and *H. trueperi*. In addition, strain MA-2^T could hydrolyse aesculin; this also differentiated it from other known *Halobacillus* species.

In terms of growth temperature range, salt tolerance, enzymic activities and fermentation of sugars, there were differences between strain MA-2^T and other species of the genus (Table 1). On the basis of the evidence obtained from the phenotypic data, phylogenetic characteristics and DNA–DNA hybridization, it is proposed that strain MA-2^T be placed as the type strain in a novel species of the genus *Halobacillus*, *Halobacillus karajensis* sp. nov.

Description of *Halobacillus karajensis* sp. nov.

Halobacillus karajensis (ka.ra.jen'sis. N.L. masc. adj. *kara-jensis* from the region of Karaj, Iran, where the type strain was isolated).

Cells are Gram-positive rods, 2.5–4.0 × 0.8–0.9 µm, occurring singly, in pairs or in short chains (Fig. 1). Non-motile, spherical or ellipsoidal endospores are produced in the central or subterminal position. Colonies are non-pigmented (cream or white), circular, opaque and entire. Growth occurs in 1–24% (w/v) NaCl; 10% (w/v) is optimal for growth. No growth occurs in the absence of NaCl. Growth

Fig. 2. Neighbour-joining tree showing the position of *Halobacillus karajensis* sp. nov. MA-2^T among type species of the family *Bacillaceae*. The sequence of *Paenibacillus polymyxa* DSM 36^T served as an outside reference (not shown). Bar, 5 substitutions per 100 nt.

occurs at 10–49 °C (optimum 34–38 °C). The pH range for growth is 6.0–9.6 (optimum between 7.5 and 8.5). Strictly aerobic. Catalase and oxidase are produced. Aesculin, casein, gelatin, starch and DNA are hydrolysed. Negative for hydrolysis of Tweens 80 and 20. Indole, methyl red, Voges–Proskauer and Simmons' citrate tests are negative. Urease is not produced. H₂S and phenylalanine deaminase are not produced. Acid is produced from D-glucose, D-fructose, maltose, mannitol, mannose and raffinose; D-arabinose, D-galactose, D-xylose and sucrose are not hydrolysed. The following compounds are utilized as sole carbon and energy sources: D-glucose, glucose 6-phosphate, D-cellobiose, starch, dextrin, maltose, D-melibiose, *myo*-inositol, acetate, succinate and propionate. No growth occurs on D-sorbitol, inulin, salicin or citrate. Susceptible to ampicillin (10 µg), amikacin (30 µg), cephalotin (30 µg), chloramphenicol (30 µg), nalidixic acid (30 µg), penicillin G (10 U), rifampicin (5 µg) and tetracycline (30 µg); resistant to erythromycin (15 µg) and streptomycin (10 µg); semi-susceptible to novobiocin (5 µg). Other phenotypic features of this strain are shown in Table 1. The cell wall contains peptidoglycan of the Orn–Asp type. The major respiratory lipoquinone is MK-7.

The type strain, MA-2^T (= DSM 14948^T = LMG 21515^T), was isolated from saline soil from Karaj, Iran. The G + C content of the DNA of this strain is 41.3 mol%.

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