Bacillus halochares sp. nov., a halophilic bacterium isolated from a solar saltern

A. Pappa,¹ C. Sánchez-Porro,² P. Lazoura,¹ A. Kallimanis,¹ A. Perisynakis,¹ A. Ventosa,² C. Drinas¹ and A. I. Koukkou¹

Correspondence
A. I. Koukkou
akukku@cc.uoi.gr

¹Sector of Organic Chemistry and Biochemistry, Department of Chemistry, University of Ioannina, 45110 Ioannina, Greece

²Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

A novel halophilic bacterium, designated strain MSS4^T, was isolated from the solar salterns of Mesolongi, Greece. The micro-organism, a motile, Gram-stain-positive, aerobic rod, proliferated at salinities of 1.0–4.0 M NaCl, with optimal growth at 2.5 M NaCl. Endospores were not observed. Strain MSS4^T showed optimal growth at 37 °C and pH 8.0. The G+C content of its DNA was 47.2 mol%. The polar lipid pattern of strain MSS4^T consisted of diphosphatidylglycerol, phosphatidylglycerol, phosphatidic acid and phosphatidylethanolamine. It possessed anteiso-C_{15:0}, C_{18:0}, C_{16:0} and anteiso-C_{17:0} as the major fatty acids (altogether representing 84.7 % of the total). The predominant isoprenoid quinone was MK-7. The cell-wall peptidoglycan contained meso-diaminopimelic acid. 16S rRNA gene sequence analysis showed that the new isolate has 96.1 % similarity to *Bacillus qingdaonensis* CM1^T and *Bacillus aidingensis* 17-5^T, 95.5 % to *Bacillus salarius* BH169^T and lower similarity to other *Bacillus* species. These results justify the assignment of strain MSS4^T to a novel species within the genus *Bacillus*, for which the name *Bacillus halochares* sp. nov. is proposed. The type strain is MSS4^T (=LMG 24571^T =DSM 21373^T).

Life exists over the whole range of salt concentrations encountered in natural habitats, from freshwater environments to hypersaline lakes (Oren, 2002a). Halophiles, salt-loving organisms that inhabit hypersaline environments, include mainly prokaryotic and eukaryotic micro-organisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts (Oren, 2002a; Ventosa, 2006). The heterotrophic conditions at near-saturating salt concentrations (3–5 M NaCl) usually favour extremely halophilic haloarchaea (Oren, 2002b). The decreased microbial diversity in such environments has been studied with both culture-dependent and molecular methods (Burns *et al.*, 2004). Indications that extremely halophilic micro-organisms other than haloarchaea may thrive in environments with salt concentration close to saturation have been provided recently (Antón *et al.*, 2000, 2002; Eder *et al.*, 2002; Maturrano *et al.*, 2006a, b; Kharroub *et al.*, 2006; Moreno *et al.*, 2009). In an attempt to evaluate the diversity of halophilic micro-organisms in Greek solar salterns, the Mesolongi thalassohaline salt works were chosen.

The Mesolongi salt works are located west of the town of Mesolongi (western central Greece). It is the biggest saltern

in Greece, occupying an area of 1240 ha. Part of the saltern area has been used for salt production for several centuries, whereas a considerable expansion took place in the 1960s. The wetland type could be summarized as a semi-artificial saline ecosystem with an ample gradient of characteristics starting from a regular saline wetland (pre-basins) up to extreme hypersaline meadows (crystallizers). Salt is produced mainly during summer, from July to early October, by solar evaporation. Sample collection took place from soil as well as from low- and high-salinity concentrator pans (salinity 20 and 30 %, respectively) in August. Cultivation-dependent methods in combination with molecular techniques (16S rRNA gene sequence analysis) revealed that the higher salinity concentrator pan favoured growth of members of the haloarchaea (*Haloterrigena*, *Haloferax* and *Halobacterium*; unpublished results). Here, we report the isolation of a halophilic bacterium belonging to the genus *Bacillus* and its taxonomic characterization using a polyphasic approach.

Strain MSS4^T was isolated from a sample collected from soil of a crystallizer pond in August 2004. A soil sample of 20 g was inoculated in liquid medium SW 4 M NaCl and incubated for 7 days at 37 °C. Medium SW 4 M NaCl contained (w/v) 23.4 % NaCl, 3.9 % MgCl₂·6H₂O, 6.1 % MgSO₄·7H₂O, 0.1 % anhydrous CaCl₂, 0.6 % KCl, 0.02 % NaHCO₃ and 0.5 % yeast extract (Lab M); MgCl₂ and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain MSS4^T is AM982516.

CaCl₂ were prepared separately and added to the solution after sterilization. The pH was adjusted to 7.2 ± 0.2 with 1 M NaOH prior to autoclaving. Dilutions of 100 µl of the cultivation were plated on SW 4 M NaCl agar plates. Solid media were prepared by adding 2 % (w/v) agar (Lab M). Colonies were picked and subcultured repeatedly, so as to establish pure single-colony isolates. Cells of strain MSS4^T were grown in 100 ml liquid medium SW at five salinities, 0.5, 1.0, 1.5, 2.5 and 4.0 M NaCl, in 250 ml Erlenmeyer flasks with shaking (150–200 r.p.m.) at 37 °C. Growth was monitored by measuring the OD₆₀₀ throughout the growth curve until stationary phase was reached.

Cell morphology and motility were examined using a microscope equipped with phase-contrast optics. Biochemical tests were performed as outlined elsewhere (Smibert & Krieg, 1994; Cappuccino & Sherman, 1996; Sánchez-Porro *et al.*, 2009) using cultures grown at 37 °C in the appropriate medium. *Bacillus qingdaonensis* CIP 109642^T, *Bacillus aidingensis* DSM 18341^T and *Bacillus salarius* DSM 16461^T were used as reference strains and their features were compared with those of strain MSS4^T under the same laboratory conditions.

Template DNA was extracted from colonies of strain MSS4^T using the Nucleospin Tissue kit (Macherey-Nagel) according to the manufacturer's recommendations. The 16S rRNA gene was amplified from genomic DNA using the primers 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (5'-AAGGAGGTGATCCAGCC-3') respectively corresponding to positions 9–27 and 1525–1542 of the 16S rRNA gene sequence of *Escherichia coli* (Stackebrandt & Liesack, 1993). Hot-start PCR at 95 °C for 5 min was followed by 30 cycles of 94 °C for 1 min, 60 °C for 2 min and 72 °C for 3 min, with a final chain-elongation step at 72 °C for 10 min. The amplified DNA fragment of 1.5 kb was purified using the Nucleospin Extract II kit (Macherey-Nagel) following electrophoresis on a 0.7 % agarose gel and sequenced using primers 16SF and 16SR by Macrogen (Seoul, Korea).

16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig *et al.*, 2004). The 16S rRNA gene sequence was aligned with published sequences of

closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were constructed using three different methods, the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971) and neighbour-joining (Saitou & Nei, 1987) algorithms integrated in the ARB software for phylogenetic inference. 16S rRNA gene sequences used for phylogenetic comparisons were obtained from GenBank; strain designations and accession numbers are shown in Fig. 1.

An almost-complete 16S rRNA gene sequence (1426 bp) of strain MSS4^T was obtained and used for initial BLAST searches in GenBank and phylogenetic analysis. Identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon server (<http://www.eztaxon.org/>; Chun *et al.*, 2007). Phylogenetic analysis, based on the maximum-parsimony algorithm, revealed that strain MSS4^T formed a phyletic group with *Bacillus qingdaonensis* CM1^T and *Bacillus aidingensis* 17-5^T (96.1 % similarity) as well as *Bacillus salarius* BH169^T (95.5 % similarity) (Fig. 1). The 16S rRNA gene sequence similarity between strain MSS4^T and members of other *Bacillus* species was ≤91.5 %. Neighbour-joining and maximum-likelihood methods resulted in highly similar tree topologies, and only the maximum-parsimony results are shown. Based on the sequence divergence, it was evident that strain MSS4^T constituted a different taxon separated from other *Bacillus* species.

Cells of isolate MSS4^T were Gram-stain-positive, aerobic, motile rods. Endospores were not observed. Growth was observed at 1.0–4.0 M NaCl, with an optimum at 2.5 M. No growth was observed on SW agar without NaCl. The temperature range was 26–45 °C, with optimum growth at 37 °C. The pH range was 6.0–9.0, with optimum growth at pH 8.0. Standard bacteriological and biochemical methods described above were applied for phenotypic characterization. All media used were supplemented with 2.5 M NaCl. Catalase and oxidase tests were positive, whereas tests for urease, amylase and gelatinase activities and nitrate reduction as well as H₂S production were negative. Other

Fig. 1. Maximum-parsimony phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of strain MSS4^T and the type strains of related taxa. GenBank accession numbers are shown in parentheses. *Brevibacillus brevis* JCM 2503^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

phenotypic features are included in the species description and in Table 1.

Phospholipid and fatty acid (FAME) analysis were performed as described by Kallimanis *et al.* (2007) and references therein. Cells were cultured on SW medium at pH 7.2, 37 °C for 24 h (OD₆₀₀ 0.7). Analysis of quinones and cell-wall peptidoglycan was carried out by the Identification Service and Dr B. J. Tindall, DSMZ, Braunschweig, Germany. The major fatty acids were anteiso-C_{15:0} (35.5%), C_{18:0} (21.9%), C_{16:0} (16.9%) and anteiso-C_{17:0} (10.4%). The major phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidic acid and phosphatidylethanolamine (35.6 ± 4.1, 35.0 ± 3.9, 4.9 ± 1.2 and 4.8 ± 0.9% respectively). The major isoprenoid quinone of strain MSS4^T was menaquinone MK-7 and the cell-wall type was based on *meso*-diaminopimelic acid. The respiratory lipoquinone and peptidoglycan type

of the cell wall of strain MSS4^T were typical of those found in members of the genus *Bacillus* (Priest *et al.*, 1988; Heyrman *et al.*, 2004, 2005; Wieser *et al.*, 2005; Lim *et al.*, 2006).

The G+C content of the genomic DNA was determined from the midpoint value of the thermal denaturation profile (Marmur & Doty, 1962) using the equation of Owen & Hill (1979), as described previously in detail (Ventosa *et al.*, 1999). The G+C content of the DNA of strain MSS4^T was 47.2 mol%. This value is close to those reported for *B. qingdaonensis* (Wang *et al.*, 2007) and *B. ainingensis* (Xue *et al.*, 2008), but quite different from that of *B. salarius* (Lim *et al.*, 2006) (Table 1).

Overall, our data show that strain MSS4^T is placed phylogenetically within the radiation of the genus *Bacillus*, but the 16S rRNA gene sequence similarity with

Table 1. Characteristics that distinguish strain MSS4^T from type strains of related species of the genus *Bacillus*

Strains: 1, MSS4^T; 2, *B. qingdaonensis* CIP 109642^T (Wang *et al.*, 2007); 3, *B. ainingensis* DSM 18341^T (Xue *et al.*, 2008); 4, *B. salarius* DSM 16461^T (Lim *et al.*, 2006). Data for reference type strains were taken from the publications listed unless indicated.

Characteristic	1	2	3	4
Cell width (µm)	0.5–0.7	0.3–0.4	0.2–0.4	0.5–0.7
Cell length (µm)	1.7–3.5	1.8–3.2	1.3–2.5	1.3–1.9
Pigmentation*	C	C	C–WH	C–WH to Y
Endospore formation	–	–	+	+
Spore shape†	NA	NA	E	ST
Oxidase	+	–	–	+
Motility	+	–	+	–
NaCl concentration for growth (% w/v)				
Range	6–23	2.5–20	8–33	3–20
Optimum	15	12	12	10–12
Temperature for growth (°C)				
Range	26–45	25–45	22–44	15–40
Optimum	37	37	37	30
pH for growth				
Range	6.0–9.0	6.5–10.5	6.0–9.5	6.8–9.5
Optimum	8.0	9	7.2	8.0
Acid production from:				
D-Arabinose	–	–‡	+‡	+§
Lactose	–	+	–	+§
Maltose	–	–‡	+§	+
Sucrose	+	+	–	–‡
D-Xylose	+	+	–	+
Nitrate reduction	–	+§	+	–§
Hydrolysis of gelatin	–	–	+§	–‡
Urease activity	–	+§	–	–
DNA G+C content (mol%)	47.2	48	48.1	43

*C, Cream; WH, white; Y, yellow.

†E, Ellipsoidal; ST, spherical, terminal; NA, not applicable.

‡Data from this study.

§Data from this study; a different reaction was reported in the original description.

the type strains of the closest *Bacillus* species suggests that it could represent a different species; it also shows several phenotypic differences from the most closely related species (Table 1), justifying the proposal of a novel species, for which we suggest the name *Bacillus halochares* sp. nov.

Description of *Bacillus halochares* sp. nov.

Bacillus halochares [ha.lo.cha'res. Gr. masc. n. *hals*, *halos* salt; N.L. part. adj. *charēs* from Gr. v. *chairō* to rejoice at, to delight in; N.L. part. adj. *halocharēs* finding pleasure in salt (salty environments)].

Cells are Gram-stain-positive, motile rods, $0.5\text{--}0.7 \times 1.7\text{--}3.5\text{ }\mu\text{m}$. Endospores are not observed. Colonies are circular, entire, cream and approximately 1 mm in diameter after cultivation on SW medium at $37\text{ }^{\circ}\text{C}$ for 24 h. Strictly aerobic. Moderately halophilic. Growth occurs at NaCl concentrations of 1.0–4.0 M (optimum 2.5 M NaCl). No growth occurs in the absence of NaCl. Temperature range for growth is $26\text{--}45\text{ }^{\circ}\text{C}$ (optimum $37\text{ }^{\circ}\text{C}$). Grows at pH 6.0–9.0 (optimum pH 8.0). Catalase- and oxidase-positive, urease-negative. Aesculin is hydrolysed but not amylase, gelatin, casein, Tween 80 or DNA. Nitrate is not reduced. H_2S is not produced. Acid is produced from D-fructose, D-galactose, D-glucose, glycerol, D-mannitol, D-mannose, sucrose, trehalose and D-xylose but not from D-arabinose, lactose, maltose, raffinose or sorbitol. The following compounds are utilized as sole carbon and energy sources: starch, cellobiose, lactose, maltose, ribose, sucrose, glycerol, D-mannitol and acetate. The following compounds are not utilized as sole carbon and energy sources: D-arabinose, D-fructose, D-fucose, D-galactose, D-glucose, D-mannose, melibiose, raffinose, trehalose, D-xylose, butanol, dulcitol, ethanol, *myo*-inositol, propanol, D-sorbitol, xylitol, methanol, benzoate, citrate, formate, fumarate, propionate, succinate and valerate. The following compounds are not used as sole carbon, nitrogen and energy sources: L-arginine, aspartic acid, L-cysteine, L-phenylalanine, glutamic acid, L-methionine, L-ornithine, L-serine and tryptophan. L-Alanine is used as a sole source of carbon, nitrogen and energy. The major menaquinone is MK-7. Major fatty acids are anteiso- $\text{C}_{15:0}$, $\text{C}_{18:0}$, $\text{C}_{16:0}$ and anteiso- $\text{C}_{17:0}$. The major phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidic acid and phosphatidylethanolamine. The diagnostic diamino acid in the cell-wall peptidoglycan is *meso*-diaminopimelic acid. The DNA G+C content of the type strain is 47.2 mol% (T_m).

The type strain, MSS4^T (=LMG 24571^T =DSM 21373^T), was isolated from soil of a crystallizer pond of the solar salterns at Mesolongi (Greece).

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