***Bacillus marismortui* sp. nov., a new moderately halophilic species from the Dead Sea**

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A group of 91 moderately halophilic, Gram-positive, rod-shaped strains were isolated from enrichments prepared from Dead Sea water samples collected 57 years ago. These strains were examined for 117 morphological, physiological, biochemical, nutritional and antibiotic susceptibility characteristics. All strains formed endospores and were motile, strictly aerobic and positive for catalase and oxidase. They grew in media containing 5–25% (w/v) total salts, showing optimal growth at 10% (w/v). Eighteen strains were chosen as representative isolates and were studied in more detail. All these strains had meso-diaminopimelic acid in the cell wall and a DNA G+C content of 39.0–42.8 mol%; they constitute a group with levels of DNA–DNA similarity of 70–100%. The sequences of the 16S rRNA genes of three representative strains (strains 123^T, 557 and 832) were almost identical (99.9%), and placed the strains in the low G+C content Gram-positive bacteria. On the basis of their features, these isolates should be regarded as members of a new species of the genus *Bacillus*, for which the name *Bacillus marismortui* sp. nov. is proposed. The type strain is strain 123^T (= DSM 12325^T = ATCC 700626^T = CIP 105609^T = CECT 5066^T).

Keywords: *Bacillus marismortui*, taxonomy, DNA–DNA similarity, cell wall type, 16S rRNA sequence

INTRODUCTION

Studies on the microbiology of the Dead Sea have demonstrated the presence of a variety of micro-organisms adapted to the high salt concentration in the lake: protozoa, unicellular algae, cyanobacteria, and aerobic and anaerobic chemo-organotrophic prokaryotes (Javor, 1989; Ventosa *et al.*, 1999). Among the aerobic prokaryotes, the majority of the isolates from the Dead Sea belong to the domain *Archaea* and are placed in the family *Halobacteriaceae* (Oren, 1993). On the other hand, in the domain *Bacteria* there have been a few reports about Gram-negative, moderately halophilic species (Oren, 1988). In contrast, there has been no description of aerobic, Gram-positive, moderately halophilic bacteria.

In this study, a group of 91 Gram-positive, moderately halophilic, endospore-forming rods was isolated from enrichments obtained from Dead Sea water samples used by Volcani in 1936 and kept by him for more than 50 years in closed bottles. In a recent study, it has been shown that extremely halophilic archaea belonging to the genera *Halobacterium*, *Haloarcula* and *Haloferax* could still be isolated from these samples (Arahal *et al.*, 1996). The strains isolated in the present study were characterized phenotypically, as well as with respect to their DNA base composition, levels of DNA–DNA similarity and cell wall composition. Three representative strains were selected for phylogenetic analysis of their 16S rRNA gene sequences. Our results suggest that these strains may represent a new species of the genus *Bacillus*, for which the name *Bacillus marismortui* sp. nov. is proposed.

METHODS

Strain isolation and culture conditions. The source of our isolates was seven enrichments obtained from water samples taken in 1936 by B. E. Volcani at surface level in the

Abbreviations: S_j, Jaccard coefficient; UPGMA, unweighted pair group method with averages.

The EMBL accession number for the 16S rRNA gene sequence of strain 123^T is AJ009793.

§Deceased 6 February 1999.

Table 1. DNA G + C contents and levels of DNA–DNA relatedness for some strains of *Bacillus marismortui* and related genera

DNA similarities equal to or greater than 70 % are given in bold.

Source of unlabelled DNA*	G + C content (mol %)	Homology (%) with ³ H-labelled DNA from strain:		
		123 ^T	557	832
<i>Bacillus marismortui</i> strains				
104	40.9	91	96	99
114	40.1	91	88	86
116	42.4	97	83	95
120	42.0	91	91	88
123 ^T	40.7	100	80	85
135	42.4	95	93	83
511	40.1	98	93	74
517	39.3	89	96	79
526	40.5	95	99	78
532	40.1	95	92	97
539	40.1	98	100	91
544	39.9	97	98	97
550	40.1	90	84	85
557	42.8	87	75	100
720	40.7	92	96	79
811	41.7	93	86	93
821	42.2	97	93	84
832	39.0	72	100	70
<i>Bacillus alcalophilus</i> ATCC 27647 ^T	47.0†	0	0	10
<i>Bacillus dipsosauri</i> DSM 11125 ^T	40.1	11	8	10
<i>Bacillus globisporus</i> ATCC 23301 ^T	49.0†	27	7	12
<i>Bacillus halophilus</i> ATCC 49085 ^T	50.8	31	20	25
<i>Bacillus marinus</i> ATCC 29841 ^T	37.6†	0	0	6
<i>Bacillus salexigens</i> DSM 11483 ^T	39.5†	41	41	45
<i>Halobacillus halophilus</i> DSM 2266 ^T	40.8†	15	0	18
<i>Halobacillus litoralis</i> DSM 10405 ^T	42.0†	19	16	18
<i>Halobacillus trueperi</i> DSM 10404 ^T	43.0†	34	44	45
<i>Paenibacillus polymyxa</i> DSM 36 ^T	44.3†	19	27	18
<i>Virgibacillus pantothenicus</i> DSM 26 ^T	36.9†	33	28	30

* ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

† Data from Garabito *et al.* (1997), Fahmy *et al.* (1985) and Spring *et al.* (1996).

Northern basin of the Dead Sea by sampling at different locations close to the mouth of the Jordan river (Volcani, 1936, 1940). The enrichments, prepared with Dead Sea water plus 1.0 % (w/v) peptone, were kept in closed 500 ml bottles under sterile conditions and stored in the dark in a dry place at 18–20 °C. These enrichments remained unopened over the entire storage period. Inocula were removed in 1993 from the bottles under sterile conditions. Several isolation media were used with various total salt concentrations and compositions. SW-10 medium (Nieto *et al.*, 1989) contained a final salt concentration of about 10 % (w/v) and 0.5 % (w/v) yeast extract (Difco). HM medium (Ventosa *et al.*, 1982) contained a balanced mixture of salts, giving a final concentration of about 18 % (w/v), and was supplemented with 1 % (w/v) yeast extract (Difco), 0.5 % (w/v) proteose

peptone no. 3 (Difco) and 0.1 % (w/v) glucose. The last medium used, designated M4, contained (w/v): 10.3 % NaCl, 1.8 % MgSO₄·7H₂O, 0.025 % CaCl₂·2H₂O, 0.019 KCl and 0.05 % peptone (Difco). The pH was always adjusted to 7.2. Cultures were incubated at 37 °C in an orbital shaker (New Brunswick Scientific) at 200 strokes min⁻¹. For the phenotypic characterization studies, the following reference strains were used: *Bacillus alcalophilus* ATCC 27647^T; *Bacillus badius* ATCC 14574^T; *Bacillus circulans* ATCC 4513^T; *Bacillus coagulans* ATCC 7050^T; *Bacillus globisporus* ATCC 23301^T; *Bacillus halophilus* ATCC 49085^T; *Bacillus insolitus* ATCC 23299^T; *Bacillus licheniformis* ATCC 14580^T; *Bacillus marinus* ATCC 29841^T; *Bacillus pallidus* DSM 3670^T; *Bacillus pumilus* ATCC 7061^T; *Bacillus salexigens* DSM 11483^T; *Bacillus smithii* DSM

Table 2. Phenotypic characteristics of the 91 isolates of *Bacillus marismortui* and the type strain of this species

All strains were strictly aerobic, grew in the presence of 5–20% (w/v) salts, at pH 6.5–8.0 and at 15–50 °C. They were positive for catalase and negative for indole and phenylalanine deaminase. They did not produce acid from D-arabinose, D-galactose or D-xylose and they did not hydrolyse Tween 80. They utilized maltose, D-mannose and pyruvate but lactate was not utilized. They were susceptible to erythromycin (15 µg) and resistant to nalidixic acid (30 µg) and neomycin (30 µg).

Character	No. of strains positive	<i>Bacillus marismortui</i> strain 123 ^T
Terminal spores	67	+
Subterminal spores	24	+
Sporangium swollen	78	+
Growth in salts at (% w/v):		
0.9	13	—
3	44	+
25	45	—
Growth at 55 °C	3	—
Growth at pH:		
5.5	6	—
6.0	87	+
8.5	87	+
9.0	77	—
9.5	39	—
Hydrolysis of:		
Casein	58	+
Gelatin	87	+
Starch	3	—
DNase	87	+
Phosphatase	83	+
Acid production from:		
D-Fructose	89	+
D-Glucose	89	+
Glycerol	88	+
Lactose	4	—
Maltose	88	+
D-Mannitol	4	—
Sucrose	5	—
D-Trehalose	7	—
Methyl red	79	+
Voges-Proskauer	6	—
Nitrate reduction	89	+
Nitrite reduction	31	—
Citrate utilization	18	—
H ₂ S production	22	+
Arginine dehydrolase	5	—
Urease	80	+
Utilization of:		
D-Arabinose	24	—
D-Cellobiose	22	+
D-Fructose	85	+
D-Galactose	17	+
D-Glucose	18	+
Inulin	42	+

Table 2 (cont.)

Character	No. of strains positive	<i>Bacillus marismortui</i> strain 123 ^T
Lactose	14	+
D-Raffinose	87	+
D-Rhamnose	62	+
D-Ribose	5	—
Salicin	27	—
Starch	31	—
Sucrose	7	—
D-Trehalose	3	—
D-Xylose	4	—
Glycerol	4	—
myo-Inositol	18	—
D-Mannitol	59	—
D-Sorbitol	15	—
Acetate	21	—
Butyrate	10	—
Citrate	19	—
Fumarate	22	—
L-Glutamate	30	—
Hippurate	23	—
Malate	14	—
Propionate	10	+
Succinate	68	+
L-Alanine	16	—
DL-Arginine	29	—
L-Asparagine	89	+
L-Aspartic acid	8	—
L-Glutamine	4	—
L-Isoleucine	15	—
L-Leucine	14	—
L-Ornithine	57	+
L-Phenylalanine	19	—
L-Proline	17	—
L-Serine	10	—
L-Threonine	43	+
Susceptibility to:		
Chloramphenicol (30 µg)	22	—
Novobiocin (30 µg)	89	+
Penicillin G (10 U)	3	—
Rifampin (5 µg)	90	+
Streptomycin (10 µg)	2	—
Tetracycline (30 µg)	5	—

4216^T; *Bacillus subtilis* ATCC 6051^T; and *Virgibacillus pantothenicus* ATCC 14576^T. For the DNA-DNA hybridization studies, the reference strains shown in Table 1 were used. The recommended media and culture conditions were used to grow these organisms.

Phenotypic characterization. Tests for 117 characteristics, including morphological, cultural, physiological, biochemical, nutritional and antibiotic susceptibility features, were carried out (Table 2). All test procedures have been described previously (García *et al.*, 1987; Quesada *et al.*, 1984; Ventosa

Table 3. Bacterial strains and accession numbers of sequences used in 16S rRNA analysis

Species	Strain*	EMBL accession no.
<i>Aneurinibacillus aneurinolyticus</i>	NCIMB 10056 ^T	X60645
<i>Bacillus alcalophilus</i>	ATCC 27647 ^T	X60603
<i>Bacillus badius</i>	ATCC 14574 ^T	D78310
<i>Bacillus cereus</i>	ATCC 14579 ^T	X55060
<i>Bacillus circulans</i>	NCDO 1775 ^T	X60613
<i>Bacillus coagulans</i>	NCDO 1761 ^T	X60614
<i>Bacillus cohnii</i>	DSM 6307 ^T	X76437
<i>Bacillus dipsosauri</i>	NCFB 3027 ^T	X82436
<i>Bacillus globisporus</i>	NCIMB 11434 ^T	X60644
<i>Bacillus insolitus</i>	DSM 5 ^T	X60642
<i>Bacillus licheniformis</i>	NCDO 1772 ^T	X60623
<i>Bacillus megaterium</i>	ATCC 14581 ^T	X60629
<i>Bacillus pallidus</i>	DSM 3670 ^T	Z26930
<i>Bacillus pasteurii</i>	NCIMB 8841 ^T	X60631
<i>Bacillus pumilus</i>	NCDO 1776 ^T	X60637
<i>Bacillus salexigens</i>	ATCC 700290 ^T	Y11603
<i>Bacillus smithii</i>	DSM 4216 ^T	Z26935
<i>Bacillus stearothermophilus</i>	ATCC 12980 ^T	X60640
<i>Bacillus subtilis</i>	NCDO 1769 ^T	X60646
<i>Brevibacillus brevis</i>	NCIMB 9372 ^T	X60612
<i>Brevibacillus laterosporus</i>	NCDO 1763 ^T	X60620
<i>Halobacillus halophilus</i>	ATCC 35676 ^T	X62174
<i>Halobacillus litoralis</i>	DSM 10405 ^T	X94558
<i>Marinococcus albus</i>	DSM 20748 ^T	X90834
<i>Paenibacillus amylolyticus</i>	NCIMB 8144 ^T	X60606
<i>Paenibacillus larvae</i>	ATCC 9545 ^T	X60619
<i>Paenibacillus macerans</i>	ATCC 8244 ^T	X60624
<i>Paenibacillus polymyxa</i>	ATCC 842 ^T	X60632
<i>Sporosarcina ureae</i>	NCIMB 9251 ^T	X62174
<i>Virgibacillus pantothenicus</i>	ATCC 14576 ^T	X60627

* NCIMB, National Collection of Industrial and Marine Bacteria; ATCC, American Type Culture Collection; NCDO, National Collection of Dairy Organisms; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; NCFB, National Collection of Food Bacteria.

et al., 1982). Unless otherwise indicated, the tests were carried out in media containing 10% (w/v) salts, at pH 7.5 and incubated at 37 °C.

Numerical analysis. Of the 117 phenotypic characteristics studied, 106 were differential features and used for numerical analysis. Positive and negative results were coded as 1 and 0, respectively; non-compatible or missing data were coded as 9. Strain similarities were estimated with the Jaccard coefficient (S_j) (Jaccard, 1908) and cluster analysis was carried out by the unweighted pair group method with averages (UPGMA) (Sneath & Johnson, 1972). Test error was estimated by examining 10 strains in duplicate (Sneath & Johnson, 1972). Cophenetic correlation was also evaluated (Sneath & Sokal, 1973). These computations were done using the NTSYS-pc version 1.80 program of Rohlf (1993) on a PC computer.

DNA extraction and purification. Cells from 18 representative strains selected for this study and 13 reference strains were harvested, washed, suspended in 0.15 M NaCl/0.1 M EDTA

buffer (pH 8.0) (5 g wet weight in 50 ml buffer), and lysed with lysozyme (approx. 10 mg) at 37 °C and with SDS at a final concentration of 2% (w/v) at 60 °C. The DNA was extracted and purified by the method of Marmur (1961). The purity was assessed from the A_{260}/A_{280} and A_{230}/A_{260} absorbance ratios (Johnson, 1994).

DNA base composition. The G + C content of the DNA was determined from the midpoint value of the thermal denaturation profile (Marmur & Doty, 1962) obtained with a model UV-Vis 551S spectrophotometer (Perkin-Elmer) at 260 nm; this instrument was programmed for temperature increases of 1.0 °C min⁻¹. The G + C content was calculated from the thermal denaturation temperature with the equation of Owen & Hill (1979). The G + C content of reference DNA from *Escherichia coli* NCTC 9001 was taken to be 51 mol% (Owen & Pitcher, 1985).

Preparation of ³H-labelled DNA and DNA-DNA hybridization experiments. DNA was radioactively labelled by the multiprime system with a commercial kit (RPN 1601Y;

Fig. 1. Simplified dendrogram showing the clustering of the strains into a phenon based on S_1 coefficient and unweighted mean linkage clustering (UPGMA) for 91 moderately halophilic Gram-positive endospore-forming rods and 15 species belonging to *Bacillus* and *Virgibacillus*.

Amersham), using [$1',2',5\text{'}$ - ^3H]dCTP (Amersham). The mean specific activity obtained with this procedure was 8.8×10^6 c.p.m. ($\mu\text{g DNA}^{-1}$). The labelled DNA was denatured before hybridization by heating at 100°C for 5 min and then placed on ice. DNA-DNA similarity was studied by the competition procedure described by Johnson (1994). Competitor DNAs were sonicated (Braun Melsungen) at 50 W for two 15 s time intervals. Membrane filters (HAHY; Millipore) containing reference DNA (approx. $25 \mu\text{g cm}^{-2}$) were placed in 5 ml screw-cap vials which contained the labelled, sheared, denatured DNA and the denatured and sheared competitor DNA. The ratio of the concentration of competitor DNA to the concentration of labelled DNA was at least 150:1. The final reaction concentrations were $2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M sodium chloride plus 0.015 M sodium citrate) and 30% formamide, and the final volume was 140 μl . The hybridization experiments were carried out under optimal conditions, with temperatures ranging between 45.3 and 47.5°C , which is within the limits of validity for the filter method (De Ley & Tijtgaat, 1970). The vials were shaken slightly for 18 h in a water bath (Grant Instruments); these procedures were done in triplicate. After hybridization, the filters were measured with a liquid scintillation counter (Beckman Instruments) and the percentage similarity was calculated as described by Johnson (1994). At least two independent determinations were carried out for each experiment and the mean values are reported here.

Determination of isomers of diaminopimelic acid. The isomers of diaminopimelic acid in the cell walls of the 18 representative strains were detected by the method of Stanek & Roberts (1974). Dried cells (approx. 10 mg) were hydrolysed with 1 ml 10 M HCl at 100°C for 20 h in a screw-cap tube, and 1 ml filtered hydrolysate was subjected to TLC on cellulose thin-layer (Merck) in parallel with a reference amino acid mixture. For separation, methanol/distilled water/10 M HCl/pyridine (80:26:4:10 by vol.) was used as the solvent. Then the chromatogram was dried at room temperature, sprayed with a 5% ninhydrin/butanol solution and heated at 100°C for 3 min.

Phylogenetic analysis. Isolation of genomic DNA from strains 123^T, 557 and 832, which were used as representatives of our isolates, *in vitro* amplification of the 16S rRNA gene, and direct sequencing of the PCR-amplified 16S rDNA fragments were carried out using previously described methods (Springer *et al.*, 1993).

Data analyses, including more than 4000 16S rRNA sequences available from rRNA sequence databases (Maidak *et al.*, 1996; Van de Peer *et al.*, 1994), were performed as described previously (Arahal *et al.*, 1996). The most closely related sequences (Table 3) were chosen for constructing a similarity matrix; only those positions at which there were data for 90% of the 16S rRNA sequences of the strains were used. The similarity matrix was corrected

for multiple base changes at single positions by the method of Saitou & Nei (1987).

RESULTS

Reproducibility of phenotypic characters

The inclusion of the pairs of randomly chosen duplicate strains in the analysis enabled experimental test error to be calculated. The mean probability of an erroneous result was 4.0%. A small number of tests was responsible for most of the test error. These tests were hydrolysis of casein, acid production from arabinose and D-mannitol, utilization of citrate, sucrose, D-mannitol and butyrate as sole source of carbon and

energy, and utilization of L-alanine and DL-arginine as sole source of carbon, nitrogen and energy.

Numerical analysis

The results of the numerical study of the strains grouped by means of the S_j coefficient and UPGMA clustering yielded the dendrogram shown in Fig. 1. The cophenetic correlation was 0.879.

In the dendrogram obtained, all the isolates grouped in a single phenon at a 58% similarity level but none of the reference strains were included in that phenon. Table 2 shows the characteristics which differentiate the 91 isolates studied from one another. Other

Fig. 2. Unrooted phylogenetic tree derived from the analysis of the 16S rRNA sequences of *Bacillus marismortui* strains 123^T, 557 and 832, some *Bacillus* species and other related species belonging to the Gram-positive bacteria with low DNA G+C content branch.

phenotypic characteristics are included below in the species description.

DNA base composition

The G + C contents of the DNAs of 18 strains chosen as representative isolates were 39.0–42.8 mol % (Table 1).

DNA–DNA hybridization

The results of the DNA–DNA hybridization experiments are shown in Table 1. The levels of relatedness of labelled DNAs from three representative strains to DNAs from the other isolates were 70–100 %. On the other hand, low levels of relatedness (0–45 %) were found between the three reference strains and the other strains belonging to related species used for comparison.

Determination of diaminopimelic acid in the cell wall

The cell walls of 18 representative strains contained *meso*-diaminopimelic acid in the peptidoglycan.

Phylogeny

The almost complete 16S rRNA sequences (about 1560 bases) of strains 123^T, 557 and 832 were determined. These sequences were almost identical to each other (99.9 %). However, they did not show high similarity with other sequences available from the database of other bacterial species. The highest levels of similarity were with the 16S rRNA sequence of *V. pantothenicus* (97.8 %), *B. salexigens* (96.6 %), *B. circulans* (95.9 %), *Halobacillus litoralis* (95.7 %) and *B. halophilus* (94.6 %).

The phylogenetic tree in Fig. 2 shows the relationship between our isolates and some representative *Bacillus* species and other related micro-organisms. The accession numbers for the 16S rRNA sequences of these reference strains are shown in Table 3. *B. marismortui* and *B. salexigens* are on the same phylogenetic branch, together with *V. pantothenicus* and close to *H. litoralis*, *Halobacillus halophilus*, *Marinococcus albus* and *Bacillus dipsosauri*.

DISCUSSION

All 91 Dead Sea isolates studied were grouped in a single phenon, indicating that they have very similar phenotypic characteristics (Table 2). On the other hand, these strains are quite different from the moderately halophilic Gram-positive rods described previously (Fendrich *et al.*, 1990; Spring *et al.*, 1996; Ventosa *et al.*, 1989).

All strains are Gram-positive, motile, endospore-forming rods and strictly aerobic. On the basis of their

phenotypic features, cell wall peptidoglycan (*meso*-diaminopimelic acid type) and DNA G + C contents, these strains can be considered as members of the genus *Bacillus sensu lato* (Claus & Berkeley, 1986). Their salt requirements indicate that they should be included in the group of moderately halophilic bacteria, defined as those organisms able to grow optimally in media containing 5–15 % (w/v) salts (Kushner & Kamekura, 1988).

Very recently, Heyndrickx *et al.* (1998) reported that *Bacillus pantothenicus* represents a phylogenetic group sufficiently different from other *Bacillus* species to warrant the status of a separate genus, for which they have proposed the name *Virgibacillus*, with the single species *V. pantothenicus*. This species can be distinguished from members of *Bacillus* rRNA group 1 (*Bacillus sensu stricto*) and from members of other aerobic endospore-forming bacteria by phenotypic characteristics. There are many phenotypic and genotypic differences between *V. pantothenicus* and our isolates (Table 4), for instance, their salt requirements (our isolates are unable to grow in media without NaCl), growth in anaerobic conditions (achieved by *V. pantothenicus* but not by our isolates), and G + C content, the values of our isolates (39.0–42.8 mol %) being somewhat higher than those of *V. pantothenicus* (36.9–38.3 mol %) (Heyndrickx *et al.*, 1998).

Phenotypically, our isolates were different in many characteristics with respect to the moderately halophilic species of the genus *Bacillus*, *B. halophilus* and *B. salexigens* (Ventosa *et al.*, 1989; Garabito *et al.*, 1997), and from moderately halophilic species of the genus *Halobacillus*, *Halobacillus trueperi* and *H. litoralis* (Spring *et al.*, 1996) (Table 4).

The DNA base composition of our isolates (39.0–42.8 mol %) was similar to that of *H. litoralis* (42.0 mol %) (Spring *et al.*, 1996), but lower than the values described for *H. trueperi* (43.0 mol %) (Spring *et al.*, 1996) and *B. halophilus* (51.5 mol %) (Ventosa *et al.*, 1989), and somewhat higher than those of *B. salexigens* (36.3–39.5 mol %) (Garabito *et al.*, 1997). Cell wall analysis of our strains showed that their peptidoglycan contained *meso*-diaminopimelic acid, as found in *B. halophilus* and *B. salexigens* (Ventosa *et al.*, 1989; Garabito *et al.*, 1997) as well as most other *Bacillus* species (Claus & Berkeley, 1986), but which differed from the cell wall peptidoglycan (Orn-D-Asp type) described for *H. trueperi* and *H. litoralis* (Spring *et al.*, 1996) (Table 4). Other traits which distinguish the strains studied from other related species are shown in Table 4.

The DNA relatedness experiments showed that our isolates form a single DNA similarity group with similarity values equal to or greater than 70 %. Most authors suggest that, among strains of the same species, the levels of DNA–DNA similarity should be equal to or greater than 70 % (Owen & Pitcher, 1985; Wayne *et al.*, 1987; Stackebrandt & Goebel, 1994).

Table 4. Characteristics useful for distinguishing *Bacillus marismortui* from other related Gram-positive endospore-forming rods

Data from Claus & Berkeley (1986), Garabito *et al.* (1997), Heyndrickx *et al.* (1998), Spring *et al.* (1996), Ventosa *et al.* (1989) and this study. 1, *Bacillus marismortui*; 2, *Virgibacillus pantothenicus*; 3, *Bacillus halophilus*; 4, *Bacillus salexigens*; 5, *Halobacillus litoralis*; and 6, *Halobacillus trueperi*. ND, Not determined; +, positive; —, negative.

Character	1	2	3	4	5	6
Pigmentation	Cream	Cream	None	None	Orange	Orange
Spore shape*	E	E/S	E	E	E/S	E/S
Spore position*	T/ST	T	C	ST/C	C/ST	C/ST
Sporangium swollen	+	+	—	+	ND	ND
Growth in salts at (% w/v):						
0	—	+	—	—	—	—
0.9	—	+	—	—	+	+
3	+	+	+	—	+	+
20	+	—	+	+	+	+
25	—	—	+	—	+	+
30	—	—	+	—	—	+
Optimal growth at 10% salts	+	—	—	+	+	+
Growth at 50 °C	+	+	+	—	—	—
Growth at pH:						
8.5	+	+	—	+	+	+
10.0	—	+	—	+	—	—
Anaerobic growth	—	+	—	—	—	—
Hydrolysis of:						
Casein	+	+	—	+	—	—
Gelatin	+	+	—	+	+	+
Starch	—	+	—	—	—	—
Tween 80	—	+	+	—	—	—
Phosphatase	+	—	—	—	—	—
Acid production from:						
D-Fructose	+	+	—	+	+	+
D-Galactose	—	+	—	—	—	+
Maltose	+	+	—	+	+	+
D-Mannose	—	+	+	+	ND	ND
Sucrose	—	+	+	ND	+	+
D-Trehalose	—	+	+	—	+	+
D-Xylose	—	—	+	—	+	—
Methyl red	+	—	—	—	ND	ND
Nitrate reduction	+	+	—	—	—	—
H ₂ S production	+	—	—	+	ND	ND
Arginine dehydrolase	—	+	—	—	—	—
Urease	+	—	+	ND	—	—
Cell wall type†	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	<i>m</i> -Dpm	Orn-D-Asp	Orn-D-Asp
G + C content (mol%)	40.7	36.9	51.5	39.5	42.0	43.0

* E, Ellipsoidal; S, spherical; C, central; ST, subterminal; T, terminal.

† *m*-Dpm, *meso*-Diaminopimelic acid.

Thus, on the basis of DNA relatedness data, our isolates form a group of organisms that are sufficiently related as to constitute a genospecies. On the other hand, low levels of DNA–DNA similarity, with values of 0–45 %, were obtained between the three representative strains and all the moderately halophilic, marine or non-halophilic bacteria studied (Table 1).

According to the comparative analysis of the 16S rRNA sequences, strains 123^T, 557 and 832 show a close phylogenetic relationship and are included in the phylogenetic group containing the low G + C content Gram-positive bacteria, closely related to the species belonging to phylogenetic group I of the genus *Bacillus* (Fig. 2) described by Ash *et al.* (1991). The recently

Fig. 3. Phase-contrast micrograph of strain 123^T showing spore-forming rods. Bar, 10 µm.

described moderately halophilic species *B. salexigens* (Garabito *et al.*, 1997) is also phylogenetically close to our isolates. The higher levels of sequence similarity between strains 123^T, 557 and 832 and the species included in the phylogenetic analysis correspond to those of *V. pantothenicus* (97.8% similarity) and *B. salexigens* (96.6% similarity). The rest of the 16S rRNA similarity values obtained were lower than 96%. Stackebrandt & Goebel (1994) stated that levels of similarity between 16S rRNA sequences that are lower than 97% suggest that strains do not belong to the same species; therefore our isolates can be considered a different species to *B. salexigens*.

These results agree with the DNA–DNA similarity studies and overall, they show that our isolates should be classified in a new species. Although 16S rRNA sequence comparison shows that they are most closely related to *V. pantothenicus*, they do not match with the description of the newly proposed genus *Virgibacillus* and thus, it is proposed that they are placed in the genus *Bacillus* as *Bacillus marismortui*. Other molecular data should be compiled in order to elucidate the taxonomic position of this and other *Bacillus* species, including *B. salexigens*.

Description of *Bacillus marismortui* Arahal, Márquez, Volcani, Schleifer and Ventosa sp. nov.

Bacillus marismortui (ma.ris.mor'tu.i. L. gen. n. *maris* of the sea; L. adj. *mortuus* dead; M.L. gen. n. *marismortui* of the Dead Sea).

Gram-positive rods, 2.0–3.6 by 0.5–0.7 µm, occurring singly, in pairs or in short chains (Fig. 3). Motile, oval endospores are produced at a terminal or subterminal position in swollen sporangia. Colonies are pigmented (cream), circular, opaque and entire. Growth occurs at 5–25% (w/v) total salts, optimal growth at 10% (w/v). No growth occurs in the absence of salts. Grows at 15–50 °C (optimal at 37 °C) and at pH 6.0–9.0

(optimal at pH 7.5). Strictly aerobic. Catalase and oxidase are produced. Acid is produced from D-fructose, D-glucose, glycerol and maltose; acid is not produced from D-arabinose, D-galactose, lactose, D-mannitol, sucrose, D-trehalose or D-xylose. Casein, DNA and gelatin are hydrolysed; starch or Tween 80 are not hydrolysed. Methyl red test is positive. Nitrate but not nitrite is reduced. Indole, Voges–Proskauer and Simmons citrate tests are negative. Urease is produced; H₂S, arginine dehydrolase or phenylalanine deaminase are not produced. The following compounds are utilized as sole carbon and energy sources: D-fructose, inulin, maltose, D-mannitol, D-mannose, pyruvate, D-raffinose, D-rhamnose and succinate. Susceptible to chloramphenicol, erythromycin, penicillin G, streptomycin and tetracycline; resistant to nalidixic acid, neomycin, novobiocin and rifampin. Other phenotypic features of this species are shown in Table 2. DNA G+C content is 39.0–42.8 mol% (*T_m* method). The cell wall contains peptidoglycan of the *meso*-diaminopimelic acid type. Isolated from the Dead Sea. The type strain is strain 123^T (= DSM 12325^T = ATCC 700626^T = CIP 105609^T = CECT 5066^T).

Description of the type strain. The description of the type strain is the same as that of the species, except that the type strain grows at 3–20% (w/v) total salt and pH 6.0–8.5. The DNA G+C content is 40.7 mol% (*T_m* method). Other characteristics of this strain are shown in Table 2.

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This article is dedicated to the memory of Professor B. E. Volcani, who died on 6 February 1999 from kidney failure. He pioneered studies on halophilic micro-organisms and described new halobacteria and moderately halophilic bacteria. In 1936 he reported the presence of a variety of micro-organisms in the Dead Sea, a hypersaline environment previously considered to be sterile due to its high salinity. His early studies on the Dead Sea were recently continued, since some of old samples from the 1930s were preserved by Professor Volcani.

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