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Phylogenetic Analyses of Some Extremely Halophilic Archaea Isolated from Dead Sea Water, Determined on the Basis of Their 16S rRNA Sequences

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Twenty-two extremely halophilic aerobic archaeal strains were isolated from enrichments prepared from Dead Sea water samples collected 57 years ago. The isolates were phenotypically clustered into five different groups, and a representative from each group was chosen for further study. Almost the entire sequences of the 16S rRNA genes of these representatives, and of *Haloarcula hispanica* ATCC 33960, were determined to establish their phylogenetic positions. The sequences of these strains were compared to previously published sequences of 27 reference halophilic archaea (members of the family *Halobacteriaceae*) and two other archaea, *Methanobacterium formicicum* DSM 1312 and *Methanospirillum hungatei* DSM 864. Phylogenetic analysis using approximately 1,400 base comparisons of 16S rRNA-encoding gene sequences demonstrated that the five isolates clustered closely to species belonging to three different genera—*Haloferax*, *Halobacterium*, and *Haloarcula*. Strains E1 and E8 were closely related and identified as members of the species *Haloferax volcanii*, and strain E12 was closely related and identified as a member of the species *Halobacterium salinarum*. However, strains E2 and E11 clustered in the *Haloarcula* branch with *Haloarcula hispanica* as the closest relative at 98.9 and 98.8% similarity, respectively. Strains E2 and E11 could represent two new species of the genus *Haloarcula*. However, because strains of these two new species were isolated from a single source, they will not be named until additional strains are isolated from other sources and fully characterized.

The Dead Sea is a hypersaline lake with a neutral pH from which a large number of extremely halophilic archaea have been isolated. The first microbiological studies were carried out in 1940 by B. E. Volcani (44), who described several new halophilic species distinct from other species in other categories of halophilic microorganisms. Years later, most of the cultures were lost. Since then, many other isolates from the Dead Sea have provided some novel members of the family *Halobacteriaceae*, such as *Haloferax volcanii* (26), *Haloarcula marismortui* (32), *Halorubrum* (formerly *Halobacterium*) *sodomense* (23, 31), and, very recently, *Halobaculum gomorrense* (33). The purpose of our study was to isolate and characterize pure cultures of extreme halophiles 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. Inocula were taken from these old enrichments, and by using appropriate media and growth conditions, we isolated 22 extremely halophilic microorganisms. Among these, five strains were selected for phylogenetic analysis of their 16S rRNA gene sequences. The 16S rRNA gene sequence of *Haloarcula hispanica* ATCC 33960 was also determined since it has not been previously reported.

MATERIALS AND 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 north basin of the Dead Sea by sampling at different spots close to the mouth of the Jordan river (42–48). The enrichments, prepared with Dead Sea water plus 1.0% (wt/vol) peptone, were kept in closed 500-ml bottles under sterile conditions and stored in the dark in a dry place at 18 to 20°C. These enrichments remained unopened over the entire storage period. Inocula were obtained from the bottles under sterile conditions. Several isolation media were used, varying the total salt concentration and its composition—especially the amount of Mg^{2+} ion. SWYE medium contained a final total salt concentration of ca. 25% (wt/vol) and 0.5% (wt/vol) yeast extract (Difco Laboratories, Detroit, Mich.) (28). HM medium (40) contained 1% (wt/vol) yeast extract (Difco), 0.5% (wt/vol) Proteose Peptone no. 3 (Difco), and 0.1% (wt/vol) glucose. This medium was supplemented with a balanced mixture of salts, giving a final concentration of 25% (wt/vol) (36), and with 500 IU of sodium penicillin G ml^{-1} to inhibit the growth of moderately halophilic and halotolerant bacteria (24). The last medium used, designated HS, was described by Oren for the isolation of *Halorubrum* (*Halobacterium*) *sodomense* from the Dead Sea. This medium contained (wt/vol) 12.5% NaCl, 16.0% $MgCl_2 \cdot 6H_2O$, 0.5% K_2SO_4, 0.01% $CaCl_2 \cdot 2H_2O$, 0.1% yeast extract (Difco), 0.1% Proteose Peptone no. 3 (Difco), and 0.2% soluble starch (31). The pH was always adjusted to 7.2. Incubation was done at 37°C in an orbital shaker (New Brunswick Scientific Co. Inc., Edison, N.J.) at 200 strokes per min. Twenty-two strains, designated E1 to E22, were isolated between March and September 1993. For comparison, the following culture collection strains were used in this study: *Haloarcula hispanica* ATCC 33960^T (T = type strain), *H. japonica* JCM 7785^T, *H. marismortui* ATCC 43049^T, *H. vallismortis* ATCC 29715^T, *Halobacterium distributum* VKM B-1733^T, *H. salinarum* CCM 2148, *Halococcus morrhuae* CCM 537^T, *H. saccharolyticus* ATCC 49257^T, *Haloferax denitrificans* DSM 4425^T, *H. gibbonsii* ATCC 33959^T, *H. mediterranei* CCM 3361^T, *H. volcanii* NCIMB 2012^T, *Halorubrum lacusprofundi* DSM 5036^T, and *H. saccharovorum* NCIMB 2081^T. SWYE medium was used for cultivation and maintenance of the strains. When necessary, plates and agar slants were prepared by adding 2% (wt/vol) Bacto Agar (Difco) to this medium.

Phenotypic characterization. The tests conducted for this study are listed in Table 1. Cell morphology and motility were examined on freshly prepared wet mounts by phase-contrast microscopy of exponentially growing liquid cultures. Gram staining was performed by using acetic acid-fixed samples as described by Dussault (2). Growth rates at different salt concentrations were determined by using maintenance medium prepared with salt concentrations of 0.5, 3, 5, 10, 15, 20, 25, and 30% (wt/vol) (35). Catalase and oxidase activities and starch hydrolysis were tested by using standard procedures (3, 15, 41). Gelatin hydrolysis experiments were performed as recommended by González et al (4). Procedures for determination of caseinase, phosphatase, and DNase activities have been

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TABLE 1. Phenotypic features of 22 isolates studied

Characteristic	Strain									
	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10
Cell shape	Flattened disks, cups	Irregular rods	Pleomorphic rods	Irregular rods, triangles	Pleomorphic rods	Irregular rods	Irregular rods, disks	Pleomorphic rods	Irregular triangles, rectangles	Rods, triangles, rectangles
Motility	+	+	+	+	+	+	+	+	+	+
Salt requirements[a]:										
Range for growth	15–25	20–30	20–30	20–30	20–25	20–30	15–20	15–25	15–30	20–30
Optimal growth	20	25	25	25	20	25	20	20	20	25
Pigmentation	Red	Red	Red	Red	Red	Red	Red	Pink	Red	Red
Oxidase	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	−	−	−	+	−	−	−	+	+	+
Starch hydrolysis	−	−	−	+	−	−	−	+	+	+
Casein hydrolysis	−	−	−	+	−	−	−	−	+	+
Phosphatase	−	−	−	−	−	−	−	−	−	−
DNase	−	−	−	−	−	−	−	−	−	−
Acid production[b] from:										
D-Arabinose	+	+	+	±	+	+	+	−	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+
D-Galactose	−	−	−	−	−	−	−	−	−	±
D-Glucose	+	+	±	+	+	+	+	+	+	+
Glycerol	+	−	±	−	−	−	±	+	−	±
Lactose	−	−	−	−	−	−	−	−	−	−
Maltose	−	+	+	+	+	±	±	±	+	+
D-Mannitol	−	−	−	−	−	−	−	−	−	−
Sucrose	±	−	−	−	−	−	−	+	−	±
D-Trehalose	−	−	−	−	−	−	−	±	−	−
D-Xylose	+	+	+	+	+	+	+	±	+	+

[a] Concentration (%, wt/vol) of total salts.
[b] +, positive; −, negative; ±, intermediate.

described elsewhere (3, 36, 39). Production of acid from sugars and other compounds was determined on maintenance medium modified as follows: yeast extract was reduced to 0.5% (wt/vol), and 0.001% (wt/vol) phenol red was added. The compounds tested were D-arabinose, D-fructose, D-galactose, D-glucose, glycerol, lactose, maltose, D-mannitol, sucrose, D-trehalose, and D-xylose. Each of these compounds was added to a final concentration of 1% (wt/vol) by filtration through a 0.45-μm-pore-size membrane filter (Millipore Corp., Bedford, Mass.) onto the sterile medium. Starch hydrolysis was tested by flooding colonies grown on agar plates with an iodine solution. Plates for the DNase test were flooded with a 1 N HCl solution, and the reaction was considered positive if a halo formed around the colonies (3).

Extraction of DNA. Cells were cultured to approximately the late exponential phase and then harvested by centrifugation. The cells were lysed by adding 200 μl of 5% lysozyme, 20 μl of 1% pronase, 8 μl of mutanolysin (500 U/ml), and 4 μl of 1% RNase. DNA was extracted by adding 250 μl of extraction buffer (20 mM Tris-HCl, 100 mM EDTA, 1% sodium dodecyl sulfate, 0.01% proteinase K). After extraction, DNA was precipitated with 3 M Na-acetate (1/10 of the final volume) and cold ethanol (twice the volume), centrifuged, dried, and finally dissolved for spectrophotometric quantification (1).

Amplification of 16S rRNA gene sequences. 16S rRNA genes were amplified with primers D30 and D56 (Table 2). PCRs were performed with thin-walled tubes by using a Perkin-Elmer 480 thermal cycler. Three microliters of 1/1,000-diluted DNA and 1 μM primers were added to the reaction mixture to a final volume of 82 μl. The following conditions were used for amplification: denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 1.5 min with an additional 5 s added for each cycle for a total of 30 cycles, followed by a final elongation step at 72°C for 15 min. The purity of the amplified product was determined by electrophoresis in a 1% agarose gel (FMC Bioproducts). DNA was stained with ethidium bromide and observed under short-wavelength UV light.

Purification of PCR products. The amplified DNA was purified by precipitation with polyethylene glycol 8000 (16). After removal of Ampliwax, 0.6 volume of 20% polyethylene glycol 8000 (Sigma) in 2.5 M NaCl was added and the mixture was incubated at 37°C for 10 min. The sample was centrifuged for 15 min at 15,000 × *g* and then washed with 80% cold ethanol and pelleted as before. The pellet was air dried and dissolved in 30 μl of distilled water.

Sequencing of 16S rRNA-encoding DNA. The purified DNA amplicons were sequenced by using a TAQuence Cycle Sequencing Kit (United States Biochemical Corp.) in accordance with the manufacturer's protocol. The seven sequencing primers used are given in Table 1. Primers were end labeled with ^{33}P (NEN/Dupont) in accordance with the manufacturer's protocol. Approximately 100 ng of purified DNA from the PCR was used for sequencing. Reaction

TABLE 2. Oligonucleotide primers used for PCR amplification and sequencing of 16S rRNA-encoding genes

Primer designation	Type	Sequence (5′–3′)[a]	Position[b]	Orientation
D30[c]	PCR, sequencing	ATTCCGGTTGATCCTGC	6–22	Forward
D33[c]	Sequencing	TCGCGCCTGCGCCCCGT	344–360	Reverse
B99	Sequencing	GTGTTACCGCGGCTGCTG	519–536	Reverse
B36	Sequencing	GGACTACCAGGGTATCTA	789–806	Reverse
D34[c]	Sequencing	GGTCTCGCTCGTTGCCTG	1096–1113	Reverse
X10	Sequencing	ACGGGCGGTGTGTRC	1392–1406	Reverse
D56	PCR, sequencing	GYTACCTTGTTACGACTT	1492–1509	Reverse

[a] Base codes are standard International Union of Biochemistry codes for bases and ambiguity.
[b] Numbering is based upon that of *Escherichia coli.*
[c] Specific for archaea.

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TABLE 1—*Continued*

Strain											
E11	E12	E13	E14	E15	E16	E17	E18	E19	E20	E21	E22
Rods, triangles, rectangles	Rods	Pleomorphic rods	Rods, triangles, rectangles	Rods	Pleomorphic rods	Irregular rods, disks	Irregular, flat disks, rods	Flattened disks	Irregular rods	Flattened disks	Irregular rods, disks
+	+	+	+	+	+	+	+	+	+	+	+
15–25	20–30	15–25	20–30	25–30	20–30	15–25	15–30	15–25	15–25	15–25	15–25
20	25	20	25	25	25	20	25	20	20	20	20
Red	Red	Pink	Red	Red	Red	Pink	Red	Red	Pink	Red	Red
+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	−	+	+	−	+	−	−
+	−	+	+	−	−	+	+	−	+	−	−
+	−	−	+	−	−	−	+	−	−	−	−
−	+	−	−	+	−	−	−	−	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−
+	−	−	+	−	+	−	+	+	−	+	+
+	−	+	+	−	+	+	+	+	+	+	+
−	−	−	−	−	−	−	−	−	−	−	−
+	−	+	+	±	+	+	+	+	+	+	+
−	−	+	±	−	−	+	−	+	+	±	+
−	−	−	−	−	−	−	−	−	−	−	−
+	−	±	+	−	+	±	+	−	+	±	±
−	−	−	−	−	−	−	−	−	−	−	−
−	−	+	−	−	−	+	−	±	+	±	−
−	−	±	−	−	−	±	−	−	−	−	−
+	−	±	+	−	+	±	+	+	±	+	+

products were loaded onto 8% polyacrylamide–urea gels, electrophoresed, and detected by exposure to X-ray film for 24 h.

16S rRNA gene sequence analysis. A program set for entry of data, editing, sequence alignment, secondary-structure comparison, similarity matrix generation, and dendrogram construction for 16S rRNA data was written in Microsoft Quick BASIC for use on IBM PC-AT and compatible computers (34). Our sequence database contains the archaeal sequences determined in our laboratory and 32 more obtained from GenBank or the Ribosomal Database Project (20). The reference strains used in the 16S rRNA analysis are shown in Table 3. Similarity matrices were constructed from the aligned sequences by using only those positions for which there were data for 90% of the strains. The similarity matrices were corrected for multiple base changes at single positions by the method of Jukes and Cantor (10). Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (38).

Nucleotide sequence accession numbers. The GenBank and culture collection accession numbers of the strains examined in this study are given in Table 3.

RESULTS AND DISCUSSION

A total of 22 strains were isolated from the 57-year-old enrichments obtained from Dead Sea water samples. All isolates produced colonies on solid media that were pink to red pigmented. Since all isolates had salt growth requirements that were always above 15% (wt/vol) salts with an optimal concentration of 20 to 25%, they were considered extreme halophiles. These isolates were presumptively identified as members of the family *Halobacteriaceae* (5) on the basis of phenotypic characteristics (Table 1). Some of the strains could not be differentiated on the basis of the characteristics examined. All strains were gram negative, motile, and catalase and oxidase positive but were not able to degrade DNA. On the basis of the phenotypic features tested, the isolates were divided into five groups. Group I comprised strains E1, E7, E19, E21, and E22; group II comprised strains E2, E3, E5, E6, and E16; group III comprised strains E4, E9, E10, E11, E14, and E18; group IV comprised strains E8, E13, E17, and E20; and group V comprised strains E12 and E15. Strains in groups I, II, and V showed phenotypic features resembling members of the genera *Haloferax*, *Haloarcula*, and *Halobacterium*, respectively (5, 9). Strains in groups III and IV did not clearly fit the descriptions of known genera in the family *Halobacteriaceae*. The 16S rRNA gene sequence from a representative of each of the five groups was obtained to determine phylogenetic identities. These representative isolates were strains E1, E2, E8, E11, and E12.

Approximately 96% (ca. 1,420 bases) of the total 16S rRNA gene sequence was determined for each of the five strains examined and for *Haloarcula hispanica* ATCC 33960. These sequences were compared with a database of more than 3,000 prokaryotic sequences (20). The similarity matrix for these comparisons is presented in Table 4. A phylogenetic tree constructed by the neighbor-joining method is presented in Fig. 1. Comparison of the *Haloarcula hispanica* sequence with sequences for other halobacteria in the database indicates that is most closely related to the other species of the genus *Haloarcula*. The similarities between this species and *H. marismortui* (*rrnB* gene) and *H. vallismortis* were 97.8 and 97.6%, respectively. Thus, our study supports its current taxonomic position. Strains E1 (group I) and E8 (group IV) differ phenotypically in the ability to hydrolyze gelatin and starch, as well as in the ability to produce acid from some sugars. The similarities between these two strains and *Haloarcula volcanii* ATCC 29605 were 99.9 and 99.7%, respectively, and they were 99.6% similar to each other. Strain E12 (group V) was very similar to *H. salinarum* DSM 671 and also to an unidentified strain belonging to the genus *Halobacterium*, strain Y12 (13). These results agree with the phenotypic characteristics studied for these strains. With respect to strains E2 (group II) and E11 (group III), on the basis of their phenotypic features (Table 1) and

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TABLE 3. Sources and accession numbers of strains studied

Organism	Strain[a]	Accession no.[b]	Abbreviation[c]	Reference[d]
Isolates				
Haloarcula sp.	E2	U68539	HAE2	This study
Haloarcula sp.	E11	U68537	HAE11	This study
Halobacterium salinarum	E12	U68538	HBSE12	This study
Haloferax volcanii	E1			This study
Haloferax volcanii	E8	U68540	HFVE8	This study
Reference species				
Haloarcula hispanica	ATCC 33960^T	U68541	HAHIS	This study
Haloarcula japonica	JCM 7785^T	D28872	HAJAP	7
Haloarcula marismortui (*rrnA* gene)	Ginzburg strain	X61688	HAMARA	27
Haloarcula marismortui (*rrnB* gene)	Ginzburg strain	X61689	HAMARB	27
"*Haloarcula sinaiiensis*" (major gene)	ATCC 33800^T	D14130	HASIN1	13
"*Haloarcula sinaiiensis*" (minor gene)	ATCC 33800^T	D14129	HASIN2	13
Haloarcula vallismortis	ATCC 29715^T	U17593	HAVALL	11
Halobacterium salinarum (*halobium*)	DSM 671	M38280	HBSAL	21
Halobacterium trapanicum	NCIMB 767	D14125	HBTRAP	13
Halorubrum coriense	Ch2	L00922	HRCOR	30
Halobacterium sp.	Y12	D14127	HBY12	13
Halobaculum gomorrense	DSM 9297^T	L37444	HBLGOM	33
Halococcus morrhuae	NRC 16008	D11106	HCMORR	12
Halococcus morrhuae	ATCC 17082^T	X00662	HCMO-T	18
Haloferax denitrificans	ATCC 35960^T	D14128	HFDEN	13
Haloferax gibbonsii	ATCC 33959^T	D13378	HFGIBB	13
Haloferax mediterranei	ATCC 33500^T	D11107	HFMED	12
Haloferax volcanii	ATCC 29605^T	K00421	HFVOLC	6
Halorubrum lacusprofundi	ACAM 34^T	X82170	HRLAC	23
Halorubrum saccharovorum	NCIMB 2081^T	X82167	HRSAC	23
Halorubrum sodomense	ATCC 33755^T	X82169	HRSOD	23
Halorubrum trapanicum	NRC 34021	X82168	HRTRA	23
Natrialba asiatica	172P1	D14123	NA172P1	13
Natrialba asiatica	B1T	D14124	NAB1T	13
Halophilic strain	L-11	D14126	L-11	13
Natronobacterium magadii	NCIMB 2190^T	X72495	NBMAG	19
Natronococcus amylolyticus	JCM 9655^T	D43628	NCAMYL	8
Natronococcus occultus	NCIMB 2192^T	Z28378	NCOCC	22
Methanobacterium formicicum	DSM 1312^T	M36508	MBFOR	17
Methanospirillum hungatei	DSM 864	M60882	MSHUN	49

[a] ACAM, Australian Collection of Antarctic Microorganisms, Departament of Agricultural Science, University of Tasmania, Hobart, Tasmania, Australia; ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Sammlung von Microorganismen und Zellkulturen GmbH, Braunschweig, Germany; JMC, Japan Collection of Microorganisms, Tokyo; NCIMB, National Collection of Industrial and Marine Bacteria, Ltd., Aberdeen, Scotland. NRC, National Research Council of Canada, Ottawa, Ontario.

[b] Accession number of 16S rRNA sequence for electronic retrieval from database.

[c] Abbreviation code used in Table 4.

[d] Reference in which the 16S rRNA gene sequence is described.

their phylogenetic relationship (Fig. 1), it is clear that both of them belong to the genus *Haloarcula*. The closest relative of strains E2 and E11 is *H. hispanica*, with sequence similarities of 98.9 and 98.8%, respectively. The sequence similarity between strains E2 and E11 is 98.7%. Since other species within this genus are separated by comparable sequence similarities, it is likely that E2 and E11 represent two new species. However, before additional species are specified, other methods, such as DNA-DNA hybridization, should be used to verify that separate species designation are warranted.

On the basis of phenotypic characterizations in 1940, Volcani (44) described an organism similar to *H. salinarum* and another one closer to members of the genus *Halococcus* (formerly *Sarcina*) that were isolated from the same samples used in the present study. Strains in group V, E12 and E15, showed phenotypic characteristics similar to those of *H. salinarum*. This species is more frequently isolated when the salinity is above 25% (wt/vol) (37). The similarity between E12 and *H. salinarum* was 99.7% (Table 4). However, none of our isolates resembled *Halococcus* sp., probably because of their relatively low numbers in hypersaline environments compared with other halobacteria (24). We must also consider the possibility that halococci lack long-term survival ability.

Haloferax volcanii was first isolated from the Dead Sea and described as *Halobacterium volcanii* in 1975 by Mullakhanbhai and Larsen (26). Strains included in group I showed phenotypic features similar to those of *H. volcanii*, and the 16S rRNA gene sequence of strain E1, the representative of the group, was almost identical to the sequence of *H. volcanii*. While strains in group IV were phenotypically different from other groups, the 16S rRNA gene sequence of strain E8, the representative of the group, was very similar to the sequences of *H. volcanii* and strain E1. Another species isolated originally from the Dead Sea was *Haloarcula marismortui* (32), and strains clustered in group II resembled it in terms of phenotypic characteristics. The similarity between E2, the representative of the group, and *H. marismortui* (gene *rrnB*) sequences was 97.8% (Table 4). None of our strains resembled *Halorubrum sodo-*

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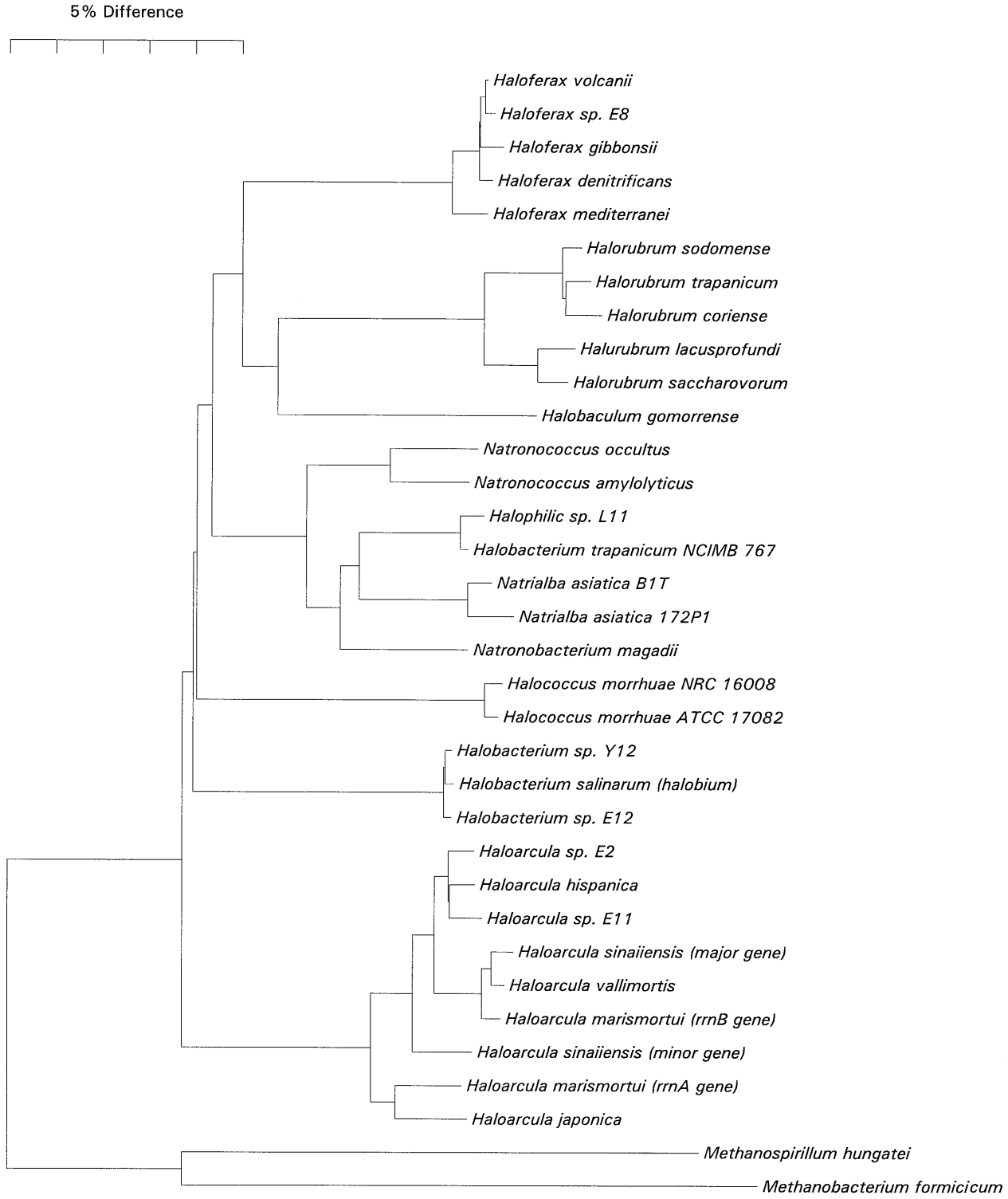

FIG. 1. Phylogenetic tree based on 16S rRNA sequence similarity data for the five isolates of our study, *Haloarcula hispanica*, and other species of the family *Halobacteriaceae*. *Methanobacterium formicicum* and *Methanospirillum hungatei* are also included as representatives of nonhalophilic archaea. The scale bar represents a 5% nucleotide sequence difference as determined by measuring the lengths of horizontal lines connecting any two species.

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TABLE 4. Similarity matrix based on 16S rRNA gene sequence comparisons

No. and abbreviation	% Similarity or difference[a]															
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. HFVOLC		99.7	99.5	99.2	98.4	88.4	88.8	89.0	89.2	89.1	89.1	90.3	89.8	89.6	89.3	90.1
2. HFVE8	0.3		99.4	99.2	98.3	88.2	88.6	88.8	89.0	88.9	88.8	90.1	89.6	89.4	89.1	89.9
3. HFDEN	0.5	0.6		99.2	98.4	88.3	88.6	88.8	89.1	89.0	88.9	90.0	89.5	89.4	89.1	89.6
4. HFGIBB	0.8	0.8	0.8		98.2	88.0	88.3	88.5	88.7	88.5	88.6	90.0	89.7	89.7	89.4	89.9
5. HFMED	1.6	1.7	1.7	1.8		87.8	88.2	88.4	88.7	88.5	89.1	90.0	89.8	89.5	89.2	90.0
6. HRCOR	12.6	12.9	12.7	13.1	13.3		98.7	98.8	95.6	95.8	88.7	87.4	87.0	86.2	86.0	86.6
7. HRTRA	12.2	12.4	12.4	12.7	12.9	1.3		99.0	95.6	96.0	88.8	87.9	87.4	86.5	86.3	86.5
8. HRSOD	11.9	12.2	12.1	12.5	12.6	1.3	1.0		96.0	96.3	89.2	88.0	87.6	86.8	86.6	86.8
9. HRLAC	11.6	11.9	11.7	12.3	12.3	4.5	4.5	4.1		98.5	88.5	87.5	87.1	87.3	87.0	87.7
10. HRSAC	11.8	12.0	11.9	12.4	12.5	4.4	4.1	3.8	1.5		88.8	87.6	87.1	87.5	87.4	87.5
11. HBLGOM	11.8	12.1	12.0	12.3	11.8	12.3	12.1	11.7	12.4	12.1		87.0	86.4	87.4	87.0	87.4
12. HBTRAP	10.4	10.6	10.8	10.7	10.8	13.8	13.2	13.1	13.7	13.5	14.3		99.3	95.0	94.5	94.7
13. L-11	11.0	11.2	11.3	11.1	11.0	14.3	13.7	13.6	14.1	14.2	15.0	0.7		94.7	94.2	94.1
14. NABIT	11.2	11.4	11.4	11.1	11.3	15.2	14.9	14.5	13.9	13.7	13.8	5.2	5.5		98.5	94.4
15. NA172P1	11.6	11.7	11.8	11.4	11.6	15.5	15.2	14.8	14.2	13.9	14.3	5.7	6.1	1.5		93.7
16. NBMAG	10.6	10.8	11.2	10.9	10.7	14.8	14.9	14.5	13.5	13.7	13.8	5.5	6.1	5.8	6.6	
17. NCOCC	11.3	11.5	11.7	11.8	11.2	13.5	13.2	12.8	12.8	12.4	13.5	7.1	7.3	6.7	7.3	7.9
18. NCAMYL	10.9	11.1	11.1	11.1	10.9	13.8	13.6	13.4	12.3	12.3	14.3	6.7	7.0	7.2	7.9	7.1
19. HCMORR	12.7	12.8	12.9	13.0	13.0	17.0	16.1	16.2	15.9	15.5	14.1	11.8	12.0	12.8	13.0	12.3
20. HCMO-T	12.5	12.6	12.6	12.7	13.1	16.7	15.7	15.8	15.5	15.3	14.0	12.0	12.2	12.8	13.2	12.2
21. HBSAL	13.0	13.1	13.0	13.3	13.4	14.2	14.2	13.5	13.4	12.7	12.6	11.2	11.7	11.5	12.2	11.0
22. HBY12	12.9	13.0	13.0	13.2	13.3	14.2	14.2	13.5	13.4	12.8	12.5	11.1	11.6	11.2	12.0	10.9
23. HBSE12	13.0	13.1	13.0	13.3	13.4	14.0	14.1	13.4	13.3	12.6	12.7	11.1	11.7	11.5	12.2	10.8
24. HAJAP	12.5	12.7	12.5	13.2	12.5	16.0	15.7	15.9	15.9	15.4	14.2	12.2	12.3	12.6	13.0	11.7
25. HAMARA	12.5	12.7	12.6	13.1	12.5	15.4	15.1	15.3	15.1	14.7	12.7	11.9	12.3	11.9	12.6	11.3
26. HASIN1	13.0	13.1	12.9	13.4	12.8	15.3	15.1	15.3	15.5	15.4	13.6	12.8	13.1	13.7	14.0	12.6
27. HASIN2	13.9	14.1	13.9	14.2	13.7	15.7	15.6	15.6	15.6	15.5	15.1	13.7	13.7	14.5	14.8	13.6
28. HAVALL	13.6	13.8	13.7	14.0	13.4	15.1	15.0	15.1	15.2	15.0	14.7	13.2	13.0	14.2	14.8	13.1
29. HAMARB	13.6	13.8	13.7	14.0	13.5	15.3	15.0	15.1	15.1	15.0	14.7	13.2	13.6	14.0	14.8	13.0
30. HAE2	12.8	12.8	12.8	13.1	12.6	15.5	15.2	15.3	15.2	15.1	14.1	12.4	12.8	13.0	13.7	12.2
31. HAE11	12.9	12.9	12.8	13.2	12.7	15.1	14.9	15.0	15.2	15.1	13.9	12.5	12.9	13.2	13.8	12.5
32. HAHIS	13.0	13.1	12.9	13.3	13.0	15.2	15.0	15.1	15.4	15.2	14.1	12.7	13.1	13.3	13.9	12.8
33. MSHUN	25.0	25.1	25.2	25.0	24.8	27.2	26.9	26.5	25.8	26.3	25.4	26.1	26.3	26.1	26.2	26.8
34. MBFOR	27.8	27.8	27.6	28.2	27.3	26.3	26.8	26.1	25.9	26.6	26.7	26.5	26.7	26.6	26.5	27.1

[a] Percentages above the diagonal represent similarity, and those below the diagonal represent differences corrected for multiple base changes by the method of Jukes and Cantor (10).

mense or *Halobaculum gomorrense*, two other species isolated from the Dead Sea (23, 31, 33).

Our study demonstrates that samples from enrichments collected almost 60 years ago from the Dead Sea still contained viable microorganisms. Previous microbiological studies have also described this evidence of the tenacity of life in regard to many different kinds of samples, length of storage, and types of microorganisms found—including some members of the family *Halobacteriaceae* (14, 29). A possible answer for long-term preservation like that in the present study can be that microorganisms thrive at extremely low rates, since they are present in a nutrient-poor environment and achieve a starvation survival state, as it has been called (25). In fact, it has been reported that preservation and revival of microorganisms after long storage periods, even longer than 50 years, are more common than previously thought (14).

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TABLE 4—*Continued*

% Similarity or differences[a]																	
17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
89.5	89.9	88.3	88.5	88.1	88.1	88.1	88.5	88.5	88.1	87.3	87.5	87.5	88.3	88.2	88.1	78.7	76.8
89.3	89.7	88.2	88.4	88.0	88.1	88.0	88.3	88.3	87.9	87.2	87.4	87.4	88.2	88.1	88.0	78.7	76.8
89.2	89.7	88.2	88.4	88.0	88.0	88.1	88.5	88.4	88.1	87.3	87.5	87.5	88.2	88.2	88.2	78.6	76.9
89.1	89.6	88.1	88.3	87.8	87.9	87.8	87.9	88.0	87.7	87.0	87.3	87.3	88.0	87.9	87.8	78.7	76.5
89.6	89.9	88.1	88.0	87.7	87.8	87.7	88.5	88.5	88.2	87.5	87.7	87.7	88.4	88.3	88.1	78.9	77.1
87.7	87.4	84.8	85.1	87.1	87.1	87.2	85.6	86.1	86.1	85.8	86.3	86.2	86.0	86.3	86.2	77.2	77.8
87.9	87.6	85.5	85.8	87.1	87.0	87.1	85.8	86.3	86.3	85.9	86.4	86.4	86.3	86.5	86.4	77.4	77.5
88.2	87.8	85.5	85.8	87.6	87.6	87.7	85.7	86.2	86.2	85.9	86.3	86.3	86.1	86.4	86.3	77.7	78.0
88.2	88.6	85.7	86.0	87.7	87.7	87.8	85.7	86.3	86.0	85.9	86.3	86.3	86.2	86.3	86.1	78.2	78.1
88.6	88.6	86.0	86.1	88.3	88.3	88.4	86.1	86.6	86.1	86.0	86.4	86.4	86.3	86.3	86.3	77.8	77.6
87.6	86.9	87.2	87.2	88.4	88.5	88.3	87.0	88.3	87.6	86.3	86.6	86.7	87.1	87.3	87.1	78.4	77.5
93.2	93.6	89.0	88.9	89.6	89.7	89.7	88.8	89.0	88.2	87.4	87.9	87.9	88.6	88.5	88.3	78.0	77.7
93.0	93.3	88.9	88.8	89.2	89.2	89.2	88.7	88.7	88.0	87.5	88.1	87.6	88.2	88.1	87.9	77.8	77.6
93.6	93.1	88.2	88.3	89.4	89.6	89.3	88.4	89.0	87.5	86.8	87.0	87.2	88.0	87.9	87.8	78.0	77.6
93.1	92.5	88.1	87.9	88.7	88.9	88.7	88.1	88.4	87.2	86.5	86.6	86.5	87.5	87.4	87.3	77.9	77.7
92.5	93.3	88.7	88.8	89.8	89.9	89.9	89.2	89.5	88.4	87.6	88.0	88.0	88.8	88.5	88.2	77.5	77.3
	96.5	88.2	88.5	90.0	90.0	89.9	88.0	88.2	88.0	87.3	87.8	87.8	88.4	88.3	88.2	78.3	77.7
3.6		89.2	89.3	89.3	89.3	89.3	88.5	88.7	88.6	87.9	87.9	88.0	88.7	88.4	88.3	78.3	76.9
12.8	11.7		99.3	88.8	88.8	88.8	88.3	89.3	88.0	86.9	87.1	87.1	87.6	87.7	87.3	78.4	77.0
12.5	11.5	0.7		88.6	88.6	88.6	88.2	89.4	88.1	87.0	87.1	87.1	87.7	87.8	87.4	78.7	77.0
10.8	11.6	12.2	12.3		99.7	99.7	89.3	90.3	88.6	87.4	87.5	87.8	88.7	88.5	88.3	79.3	78.3
10.7	11.5	12.2	12.3	0.3		99.6	89.3	90.3	88.6	87.5	87.6	87.8	88.8	88.6	88.3	79.2	78.2
10.8	11.6	12.2	12.3	0.3	0.4		89.3	90.4	88.7	87.5	87.7	87.9	88.9	88.7	88.5	79.3	78.4
13.1	12.5	12.7	12.8	11.5	11.5	11.5		97.1	96.0	94.7	94.8	94.9	96.0	95.9	96.1	77.5	77.2
12.8	12.2	11.5	11.4	10.4	10.4	10.3	3.0		96.2	94.3	94.6	95.0	96.4	95.8	95.8	79.3	77.8
13.0	12.3	13.0	13.0	12.4	12.4	12.3	4.1	3.9		97.3	97.2	97.0	97.3	97.0	97.5	78.9	77.5
14.0	13.2	14.4	14.3	13.8	13.7	13.7	5.5	6.0	2.8		99.2	98.7	97.3	97.4	97.5	78.3	77.6
13.4	13.1	14.1	14.1	13.6	13.6	13.5	5.4	5.6	2.9	0.8		99.3	97.6	97.4	97.6	78.3	77.5
13.3	13.1	14.2	14.1	13.4	13.3	13.2	5.3	5.2	3.0	1.3	0.7		97.8	97.6	97.8	78.3	77.4
12.6	12.3	13.6	13.5	12.2	12.1	12.0	4.2	3.7	2.8	2.7	2.4	2.2		98.7	98.9	79.3	77.7
12.7	12.6	13.4	13.3	12.4	12.4	12.3	4.2	4.3	3.1	2.7	2.6	2.4	1.3		98.8	79.1	77.9
12.8	12.8	13.9	13.8	12.7	12.7	12.5	4.0	4.3	2.6	2.6	2.5	2.3	1.1	1.3		78.9	77.7
25.6	25.6	25.4	25.1	24.2	24.3	24.2	26.7	24.2	24.8	25.7	25.7	25.6	24.2	24.6	24.8		79.8
26.5	27.6	27.5	27.5	25.6	25.8	25.4	27.1	26.3	26.7	26.6	26.8	26.9	26.5	26.2	26.4	23.6	

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