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Microbial Diversity of the Brine-Seawater Interface of the Kebrit Deep, Red Sea, Studied via 16S rRNA Gene Sequences and Cultivation Methods

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The brine-seawater interface of the Kebrit Deep, northern Red Sea, was investigated for the presence of microorganisms using phylogenetic analysis combined with cultivation methods. Under strictly anaerobic culture conditions, novel halophiles were isolated. The new rod-shaped isolates belong to the halophilic genus *Halanaerobium* and are the first representatives of the genus obtained from deep-sea, anaerobic brine pools. Within the genus *Halanaerobium*, they represent new species which grow chemoorganotrophically at NaCl concentrations ranging from 5 to 34%. The cellular fatty acid compositions are consistent with those of other *Halanaerobium* representatives, showing unusually large amounts of Δ7 and Δ11 16:1 fatty acids. Phylogenetic analysis of the brine-seawater interface sample revealed the presence of various bacterial 16S rRNA gene sequences dominated by cultivated members of the bacterial domain, with the majority affiliated with the genus *Halanaerobium*. The new *Halanaerobium* 16S rRNA clone sequences showed the highest similarity (99.9%) to the sequence of isolate KT-8-13 from the Kebrit Deep brine. In this initial survey, our polyphasic approach demonstrates that novel halophiles thrive in the anaerobic, deep-sea brine pool of the Kebrit Deep, Red Sea. They may contribute significantly to the anaerobic degradation of organic matter enriched at the brine-seawater interface.

Hypersaline ecosystems are one of the most unusual and extreme environments on earth (15, 25, 36). Anaerobic, deep-sea brine pools, which are located along various tectonic rift systems, represent a special type of hypersaline environment. During the last 50 years about 25 deep-sea brine pools (Fig. 1) with highly saline waters were identified in the Red Sea, an ocean in statu nascendi within the East African Rift Valley system (1, 6, 11, 13, 14, 18, 46, 53, 60, 65). The brines of the Red Sea are typical athalassohaline waters which are in the main a reflection of the geology, geography, and topography of the areas where they develop (18, 25, 27). The high salinity is formed when seawater circulates through subbottom Miocene evaporite deposits, obtaining geothermal heat and dissolved solids before surfacing in the depression of the deeps (1, 12, 62, 72). Characteristic of the brine pools is the formation of gradients along the brine-seawater interface, e.g., salinity, pH, temperature, and oxygen gradients. Brine pools of different origin are also found in the Gulf of Mexico (e.g., the Orca Basin) and in the Mediterranean Sea (e.g., the Tyro, Bannock, or Urania Basin) (19, 37, 44, 61, 63).

The Kebrit Deep (in Arabic, kebrit means sulfur) in the northern Red Sea was first explored during a *Valdivia* cruise in 1971 and consists of a basin of approximately 1 by 2.5 km in size (27, 52, 59). The deep is filled with a brine of 84 m in thickness at a maximum depth of 1,549 m (27). At the brine-seawater interface of the Kebrit Deep there is a steep increase in salinity from 4 to 26% (wt/vol) NaCl (within only 3 m), an increase in temperature from 21.6 to 23.4°C (within about 7 m), an increase of the CH_4 concentration from 50 nl/liter to 22 ml/liter, and a measurable brine pool H_2S content of up to 12 to 14 mg of S/liter. Over the same interface the pH drops from 8.1 to 5.5 and the O_2 concentration decreases from 3.2 ml of O_2/liter to zero (23, 27, 64). At the same time, the density gradient created at the brine-seawater interface acts as an in situ particle trap for organic and inorganic materials from the Red Sea water (27–29, 40, 57, 60, 66).

During the last 30 years, detailed geological and geochemical investigations were carried out in the Kebrit Deep. In contrast, information about the microbial communities of this deep is very rare. Recently, novel bacterial and archaeal 16S rRNA gene sequences have been retrieved from brine sediments (21, 48, 50). These investigations showed that novel groups of *Archaea* and *Bacteria* (KB1 sequence group) thrive in the extreme environment of the Kebrit Deep (21). The presence of archaeal methanogenesis is also suggested by the biochemical characterization of C_{40} isoprenoids, an archaeal biomarker, in sedimentary organic matter (45), and an apparent biotic methane oxidation at the brine-seawater interface (23). Biochemical investigations in similar brine pools (Orca and Bannock Basins) indicated a high microbial potential at the brine-seawater interface and suggest the presence of halophilic microorganisms within the brine (17, 22, 39, 40, 68).

A great diversity of microorganisms have been isolated from high-salinity environments, including aerobic and anaerobic organisms of the bacterial and archaeal domains (25). These halophilic *Bacteria* include sulfate reducers (reference 4 and

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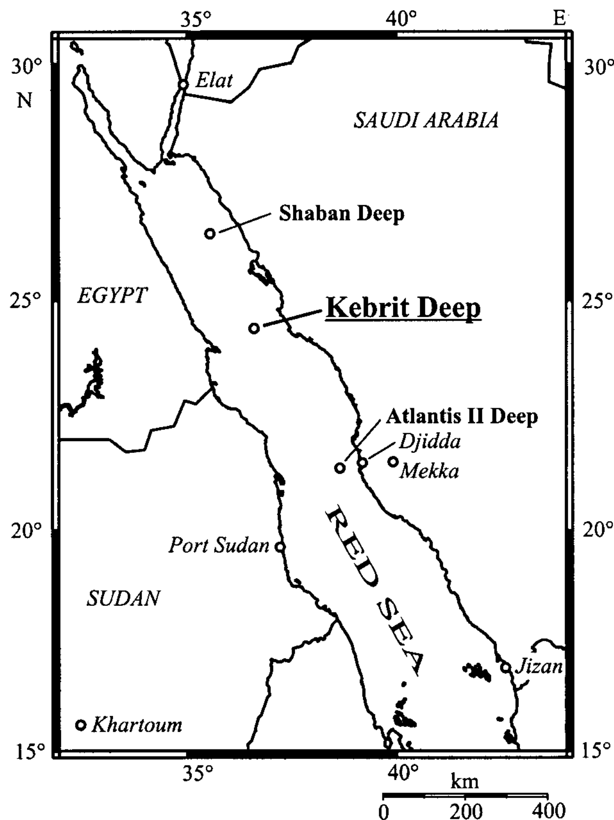

FIG. 1. Outline of the Red Sea, showing representative brine pools. The topographical map was generated with the Online Map Creation program (Geomar, Kiel, Germany [http://www.aquarius.geomar.de]).

references therein) and anaerobic phototrophs, gram-positive heterotrophs, and cyanobacteria (references 8 and 25 and references therein). Some isolates, like *Flexistipes sinusarabici*, represent separate lineages (24). In addition, the *Halanaerobiaceae*, which represent a monophyletic lineage within the *Bacteria*, are specifically adapted to their high-salt milieu (49, 55, 71). The halophilic *Archaea* known to date comprise aerobic halophiles of the family *Halobacteriaceae* and anaerobic methanogens of the family *Methanosarcinaceae* (16, 26, 47).

The goal of this research was to assess, for the first time, the bacterial diversity of the brine-seawater interface of the Kebrit Deep, Red Sea. In this initial survey, which is preliminary, a twofold approach was used. This includes (i) phylogenetic analysis of 16S rRNA gene sequences as indicators of prokaryotic diversity and (ii) isolation and cultivation of halophilic representatives to establish physiological and function potential within the ecosystem community.

MATERIALS AND METHODS

Sampling. Brine from the brine-seawater interface of the Kebrit Deep, Red Sea (Fig. 1), was sampled during RV *Sonne* cruise SO 121 in 1997 using a rosette sampler equipped with 24 niskin bottles (10 liters) and a conductivity-temperature-depths (CTD) unit for monitoring salinity, temperature, transmission, and pressure (Sea-Bird Electronic, Bellevue, Wash.). The salinity of brine samples was measured with a hand refractometer (Atago, Tokyo, Japan). Microorganisms were concentrated by pumping anaerobic brine across a crossflow tangential filtration unit (Pellicon Kasettensystem; Millipore, Eschborn, Germany) (Fig. 2) under a CO_2 protective atmosphere (cell concentration factor, 400-fold). Concentrated brine sample KT-2 was reduced with sodium dithionite (about 0.1 μM), and KT-3 was used without further treatment. In addition, sample KT-8 was taken from surface sediment of the Kebrit Deep by a chain-sack dredge (station no. 17034-2). The sample located near the brine-seawater interface consisted of oily ore rocks and brine (salinity, 15.6%). The samples were transported to the laboratory by air at ambient temperature and were stored at 4°C.

Strains. *Halanaerobium praevalens* DSM 2228, *Halothermothrix orenii* DSM 9562, and *Halanaerobacter lacunaris* DSM 6640 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany, and were cultivated by using the indicated DSMZ media.

Culture conditions. Enrichment cultures from samples KT-2 and KT-3 (KT-2/3) and KT-8 were established in 28-ml serum tubes on board the RV *Sonne*. Brine used for enrichments was supplemented with various sterile organic and inorganic compounds (e.g., yeast extract, peptone, $NaNO_3$, and Na_2SO_4) and chemically reduced by the addition of 1 ml of 50% (vol/vol) H_2S per 10 ml of brine. In the laboratory, positive enrichments were transferred several times in supplemented brine and then grown in a synthetic medium whose composition

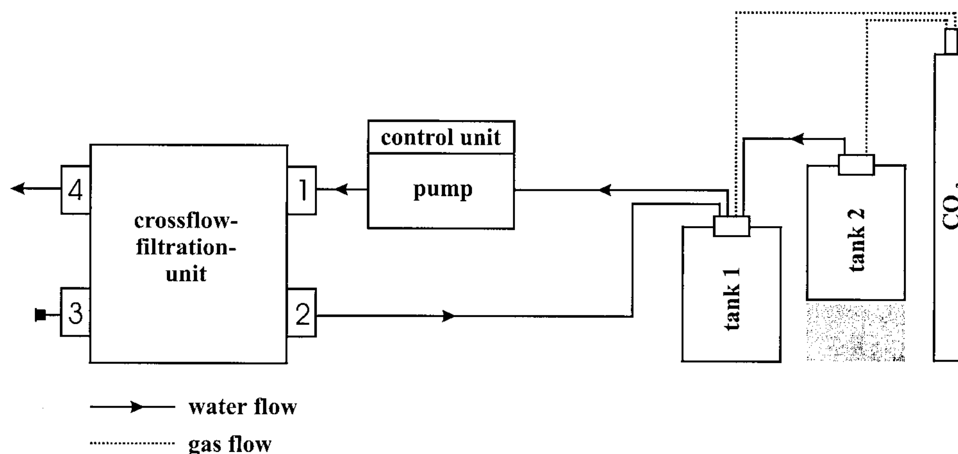

FIG. 2. Schematic drawing of the anaerobic filtration system. The crossflow filtration unit was equipped with five Durapore membranes (pore size, 0.2 μm; filter surface, 0.46 m^2) (Millipore). The cells were kept in suspension and were pumped under pressure over the filter surface. Before filtration, the complete system was flushed for 10 min with CO_2 to remove oxygen. During the filtration procedure, a protective atmosphere of CO_2 was maintained to prevent cellular damage due to oxygen sensitivity, to prevent precipitation of inorganic compounds, and to keep the pH of the sample constant. The concentrated brine was collected in tank 1; tank 2 was for storage. Brine flowed into the filtration unit at valve 1, concentrated cells exited at valve 2, and the filtrate outflow was at valve 4. Valve 3 is a closed valve.

was based on a chemical analysis of a Red Sea brine-seawater interface (brine interface medium [BI medium]) (D. Garbe-Schönberg [Institut für Geowissenschaften, Universität Kiel, Germany], personal communication). BI medium contained (per liter) 100 g of NaCl, 5.11 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of $CaCl_2 \cdot 2H_2O$, 107 mg of $MgCl_2 \cdot 6H_2O$, 19 mg of $MnSO_4 \cdot H_2O$, 10 mg of $SrCl_2 \cdot 6H_2O$, 2.7 mg of $FeCl_2 \cdot 4H_2O$, 0.075 mg of $NaNO_3$, 0.7 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of $Na_2MoO_4 \cdot 2H_2O$, 100 mg of KH_2PO_4, and 1.0 g of $NaHCO_3$. The pH was adjusted to 6.5 with HCl. The gas phase over the medium in incubation tubes was replaced with N_2, and residual O_2 was chemically reduced by the addition of 1 ml of 50% (vol/vol) H_2S per 10 ml of medium. Growth was determined by direct cell counting with a Thoma chamber (depth, 0.02 mm).

Cell masses of *Halanaerobium praevalens*, *Halothermothrix orenii*, *Halanaerobacter lacunaris,* and the novel isolates were obtained by growth at 30°C (60°C for *Halothermothrix orenii*) with stirring (100 rpm) in an 80-liter enamel-protected fermentor (Bioengineering, Wald, Switzerland) pressurized with 300 kPa of N_2 (N_2-CO_2 [80:20, vol/vol] for *Halanaerobacter lacunaris* and *Halothermothrix orenii*).

Light and electron microscopy. Light microscopy, electron microscopy, and photography were carried out as described elsewhere (31).

Lipid analyses. Freeze-dried cells (about 1 g) were extracted twice using chloroform-methanol (2:1, vol/vol) under reflux for 1 h. Fatty acid methyl esters (FAME) were prepared from a portion of the total lipid extract by a modification of the mild alkaline methanolysis procedure of White et al. (67), which involves heating at 37°C for 1 h and extraction with hexane-chloroform (4:1, vol/vol). FAME were separated by thin-layer chromatography on Silica Gel G plates (Merck, Darmstadt, Germany) using methylene chloride as the solvent. Gas chromatographic analysis was done using a Perkin-Elmer model Sigma 3B equipped with a flame ionization detector and a DB-5 megabore column (J&W Scientific, Folsom, Calif.), with methyl tricosanoate as an internal standard. Double-bond positions, *cis-trans* configurations, and confirmations of cyclopropane rings were determined with dimethyl disulfide adducts (69) using gas chromatography-mass spectrometry (35).

DNA extraction, PCR, and cloning. Nucleic acids were extracted from 120 ml of the brine-seawater interface sample KT-2 with the IsoQuick Nucleic Acid Extraction kit (ORCA Research, Bothell, Wash.) according to the manufacturer's instructions, followed by RNase treatment for 30 min and precipitation of the nucleic acids with 1 volume of isopropanol. The nucleic acids of the halophilic isolates were extracted as described elsewhere (7). PCR amplifications of the rRNA genes between *Escherichia coli* positions (5) 9 and 1406 or 9 and 1512 (9bF, bacterial primer; 1406uR and 1512uR, universal primers) were carried out as described previously (21). PCR products of sample KT-2 were purified (Microcon 100; Amicon, Witten, Germany), and the 16S rRNA gene fragments were cloned into the pCR2.1 vector (Invitrogen, Leek, The Netherlands) according to the manufacturer's instructions. The resulting ligation products were used to transform *E. coli* TOP10F′ cells. The presence of inserts of the appropriate size in the transformants was identified by direct PCR screening; amplified ribosomal DNA (rDNA) restriction analysis was performed as described previously (21). Representative transformants were selected based on the fingerprinting pattern of the rRNA gene clones, and the corresponding plasmid DNAs were obtained using a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany).

Sequencing of rRNA genes. 16S rRNA gene sequences of the halophilic isolates and 16S rDNA clone sequences of sample KT-2 were sequenced with an ABI Prism 310 capillary DNA sequencer (PE Applied Biosystems, Foster City, Calif.), using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer), at the Institute for Genetics, University of Regensburg, Regensburg, Germany. The bacterial sequences were determined using a set of specific and universal primers (21).

Phylogenetic analyses. For the analyses, an alignment of about 11,000 homologous full and partial primary sequences available in public databases (ARB project [41; W. Ludwig and O. Strunk, http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/arb.ps]) was used. The new bacterial 16S rRNA gene sequences (1,365 to 1,473 nucleotides) were fitted in the 16S rRNA tree by using the automated tools of the ARB software package (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/documentation/arb.ps). Distance matrix (Jukes-Cantor correction), maximum-parsimony, and maximum-likelihood (fastDNAml) methods were applied as implemented in the ARB software package (42, 51). Insignificant branching points were shown by multifurication. Phylogenetic distances were determined using distance matrix analysis without applying a correction factor. Each sequence alignment was checked manually, and the sequences were analyzed with the CHECK_CHIMERA program of the Ribosomal Database Project (43) to detect the presence of possible chimeric artifacts.

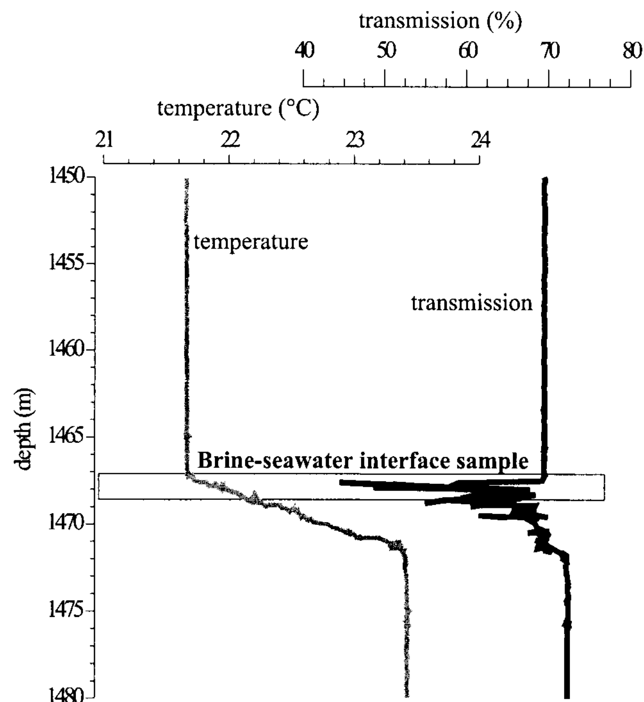

FIG. 3. Transmission-temperature-depth profile of the brine-seawater interface of Kebrit Deep, Red Sea. The salinities at water depths of 1,465, 1,467, 1,469, 1,470, and 1,471 m (corrected depths) are 4.0, 4.2, 17.2, 24.5, and 26.0% (wt/vol) NaCl, respectively.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences were submitted to EMBL and have been assigned accession numbers AJ309519 to AJ309527.

RESULTS

Transmission-temperature-depth profile of the brine-seawater interface. The data were collected during RV *Sonne* cruise SO 121. Figure 3 shows the transmission-versus-depth profile of the Kebrit Deep brine-seawater interface, including data for temperature. At a depth of 1,468 ± 3 m, 240 liters of brine was obtained (station no. 17029-8; 24°43.16′N, 36°16.42′E). The original sample temperature was 22°C, with a pH of 6.5. O_2 was not detected, and the water smelled strongly of H_2S. Over the 1-m length of the niskin bottles the salinity varied from 4.6% at the top to 9.6% at the bottom. Samples KT-2/3 were retrieved from the upper part of this brine-seawater interface, indicated by the beginning of an increase in temperature and salinity (4.6 to 9.6%) and an decrease in transmission (Fig. 3).

Enrichment and isolation. In order to isolate representative microorganisms from the Kebrit brine, enrichment cultures were established in 28-ml serum tubes on board ship by adding different sterile organic and inorganic nutrients directly to the brine. Two successful enrichments designated KT-2/3-3 (from the brine-seawater interface, 1,468 ± 3 m) and KT-8-13 (from a brine surface sediment near the brine-seawater interface) were grown anaerobically in original brine supplemented with 0.1% (wt/vol) thiosulfate and 0.01% (wt/vol) of a mixture of equal parts of yeast extract, meat extract, peptone, and brain heart infusion (C-Org). In the laboratory, the enrichment cul-

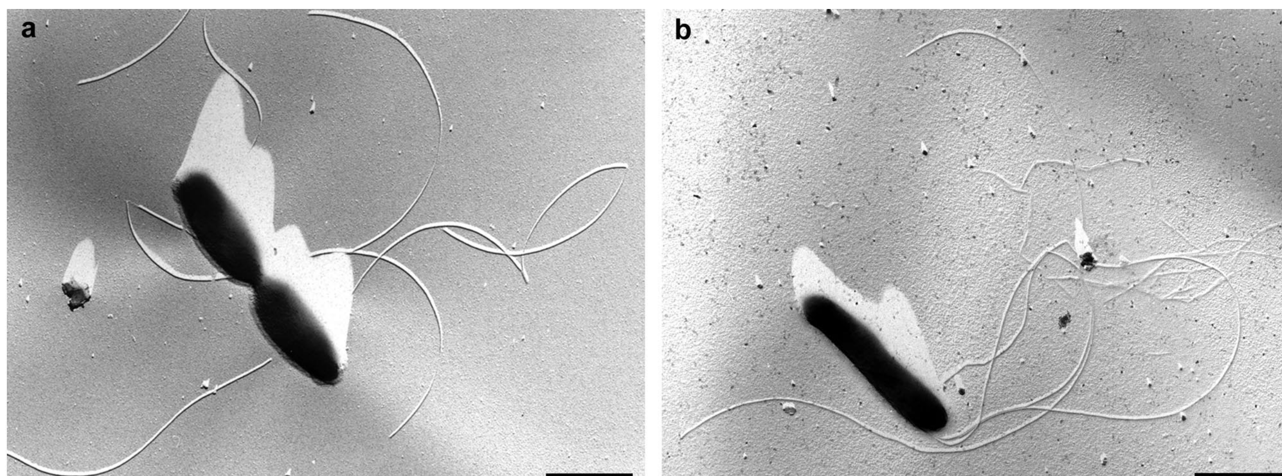

FIG. 4. Transmission electron micrographs of a dividing cell of *Halanaerobium* sp. strain KT-2/3-3 showing flagella (a) and of a single flagellated cell of *Halanaerobium* sp. strain KT-8-13 (b). The cells were air dried and platinum shadowed. Bars, 1 μm.

tures were transferred several times in the original brine and then into synthetic BI medium. From each enrichment culture, a single cell, which was optically trapped by using a strongly focused infrared laser beam ("optical tweezers"), was separated under visual control from the mixed culture and was grown as a pure culture (selected cell cultivation [2, 30, 32]). The pure cultures KT-2/3-3 and KT-8-13 (for simplicity, designated the enrichment cultures) were chosen as representatives for the experiments described below. Unless indicated otherwise, the novel isolates were cultivated in BI medium supplemented with 0.01% C-Org. Several further enrichment cultures resulted in single clones after selected cell cultivation, but they showed 16S rRNA gene sequences identical to that of either KT-2/3-3 or KT-8-13 and therefore were not further characterized (data not shown).

Morphology. Cells of isolates KT-2/3-3 and KT-8-13 were gram-negative, motile rods with rounded ends; the average cell length was 1 to 2 μm, and the average cell width was 0.3 to 0.4 μm. Addition of glucose (0.1%, wt/vol) resulted in an increase of the cell length up to 6.5 μm (for KT-8-13). During growth, cells of the two isolates appeared singly or in pairs. No evidence of spore formation was observed. In transmission electron micrographs, the new isolates exhibited monopolar, polytrichous flagellation with up to three flagella per cell (Fig. 4).

Growth and physiological characterization. Isolates KT-2/3-3 and KT-8-13 grew chemoorganoheterotrophically under strictly anaerobic culture conditions. Both isolates were obligate halophiles, with an optimal NaCl requirement of between 10 and 20% (wt/vol). They grew at NaCl concentrations of between 5 and 34% at pH 6.5. In the presence of H_2 (5%, vol/vol), thiosulfate or S^0 (but not sulfate) was reduced to H_2S without stimulating the growth rate.

Isolate KT-8-13 grew at temperatures of between 18 and 48°C, with an optimum of between 30 and 45°C. KT-8-13 was able to grow heterotrophically on C-Org (0.001 and 0.1%) and brain heart infusion (0.01%). No growth was observed on glucose (0.1%), fructose (0.1%), saccharose (0.1%), maltose (0.1%), lactose (0.1%), xylose (0.1%), betaine (0.1%), cholesterol (0.1%), acetate (0.01%), yeast extract (0.01%), peptone (0.01%) (Merck), Trypticase-peptone (0.01%) (BBL), meat extract (0.01%), or Casamino Acids (0.01%). H_2S concentrations of 0.5 to 50% (vol/vol) were tolerated by isolate KT-8-13.

Cellular fatty acid composition. The cellular fatty acid compositions of isolates KT-2/3-3 and KT-8-13 in comparison with those of representatives of three different halophilic genera are given in Table 1. The novel bacterial halophiles *Halanaerobium praevalens*, *Halothermothrix orenii,* and *Halanaerobacter lacunaris* were cultured under optimal growth conditions (see Materials and Methods) and were harvested in the exponential growth phase. The FAME analysis of the Kebrit *Halanaerobium* isolates, KT-2/3-3 and KT-8-13, showed large amounts of 16:0 and 16:1 fatty acids and minor amounts of 18:0 and 18:1 fatty acids, consistent with the data for *Halanaerobium praevalens* (Table 1) (71). The two Kebrit isolates contained a relatively large proportion of monounsaturated 16:1 with unusual bond positions at Δ7 and Δ11, closely matching the 16:1 isomer composition of *Halanaerobium praevalens. Halothermothrix orenii* contained little unsaturated FAME, while *Halanaerobacter lacunaris* had 16:1 with the commonly encountered Δ9 position but an unusual 18:1 for a bacterium, with the bond at Δ9. Hopanoids were not detected in the organisms analyzed (data not shown).

16S rRNA phylogeny. (i) Halophilic isolates. A comparative analysis of the 16S rRNA gene sequences revealed that isolates KT-2/3-3 and KT-8-13 belong to the *Halanaerobiaceae* within the monophyletic lineage of the *Halanaerobiales* (49, 55). Further sequence alignments and phylogenetic analyses showed the taxonomic and phylogenetic positions of the new isolates to be among those of the members of the genus *Halanaerobium* (Fig. 5). The sequence similarity of isolates KT-2/3-3 and KT-8-13 is 97.1%. The sequence similarities of KT-2/3-3 and KT-8-13 to other *Halanaerobium* species ranged from 95.7 to 99% and from 95.5 to 99.4%, respectively.

(ii) Brine-seawater interface. Specific (9bF) and universal (1406uR) 16S rRNA primers were used to amplify bacterial sequences from bulk DNA derived from the brine-seawater interface sample KT-2 (salinity, 4.6 to 9.6% NaCl). About 40 clones were obtained from the extracted nucleic acids. After

TABLE 1. Comparison of the cellular fatty acid compositions of isolates KT-2/3-3 and KT-8-13 with those of *Halanaerobium praevalens, Halothermothrix orenii*, and *Halanaerobacter lacunaris*

Fatty acid[a]	% in:				
	KT-2/3-3	KT-8-13	*Halanaerobium praevalens*	*Halothermothrix orenii*	*Halanaerobacter lacunaris*
14	0.8	1.2	9.6	3.5	3.7
14:1, Δ7	0.2	0.2	2.9	ND	0.5
14:1, Δ9	0.1	0.0	0.9	ND	ND
*i*15	0.1	<0.1	0.1	0.1	0.2
*a*15	0.1	<0.1	<0.1	0.1	0.2
15	0.2	0.4	0.3	0.2	0.6
15:1, Δ7	0.1	0.1	0.2	ND	ND
15:1, Δ9	0.1	0.1	0.4	ND	ND
*i*16	0.2	0.1	<0.1	0.4	0.2
16	17.3	24.8	17.9	54.9	20.9
16:1, Δ7*cis*	7.3	10.1	12.3	ND	1.2
16:1, Δ9*cis*	34.2	35.0	28.3	1.4	34.2
16:1, Δ9*trans*	8.5	2.2	4.8	0.5	4.9
16:1, Δ11*cis*	20.4	13.3	17.5	ND	1.1
*i*17	0.2	<0.1	0.1	0.2	0.1
*a*17	0.2	0.2	0.1	0.3	ND
17	0.2	0.2	<0.1	0.6	0.5
*cy*17	1.7	1.6	0.2	0.2	0.6
17:1	0.2	0.6	0.1	ND	2.6
*i*18	ND[b]	<0.1	0.1	0.1	ND
*a*18	ND	ND	<0.1	<0.1	ND
18	2.5	1.9	2.7	29.4	5.3
18:1, Δ9*cis*	0.5	1.8	0.9	3.3	10.3
18:1, Δ9*trans*	0.5	0.3	0.1	2.0	2.2
18:1, Δ11*cis*	2.0	5.0	0.4	1.0	1.0
18:1, Δ11*trans*	0.6	ND	ND	0.1	0.4
18:1, Δ13*cis*	1.4	0.4	ND	ND	ND
*i*19	ND	ND	ND	0.3	1.4
*a*19	0.5	0.4	0.1	1.2	7.5
19	ND	ND	<0.1	0.2	0.4

[a] Fatty acids are designated by total number of carbon atoms:number of double bonds. The number after Δ indicates the position of the double bond relative to the carboxylic (Δ) end of the molecule, with *cis* and *trans* geometry indicated. *cy*, cyclopropyl ring; *i* and *a*, *iso*- and *anteiso*-branched fatty acids, respectively. The concentrations of total fatty acids of KT-2/3-3, KT-8-13, *Halanaerobium praevalens, Halothermothrix orenii*, and *Halanaerobacter lacunaris* were 160.9, 228.2, 1,017.4, 187.3, and 45.6 μg g (dry weight)$^{-1}$, respectively.
[b] ND, none detected.

cloning, the 16S rRNA gene fragments were further characterized by restriction endonuclease digestion. Based on a comparison of the restriction patterns on agarose gels, eight different bacterial groups were identified. From a representative of each restriction pattern group, the 16S rRNA gene sequence was determined and aligned with 16S rRNA sequences derived from the ARB database. Clone sequences KT-2K20 and KT-2K29 (representing 26% of the clones) were chimeras: KT-2K20 of KT-2K1 and KT-2K23/KT-2K28, and KT-2K29 of KT-2K1 and KT-2K38/KT-2K12.

The analysis of the clone sequences KT-2K1, KT-2K12, KT-2K23, KT-2K28, KT-2K34, and KT-2K38 showed a high phylogenetic diversity within the bacterial domain. The phylogenetic positions of the derived clone sequences were supported by the different tree reconstruction methods (see Materials and Methods). KT-2K23 and KT-2K28 (together, 41% of the derived clones) were affiliated with cultivated members of the genus *Halanaerobium* (Fig. 5). They showed highest sequence similarity to *Halanaerobium* sp. strain KT-8-13 (99.9%), *Halanaerobium fermentans* (99.3%), and *Halanaerobium* sp. strain KT-2/3-3 (97%). Sequence clones KT-2K1, KT-2K12, and KT-2K38 (representing 26% of the clones) showed the closest relationship to *Clostridium subterminale* or *Propionibacterium acnes*, whereas KT-2K34 (7% of the clones) is related to sequence clone PVB OTU 4 (U15116) within the γ-proteobacteria. The G+C contents of the rRNA gene sequences range from 51 to 57%.

DISCUSSION

In brine-filled Red Sea deeps, the brine-seawater interface represents a unique microbial ecosystem mainly determined by a steep salt gradient (4 to 26% NaCl) within only a few meters. The combination of salt, oxygen, and pH gradients may be responsible for organisms specifically adapted to this environment. To date, nothing is known about the morphological and physiological features of these organisms. The presence of microorganisms in hypersaline brines (e.g., the Gulf of Mexico and the Mediterranean Sea) was suggested by biochemical investigations which included measurements of ATP or CH_4, lipid analysis, and epifluorescence microscopy (17, 20, 40, 54, 68). The novel halophilic isolates from the Kebrit Deep (KT-2/3-3 and KT-8-13) are the first organisms to have been cultivated and isolated from a brine-seawater interface or the deeper anaerobic, brine pool.

Based on 16S rRNA gene sequence comparisons, these Ke-

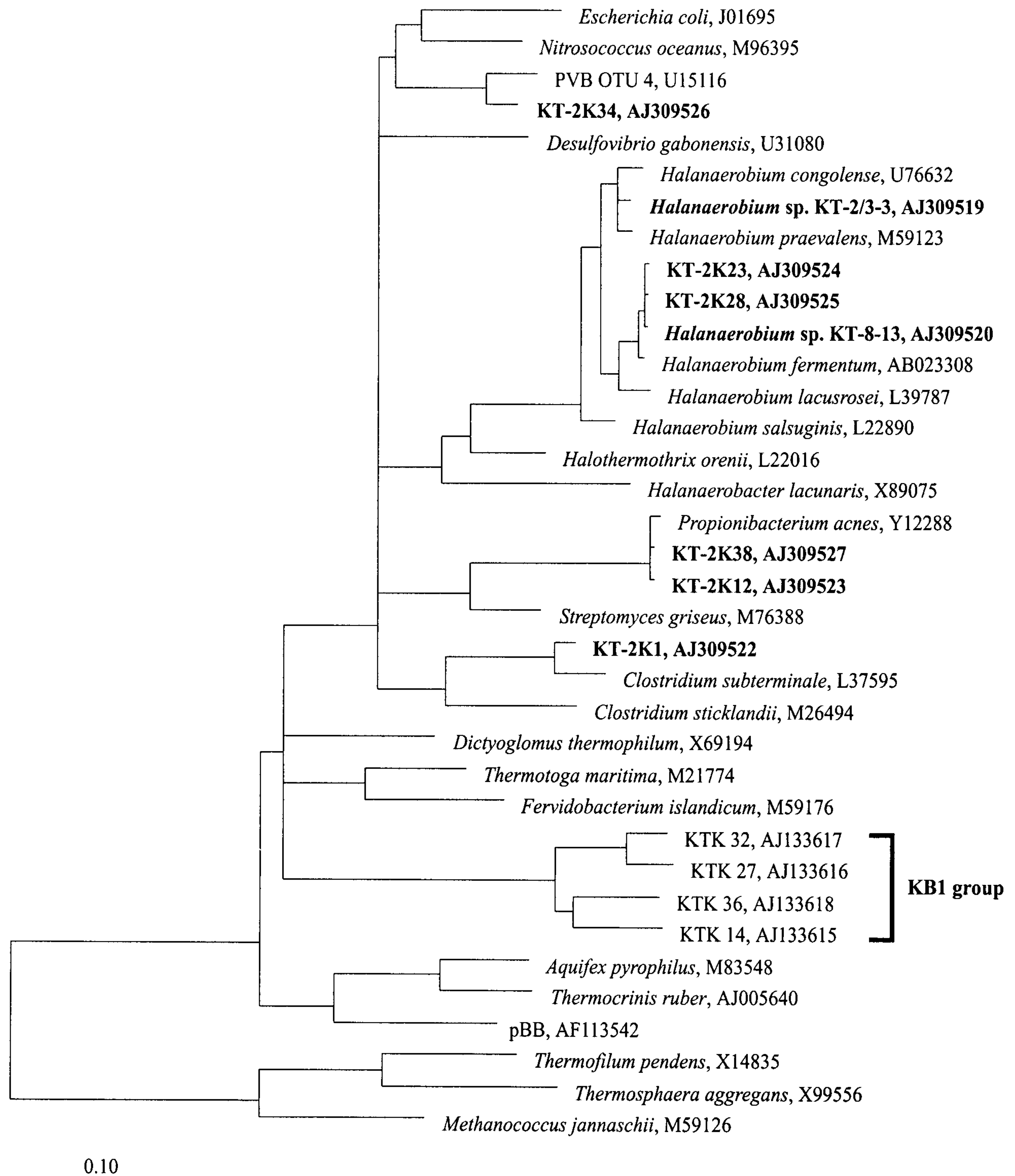

FIG. 5. 16S rRNA gene-based phylogenetic tree of the bacterial domain, including the 16S rDNA sequences from brine-seawater interface sample KT-2 and the 16S rRNA gene sequences of the new halophilic isolates from Kebrit Deep, Red Sea. The KB1 group marks a cluster of closely related environmental sequences, which were obtained from a sediment sample (depth, 1,515 m) of the Kebrit Deep (21). The topology of the tree is based on results of a maximum-parsimony analysis. Reference sequences were chosen to represent the broadest diversity of *Bacteria*. Only sequence positions that share 50% or more residues identical to the 16S rRNA sequences of a corresponding group were included for tree reconstruction. Accession numbers for the sequences are indicated. The scale bar represents 0.10 fixed mutation per nucleotide position.

brit isolates were identified as members of the *Halanaerobiales*, a group of anaerobic, halophilic, fermentative bacteria. The *Halanaerobiales* represent a separate lineage in the bacterial domain (49, 55). Within this order, the isolates KT-2/3-3 and KT-8-13 showed the closest relationship to cultivated members of the genus *Halanaerobium* and most likely comprise new species in the genus (3, 9, 38, 49, 55, 56, 71). Prior to our investigations, members of the genus *Halanaerobium* have been shown to occur only in sediments from salt lakes and offshore oil fields; this study thus increases our knowledge of the ecological distribution of these organisms (3, 9, 55, 56). The results of the FAME analysis for the new *Halanaerobium* species are consistent with the 16S rRNA data in that the new species exhibit fatty acid patterns similar to those of *Halanaerobium praevalens* (71). The FAME are dominated by 16:0 and 16:1 and minor amounts of 18:0 and 18:1. In contrast to *Halothermothrix orenii*, the new isolates show the presence of large amounts of unsaturated fatty acids (Table 1) (10). The presence of relatively large amounts of Δ7 and Δ11 16:1 is very unusual and may serve as a biomarker for *Halanaerobium*. Small amounts of Δ11 16:1 are also present in type I methanotrophs; however, these bacteria also contain Δ8, Δ9, and Δ10 16:1 (33, 34). Like most other *Halanaerobium* representatives, *Halanaerobium* sp. strains KT-2/3-3 and KT-8-13 grew over a wide NaCl range (5 to 34% NaCl). This physiological flexibility allows the organisms to grow within the salt gradient of the brine-seawater interface and in the highly saline lower brine pool. The brine-seawater interface also exhibits a density gradient which acts as an in situ particle trap for organic material (Fig. 3) (29, 40, 57, 58, 66), providing an appropriate environmental niche for heterotrophic bacteria. Indeed, these chemoorganotrophic halophiles may contribute significantly to the anaerobic degradation of suspended organic material enriched at the brine-seawater interface of the Kebrit Deep. However, the occurrence of *Halanaerobium* representatives is not restricted to the Kebrit Deep. Recently, a new member of the genus *Halanaerobium* (isolate S5L4, AJ309521) was obtained from the brine-seawater interface (salinity, 24.2%) of the Shaban Deep, Red Sea (W. Eder and R. Huber, unpublished results).

For phylogenetic analysis, the brine sample was concentrated anaerobically on board ship using a crossflow tangential filtration unit operated under a protective CO_2 atmosphere (Fig. 2). Two hundred forty liters of water of the brine-seawater interface was concentrated about 400-fold. Anaerobic sampling of the brine is essential to circumvent the precipitation of reduced inorganic compounds such as Fe(II) in the presence of O_2 or the toxic effect of O_2 on strict anaerobes like methanogens (60, 70). From the concentrated brine sample KT-2, the nucleic acids were extracted, and the 16S rRNA gene sequences were PCR amplified and cloned. The 16S rRNA gene fragments were further characterized by amplified rDNA restriction analysis, and different restriction pattern groups were identified. The phylogenetic analysis of a representative of each restriction group showed that the majority of the sequences had high sequence similarity to cultivated microorganisms within the bacterial domain. From the same bulk DNA, archaeal PCR products were obtained, but these were not further investigated for this study (Eder and Huber, unpublished).

Using different tree reconstruction methods (see Materials and Methods), the majority of the bacterial clone sequences represented by KT-2K23 and KT-2K28 showed the closest relationship with members of the genus *Halanaerobium* (55) and exhibited the highest sequence similarity to the isolated *Halanaerobium* sp. strain KT-8-13. Although KT-8-13 was enriched from the sedimentary portion of the brine pool, this sequence was not detected in a previous in situ analysis of the brine-sediment interface (21). That phylogenetic analysis identified a deep-branching KB1 sequence group (Fig. 5) (21). This KB1 group could not be retrieved from the brine-seawater interface sample, suggesting that representatives of the KB1 group are specifically adapted to the higher salinities within the lower brine body (Fig. 3) and the sediments (the salinity of the pore water is up to 26%), while KT-8-13, like KT-2/3-3, is more naturally adapted to the interface environment.

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