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Community Composition of a Hypersaline Endoevaporitic Microbial Mat

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A hypersaline, endoevaporitic microbial community in Eilat, Israel, was studied by microscopy and by PCR amplification of genes for 16S rRNA from different layers. In terms of biomass, the oxygenic layers of the community were dominated by *Cyanobacteria* of the *Halothece*, *Spirulina*, and *Phormidium* types, but cell counts (based on 4′,6′-diamidino-2-phenylindole staining) and molecular surveys (clone libraries of PCR-amplified genes for 16S rRNA) showed that oxygenic phototrophs were outnumbered by the other constituents of the community, including chemotrophs and anoxygenic phototrophs. Bacterial clone libraries were dominated by phylotypes affiliated with the *Bacteroidetes* group and both photo- and chemotrophic groups of α-proteobacteria. Green filaments related to the *Chloroflexi* were less abundant than reported from hypersaline microbial mats growing at lower salinities and were only detected in the deepest part of the anoxygenic phototrophic zone. Also detected were nonphototrophic γ- and δ-proteobacteria, *Planctomycetes*, the TM6 group, *Firmicutes*, and *Spirochetes*. Several of the phylotypes showed a distinct vertical distribution in the crust, suggesting specific adaptations to the presence or absence of oxygen and light. *Archaea* were less abundant than *Bacteria*, their diversity was lower, and the community was less stratified. Detected archaeal groups included organisms affiliated with the *Methanosarcinales*, the *Halobacteriales*, and uncultured groups of *Euryarchaeota*.

Photosynthetic communities containing distinct horizontal layers of oxygenic and anoxygenic phototrophs often cover the bottom of hypersaline ponds used for the production of sea salt. The physical appearance of these microbial communities varies according to salinity (55). At salinities below ca. 15%, the photosynthetic communities tend to form compact and highly active microbial mats on the surface of the pond floor, whereas in ponds containing between 15 and 25% salt, the mats are less compact since the photosynthetic layers tend to be embedded within the crystalline salt crust on the pond bottom. Such endoevaporitic gypsum- or halite-associated microbial systems have been reported from a number of solar salterns around the world, including those from the coasts of the Gulf of Mexico (55, 66), the Mediterranean Sea (12), the Baja Peninsula (60), and the Red Sea (53). In endoevaporitic microbial ecosystems associated with gypsum deposits, several layers of oxygenic and anoxygenic phototrophs are often visible, indicating some level of stratification in the populations. In photosynthetic communities growing within halite deposits, a vertical banding of the photosynthetic populations is still observed, but the complexity of the communities seems diminished compared to populations growing within gypsum (60).

The microbial ecology of hypersaline, endoevaporitic communities is interesting for a number of reasons. Some of the earliest evidence for life in the geologic record is preserved in the isotopic composition of organic carbon and sulfur species in 3.5 billion-year-old barite, originally deposited as gypsum, at North Pole, Australia (62). The endoevaporitic communities found today in solar salterns are likely modern homologues to the microbial communities once housed in these ancient sulfate deposits. The solar salterns may provide information about the prerequisites necessary for life to develop in evaporitic, sulfate-rich environments as once existed on the Martian surface (67, 68). Finally, endoevaporitic communities grow in a salinity range where numerous physiological groups are excluded, potentially altering the biogeochemical cycling in the system (48).

The endoevaporitic microbial communities in the salterns of Eilat have previously been studied in terms of light penetration, oxygen and sulfur biogeochemistry, and the salt tolerance of phototrophs, sulfate reducers, and methanogens (8, 53, 65). The upper oxygenic zone, which is typically 1 to 2 cm deep, consists of two layers of *Cyanobacteria*, sometimes separated by a white layer devoid of phototrophs, overlying a layer containing various anoxygenic phototrophs. The cell volume-specific photosynthesis rates are comparable to microbial mat communities from other environments, but the lower cell density results in lower overall oxygen production rates (8). This, and the deeper distribution of the phototrophs, results in a much decreased gas exchange with the overlying water such that the major part of oxygen produced in the crust is also consumed within the crust, mainly by heterotrophic organisms. The salinity tolerance of *Cyanobacteria*, anoxygenic phototrophs, sulfate reducers, and methanogens were in agreement with what has been observed in pure cultures (65). Thus, sulfate reducers

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and anoxygenic photoautotrophic organisms in the crust are inhibited at salinities greater than 12 and 15%, respectively, whereas methanogens and *Cyanobacteria* are not inhibited at the in situ salinity.

The purpose of the present study is to supplement these previous investigations with a broader phylogenetic overview of the organisms present in the crust. This should allow us to identify the major components of the heterotrophic community, which are not easily identified by microscopy, and to compare the phylogenetic composition of this community with other benthic phototrophic microbial systems.

MATERIALS AND METHODS

Site description and sampling. The salterns of the Israel Salt Industries, Ltd., in Eilat consist of a series of ponds through which seawater and water desalination refuse brines are passed as they increase in salinity due to evaporation. An endoevaporitic, phototrophic community develops within the gypsum crust covering the bottom of a number of these ponds, where salinities range from ca. 15 to 25%. The pond chosen for the present study had a salinity of 20%, and the crust varied in thickness between 1 and 10 cm. The microbial populations in this crust had a well-developed laminated benthic phototrophic community resembling previous descriptions (8, 53, 65). Thus, the uppermost 2 to 6 mm of the crust was brownish in color (B layer), below which a 2- to 8-mm-thick white zone appeared to be devoid of phototrophs (W layer). A bright green layer was situated underneath the white layer (G layer), and finally a purple layer, sometimes overlying a deeper thin, olive-green layer, marked the bottom of the phototrophic community (P/O layer). Underneath the purple or olive-green layer, the crust and the underlying sediment were highly odorous and black or grayish due to sulfide accumulation (S layer). Pieces of crust of ca. 15 by 15 cm were sampled by using a hammer, chisel, and saw. Individual layers were isolated with a forceps, and 1- to 2-g samples were either frozen in liquid nitrogen for later DNA extraction or fixed in paraformaldehyde for microscopic analysis.

Microscopic examinations. Unless otherwise stated, the chemicals used were purchased from Fisher Scientific. Crust samples from each layer were gently disaggregated in a mortar, and weighed subsamples were distributed into 50-ml centrifuge tubes. After centrifugation (6,000 × *g*, 10 min), the supernatant liquid was removed, and 10 ml of sterile-filtered pond water (0.2-μm pore size; Cameo25AS [Osmonics, Inc.]) containing 3% (wt/vol) paraformaldehyde was added. Each tube was shaken gently and let to sit for 4 h at 4°C. After this fixation step, the tubes were centrifuged, and the pellets were washed three times with sterile-filtered pond water. Finally, the pellet was resuspended in a 1:1 mixture of filtered pond water and ethanol and stored at −20°C. For cell counts, fixed samples were briefly vortexed and allowed to settle for ca. 30 s. Afterward, 10 to 100 μl of the water phase was collected with a pipette, mixed with 5 ml of distilled water, and filtered onto polycarbonate filters (0.22-μm pore size, 25-mm diameter [Osmonics, Inc.]) mounted in a filtering system. The filters were stained for 10 min with the DNA-specific stain 4,6-diamidino-2-phenylindole (DAPI) in a 1-μg ml^{-1} solution (56) and subsequently washed twice by filtering 5 ml of distilled water through each filter. The density of cells on each filter was quantified by using a fluorescence microscope (Leica Microsystems DMCB). The biovolume of each group was calculated from cell numbers and estimated average cell sizes.

DNA extraction procedure. Genomic DNA was extracted from samples of the W, G, P/O, and S layers. Samples of 0.1 to 0.5 g were mixed with 900 μl of lysis buffer (50 mM Tris, 25% [wt/vol] sucrose; pH 8) and 60 μl of sodium dodecyl sulfate solution (10% [wt/vol]) and subjected to three cycles of freezing-thawing in liquid nitrogen and in a water bath at 65°C. Proteinase K (42 μl of a 20-mg ml^{-1} stock solution) was added, and the sample was incubated at 55°C for 60 min. The DNA was extracted after addition of 1 volume phenol-chloroform-isoamyl alcohol (25:24:1, pH 7.9) by brief vortexing, followed by centrifugation (10,000 × *g*, 10 min). The aqueous phase from each sample was mixed with 0.1 volume of 5 M NaCl and two volumes of 96% (vol/vol) ethanol and left overnight at −80°C. Each sample was centrifuged (10,000 × *g*, 40 min, 4°C), and the DNA pellet was washed with 70% ethanol. Finally, the extracted DNA was stored at −80°C in autoclaved Milli-Q water.

Cloning and sequencing. PCRs were performed with the primers bac8f (AG(A/G)GTTTGATCCTGGCTCAG) and bac1492r (CGGCTACCTTGT TACGACTT) for amplification of bacterial genes for 16S rRNA or with the primers arc8f (TCCGGTTGATCCTGCC) and arc1492r (GGCTACCTTGTT ACGACTT) for archaeal genes. *Taq* DNA polymerase and PCR buffer B were purchased from Promega, Madison, WI. PCRs of 50 μl containing 1.5 mM Mg^{2+} were prepared with 1 U of *Taq* polymerase, 10 nmol of deoxynucleoside triphosphate, and 50 pmol of each primer (Promega). Between 1 and 5 μl of the DNA extractions was added, and PCRs were performed in an Eppendorf thermocycler using cycles consisting of a 45-s 94°C denaturing step, a 45-s annealing step at 58°C, and a 3-min elongation step at 72°C. In general, 28 and 32 PCR cycles were necessary in order to obtain sufficient DNA using the *Bacteria*- or *Archaea*-specific primers, respectively. PCR products were gel purified on 1% (wt/vol) low-melting-point agarose gels. About 50 ng of PCR product was loaded on the gel, run for 30 min at 70 V, and stained with SYBR gold (Invitrogen Corp., Carlsbad, CA). The PCR products were visualized on a Dark Reader transilluminator (Clare Chemicals, Dolores, CO) and excised, and DNA was extracted from the gel by using the QIAEXII gel extraction kit according to the manufacturer's instructions. The purified DNA samples were A-tailed to improve cloning efficiency by mixing the purified PCR product (40 μl) with 5 μl of adenosine deoxynucleoside triphosphate (2 mM), 5 μl of 10× PCR buffer, and 1 U of *Taq* DNA polymerase (Promega). The mixture was incubated at 72°C for 10 min, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and centrifuged at 6,000 × *g* for 5 min. The aqueous phase was then transferred to a fresh microcentrifuge tube and precipitated with 2.5 volumes of ethanol and 0.1 volume of 5 M NaCl. After centrifugation (10,000 × *g*, 10 min, 4°C), the pellet was washed once with 20 μl of 70% ethanol, air dried, and resuspended in 4 μl of PCR water. These PCR products were cloned using the TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA) and transformed by electroporation according to the manufacturer's specifications. After plating on LB agar and overnight incubation at 37°C, individual colonies were picked randomly for sequencing of plasmid inserts. Sequence data were obtained at the sequencing facility at Marine Biological Laboratory in Woods Hole, with the sequencing primers M13F (5′-GTA AAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′). Forward and reverse reads were assembled, aligned, and checked for chimeric structures by using the Bellerophon program (26), as well as the CHIMERA_CHECK application on the rdp Web site (http://rdp.cme.msu.edu/index.jsp).

Phylogenetic analysis. Sequence data were BLAST analyzed against the GenBank 16S rRNA database (1). The sequences were then aligned with their closest relatives and representative cultured and uncultured *Bacteria* and *Archaea* by using the CLUSTAL W program, followed by manual alignment in Seqpup (21). Phylogenetic trees were constructed from the alignment sequences by using Jukes-Cantor distance matrices for inferring the tree topology and neighbor joining and maximum-parsimony for bootstrap analysis (1,000 replicates) of the branching pattern using the PHYLIP software package (17).

DGGE analysis. For denaturing gradient gel electrophoresis (DGGE)-analysis, rRNA gene fragments of about 150 bp were amplified by using primers 341f [CCTACGGG(A/G)GGCAGCAG] and 521r (ACCGCGGCTGCTGGCAC) for *Bacteria* and primers 344f [ACGGGG(C/T)GCAGCAGGCG] and 518r [GGT(A/G)TTACCGCGGCGGCTG] for *Archaea* (modified from Muyzer et al. (42) and Øvreås et al. (53a). Both forward primers were supplied with a GC-clamp (CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGG GGGG) at the 5′ end. The reaction mixture and PCR program were as described above, except for the duration of the elongation step, which was decreased to 1 min, and the annealing temperature, which was increased to 60°C. The PCR products were separated by DGGE on a Bio-Rad Dcode system (42). Two stock solutions were prepared, representing 0 and 100% denaturing agent, respectively. The 0% solution consisted of 10% (wt/vol) acrylamide-bisacrylamide (37.5:1) in 0.5× Tris-acetic acid-EDTA buffer (TAE), and the 100% solution consisted of 10% (wt/vol) acrylamide-bisacrylamide, 420 g of urea $liter^{-1}$, and 400 ml of formamide $liter^{-1}$ in 0.5× TAE. The DGGE gels were cast by using mixtures of these stock solutions in linear denaturing gradients with 40% denaturing agent in the top and 70% in the bottom of the gels. The wells in each gel were loaded with 10 μl of PCR products, and the gels were run for 18 h at 70 V and 60°C. The gels were stained for 30 min in 0.5× TAE buffer containing 1:10,000 SYBR-gold (Bio-Rad) and evaluated on a Dark Reader transilluminator (Clare Chemicals, Dolores, CO). Individual bands were excised and reamplified, and 5 μl of the new PCR products was run on another DGGE gel together with the original sample to confirm the identity and purity of the excised DNA. Reamplified bands were sequenced at the DNA sequencing facility at the School of Medicine at the University of North Carolina at Chapel Hill.

RESULTS

Microscopy and enumeration of the microorganisms in the crust. We divided the organisms into the following categories:

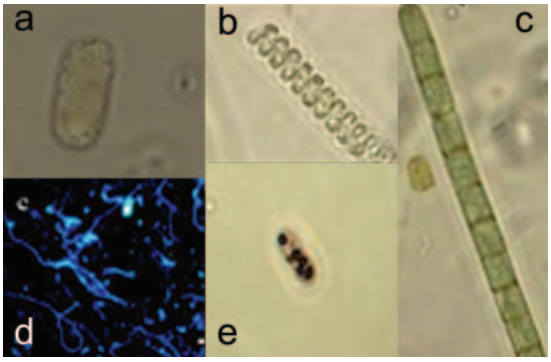

FIG. 1. Phase-contrast and epifluorescence micrographs of organisms in the gypsum crust. (a) *Halothece*-like unicellular cyanobacterium from the upper brown layer; (b) *Spirulina*/*Halospirulina*-like cyanobacterium from the green layer; (c) *Phormidium*-like and *Halothece*-like *Cyanobacteria* from the green layer; (d) DAPI stain of a P/O layer sample showing the abundant thread-like organisms and various small morphotypes; (e) *Halochromatium*-like bacterium with internal granules (presumably sulfur).

(i) unicellular *Halothece*-like *Cyanobacteria* (20); (ii) green filaments with red autofluorescence during epifluorescence microscopy; (iii) organisms resembling *Halochromatium* (28) with intracellular globules (presumably sulfur); (iv) thread-like organisms with no autofluorescence; and (v) unicellular, small morphotypes, including vibrios, cocci, and oval cell shapes. Representatives from each of these groups are shown in Fig. 1. Each group was counted after DAPI staining, and the resulting cell numbers and estimated biovolumes are summarized in Table 1.

Small unicellular or thread-like organisms constituted 88% of the population in the B layer. However, the *Cyanobacteria* accounted for the major part of the biovolume in the layer due to their larger cell size (Table 1). All of the *Cyanobacteria* observed in this layer resembled members of the *Halothece* cluster. The W layer that separated the two oxygenic layers in the crust was comprised entirely of small unicellular prokaryotes, and no obvious phototrophs were observed. The brightly colored G layer, located just below the W layer, consisted of 28% by numbers of *Cyanobacteria*, 24% thread-like, nonfluorescing organisms resembling their counterparts from the brown layer, and 48% small organisms of various morphotypes. The filamentous *Cyanobacteria* were straight or coiled with only one trichome per sheath and cell diameters from 1 to 6 μm. Heterocysts were not observed. Thus, they appeared to be members of the *Oscillatoriales* (75). The unicellular *Cyanobacteria* in this layer resembled those from the upper brown layer. As in the B layer above, the *Cyanobacteria* dominated in terms of biovolume. In the P/O layer, prokaryotes resembling purple anoxygenic phototrophic *Halochromatium* spp. with internal sulfur granules constituted 6% of the prokaryotes. Green, usually autofluorescing filaments ranging in diameter from <1 μm to about 6 μm accounted for another 4% of the community. Small, unicellular morphotypes made up more than 99% of the community in the permanently sulfidic S layer.

Cloning and sequencing of bacterial 16S rRNA genes. A total of 576 clones were sequenced, yielding 407 high-quality sequences, of which 147, 133, and 127 were from the W, G, and P/O layers, respectively. Sequences that were >99% similar were considered to have the same phylotype. The bacterial phylotypes detected are summarized in Tables 2 to 4, and phylogenetic trees constructed by Jukes-Cantor and neighbor-joining are shown in Fig. 2. Both in terms of absolute numbers and diversity, the *Bacteroidetes* and the α-proteobacteria were the two most prominent groups of organisms represented in the clone libraries. Together, these two groups accounted for more than three-quarters of the clones and phylotypes detected in any layer. As is evident in Fig. 2A and B, including the *Bacteroidetes*-related and α-proteobacteria-related phylotypes, there was considerable diversity within each of the groups, although a few phylotypes were particularly abundant in the clone libraries. Phylotype E2aA01 affiliated with the "*Flavobacteriales*" constituted 73% of the *Bacteroidetes*-related clones and 37% of the total clone library from the white layer but was rarely detected among clones from the layers below. E4aG09, a close relative of the halophilic, aerobic genus *Salinibacter*, typically found in the hypersaline water of the salterns (2, 51), was abundant in the clone library form the G layer but rare in the clone library from the permanently anoxic P/O layer. The α-proteobacteria-related phylotype E6aD01 dominated the P/O layer with 34% of the total number of clones and 61% of the α-proteobacteria-related clones. This phylotype was affiliated with the genus *Roseospira* of phototrophic purple nonsulfur bacteria. Another α-proteobacterial phylotype, E6aD10, was abundant in the W and G layers of the crust. Together with some less frequent phylotypes, it formed a phylogenetically coherent cluster, branching from the root of the "*Rhodobacterales*."

A number of γ- and δ-proteobacteria were also detected in the crust. The phylotypes of δ-proteobacteria were members of predominantly sulfate-reducing groups such as the "*Desulfovibrionales*" and the "*Desulfobacterales*." They constituted 1, 7, and 4% of clone libraries from the W, G, and P/O layers, respectively.

TABLE 1. Results of cell counts from different layers of the crust[a]

Layer[b]	Total cell no. (10^6 ml^{-1})	Unicellular *Cyanobacteria*		Green filaments		*Halochromatium*		Thread-like cells		Other cells	
		% No.	% Vol	% No.	% Vol	% No.	% Vol	% No.	% Vol	% No.	% Vol
B	19	12	79					36	18	52	3
W	22									100	100
G	70	2	3	26	92			24	4	48	1
P/O	312			4	54	6	17	45	26	45	3
S	93							<1	<1	>99	>99

[a] % No., fraction of cell number; % Vol, fraction of biovolume.
[b] B, surface brown layer; W, white layer; G, green layer; P/O, purple/olive-green layer; S, deep sulfidic layer.

TABLE 2. Summary of *Bacteroidetes*-affiliated phylotypes

Phylotype	GenBank accession no.	No. of times encountered[a]			Closest relative in BLAST search[b]	% Similarity[c]
		W (n = 75)	G (n = 41)	P/O (n = 27)		
Salinibacter and relatives						
E4aG09	DQ103651	7	23	1	*Salinibacter ruber*, AF323499	91
"*Sphingobacteriales*"						
E2aB05	DQ103648	4	1		Soil clone 67C12, AF245039	89
E2aC08	DQ103646	1	1		Plankton clone GKS2-217, AJ290034	90
E2aE07	DQ103649	1			*Flexibacter tractuosus*, AB078074	85
E2aH11	DQ103633	1			Unc. soil bact. C1120, AF507626	90
E2bA03	DQ103636	2			Sediment clone EKHO-12, AF142895	87
E4aE03	DQ103650		1		*Flexibacter aggregans*, AB078038	88
E4bF06	DQ103635		2		Biofilter clone Blfdi48, AJ318130	89
E6aA03	DQ103634			3	Mono Lake clone ML617.5J-5, AF507867	96
E6aC11	DQ103647			2	Unc. bact. PHOS-HE19, AF314428	90
"*Flavobacteriales*"						
E2aA01	DQ103638	55	2	3	Mono Lake clone ML602J-15, AF507868	91
E2aA05	DQ103642	3	6	4	Atacama clone NCh17-AT12, AJ487527	97
E4aA01	DQ103640		1		Atacama clone NCh17-AT12, AJ784527	93
E4aE04	DQ103637	1	2	5	Mono Lake clone ML617.5J-5, AF507867	96
E4aF11	DQ103639		1	3	Consortium clone SB-5, AF029041	90
E6aC02	DQ103645		1	2	Unc. bact. 44-UMH 22% pond, AF477878	95
E6aF03	DQ103644			2	Consortium bact. SHA-38, AJ249105	87
E6aH07	DQ103641			1	Arctic bact. ARK10144, AF468421	91
"*Bacteroidales*"						
E6aE11	DQ103643			1	Hypersaline clone BBBB-40, AY226247	89

[a] n = total number. W, G, and P/O are as defined in Table 1.
[b] Unc., uncultured; bact., bacterium.
[c] Calculated based on the entire query or result sequence without truncation.

The most abundant phylotype affiliated with the γ-proteobacteria, E2aA03, was detected only in the white layer. It was a member of the genus *Thioalkalivibrio*, which consist of chemoautotrophic sulfur-oxidizing organisms (63). Another phylotype, E2bG06, was also a member of the *Ectothiorhodospiraceae* but could not be assigned to a specific genus.

Three phylotypes were affiliated with the *Cyanobacteria*. These included E4aB08 and E4bG02, both affiliated with the *Halothece* cluster, and E6aG07, with a more uncertain affiliation (Fig. 2C). Other components of the community detected included organisms related to the *Spirochetes*, *Chloroflexi*, *Planctomycetes*, candidate division TM6 (5), and the order *Halanaerobiales* of the phylum *Firmicutes* (49).

Cloning and sequencing of archaeal 16S rRNA genes. Archaeal genes for 16S rRNA were amplified from the P/O layer. A total of 96 clones were sequenced, resulting in 53 archaeal sequences that were distributed among 16 phylotypes, all affiliated with the *Euryarchaeota* (Table 5 and Fig. 3). The phylotypes ArcG01, ArcF12, ArcH05, ArcA11, ArcH07, and ArcG09 were members of the order *Halobacteriales* and accounted for 19% of the archaeal clones sequenced. One phylotype (ArcB06), encountered three times among the 53 sequences, was closely related to the genus *Methanohalophilus* within the *Methanosarcinales* (61).

The majority of the phylotypes were affiliated with uncultured groups of *Archaea*. Thus, the most frequently encountered phylotypes were members of the marine benthic group D (MBGD) euryarchaeotes (73). Together, these phylotypes accounted for 64% of the archaeal clones. Other groups of uncultured *Archaea* detected in the present study included the marine group III (18), the MSBL-1 group (72), and phylotypes forming two clusters (halophilic clusters 1 and 2) that also included sequences from pond water in a Mediterranean solar saltern (4) and hypersaline sediments of the Kebrit Deep (15).

Molecular fingerprinting. Examples of the resulting DGGE gels are shown in Fig. 4. Gels run with DNA amplified with the *Bacteria*-specific primer set demonstrated a changing bacterial population with depth in the crust, whereas the archaeal community seemed more homogeneous with depth. Figure 5 illustrates the succession of bacterial bands through the W, G, P/O, and S layers. DGGE bands were excised, sequenced, and aligned with the phylotypes obtained from clone libraries. Three bacterial *Bacteroidetes* phylotypes and three archaeal phylotypes of the *Halobacteriales* or the halophilic archaeal cluster 2 were found again on the DGGE gels this way (Fig. 4). The DGGE band pattern suggested that phylotype E2aH11, retrieved once from the W layer, was present in the other layers as well. The band affiliated with phylotype E2aA05, which was common in all three clone libraries, was strong in the W, G, and P/O layer but barely visible in the deep, permanently sulfidic S layer. Finally, the apparent preference of phylotype E6aC02 for the deeper layers of the crust was confirmed by the DGGE pattern. The three archaeal phylotypes detected during DGGE analysis were all present throughout the crust.

TABLE 3. Summary of phylotypes affiliated with the α-proteobacteria

Phylotype	GenBank accession no.	No. of clones per layer[a]			Closest relative in BLAST search[b]	% Similarity[c]
		W (n = 52)	G (n = 65)	P/O (n = 71)		
"*Sphingomonadales*"						
E2aB09	DQ103610	1			Unc. bact. HOClCi63, AY328612	98
Rhodospirillaceae						
E2aC02	DQ103608	1			*Rhodovibrio salinarum*, D14432	92
E4aB02	DQ103606		5		*Rhodovibrio salinarum*, D14432	93
E4aE08	DQ103607	7	10	1	*Rhodovibrio salinarum*, D14432	97
E4aF06	DQ103605		2	1	*Rhodovibrio salinarum*, D14432	93
E4bC07	DQ103604		1		*Rhodovibrio salinarum*, D14432	92
E6aD01	DQ103603		2	43	*Roseospira* sp. strain AT2115, AJ401208	97
E6aH10	DQ103609			3	*Roseospira* sp. strain AT2115, AJ401208	89
"*Rhodobacteraceae*"						
E2aC04	DQ103626	2			*Jannaschia* sp. strain DFL-36, AJ534220	90
E2aE09	DQ103625	1			Sediment clone JTB359, AB015247	94
E2aG04	DQ103623	1			Hypersaline lake clone LA4-B3, AF513932	93
E4aB07	DQ103612	7	8	1	Hypersaline lake clone LA1-B32N, AF513928	94
E4aB11	DQ103616		1		*Roseovarius tolerans*, Y11551	96
E4aC05	DQ103627		2		*Tetracoccus cechii*, Y09610	91
E4aC11	DQ103613	1	4	1	*Rhodobacter* sp. strain AP-10, AB079681	92
E4bD01	DQ103624		1		Mixolimnion bact. ML42, AJ315683	92
E4bG07	DQ103620		1		*Oceanicola batsensis*, AY424898	92
E6aB04	DQ103622	1		2	Flagellate symbiont clone SOGA14, AJ244791	92
E6aB06	DQ103621			1	*Silicibacter pomeroyi*, AF098491	90
E6aC10	DQ103617			1	*Ruegeria* sp. strain DG898, AY258086	95
E6aG05	DQ103615	1	3	3	*Rhodobacter* sp. strain 2002-65602, AY244771	92
E6aG08	DQ103619			2	*Roseovarius tolerans*, Y11551	94
E6aH02	DQ103614	1		1	*Rhodovulum* sp., D32245	94
E6bF01	DQ103611			1	Mixolimnion bact. ML42, AJ315683	93
E6bG12	DQ103618	4		4	Dinoflagellate bact. DG941, AY258087	93
Gypsum α-cluster						
E2aB07	DQ103628	1			*Rhizobium* sp., AY500261	92
E2aG02	DQ103630	2			*Oceanicola batsensis*, AY424898	91
E2aH02	DQ103629	1	1		*Oceanicola batsensis*, AY424898	92
E2aH03	DQ103631	3	2	1	Unc. bact. DSSF4, AY328627	89
E6aD10	DQ103632	18	22	5	Unc. bact. DSSF4, AY328627	90

[a] See Table 2, footnote *a*.
[b] See Table 2, footnote *b*.
[c] See Table 2, footnote *c*.

DISCUSSION

***Cyanobacteria*.** Visually, *Cyanobacteria* dominate the brown and green layers due to their large cell size and conspicuous pigmentation, but in terms of cell numbers they amount to only a minor part of the population. We observed three main types of *Cyanobacteria* in the green layer during microscopy: unicellular bacteria resembling members of the genus *Halothece* and filamentous organisms of *Halospirulina*- or *Phormidium*-like morphologies (44, 75). Green filamentous forms were observed both in the G and in the P/O layers. The identity of the unicellular *Cyanobacteria* was confirmed by the detection of phylotypes affiliated with the *Halothece* cluster. The *Cyanobacteria* were underrepresented among the phylotypes found in the clone libraries (3%) compared to the in situ abundance of cells (28%) in the green layer. The clone libraries are built from rRNA genes within the extracted pool of genomic DNA. Apart from relative cell numbers, the abundance of 16S rRNA phylotypes in the DNA extract depends on the number of rRNA operons in each prokaryotic cell, which may vary from 1 to at least 15 (34). Further selection for or against individual or groups of organisms may occur during DNA extraction and subsequent PCR amplification.

The three types of *Cyanobacteria* observed here have previously been found to dominate the biomass in environments of similar salinity. Thus, a salinity-dependent succession was observed in the salterns of Guerrero Negro, Mexico, where *Microcoleus* dominated at salinities of <12% and *Phormidium*-like organisms and members of the *Halospirulina* and the *Halothece* cluster dominated at higher salinities (43).

***Bacteroidetes*.** Of the three main orders in this phylum, "*Sphingobacteriales*" and "*Flavobacteriales*" consist mainly of aerobic organisms while known strains of order "*Bacteroidales*" are all anaerobic (32, 59). This is consistent with the distribution of clones in the crust, where "*Bacteroidales*" phylotypes were mainly observed in the P/O layer while members of the "*Flavobacteriales*" and "*Sphingobacteriales*" with just a few exceptions were most abundant in the W and/or G layer. The results of both cloning and DGGE analysis indicate that significant physiological diversity exists among the *Bacteroidetes* in the crust.

TABLE 4. Summary of bacterial phylotypes not belonging to the *Bacteroidetes* or α-proteobacteria

Phylotype	GenBank accession no.	No. of clones per layer[a]			Closest relative in BLAST search[b]	% Similarity[c]
		W (n = 20)	G (n = 27)	P/O (n = 29)		
Planctomycetales + uncultured relatives						
E4aA08	DQ103660	1	2		Planctomycete GMD21C08, AY162119	92
E6bH02	DQ103659	1		1	Sediment clone LD1-PA40, AY114326	92
Candidate division TM6						
E4aB05	DQ103662		1		Freshwater clone PRD01a004B, AF289152	90
Cyanobacteria						
E4aB08	DQ103654		1		*Halothece* sp., AJ000709	97
E4bG02	DQ103653		2		*Halothece* sp., AJ000710	99
E6aG07	DQ103655		1	1	*Microcoleus chthonoplastes*, X70770	93
Spirochetae						
E2aA10	DQ103657	2	3	1	*Spirochaeta halophila*, M88722	90
E2aH12	DQ103658	5	3	3	*Spirochaeta halophila*, M88722	90
δ-*Proteobacteria*						
E4aE11	DQ103668		1	1	Unc. bact. SLM-DSBAC-75, AY083025	96
E6aB08	DQ103667	1	7	1	*Desulfovibrio bastinii*, U53462	88
E6aH12	DQ103652		1	2	Hypersaline clone MPD-41, AF348719	92
E6bG07	DQ103666			1	*Desulfohalobium retbaense*, X99235	92
γ-*Proteobacteria*						
E2aA03	DQ103664	8			*Thioalkalivibrio jannaschii*, AF329083	97
E2aE11	DQ103665	1	1	1	Mar. sed. clone MERTZ_0CM_353, AF424152	89
E2bG06	DQ103663	1			*Alkalispirillum mobile*, AF114783	90
Chloroflexi						
E6aA10	DQ103661		1	15	"*Ca*. Chlorothrix halophila,"AY395567	98
Halanaerobiales						
E6aF12	DQ103656		3	2	*Halanaerobium salsulginis*, L22890	89

[a] See Table 2, footnote *a*.
[b] See Table 2, footnote *b*. Mar., marine; sed., sediment.

α-Proteobacteria. Numerous phylotypes were affiliated with the *Rhodospirillales*, the "*Rhodobacterales*" or the "*Sphingomonadales*." Cultured members of the *Rhodospirillales* are predominantly phototrophic, although some reference species also exhibit chemoorganotrophic growth under oxic or microoxic conditions (24, 27, 37). Most notable among the detected phylotypes affiliated with this group was E6aD01. It dominated in the P/O layer, where it made up almost one-third of the clones, but was virtually absent in the layers above. This, and the fact that it was closely related to known halophilic species of the phototrophic genus *Roseospira*, indicates that it represents an important anoxygenic phototroph contributing to the pink coloration in the crust. Its closest relatives include curved to spiral-shaped organisms with cell diameters of <1 μm and variable cell lengths, exhibiting infrared autofluorescence (24). During epifluorescence microscopy, the organism corresponding to phylotype E6aD01 was probably counted as one of the small, unicellular organisms that were abundant in all layers.

Phylotype E6aD10, which was abundant in the W and G layers, formed a cluster with four less-common phylotypes. These phylotypes were only distantly related to known groups of α-proteobacteria and may represent a new lineage of high-salt-adapted organism.

A number of phylotypes were affiliated with the *Roseobacter* clade, a phylogenetically coherent group of marine α-proteobacteria within the "*Rhodobacterales*" (22, 39). Members of the *Roseobacter* clade are abundant in marine environments, where the group is involved in the degradation of organic sulfur compounds (23). Phototrophic members of the group have been cultured from hypersaline environments (31, 36, 69), and environmental clones have been retrieved from the water phase of marine solar salterns (4). However, the role of the group in the crust environment is uncertain. Since members of the *Roseobacter* clade are likely present in the water delivered to the ponds, their occurrence in the upper layers of the crust would be consistent with entrainment into the mat from nearshore seawater.

γ- and δ-proteobacteria. One of the γ-proteobacterial phylotypes was closely related to the nonphototrophic genus *Thioalkalivibrio* within the *Ectothiorhodospiraceae*. This organism was most abundant in the W and G layers, undergoing daily fluctuations in oxygen and sulfide content. The genus *Thioalkalivibrio* consists of sulfur-oxidizing organisms that use nitrate or oxygen as electron acceptors, and it seems likely that the phylotype detected in the crust shares this metabolism. Phototrophic γ-proteobacteria with internal sulfur globules were seen during microscopy, and organisms resembling members of the

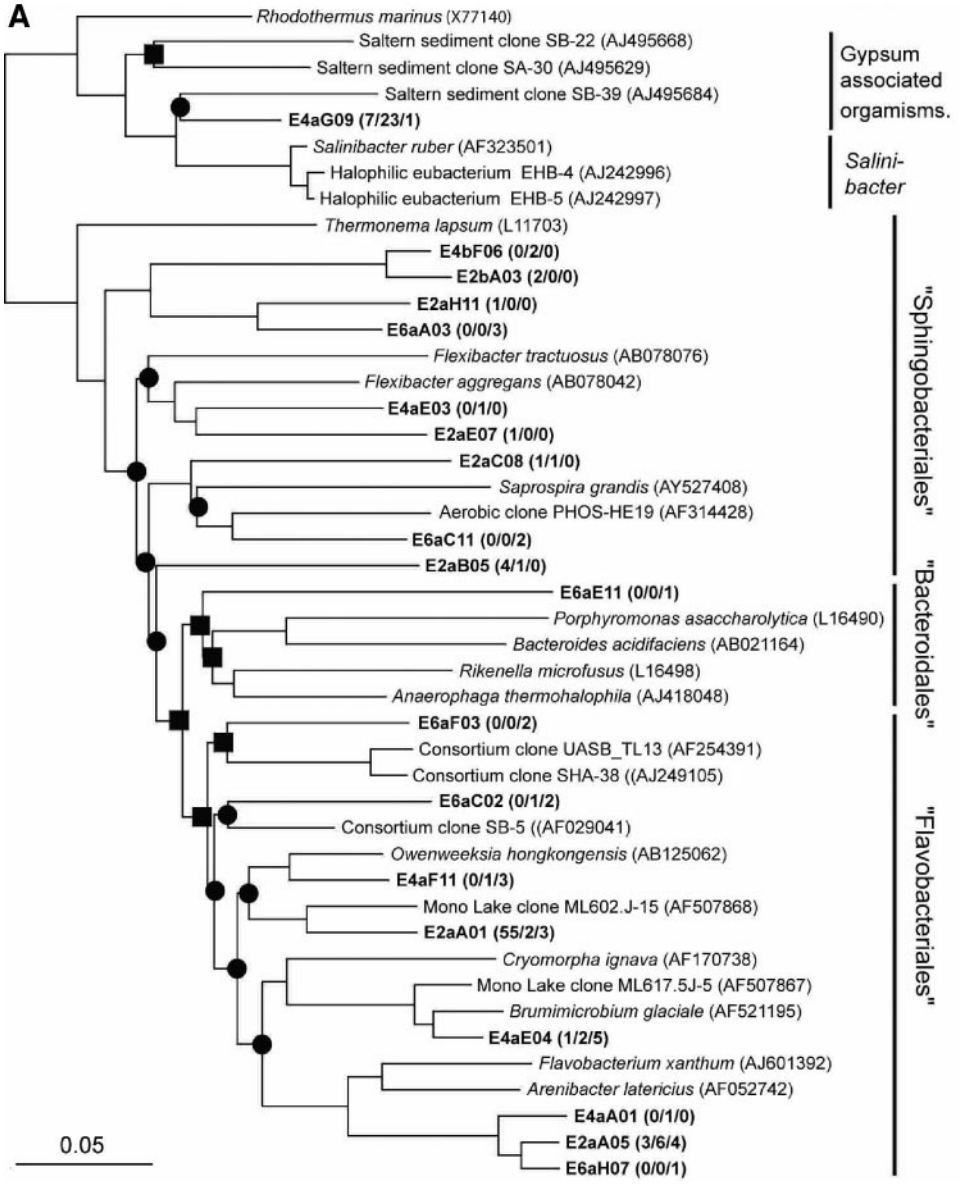

FIG. 2. Phylogenetic trees showing phylotypes affiliated with *Bacteroidetes* (A), α-proteobacteria (B), and other groups (C). The trees were constructed by using Jukes-Cantor distance calculations and neighbor joining. Bootstrap values were determined with 1,000 replicates. Nodes with bootstrap values smaller than 60% are marked with squares (40 to 60%) or circles (<40%).

Chromatiaceae and *Ectothiorhodospiraceae* were also observed in the crust prior to the present study (53). Furthermore, phototrophic halophilic members of the "*Chromatiales*" have been isolated from solar salterns (10, 11). Thus, it is surprising that sequences affiliated with phototrophic γ-proteobacteria were not observed in clone libraries constructed from genomic DNA extracts. As was discussed above for the *Cyanobacteria*, a number of factors including relatively low cell numbers of large organisms and a variable number of rRNA operons among organisms, as well as extraction and PCR bias, may lead to under-representation of phylotypes relative to their in situ abundance.

Phylotypes of δ-proteobacteria were associated with the predominantly sulfate-reducing "*Desulfobacteraceae*" and "*Desulfovibrionaceae*," members of which have previously been observed in hypersaline environments (14, 38). The salinity tolerance of isolated strains of sulfate-reducing δ-proteobacteria, as well as observations of in situ populations, indicate that the group is best adapted to much lower salinities and hence is growing at a considerable salinity stress in the gypsum crust (6, 7, 9, 35, 65). The depth distribution of the sulfate-reducing organisms, as reflected in the clone library, shows a maximum frequency of clones in the green layer and below. This is in accordance with previous measurements showing low sulfate reduction rates above the green layer (8).

***Chloroflexi*.** Phylotype E6aA10, which was highly similar (98% identity) to the filamentous green nonsulfur bacterium

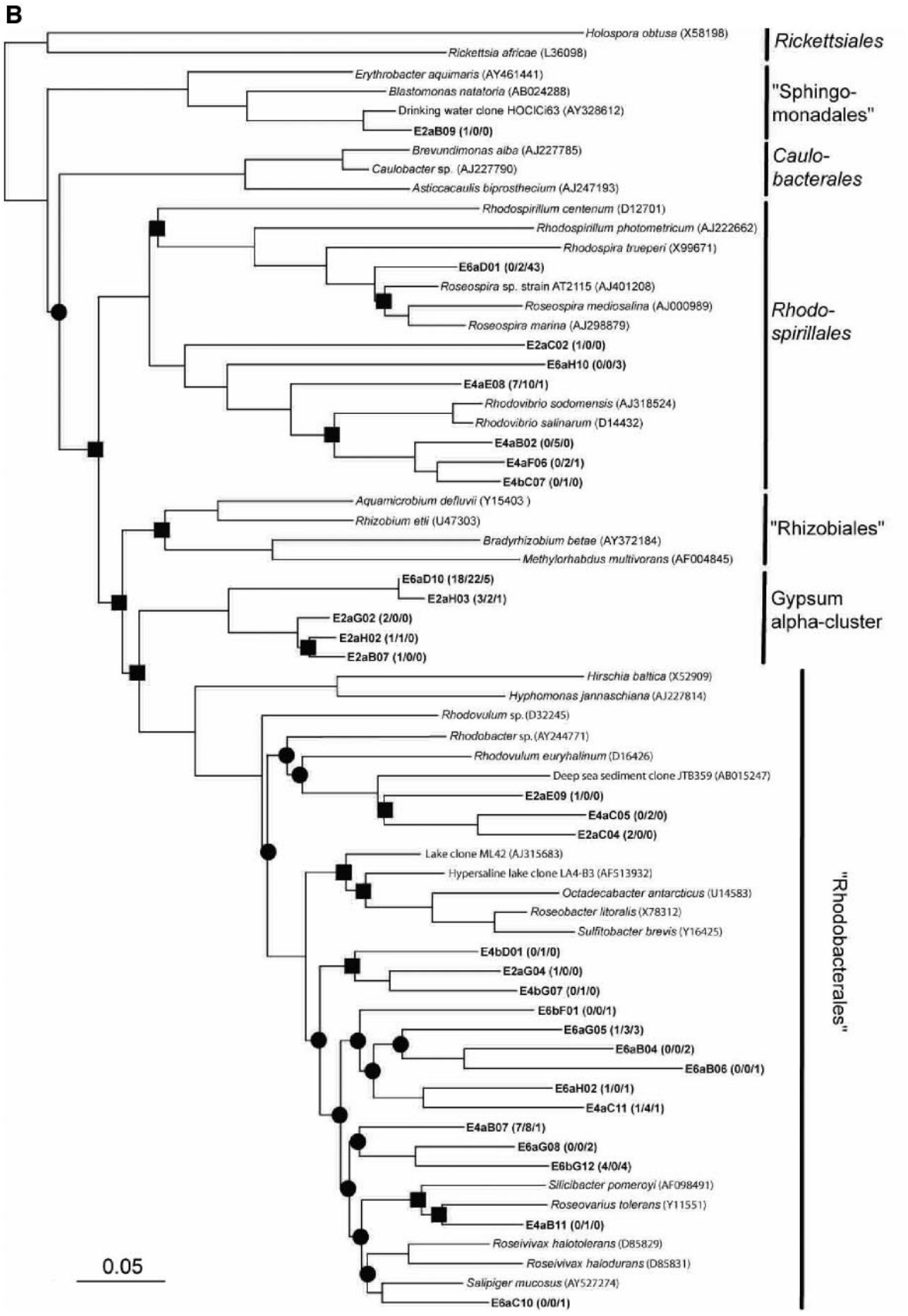

FIG. 2—*Continued*.

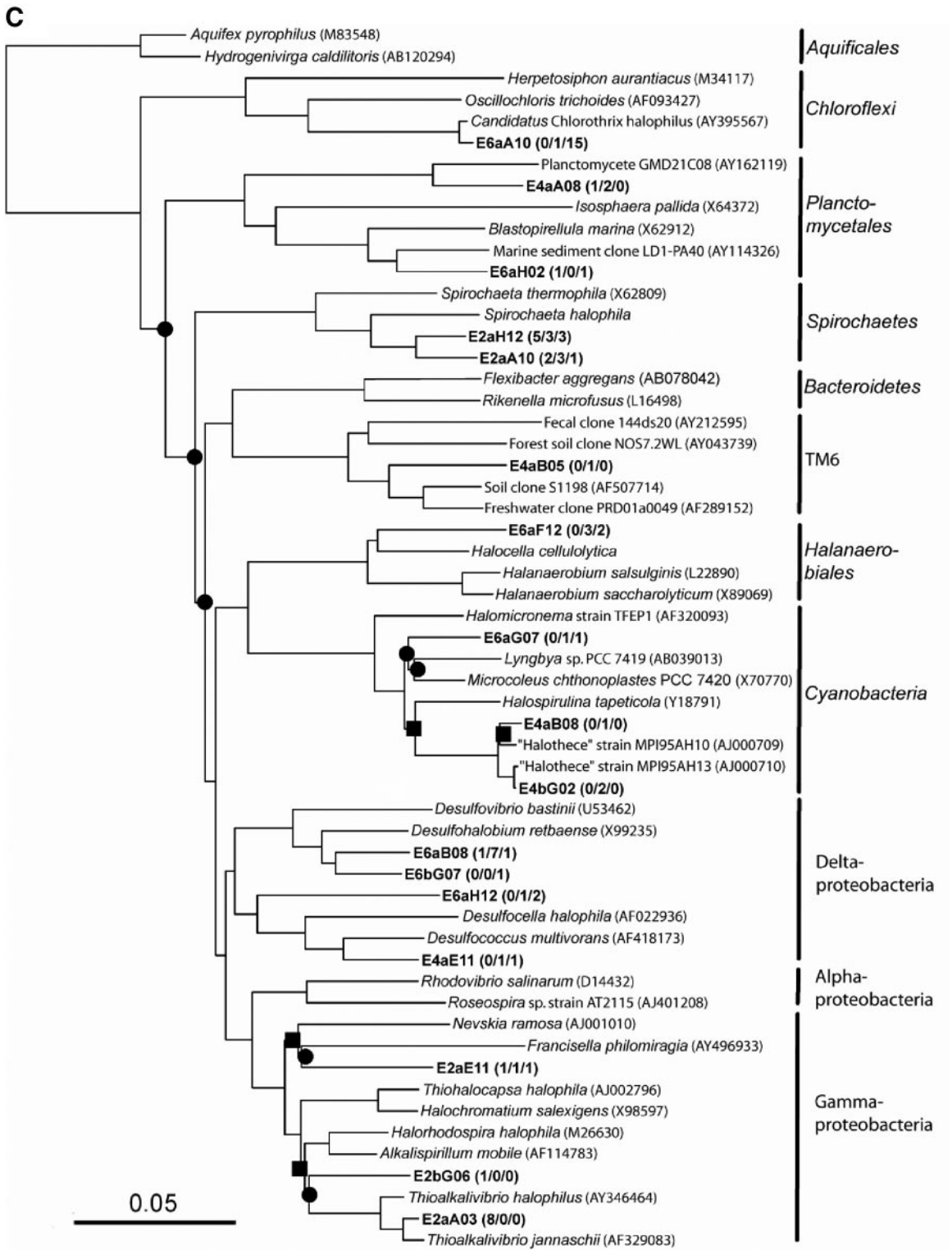

FIG. 2—*Continued*.

"*Candidatus* Chlorothrix halophilus," was abundant in the clone library from the P/O layer. "*Candidatus* Chlorothrix halophilus" is an obligately anaerobic sulfide-dependent phototrophic green nonsulfur bacterium that was cultured from a hypersaline microbial mat (33). Given the phylogenetic similarity and its affinity for the sulfidic part of the crust, phylotype E6aA10 seems likely to share this physiology.

Filamentous green nonsulfur bacteria are among the most abundant organisms in both oxic and anoxic layers of microbial mats from a variety of environments (30, 45, 46, 55, 74). They

TABLE 5. Summary of archaeal phylotypes

Phylotype	GenBank accession no.	No. of times encountered (n = 53)	Closest relative in BLAST search	% Similarity[a]
Methanosarcinales				
ArcB03	DQ103680	3	*Methanohalophilus mahii*, M59133	97
Halobacteriales				
ArcA11	DQ103682	1	"*Haloquadratum walsbyi*," AY676200	99
ArcF12	DQ103677	4	*Haloferax lucentense*, AB081732	91
ArcG01	DQ103676	1	*Haloferax volcanii*, K00421	89
ArcG09	DQ103674	2	Haloarchaeon strain, T4.2, AJ270231	91
ArcH05	DQ103672	1	Haloarchaeon CSW2.24.4, AY498650	98
ArcH07	DQ103670	1	Haloarchaeon strain BbpA.1, AJ270239	92
MBGD/DHVE1				
ArcC08	DQ103679	6	Hypersaline clone D4.75-4, AF199374	99
ArcG12	DQ103673	4	Marine clone CCA59, AY179970	90
ArcH06	DQ103671	24	Marine clone CCA59, AY179970	90
MGIII				
ArcA01	DQ103669	1	Saltern clone SA-ARCH20, AJ495709	95
MSBL1				
ArcE07	DQ103678	1	Hypersaline clone ALTA-100, AY164275	92
Halophilic archaeal cluster I				
ArcA07	DQ103684	1	Brine clone KTK 18A, AJ133623	82
ArcA12	DQ103681	1	Saltern clone 52UMH 8%, AF477910	89
Halophilic archaeal cluster II				
ArcA08	DQ103683	1	Brine clone KTK 4A, AJ133621	98
ArcG08	DQ103675	1	Brine clone KTK 4A, AJ133621	93

[a] See Table 2, footnote *c*.

include both photo- and chemotrophs, but the overall physiological capability and ecological role of the group is poorly understood. The fact that only a single phylotype was detected in the crust and only in the deeper permanently anoxic part of the photic zone may indicate that the ecological significance of the group is reduced compared to other studied microbial mats, possibly as a consequence of the higher salinity. Alternatively, DNA extraction and PCR bias may have selected against the group during construction of clone libraries as was discussed above.

Most of the green filaments making up 4% of the organisms in the P/O layer were autofluorescing in the visible red under UV light, indicating that the samples contained more *Cyanobacteria* than *Chloroflexi* relatives. The *Cyanobacteria* probably originate from the purple portion of the layer which is in close contact with the overlying G layer and may contain organisms that are buried as the crust expands through precipitation and growth of crystals. The *Chloroflexi* phylotype, on the other hand, is probably a contribution from the olive-green layer below. To confirm this, it will be necessary to separate the purple from the olive-green portion of the crust and study them separately.

***Firmicutes*, *Spirochetes*, and *Planctomycetes*.** Firmicutes of the *Halanaerobiales* are abundant in hypersaline environments, including the Great Salt Lake, the Dead Sea, oil wells, saltern ponds in France and California, and a variety of other locations (40, 47, 57, 58, 76). Known members are obligately anaerobic, moderately halophilic organisms that gain energy by fermentation of various organic compounds. In the present study they were retrieved from the G- and P/O layer, suggesting an affinity for the deeper parts of the crust, which is consistent with a fermentative mode of life. Although not observed during microscopy, *Spirochetes* related to *Spirochaeta halophila*, a facultative anaerobe, were detected in all 3 libraries. These organisms as well as two relatives of the *Planctomycetes* probably contribute to the mineralization of organic material created by phototrophic primary producers in the crust.

Methanogens. Previous measurements have documented the presence of methanogens in the crust, although methanogenesis amounts to less than 0.1% of the total anaerobic mineralization (65). Cultured members of the *Methanohalophilus* genus are methylotrophic, mesophilic halophiles, and the type species *M. mahii* grows optimally at salinities up to ca. 15% NaCl (19, 54). Earlier enrichment experiments have suggested the presence of methylotrophic methanogens in the crust. Thus, methane accumulated in enrichment cultures in which pieces of crust was added to anoxic media containing methanol (64).

***Halobacteriales*.** DNA from the *Halobacteriales* has been extracted from the Dead Sea, solar salterns, Antarctic hypersaline lakes, alkaline African hypersaline lakes, and Solar Lake, Sinai (3, 13, 41, 50). Isolated strains of this group are aerobic halophiles growing at salinities up to NaCl precipitation, although some are capable of anaerobic growth either in the light using bacteriorhodopsin or in the dark by fermentation (25, 52). Although clone libraries of archaeal 16S rRNA genes were constructed from samples of the P/O layer, the DGGE-analysis indicated that at least two of the *Halobacteriales*-

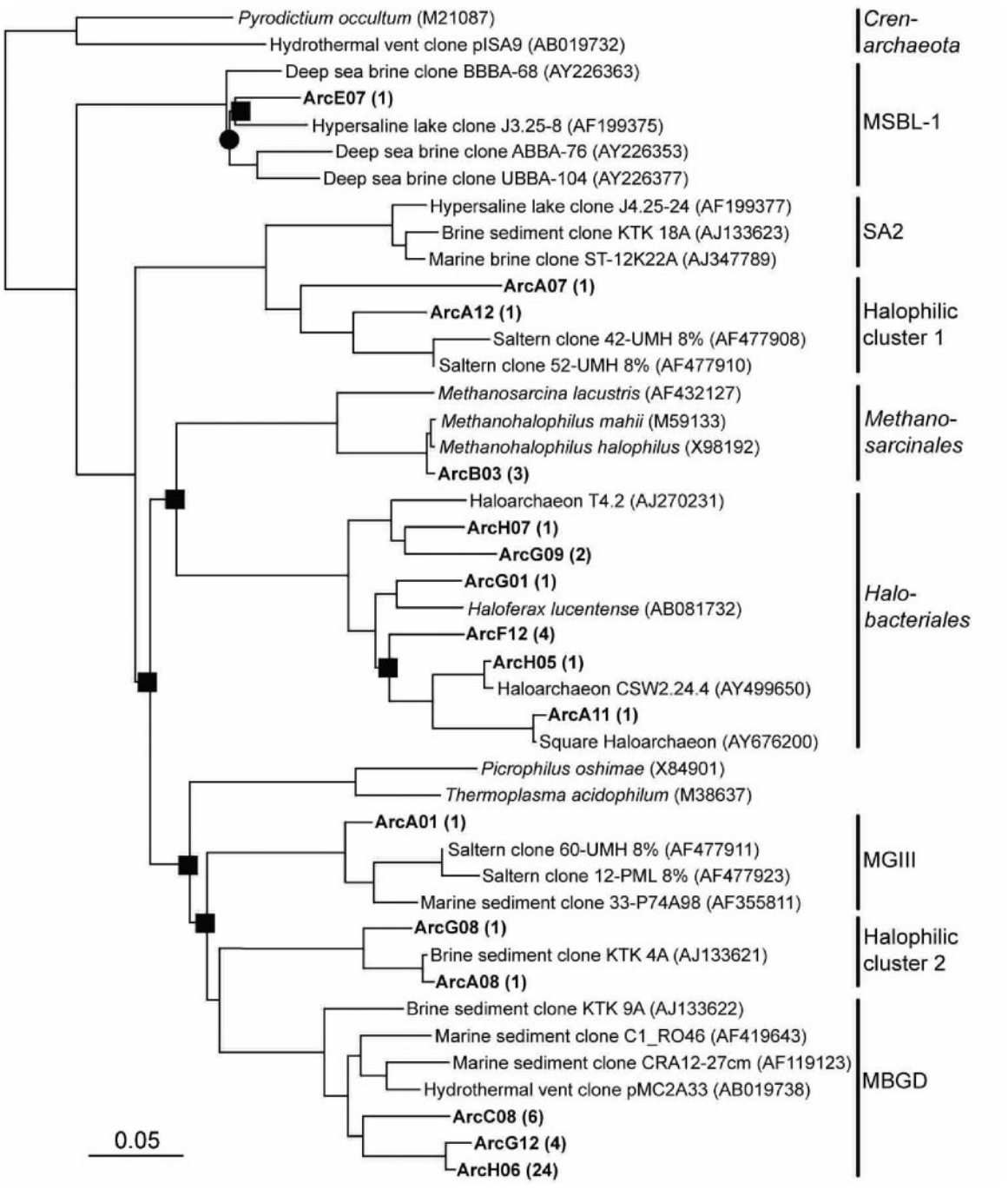

FIG. 3. Phylogenetic tree showing the affiliation of the archaeal phylotypes detected in the gypsum crust. The tree was constructed by using Jukes-Cantor distance calculations and neighbor joining. Bootstrap values were determined with 1,000 replicates. Nodes with bootstrap values smaller than 60% are marked with squares (40 to 60%) or circles (<40%). MBGD, marine benthic group D; MGIII, marine group III.

related phylotypes were also present in the layers above. This may indicate that the phylotypes detected in the P/O layer are remains of aerobic organisms either from the surface layers of the crust or from the pond water that has been entrained and buried.

Uncultured archaeal groups. The MBGD, which was the most numerous group of *Archaea* in the clone libraries, has previously been encountered in both marine (70, 71, 73) and terrestrial environments (16), and also the candidate order MSBL1 and the Halophilic Clusters 1 and 2 seem to have some affinity for marine and/or hypersaline environments. However, since no representative organisms of these groups have been cultured, their ecological significance is unknown.

Endoevaporitic benthic communities at high salinity. The groups found to dominate the clone libraries from the gypsum crust in Eilat have previously been associated with other hy-

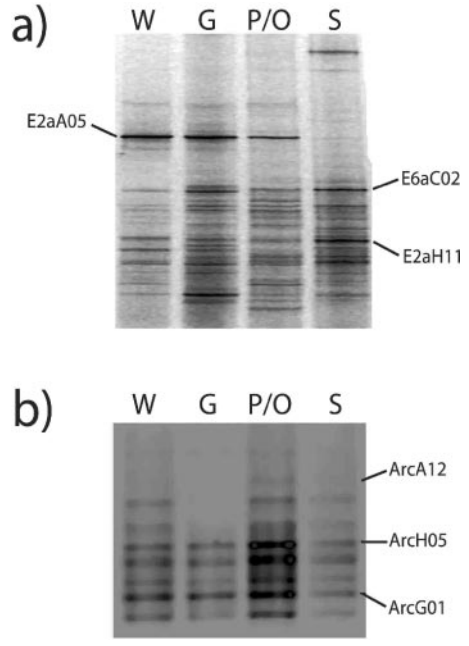

FIG. 4. Representative DGGE gels with PCR products obtained with primers specific for *Bacteria* (a) and *Archaea* (b). The gels contained 10% acrylamide-bisacrylamide and a 40 to 70% denaturing gradient. Bands that were identified as a phylotype from one of the clone libraries are indicated in the figure.

persaline environments. Thus, the cultivable aerobic heterotrophs of a hypersaline microbial mat with a salinity of 9% maintained at the Interuniversity Institute for Marine Sciences of Eilat included organisms related to the *Bacteroidetes*, α-proteobacteria of the *Roseobacter* clade or the "*Rhizobiales*," and γ-proteobacteria (29). These organisms were enriched on yeast extract or glycolate. In a molecular study of anoxic sediments below a gypsum encrusted community in Salin-de-Giraud, France, the most abundant groups were the *Bacteroidetes*, *Firmicutes*, and α-, γ-, and δ-proteobacteria (41). Finally, in a 16S rRNA survey of endoevaporitic microbial communities in the salterns of Guerrero Negro, Spear et al. (66) found a predominance of bacterial phylotypes affiliated with *Cyanobacteria*, *Bacteroidetes*, α-, γ-, and δ-proteobacteria, *Planctomycetes*, and *Firmicutes*. Green sulfur bacteria and ε-proteobacteria were also detected but in low numbers. The archaeal phylotypes included *Halobacteria* and a few methanogens. Together, these studies indicate that a few prokaryotic phyla—including *Cyanobacteria*, *Bacteroidetes*, *Proteobacteria* of the α-, γ-, and δ-classes, and *Firmicutes*—are particularly well adapted to the high-salt and variable redox conditions within the evaporites.

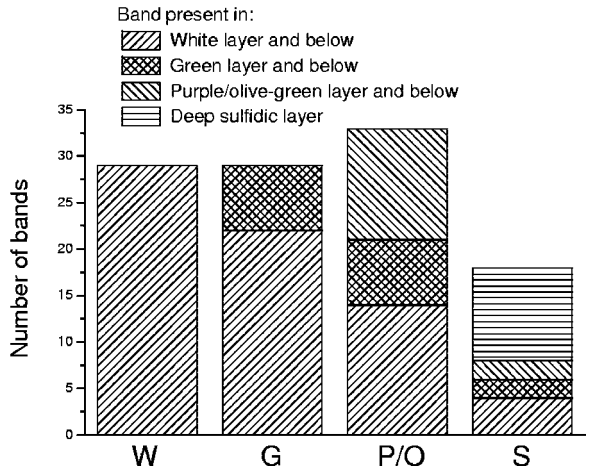

FIG. 5. Number and distribution of bands on DGGE gels loaded with bacterial PCR products from the white (W), green (G), purple/olive-green (P/O), and deep sulfidic (S) layers.

The DGGE analysis revealed a highly structured bacterial community, where organisms were occupying specific horizontal layers. This stratification was also evident from the distribution of the most abundant phylotypes of *Bacteroidetes*, *Chloroflexi*, and *Proteobacteria* in clone libraries from the different layers. Among the *Bacteroidetes*, phylotypes E2aA01 showed an affinity for the W layer, whereas phylotype E4aG09 was abundant in the G layer. Similarly, among the *Rhodospirillales*, E6aD01 had a strong affinity for the P/O layer, whereas E4aE08 was most abundant in the W and G layers. This indicates that substantial physiological and/or metabolic diversity exists within the *Bacteroidetes* and the α-proteobacteria in the crust.

The high number of PCR cycles that was necessary in order to amplify DNA with *Archaea*-specific primers, the low diversity of *Archaea* compared to *Bacteria*, and the lack of a vertical succession in the archaeal community indicate that the role of *Archaea* is limited in the endoevaporitic environment.

Conclusion. This study provides a window into the diversity and structure of endoevaporitic communities found in gypsum-precipitating saltern ponds. In spite of the high salinity, a rich prokaryotic community is thriving in the gypsum, and although *Cyanobacteri*a dominate the system in terms of biomass, they are far outnumbered by other groups of prokaryotes. The community is highly stratified with organisms inhabiting specific layers according to their metabolic needs and capabilities. Particularly members of the *Bacteroidetes* and α-proteobacteria were numerous. The *Chloroflexi* appeared to be less abundant compared to phototrophic benthic communities growing at lower salinities, and *Archaea* seem to play only a minor role in the crust.

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