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Microbial Communities in the Chemocline of a Hypersaline Deep-Sea Basin (Urania Basin, Mediterranean Sea)

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The Urania basin is a hypersaline sulfidic brine lake at the bottom of the eastern Mediterranean Sea. Since this basin is located at a depth of ~3,500 m below the sea surface, it receives only a small amount of phytoplankton organic carbon. In the present study, the bacterial assemblages at the interface between the hypersaline brine and the overlaying seawater were investigated. The sulfide concentration increased from 0 to 10 mM within a vertical interval of 5 m across the interface. Within this chemocline, the total bacterial cell counts and the exoenzyme activities were elevated. Employing 11 cultivation methods, we isolated a total of 70 bacterial strains. The 16S ribosomal DNA sequences of 32 of the strains were identical to environmental sequences detected in the chemocline by culture-independent molecular methods. These strains were identified as flavobacteria, *Alteromonas macleodii*, and *Halomonas aquamarina*. All 70 strains could grow chemoorganoheterotrophically under oxic conditions. Sixty-six strains grew on peptone, casein hydrolysate, and yeast extract, whereas only 15 strains did not utilize polymeric carbohydrates. Twenty-one of the isolates could grow both chemoorganotrophically and chemolithotrophically. While the most probable numbers in most cases ranged between 0.006 and 4.3% of the total cell counts, an unsually high value of 54% was determined above the chemocline with media containing amino acids as the carbon and energy source. Our results indicate that culturable bacteria thriving at the oxic-anoxic interface of the Urania basin differ considerably from the chemolithoautotrophic bacteria typical of other chemocline habitats.

In pelagial lakes and certain marine environments, strong salinity or temperature gradients can prevent mixing and hence lead to stratification of the water column. Steep vertical gradients of oxygen, sulfide, ammonium, and sometimes methane concentrations occur across the interface layer (22, 30, 41). In most cases, these chemical gradients across the chemocline originate from microbial activities. Compared to the microbial assemblages in the upper and lower water layers, the microbial assemblages at chemoclines are characterized not only by higher levels of bacteria, protozoans, and zooplankters (23, 41) but also by higher microbial activities, like CO_2 fixation and exoenzyme activities (31, 41, 43). In many stratified ecosystems, the chemocline is characterized by an intense cycling of sulfur compounds (30, 43, 54). The chemocline thus offers a variety of ecological niches on a small spatial scale and consequently may also harbor a highly diverse microbial community.

In the pelagic marine environment, oxic-anoxic interfaces are mostly limited to coastal lagoons or fjords; the euxinic Black Sea is a notable exception. However, several hypersaline basins have been discovered on the seafloor in different oceanographic provinces, like the Gulf of Mexico (51), the Red Sea (46), and the Mediterranean Sea (29). In the Mediterranean Sea, these so-called brine lakes most likely developed by the dissolution of 5- to 8-million year-old Messinian evaporites (9) which became exposed to seawater during the collision of the African and Eurasian tectonic plates. Alternatively, the depressions may have been formed by accrecionary processes and may have subsequently been filled with fossils and highly concentrated relics of Messinian seawater trapped in the interstitial areas of the deep-sea sediments (58).

The deep-sea anoxic hypersaline basins of the eastern Mediterranean Sea (Fig. 1) are located far below the photic zone. In addition, the primary productivity in the surface waters is extraordinarily low due to the antiestuarine circulation pattern of the Mediterranean Sea (61). Because of the low production and long sedimentation path, only a small flux of organic carbon reaches the deep-sea community at the chemoclines of the brine lakes. At the same time, the occurrence of sulfide in the Bannock and Tyro basins (20; W. Ziebis, M. E. Böttcher, A. Weber, J.-C. Miquel, S. Sievert, and P. Linke, J. Conf. Abstr. Goldschmidt Conf. 2000 **5:**1134, 2000), which are depleted in ^{34}S (33; Ziebis et al., J. Conf. Abstr. Goldschmidt Conf. 2000), may indicate that active sulfate-reducing bacteria are present. So far, little is known about the abundance, diversity, and physiological capabilities of microorganisms that thrive in these unusual chemocline systems. Only recently, microbial sulfate reduction in the water column of the deep Urania basin was directly shown by radiotracer techniques (Ziebis et al., J. Conf. Abstr. Goldschmidt Conf. 2000).

In this paper we describe the environmental conditions in the chemocline of the Urania basin in the eastern Mediterranean Sea and an analysis of the indigenous microbial community and present evidence that at least some of the numerically significant bacteria can be cultivated by using a set of different cultivation conditions.

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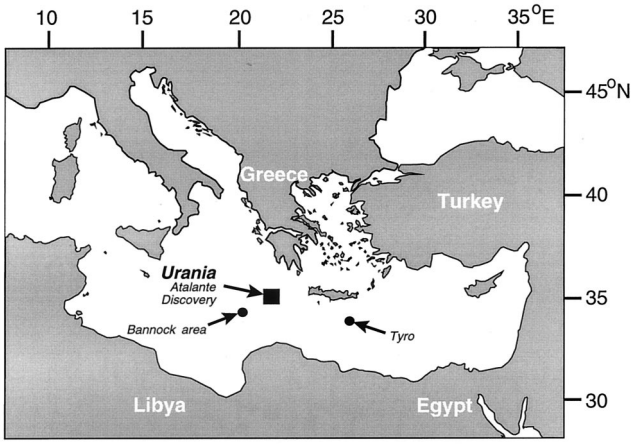

FIG. 1. Locations of the Urania basin and other hypersaline brine lakes in the eastern Mediterranean Sea.

MATERIALS AND METHODS

Sampling site. The Urania basin (Fig. 1) is a horseshoe-shaped brine lake located west of Crete in the eastern Mediterranean Sea. The surface of this brine lake lies 3,470 m below sea level (35), and the maximum brine thickness is 200 m at its southwestern end. The salinity of the brine is five times higher than that of standard seawater (58).

Sampling was performed during cruise M40/4 of the R/V *Meteor* at station 76 (35°13,83′N, 21°28,29′E) in January 1998. Water samples were taken at 5- or 10-m intervals from 16 different water depths by using a rosette sampler (HydroBios, Kiel, Germany) equipped with Niskin bottles (General Oceanics, Miami, Fl.). Sampling through the chemocline was performed at 5-m intervals. Three depths were chosen for microbiological analyses: 3,455 m (above the chemocline), 3,475 m (within the chemocline), and 3,500 m (below the chemocline). The vertical position of the chemocline was located by means of the drop in oxygen concentration recorded with a CTD probe (see below).

Chemical and physical parameters. Temperatures and oxygen concentrations in the water column were measured online during sampling by using a CTD probe (type SBE 19 CTD; Seabird Electronics, Bellevue, Wash.) connected to the rosette sampler. Depth was determined with the pressure sensor of the CTD probe.

Chloride and sulfate concentrations were measured by ion chromatography (Sykam, Gilching, Germany) (3). Because of their high salt concentrations, samples of seawater and the brine were diluted 100- to 1,000-fold in eluent prior to analysis. Standards were prepared as mixed salt solutions that had a composition corresponding to that of the brine and were diluted as well. Parallel analyses of several standards demonstrated that the standard errors due to dilution were between 0.1 and 3%. The *ortho*-phosphate content was determined photometrically by the molybdenum blue method (50). For determination of sulfide concentrations, water samples were immediately mixed with a 1 M zinc acetate solution so that the final zinc concentration was 100 mM. The samples were stored in glass ampoules which were flushed with N_2 and sealed with butyl rubber stoppers. Sulfide was assayed spectrophotometrically by using the methylene blue method of Cline (11).

Sulfur isotope values were determined by separate precipitation of sulfide and sulfate as Ag_2S and $BaSO_4$ and subsequent isotope analysis by combustion isotope ratio monitoring mass spectrometry (7). Because of the high concentrations of chloride in the brine samples, sulfide was first precipitated as ZnS; the latter was collected and acidified, and the liberated sulfide was flushed out by a stream of N_2 and reprecipitated as Ag_2S in a second trap. Isotope fractionation was calculated from ratios of ^{34}S to ^{32}S for samples (R_{sample}) and for the V-CDT sulfur standard (R_{std}), as follows: $\delta^{34}S = [(R_{sample}/R_{std}) - 1] \times 1{,}000$.

Microbiological parameters. The potential activities of the hydrolytic exoenzymes alkaline phosphatase (EC 3.1.3.1), β-glucosidase (EC 3.1.21), and leucine aminopeptidase (EC 3.4.1.1) were measured on board immediately after sampling by using fluorescently labeled substrate analogues (25, 26). The measurements for brine samples were obtained under anoxic conditions.

Total cell counts were determined by epifluorescence microscopy. Samples were preserved with filtered (pore size, 0.1 μm) glutardialdehyde at a final concentration of 2%. Bacterial cells were stained with DAPI (4′,6-diamidino-2-phenylindole) (48).

Source of organisms. In this study a total of 70 bacterial strains were investigated (Table 1). The strains were isolated from the three water depths of the Urania basin by employing 11 isolation procedures (Table 1). Besides agar plates containing peptone, hydrolyzed casein, and soluble starch (CPSm medium) (13), liquid anaerobic cultures with different substrates and sulfide-oxygen gradient tubes were employed. The basal liquid medium consisted of artificial seawater (13). One milliliter of trace element solution SL 10 (60) and 1 ml of a solution

TABLE 1. Origins, isolation conditions, and designations of bacterial strains

Depth of sample (m)	Isolation medium[a]	Strain(s)[b]
3,455	CPSm medium on agar plates, O_2	UM1, UM2a, **UM2b, UM3a, UM3b, UM4a, UM4b, UM4c, UM5b, UM6b**
	Fatty acids	U53, U54, **U56, U57**
	Acetate	U34, U36, U37
	Lactate	**U45**, U48, U49
	Amino acids	U61, U63, U64, U65
	Thiosulfate, nitrate	U13, U15
	Thiosulfate	U72, U73, **U74**
	Polysulfide-O_2 gradients	U18, U20, U21
3,475 (chemocline)	CPSm medium on agar plates, O_2	UM6c, **UM7, UM8**, UM9, **UM11**
	Fatty acids	**U51, U52**, U55, **U58**
	Acetate	U31, U35, U38
	Lactate	**U41, U42**, U43, **U44, U46, U47**
	Amino acids	U66
	Thiosulfate, O_2	**U4**, U9
	Thiosulfate, nitrate	U14, U19
	Thiosulfate	**U71**
	Polysulfide-O_2 gradients	**U6, U16**, U17, **U23**
	Fatty acids, nitrate	U1, U2, U3
3,500	Acetate	U32, U33
	Amino acids	U67
	Thiosulfate, O_2	**U7, U8, U10, U12**

[a] For the exact compositions of media see Materials and Methods. Media were incubated under anoxic conditions unless indicated otherwise.
[b] Strains which represent numerically significant phylotypes are shown in boldface type.

of selenite and tungstate (60) were added, and the pH was adjusted to 7.5. After autoclaving, 10 ml of a sterile filtered vitamin solution (4) per liter and 10 ml of a sterile sodium bicarbonate solution (20 g · liter^{-1}) per liter were added. For anoxic incubation experiments the medium was reduced with 400 μM sterile sodium sulfide. The salt concentrations of the medium used for samples from the brine and the sediment surface were elevated to twice those of seawater by adding a concentrated salt solution (290 g of NaCl per liter, 40 g of $MgCl_2 \cdot 6H_2O$ per liter).

In order to generate a variety of incubation conditions, the basal medium was supplemented with either acetate (15 mM), lactate (10 mM), a mixture of volatile fatty acids (containing 3.75 mM acetate, 1.5 mM propionate, 1.5 mM butyrate, 0.75 mM isobutyrate, 0.75 mM valerate, 0.75 mM 2-methylbutyrate, and 0.75 mM 3-methylbutyrate), or a mixture of amino acids (containing 2 mM alanine, 1 mM arginine, 1 mM asparagine, and 1 mM cysteine). Thiosulfate-disproportionating bacteria were grown with 15 mM thiosulfate and 2 mM acetate. Sulfur-oxidizing bacteria were obtained in media containing thiosulfate (10 mM) or polysulfide (15 mM S^0 and 6 mM sulfide) and either supplemented with nitrate (15 mM) or incubated under oxic conditions. All the media described above were employed in most-probable-number (MPN) dilution series. After 6 weeks, growth was monitored, viable cell counts were calculated, and strains were isolated from different parallel preparations of the highest positive dilutions by repeated application of the deep agar dilution method (60).

Gradient tubes were established by adding polysulfide to anoxic artificial seawater containing sterile molten agarose (1%). After solidification, the tubes were incubated under air.

DNA extraction. Immediately after sampling, 8-liter portions of water from the three selected depths were filtered through sterile polycarbonate filters (diameter, 45 mm; pore size, 0.2 μm; Millipore, Bedford, Mass.). DNA was extracted with buffered phenol at 55°C, phenol-chloroform, and chloroform (44). Subsequent purification was performed with an EasyPure kit (Biozym, Hessisch Oldendorf, Germany).

Genomic DNA from bacterial cultures was extracted by a freeze-thaw protocol (28). One microliter of a cell pellet was added to 50 μl of filter-sterilized 50 mM Tris buffer (pH 7.4). The suspension was heated to 100°C for 3 min and then incubated at −80°C for 3 min. This procedure was repeated five times, and 1 μl of the extract was added to 50 μl of the PCR mixture.

Amplification, separation, and sequencing of 16S rDNA fragments. DNA fragments (length, 630 bp) of the natural community and of pure cultures were amplified by using eubacterial primers GC357f containing a 40-bp GC clamp (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC TAC GGG AGG CAG CAG-3′) (37) and 907r (5′-CCG TCA ATT CCT TTG AGT TT-3′). A hot start at 96°C (4 min) was applied; during cycling, the melting temperature was set at 94°C (30 s), and the extension temperature was 72°C (1 min). A step-down procedure was applied by using an annealing temperature of 61°C for the first 10 cycles and of 56°C for the subsequent 20 cycles.

The 16S ribosomal DNA (rDNA) fragments generated were separated on the basis of their melting behavior by denaturing gradient gel electrophoresis (DGGE) (37). DGGE was carried out with the Bio-Rad D Gene system (Bio-Rad, Munich, Germany) as described previously (44). Individual DNA bands from the natural community and from strains with multiple rRNA operons were excised with a sterile scalpel and then electroeluted (Centrilutor Micro-Electroelutor; Amicon, Witten, Germany) and purified (12). 16S rRNA genes of other pure cultures which did not display multiple bands during DGGE were amplified and sequenced directly.

Sequencing was performed with a SequiTherm Long-Read kit (Epicentre, Madison, Wis.) and an automated infrared laser fluorescence sequencer (Li-Cor model 4000 DNA sequencer) (44). The primers used for sequencing were the same as those used for PCR amplification, except that the GC clamp of primer GC357f was omitted.

Phylogenetic analysis. For each 16S rDNA sequence, the most closely related sequence and the sequence of the most closely related cultured bacterial strain were retrieved from the GenBank database by using BLASTN 2.0 (1) and from the Ribosomal Database Project by using the SIMILARITY_CHECK option (34) (Table 2).

The sequences were aligned with the CLUSTAL W program (57). Nucleotide positions that differed in more than 50% of all sequences were excluded from the analysis. This resulted in a total of 452 informative nucleotide positions. Phylogenetic trees were calculated with the maximum-likelihood program (DNAML) and the parsimony program (DNAPARS) of the PHYLIP 3.57c package (19).

ERIC PCR. In order to investigate the genomic heterogeneity among isolates of the same phylotype, enterobacterial repetitive intergenic consensus (ERIC) fingerprinting (59) was carried out. The primers used were ERIC1R (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′). The PCR conditions used and the method used for visualization of products have been outlined earlier (44).

TABLE 2. Reference organisms or sequences and accession numbers

Organism or clone	Accession no.
Alteromonas macleodii IAM 12920^T	X82145
Aquaspirillum itersonii NCIMB 9070^T	Z29620
Archangium gephyra Ar g1	AJ233912
Arthrobacter globiformis DSM 20124^T	X80736
Chlorobium limicola DSM 245^T	Y10113
"*Erythrobacter citreus*" RE35F/1	AF118020
Flavobacterium uliginosum ATCC 14397^T	M62799
Fundibacter jadensis DSM 12178^T	AJ001150
Halomonas aquamarina ATCC 14400^T	M93352
Marinobacter aquaeolei ATCC 700491^T	AJ000726
Microbacterium oxydans DSM 20578^T	Y17227
Oceanospirillum kriegii IFO15467^T	AB006767
Pseudomonas stutzeri ATCC 11607	U26415
Roseobacter algicola ATCC 51440^T	X78315
Uncultured α-proteobacterium NKB7	AB013259
γ-Proteobacterial strain HTB123	AB010866
γ-Proteobacterial strain HTB148	AB010868
Humic substance-degrading bacterial strain HS296	AF231417
Obligately oligotrophic bacterial strain POO-15	AB022706
Uncultured bacterium SkelCos2-14	AF216516
Uncultured hydrocarbon seep bacterium BPC065	AF154094

The resulting ERIC band patterns were subjected to a cluster analysis. A binary 0/1 matrix was created based on the absence or presence of DNA bands. Pairwise distances were calculated with the SimQual option of the NTSYSpc 2.02j computer program (Exeter Software, Setauket, N.Y.) by employing the Dice coefficient for two-state data. For the cluster analysis we employed the SAHN option of the package using the unweighted pair group method with arithmetic average for clustering.

Nucleotide sequence accession numbers. Sequences representing each of the 12 different cultured phylotypes, five environmental sequences, and four sequences of multiple operons of isolate UM4b have been deposited in the GenBank database under accession numbers AF321058 through AF321078.

RESULTS

Chemical and physical characterization of the habitat. On our sampling date, the chemocline of the Urania basin was located at a depth of 3,470 m below the sea surface. Within a 5-m depth interval, the oxygen concentration decreased below the detection limit, whereas the sulfide concentration reached 10 mM. Similarly, the concentrations of chloride, sulfate, and phosphate increased markedly (to 2.8 M, 85 mM, and 41 μM, respectively) (Fig. 2A and B; Table 3). Across this chemocline the temperature increased slightly (by 2.5°C), and there was a significant decrease in pH from 8.6 to 6.8. The $\delta^{34}S$ values for sulfate were 20.6 ± 0.14‰ above the chemocline and 25.4‰ in the brine. The $\delta^{34}S$ value of sulfide was found to be −15.2 ± 0.2‰ in samples from the chemocline, as well as in brine samples. This confirmed earlier measurements (Ziebis et al., J. Conf. Abstr. Goldschmidt Conf. 2000).

Total and viable cell counts. The total concentration of bacterial cells reached 1.3×10^5 cells · ml^{-1} in the chemocline and increased to a maximum of 2.2×10^5 cells · ml^{-1} at 10 m below the chemocline (Fig. 2C). The concentrations within and just below the chemocline were thus slightly, but significantly ($P < 0.005$), elevated compared to the concentrations in most water layers above the chemocline and to the concentrations in the brine below the chemocline.

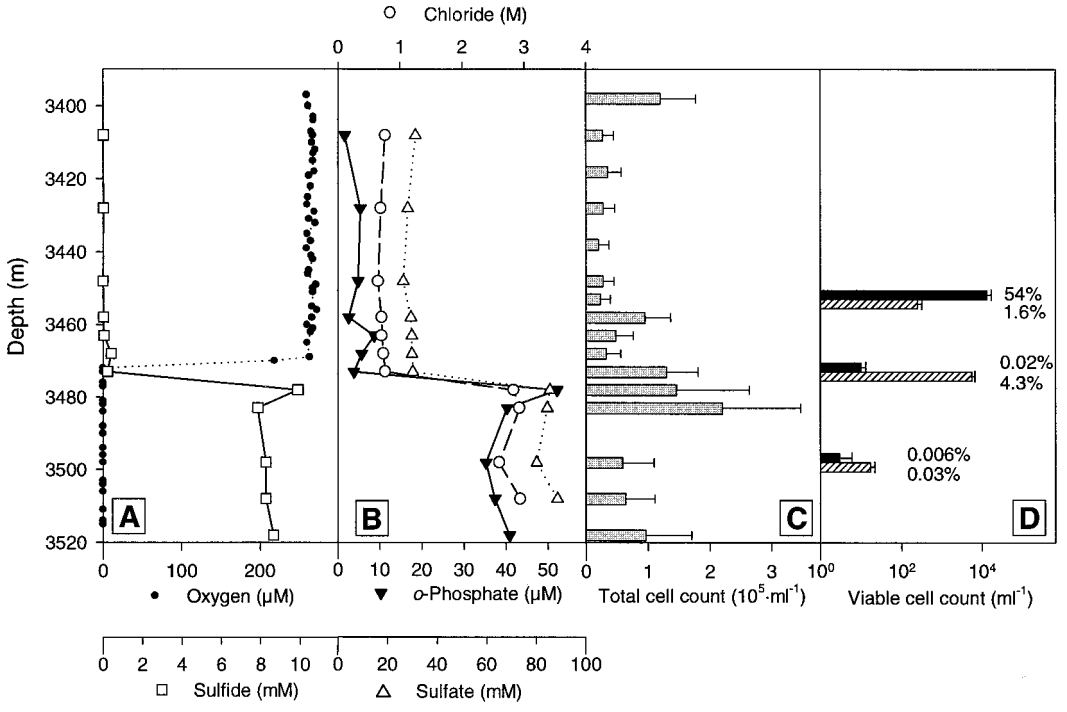

FIG. 2. Physicochemical parameters, total cell counts, and MPN across the oxic-anoxic interface of the Urania basin. (A) Oxygen and sulfide concentrations; (B) sulfate, *ortho*-phosphate, and chloride concentrations; (C) total cell counts; (D) viable cell counts in anoxic media containing the amino acid mixture (solid bars) and in oxic media containing thiosulfate (cross-hatched bars). Only values for the media which yielded maximum MPN are shown. The percentages are percentages relative to the total cell count. The error bars indicate standard deviations.

The maximum viable cell counts were obtained for samples from 3,455 m when anoxic media with alanine, arginine, asparagine, and cysteine as the sole carbon and energy sources were used. In these media up to 54% of all bacterial cells could be cultivated by the MPN technique (Fig. 2D). In contrast, the MPN values for the chemocline were highest in media supplemented only with thiosulfate and incubated under oxic conditions; a smaller fraction of the cells (4.3%) grew in these media. The culturability was substantially lower in samples from the anoxic brine, reaching 0.03% of total cell counts with thiosulfate as the substrate and 0.006% with amino acids as the substrates.

Exoenzyme activity. Within the chemocline, the activities of the exoenzymes β-glucosidase, aminopeptidase, and alkaline phosphatase were significantly increased compared to the activities in the water layers above and below (Fig. 3). The highest activity values were those observed for alkaline phosphatase, which reached 20.5 ± 1.5 nM · h^{-1}. The cell-specific exoenzyme activities in the chemocline were 15.8×10^{-17} mol · $cell^{-1}$ · h^{-1} for alkaline phosphatase and 4.58×10^{-17} and 1.57×10^{-17} mol · $cell^{-1}$ · h^{-1} for aminopeptidase and β-glucosidase, respectively (Fig. 3).

16S rDNA fingerprinting. After amplification of environmental DNA with the eubacterial primer set, 12 distinct bands were separated by DGGE (Fig. 4A). DNA samples from the three different depths examined yielded similar DGGE fingerprints; therefore, only the chemocline sample was analyzed further. After excision and sequencing, five of the bands (A1, A2, A4, A7, and A8) contained a single 16S rDNA, as judged from the absence of ambiguous base positions on sequencing gels. The five sequences were included in the phylogenetic analyses. In all cases, these molecular isolates clustered with sequences of typical marine bacteria (Fig. 5). Phylotype A4 was distantly related to the sequence of an uncultured member of

TABLE 3. Chemical compositions of different anoxic deep-sea brines and oxygenated seawater

Brine or seawater	Salinity (‰)	pH	O_2 concn (μM)	H_2S concn (mM)	SO_4^{2-} concn (mM)	PO_4^{3} concn (μM)	NH_4^+ concn (μM)	Ca^{2+} concn (mM)	Cl^- concn (mM)	References
Urania basin	200	6.8	0	8.6–11	85	41.2	ND[a]	34	2,830	20, 58; this study; Ziebis et al.[b]
Libeccio basin (Bannock area)	321	6.5	0	1.67	99	12	3,080	21	5,333	8, 16
Tyro basin	ND	6.75	0	2.15	49	10	1,260	34	5,350	8, 16
Orca basin (Gulf of Mexico)	258.1	5.5	0	0.60	47.1	82	500	32	5,000	16, 51
Oxygenated seawater (eastern Mediterranean)	39	8.2	200	0	31	0.2	0.2	11	660	8, 58

[a] ND, not determined.
[b] Ziebis et al., J. Conf. Abstr. Goldschmidt Conf. 2000. Mean values were calculated for the upper brine layers (depth, 3,490 to 3,520 m) in this study.

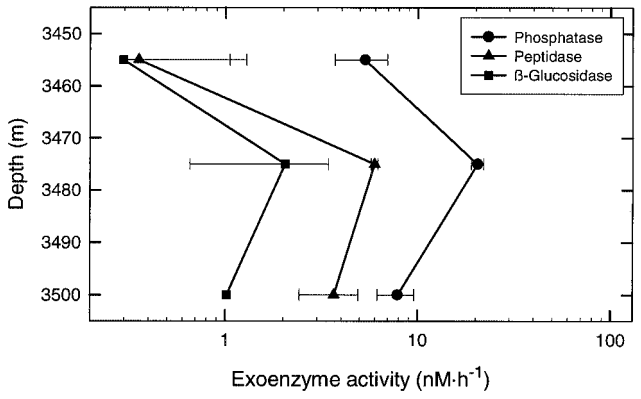

FIG. 3. Extracellular β-glucosidase, leucine aminopeptidase, and alkaline phosphatase activities in the Urania basin. The error bars indicate standard deviations.

the α subclass of the class *Proteobacteria* (α-proteobacteria) recovered from Pacific deep-sea sediments; its closest cultured relative was the marine organism *Roseobacter algicola* (Fig. 5). The partial sequences of bands A1 and A7 were identical or almost identical (99.8%) to sequences of the marine species *Halomonas aquamarina* and *Alteromonas macleodii*, respectively. The closest relative of environmental sequence A8 was an uncultured bacterium from a hydrocarbon seep. Finally, pronounced band A2 contained a 16S rDNA sequence which fell in the *Cytophaga-Flavobacterium* group and was closely related to an uncultured marine bacterium detected in the phycosphere of a marine diatom (Fig. 5). An analysis by the parsimony method fully supported the phylogenetic tree generated by the maximum-likelihood method.

Of the 70 bacterial isolates obtained in the present study, 27% were affiliated with the α-proteobacteria, 62% were affiliated with the γ-proteobacteria, 8% were affiliated with the *Cytophaga-Flavobacterium* group, and 3% were affiliated with the high-G+C-content gram-positive bacteria (Fig. 4 and 5). A comparison of the 16S rDNA fingerprints of the natural bacterial community with those of the 70 cellular isolates revealed that about one-half of our strains (32 strains) potentially represent bacteria which are also detectable by culture-independent methods in the natural bacterial community of the chemocline (Fig. 4). Sequencing confirmed that strains with 16S rDNA fingerprints corresponding to those of bands A1, A2, and A7 indeed also contained the same sequences (Fig. 5). Eighteen strains of *H. aquamarina* represent phylotype A1 of the natural community. Six cellular isolates had partial 16S rDNA sequences identical to the band A2 sequence and grouped with the *Cytophaga-Flavobacterium* division.

Eight of the strains isolated (U4, U7, U8, U10, U12, UM4b, UM7, UM8) showed multiple DGGE bands (Fig. 4B) which were indicative of the presence of multiple 16S rRNA operons (39). Sequencing the four most frequently detected bands (lowermost bands *rrnA* through *rrnD* in Fig. 4B) of two of the strains (U4, UM4b) revealed that the bands with different melting behaviors differed in only one or two bases. All sequences clustered closely with the *A. macleodii* sequence (Fig. 5); one of the bands (*rrnD*) corresponded to band A7 of the natural bacterial community.

In order to estimate the abundance of phylotypes A1, A2, and A7 in the natural community, we performed MPN-PCR (40) at fivefold dilution steps using three replicates. For each dilution, the presence of phylotypes A1, A2, and A7 among the amplification products was checked by DGGE. Amplicons of phylotypes A1 and A7 were detected in PCR assay mixtures that contained 2 ng of genomic DNA, whereas amplicons of A2 were found also with the next dilution step mixtures (containing 0.4 ng of DNA). Based on the MPN values obtained (1.6×10^{10} and 8.2×10^{10} targets per g of genomic DNA, respectively), phylotypes A1 and A7 contribute about 3.6% of the total eubacterial template in the chemocline (confidence interval, 0.003 to 17.3%) and phylotype A2 contributes about 18.3% (confidence interval, 1.4 to 88.6%).

Genetic and physiological diversity within the same phylotype. The three groups of bacterial strains which represented phylotypes of the natural community consisted of 6 to 18 identical partial 16S rDNA sequences and hence phylogenetically were very closely related or even identical. Therefore, we investigated whether the different bacterial strains of the same phylotype were genetically and physiologically different or whether members of one homogeneous population had been obtained. The genomic heterogeneity as analyzed by ERIC PCR was considerable in all three groups (Fig. 6). Only a few strains yielded identical ERIC fingerprint patterns. Even strains from the same water sample and isolated in the same medium were genetically different. Genetic diversity was highest among members of the *A. macleodii* cluster. The diversity among strains of the same phylotype was also assessed by physiological characterization of the 70 strains using 67 growth substrates and different incubation conditions (A. Sass, J. Overmann, M. Coolen, H. Cypionka, and H. Sass, submitted for publication). These tests revealed considerable physiological differences between strains of the same phylotype isolated from the same depth, and the results support the view that different bacterial ecotypes had been isolated from the chemocline of the Urania basin.

Culturability. Most notably, the success of cultivation of bacteria belonging to phylotypes A1, A2, and A7 was found to depend on the type of medium employed and also on the sampling depth (Table 1). All members of the *Cytophaga-Flavobacterium* group were obtained by cultivation on complex solid media under oxic conditions when the sample obtained above the chemocline was used. No isolate belonging to this group was obtained from the chemocline, although a corresponding molecular fingerprint was clearly detected in this sample by the culture-independent method (Fig. 4A, band A2). This result may reflect vertical differences in the viability of the bacteria. Strains of *A. macleodii* were isolated on the complex solid media, as well as in media containing only thiosulfate and O_2 (Table 1). The numerous strains of *H. aquamarina* were isolated either as aerobic organoheterotrophs on complex solid medium or as anaerobic organoheterotrophs with fatty acids or lactate and in media designed for chemolithotrophs containing polysulfides or supplemented with thiosulfate.

DISCUSSION

Representative character of the culture collection. Different statistical analyses showed that the culture collection investi-

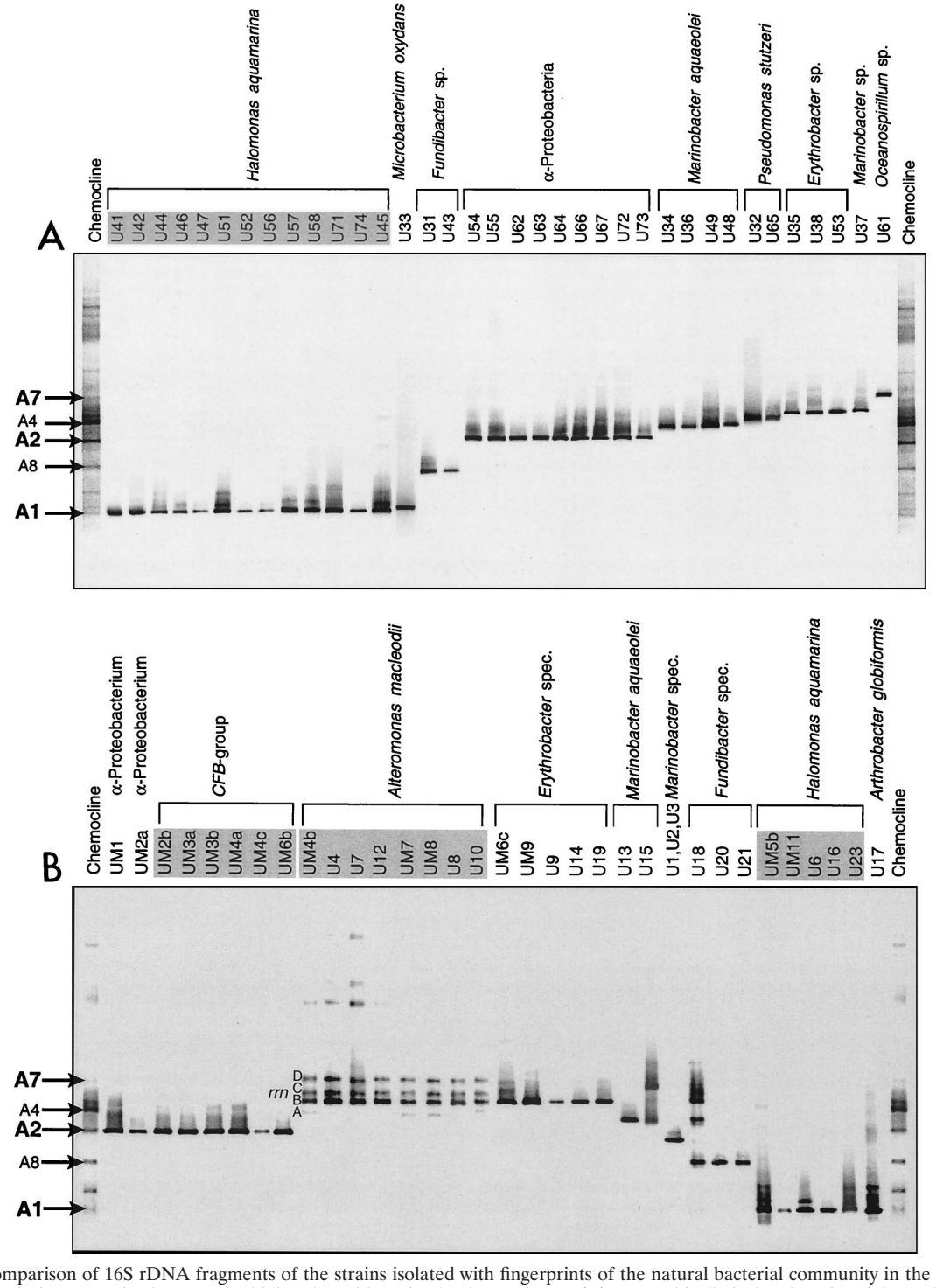

FIG. 4. Comparison of 16S rDNA fragments of the strains isolated with fingerprints of the natural bacterial community in the chemocline by DGGE fingerprinting. Negative images of ethidium bromide-stained gels are shown. (A) Anaerobically isolated strains; (B) aerobically isolated strains. Strains were assigned to species and to genera if sequence identities were >97 and >93%, respectively. Strains marked by shading had 16S rDNA sequences identical to those of band A1, A2, or A7. CFB-group, *Cytophaga-Flexibacter-Bacteroides* group.

gated in the present study is large enough to represent the majority of the bacteria which are culturable by our methods. First, the cumulative plot of the different phylotypes reached saturation (Fig. 7); a total of 13 phylotypes could be distinguished. Rarefaction analysis (Fig. 7) also revealed that continued isolation of strains would have yielded very few new phylotypes. Second, a high sample coverage value was obtained in the present study. The coverage (C) [$C = 1 - (n_1/N)$, where n_1 is the number of phylotypes which occurred only once in our culture collection and N is the total number of strains (36)] was

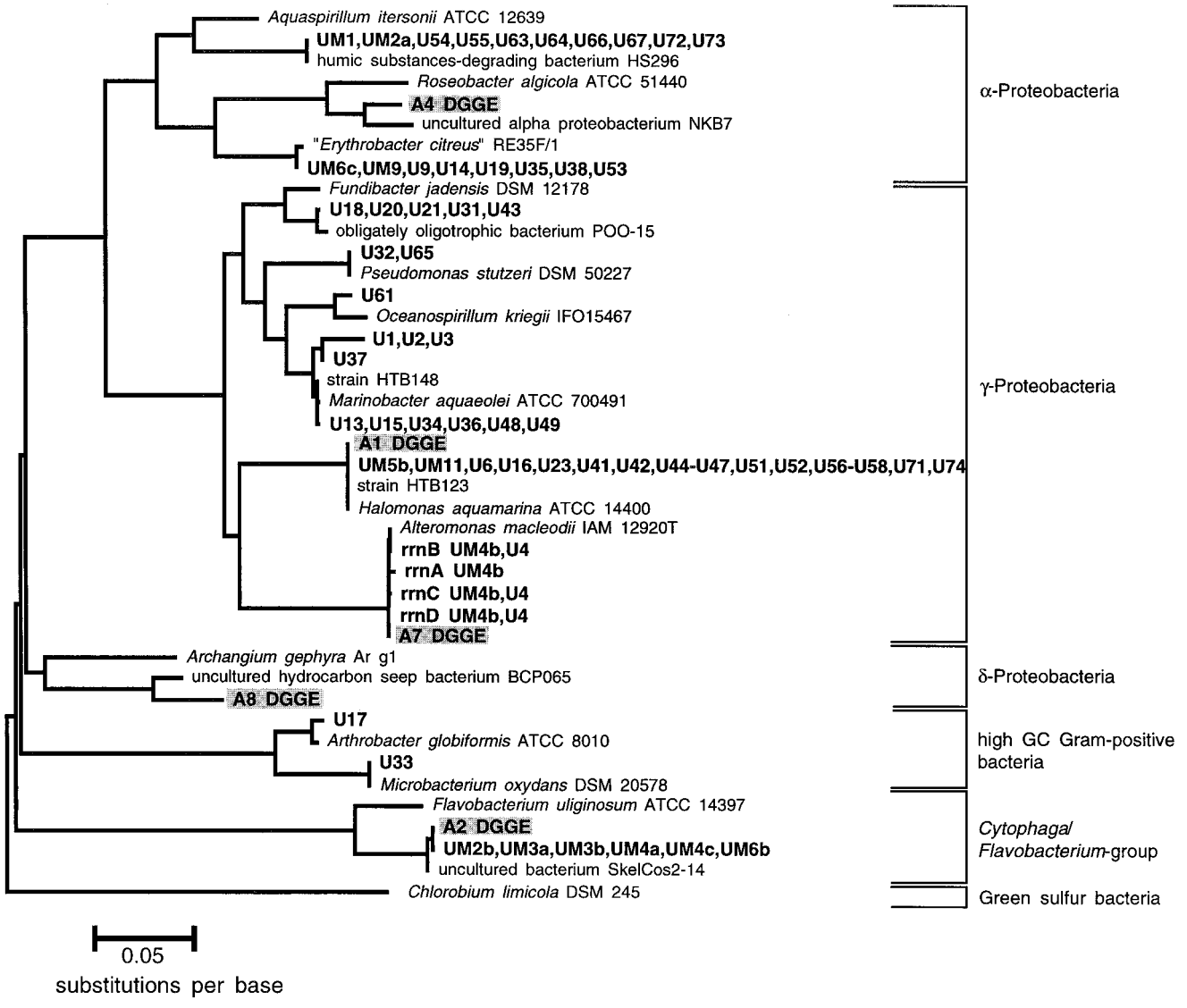

FIG. 5. Phylogenetic affiliations of the strains isolated and the molecular isolates from the chemocline bacterial community. The tree is based on 452 informative nucleotide positions of the 16S rRNA gene. *Chlorobium limicola* was used as the outgroup.

94.4%. This very high value indicates that our culture collection included almost all of the bacterial phylotypes of the natural community which grew in the 11 media used.

Bacterial culturability. One of the most pressing problems in bacterioplankton ecology is the small fraction of the total bacterial cells which can be cultivated (2, 18). Especially low values have been reported for seawater samples (0.001 to 0.1%) (2), and it has been suggested that the vast majority of the numerically important bacteria have not been cultured so far (55). In the present study, three 16S rDNA sequences detected in the natural community were also present in almost one-half of our bacterial isolates. Commonly, only those 16S rDNA sequences which constitute more than 1% of all template molecules in a natural bacterial community are detectable by the PCR-DGGE approach (38). Based on the results of our MPN-PCR analysis, phylotypes A1, A2, and A7 contributed approximately 4 to 18% of all eubacterial template molecules. The isolates of the *Cytophaga-Flavobacterium* group are especially important numerically. Our results support the view that at least some of the abundant marine bacteria are readily culturable (21, 47). Only a single sampling was performed, and limited amounts of sample material from the deep sea were available to us. Therefore, more precise quantification of phylotypes A1, A2, and A7 has to await future molecular investigations of the Urania basin chemocline.

Eight of the isolates which corresponded to a molecular isolate were identified as *A. macleodii*. This species has been shown to constitute a major fraction of bacterioplankton in eutrophied Mediterranean seawater (49), and it has been speculated that microheterogeneity occurs at the *rrn* operon level of this species. Interestingly, 16S rDNA fingerprinting revealed that all eight strains of *A. macleodii* isolated in the present study contained the same multiple *rrn* operons which differed at one or two nucleotide positions over the 452 nucleotide

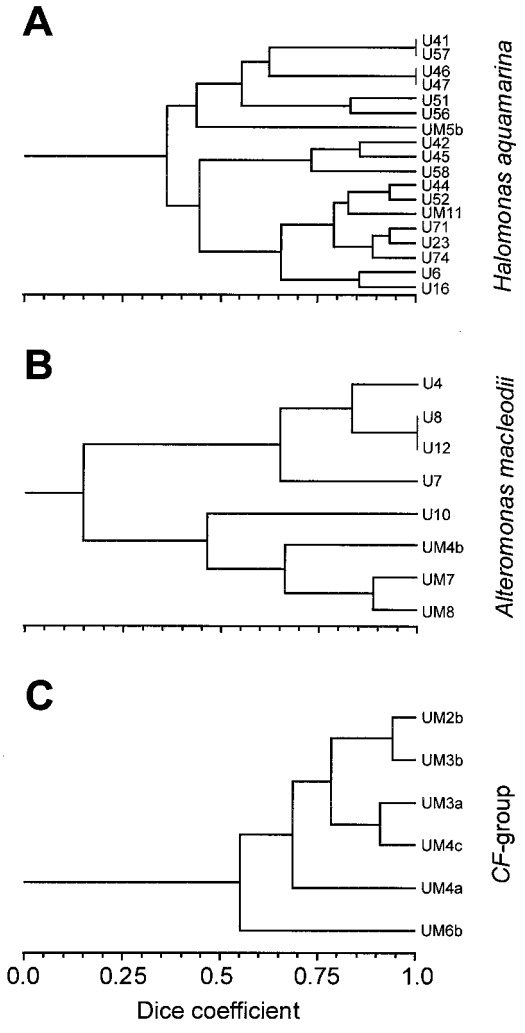

FIG. 6. Genetic diversity of the three groups of bacterial isolates which represent numerically significant phylotypes. The dendrograms are based on ERIC PCR band patterns. *CF*-group, *Cytophaga-Flavobacterium* group.

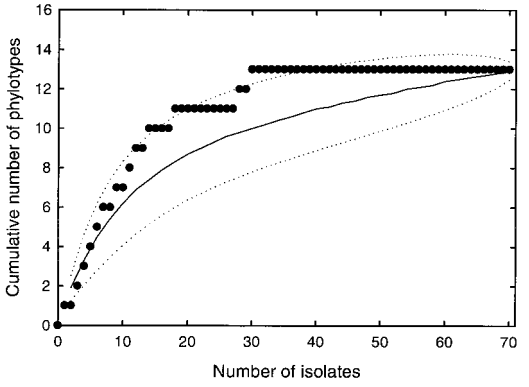

FIG. 7. Diversity of phylotypes in the collection of 70 bacterial isolates. A total of 13 phylotypes were detected. After the order of strains was randomized, sequential detection of phylotypes was plotted in a cumulative manner (●). For comparison, a rarefaction curve (solid line) was produced from the data by employing the freeware program Analytical Rarefaction 1.2 (compiled by Steven M. Holland; available at http://www.uga.edu/~strata/Software.html). The dotted lines indicate 95% confidence intervals.

positions analyzed. *A. macleodii* obviously is a widely distributed marine species which, in contrast to many other marine bacteria, is characterized by the existence of multiple, heterogeneous *rrn* operons.

Environmental conditions in the Urania basin chemocline. In the chemoclines of deep-sea hypersaline brines, the concentrations of particulate organic carbon and dissolved organic carbon are significantly increased. It has been proposed that these conditions may stimulate bacterial activity (24).

Compared to other deep-sea brine lakes, the outstanding feature of the Urania basin is its high sulfide content (Table 3). The sulfide concentration in this basin is up to 10 mM (mean concentration in the brine, 8.6 mM) and is higher than the sulfide concentration in any other marine water body investigated to date. The sulfur isotope values for sulfide and sulfate agree well with earlier measurements (Ziebis et al., J. Conf. Abstr. Goldschmidt Conf. 2000) and indicate that sulfide is formed from microbial reduction of sulfate. The apparent isotope fractionation in the brine, about −41‰, is within the range observed in pure cultures of sulfate-reducing bacteria (up to −46‰) (32). Corresponding to the high sulfide concentrations, high rates of sulfate reduction (6 to 14 μmol · liter^{-1} · day^{-1}) have been determined in the brine (Ziebis et al., J. Conf. Abstr. Goldschmidt Conf. 2000). These values are high compared to those in other anoxic pelagic systems, like the Black Sea (3 to 36 nmol · liter^{-1} · day^{-1}) (31).

The primary production in the oligotrophic eastern Mediterranean Sea is about 16 g of C · m^{-2} · year^{-1} (61). Based on the stoichiometry of sulfate reduction, which can be calculated by $2<CH_2O> + SO_4^{2-} + 2H^+ \rightarrow H_2S + 2H_2O + 2CO_2$, the primary production (if all of it reaches the chemocline) could supply a maximum of 0.24% of the carbon demand of the sulfate-reducing bacteria beneath 1 m^2 of the brine lake. Obviously, sedimentation of phytoplankton from the photic water layers is far from sufficient to explain the sulfate reduction rates observed. Hence, organic carbon must reach the Urania basin by other routes, such as lateral advection of suspended organic matter from the continental shelf or erosion of sediment layers rich in organic carbon (so-called green mud containing >2% organic carbon) (20) which become exposed to the brine water. Another possible substrate for sulfate reduction may be methane (6, 27), which has been detected at high concentrations in the brine (2.6 to 3.8 mM) (20).

Since the vertical sedimentation flux of organic carbon appears to contribute little to the overall carbon budget, chemoorganoheterotrophic bacteria are expected to have little biogeochemical significance in the chemocline of the Urania basin. In contrast, it is expected that chemolithotrophic sulfur-oxidizing bacteria constitute a major fraction of the microbial community in the chemocline of the Urania basin since a pronounced sulfide gradient exists at the interface with oxic water layers. Both our culture-independent analysis and an analysis of the culturable fraction of the bacterioplankton yielded a different result, however.

Biogeochemical implications. The activity of leucine aminopeptidase is associated exclusively with heterotrophic bacteria (10). This enzyme, unlike alkaline phosphatase and β-glucosi-

dase, is not induced by its natural substrates (polypeptides and proteins) in marine sediments; however, it is inhibited by glycine and other amino acids (5). Since our cell-specific values are comparable to maximum values determined in other environments (up to 2.97×10^{-17} mol · cell^{-1} · h^{-1} [13, 25]), a large fraction of the bacterial cells present in the chemocline must be physiologically active.

The exoenzymes alkaline phosphatase and β-glucosidase are inducible by their substrates (organic phosphoesters and cellobiose, respectively) and are subject to catabolite repression by their products (glucose and phosphate, respectively) (10, 52). Cell-specific activities of these two enzymes may thus be used as indicators of the presence of degradable biopolymers (10, 14, 15, 42). Since the cell-specific phosphatase activities determined in the present study were much lower than those of phosphate-deficient bacterial communities (3×10^{-15} to 7.7×10^{-15} mol · cell^{-1} · h^{-1} [42]), bacteria in the chemocline of the Urania basin appear not to be limited by inorganic phosphate. Obviously, the supply of phosphate from the anoxic brine is sufficient to repress the synthesis of alkaline phosphatase in the cells. In contrast to the specific activities of alkaline phosphatase, the specific activities of β-glucosidase exceeded values determined for other pelagic water samples (50×10^{-20} mol · cell^{-1} · h^{-1} [25]) by a factor of >30. It can be concluded that degradable carbohydrate biopolymers are present and are degraded by bacteria in the chemocline despite the supposedly small amount of organic matter which reaches the Urania deep-sea environment.

The strains affiliated with the *Cytophaga-Flavobacterium* group were obligate chemoorganoheterotrophs and corresponded to a major DGGE band of the natural bacterial community (band A2). All 70 strains, including the 21 strains isolated in media designed for chemolithotrophs, (Table 1), could grow chemoorganoheterotrophically under oxic conditions (Sass et al., submitted). Similar results have been obtained for marine sediments and hydrothermal vents, where liquid MPN series containing thiosulfate yielded heterotrophic isolates with the highest dilutions (56). Conversely, 15 of the strains isolated as chemoorganoheterotrophs could not grow chemolithotrophically with reduced sulfur compounds. Sixty-six strains could grow on peptone, casein hydrolysate, and yeast extract, whereas only 15 strains did not utilize polymeric carbohydrates. Together with the high specific activity of β-glucosidase, our results indicate that chemoorganotrophic bacteria constitute a significant fraction of the natural bacterial community.

The 16S rDNA sequences retrieved by the culture-independent PCR-DGGE approach did not match sequences of typical chemolithoautotrophic chemocline bacteria expected to thrive in a chemocline with oxygen and sulfide countergradients (45). However, 55 of our isolates were facultatively chemolithoautotrophic and used reduced sulfur compounds (Sass et al., submitted). Molecular isolate A4 is affiliated with the genus *Roseobacter*, which comprises many facultatively chemolithotrophic sulfide oxidizers (56). Members of the *Pseudomonas stutzeri* group (Fig. 5, compare isolates U32 and U65) have been shown to utilize thiosulfate under oxic conditions as well as under anoxic conditions in the presence of organic carbon substrates (53). Possibly, oxidation of sulfide in the chemocline of the Urania basin is mediated to a large extent by such mixotrophic bacteria. Our findings are in line with those of other studies of marine waters and sediments, in which heterotrophic sulfur-oxidizing bacteria are common (56).

The bacterial isolates obtained in the present study resemble those which are frequently obtained from the marine water column and represent a subset of the standard pelagic bacterial groups that is frequently found with molecular methods (18, 36, 47, 49, 55). It therefore appears that a major fraction of the chemocline bacteria may originate in upper water layers and reach the pycnocline by sedimentation. Even if the typical heterotrophic bacteria are of allochthonous origin, our results indicate that these organisms must still be capable of resuming active metabolism at the oxic-anoxic interface and growing to higher cell densities.

Hypersaline sediments from the Kebrit Deep at the bottom of the Red Sea harbor unknown *Euryarchaeota*, as well as *Bacteria* which are distantly related to the *Aquificales* and *Thermotogales* (17). The salt concentration in hypersaline brine sediments of the Red Sea is 4.3 M and thus exceeds the chlorinity in sediments of the Urania basin. Most of our isolates originated from significantly less saline (1 M chloride) chemocline waters of the Urania basin and were identified as proteobacteria, high-G+C-content gram-positive bacteria, or members of the *Cytophaga-Flavobacterium* group. Future research should reveal whether the composition of microbial communities changes along the salinity gradient in the Urania basin and whether *Archaea* are abundant in the hypersaline brine.

In conclusion, the present polyphasic study provided new insights into the ecophysiology of bacteria in the chemocline of the Urania basin. Physiologically active chemoorganotrophic bacteria are present and appear to be more dominant than chemolithoautotrophs. Our repeated isolation of bacteria that had the same partial 16S rDNA sequence but produced different ERIC band patterns and had distinct physiological properties could merely reflect genetic variation within one species. Alternatively, the microdiversity may indicate that there are different ecotypes which occupy different ecological microniches in the chemocline of the Urania basin.

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