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Distribution of Sulfate-Reducing Bacteria in a Stratified Fjord (Mariager Fjord, Denmark) as Evaluated by Most-Probable-Number Counts and Denaturing Gradient Gel Electrophoresis of PCR-Amplified Ribosomal DNA Fragments

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The sulfate-reducing bacterial populations of a stratified marine water column, Mariager Fjord, Denmark, were investigated by molecular and culture-dependent approaches in parallel. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA and DNA encoding rRNA (rDNA) isolated from the water column indicated specific bacterial populations in different water column layers and revealed a highly differentiated pattern of rRNA- and rDNA-derived PCR amplificates, probably reflecting active and resting bacterial populations. Hybridization of DGGE patterns with rRNA probes indicated the increased presence and activity (by at least 1 order of magnitude) of sulfate-reducing bacteria within and below the chemocline. Parallel to this molecular approach, an approach involving most-probable-number (MPN) counts was used, and it found a similar distribution of cultivable sulfate-reducing bacteria in the water column of Mariager Fjord, Approximately 25 cells and 250 cells per ml above and below the chemocline, respectively, were found. *Desulfovibrio*- and *Desulfobulbus*-related strains occurred in the oxic zone. DGGE bands from MPN cultures were sequenced and compared with those obtained from nucleic acids extracted from water column samples. The MPN isolates were phylogenetically affiliated with sulfate-reducing delta subdivision proteobacteria (members of the genera *Desulfovibrio*, *Desulfobulbus*, and *Desulfobacter*), whereas the molecular isolates constituted an independent lineage of the delta subdivision proteobacteria. DGGE of PCR-amplified nucleic acids with general eubacterial PCR primers conceptually revealed the general bacterial population, whereas the use of culture media allowed cultivable sulfate-reducing bacteria to be selected. A parallel study of Mariager Fjord biogeochemistry, bacterial activity, and bacterial counts complementing this investigation has been presented elsewhere (N. B. Ramsing, H. Fossing, T. G. Ferdelman, F. Andersen, and B. Thamdrup, Appl. Environ. Microbiol. 62:1391–1404, 1996).

Oxygen-deficient marine basins show characteristic chemical zones because of the gradual depletion of oxygen and other oxidized chemical species in the water column (10). The redox gradient in the water column is mirrored by different modes of bacterial respiration, from oxic respiration through denitrification, manganese and iron reduction, sulfate reduction, and methanogenesis (22). According to the classic picture, oxidative processes occur in the oxygenated water above the chemocline and reductive processes, i.e., denitrification and sulfate reduction, are confined to the anoxic zone. The confinement of sulfate reduction to anoxic water layers has, however, been questioned. Sulfate reduction in the presence of oxygen was observed in and above the chemocline of the Cariaco trench (18). Particle-associated methanogenic and sulfate-reducing bacteria were enriched from oxic surface water as well as from the pycnocline and bottom water of the Chesapeake Bay (48).

Mariager Fjord (Denmark) is an especially suitable marine environment to investigate the potential overlap of aerobic and anaerobic bacterial populations and their metabolic processes in a stratified water column (33). This fjord is connected to the sea by a long, narrow channel with a maximum depth of 4 m but widens and deepens in its inland part to a depth of 25 to 30 m. The narrow channel limits the exchange of oxygenated water and leaves the inland basin of the fjord anoxic from a depth of approximately 14 m to the bottom (33).

This polyphasic study of the distribution of sulfate-reducing bacteria in Mariager Fjord used cultivation-dependent and molecular approaches in parallel to investigate the bacterial ecosystem as well as the strengths and biases inherent in the methods. A geochemical evaluation of Mariager Fjord and direct bacterial counts by fluorescent in situ hybridization were performed in parallel to this study at the same location and time with the same samples as a framework for and complement to this investigation (42). Denaturing gradient gel electrophoresis (DGGE) of enzymatically amplified 16S DNA encoding rRNA (rDNA) was used as a molecular approach for the analysis of the microbial community of Mariager Fjord. PCR products that have the same lengths can be separated by DGGE on the basis of melting domain structure and nucleotide composition (13, 38, 52). This technique has recently been introduced into molecular microbial ecology to determine the genetic diversity of natural microbial communities (35) and to identify dominant community members whose phylogenetic affiliations were subsequently determined by sequencing the DGGE fragments (34, 37). Bacterial DNA and total RNA were extracted from water column samples; rRNA was transcribed into ribosomal cDNA (rcDNA) and subsequently amplified by PCR. Since metabolically active cells contain more rRNA than resting or dormant cells (29, 41, 45), the DGGE

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patterns of PCR-amplified rRNA conceptually represent the metabolically active, rRNA-rich bacterial populations. The DGGE patterns derived after amplification of the ribosomal genes represent the presence of bacterial populations but do not take metabolic activity into account. These two types of DGGE patterns differ significantly. In addition, they reveal how the composition of the bacterial community changes throughout the water column. DGGE gels were analyzed by blotting and by the reamplification and sequencing of individual DGGE bands. The resulting picture was evaluated with reference to the physical parameters of the water column, total bacterial counts, and counts of sulfate-reducing bacteria in a most-probable-number (MPN) series.

We performed MPN counts of sulfate-reducing bacteria in the water column to assess the congruence of the classical microbiological and molecular, rRNA-related approaches in the detection and quantification of sulfate-reducing bacterial populations. Special attention was paid to the enumeration and identification of sulfate-reducing bacteria from the oxic part of the water column. MPN cultures of sulfate-reducing bacteria from the oxic part of the water column were analyzed phylogenetically to evaluate the existence of a possible distinct group of oxygen-tolerant, sulfate-reducing bacteria.

MATERIALS AND METHODS

Sampling of bacteria. Water samples were taken from Station Dybet M3, approximately 1 km north of the harbor of Mariager (56°39′66″N, 09°58′56″E), on 19 and 20 August 1993. The position of the chemocline was determined with a conductivity-temperature-depth sensor, measuring pressure, temperature, conductivity, oxygen (two independent sensors), incident light, and fluorescence. The sampling scheme was devised to obtain water samples from the upper oxic water layer, from the chemocline, and from the anoxic bottom water. The chemocline was distinguished by the simultaneous presence of both O_2 and H_2S in micromolar concentrations (see Fig. 3A in reference 42). Water samples for nucleic acid extraction and MPN counts were taken from the oxic water layer (8 m deep), from the upper, central, and lower chemocline (13, 14.5, and 16 m deep), and from the anoxic water layer (20 m deep). Sediment samples were taken at a depth of 24.3 m. For nucleic acid extraction, bacteria were collected from 200 ml of seawater and from 20 ml of bottom sediment. Water and sediment samples were filtered through Durapore filters (pore size, 0.22 μm; filter diameter, 25 mm; Millipore) with a syringe filtration device to capture bacteria and biomass on the filter for subsequent nucleic acid extraction. The very soft sediment samples were concentrated from 20 ml to a total volume of 2 ml by filtration. Filters with captured bacteria and biomass were frozen immediately and stored in liquid nitrogen until nucleic acid extraction.

MPN counts of sulfate-reducing bacteria. Numbers of viable sulfate-reducing bacteria were estimated with MPN dilutions in liquid medium (2). The multipurpose medium of Widdel and Bak (55) for sulfate-reducing bacteria was used with nonchelated trace element mixture no. 1 and seawater. MPN counts were performed on lactate (20 mM), acetate (20 mM), and H_2 plus 2 mM acetate as the carbon source. The medium was prepared anaerobically in a pressure-proof modified Erlenmeyer flask (55) and dispensed into culture vials (Kimex). After the medium was dispensed, each MPN vial was immediately gassed with a gassing syringe according to the Hungate technique and sealed with a butyl stopper. Lactate and acetate MPN vials were gassed with a mixture of 90% (vol/vol) N_2 and 10% (vol/vol) CO_2. The headspaces of the hydrogen MPN vials were gassed anoxically with a mixture of 90% (vol/vol) H_2 and 10% (vol/vol) CO_2.

Sulfate reducers from 8-, 13-, 14.5-, 16-, and 20-m-deep and bottom sediment samples were counted in a threefold MPN dilution series. The MPN dilution series was carried out with 1-ml water or sediment samples that were subsequently diluted in eight 1:10 dilution steps.

Nucleic acid extraction. Nucleic acids were extracted from the filters by the method of Oelmüller et al. (39) adapted as follows. The filters were thawed and washed twice with 1 ml of ice-cold AE buffer (20 mM sodium acetate [pH 5.5], 1 mM EDTA). Each filter and buffer were added to 6 ml of hot Tris-EDTA (TE)-buffered phenol-chloroform-isoamylalcohol (25:24:1, pH 8) and 60 μl of 25% (wt/vol) sodium dodecyl sulfate (SDS). After 5 min of incubation at 60°C, the solution was cooled on ice and then centrifuged for 5 min at 4,000 × *g*. A 250-μl portion of 2 M sodium acetate, pH 5.2, was added to the aqueous phase. Proteins and lipids were removed by repeated extraction of the aqueous phase with 5 ml of TE-buffered phenol-chloroform-isoamylalcohol (25:24:1, pH 8). Nucleic acids were precipitated with 2.5 volumes of 96% (vol/vol) ethanol for 3 h at −20°C, and the precipitation was followed by 60 min of centrifugation at 4,000 × *g*. The pellet was washed with 75% (vol/vol) ethanol, dried for 20 min under a vacuum, and redissolved in 50 μl of diethyl pyrocarbonate-treated water. Nucleic acid preparations including rRNA were tested in 2% (wt/vol) agarose gels with MOPS (morpholine propanesulfonic acid) buffer (46). The total amounts of nucleic acids extracted from the 200-ml water column samples were between 5 and 10 μg. Several different protocols for nucleic acid extraction were evaluated in test experiments with Mariager Fjord sediment and water samples. However, only the protocol described above proved successful in isolating rRNA. The DNA yield by this method was equivalent to yields by methods optimized for DNA extraction (15). We therefore used only a single nucleic acid procedure to obtain both RNA and DNA. The DNA and rRNA bands of the gel picture (see Fig. 1) were scanned to quantify DNA and rRNA separately, and the ethidium bromide staining intensities were integrated with the software program Photoshop 3.0 (Adobe). The nucleic acid extracts were used for the amplification of rDNA. A 1-μl portion of each water column extract diluted 1:10 and 1 μl of the sediment extract diluted 1:100 to account for the higher concentration were used for PCR amplification (10 to 20 ng of DNA input).

DNA was extracted from MPN cultures (0.5-ml sample volume) by a proteinase K-SDS digestion step followed by phenol extraction and ethanol precipitation (46).

Preparation of rcDNA. The undiluted nucleic acid extract described above (17 μl) was mixed with 2 μl of 10× DNase buffer (400 mM Tris-HCl [pH 7.5], 60 mM $MgCl_2$, 20 mM $CaCl_2$) and 1 μl of DNase (10 U/μl; Boehringer, Mannheim, Germany) and incubated for 30 min at 37°C. The DNase was removed by phenol-chloroform extraction. After ethanol precipitation and centrifugation, the resulting RNA pellet was dissolved in 20 μl of water. Four microliters of each RNA preparation containing rRNA and mRNA was checked on a MOPS agarose gel for contamination with genomic DNA. The RNA preparations were subsequently transcribed into cDNA. Transcription was carried out with 10 μl of RNA solution. The solution was denatured at 70°C for 10 min. Subsequently, 1 μl of hexanucleotides (Boehringer Mannheim) in a 10× concentration diluted 1:50, 2 μl of 10× PCR buffer, 2 μl of 0.1 M dithiothreitol, and 4 μl of deoxynucleoside triphosphates (dNTP) (2.5 mM each) were added. The mixture was incubated for 2 min at 37°C before 1 μl of SuperscriptTM reverse transcriptase (200 U/μl; Gibco BRL Life Technologies Inc.) was added. The samples were incubated at 37°C for 1 h and subsequently heated to 95°C for 5 min to stop the reaction. The cDNA preparations were diluted 1:1,000 with water. Of these diluted preparations, 1 μl was used in the PCR (50-μl volume). Random priming of rcDNA synthesis with a hexanucleotide mix prevents the premature termination of 16S rcDNA synthesis because of modified nucleotides, such as those at positions 966 and 967 of the 16S rRNA (54). Random hexamers are consistently more efficient for cDNA synthesis than target site-directed primers, as was shown by cDNA PCR (26).

PCR amplification of rDNA fragments. The 16S rRNA genes from mixed bacterial DNA or cDNA were amplified by PCR. The primer combination GM5F-GC clamp and DS907R amplified a 550-bp fragment of the 16S rRNA suitable for subsequent DGGE analysis, membrane hybridization, and sequencing (Table 1). This PCR amplificate yields sufficient sequence information for phylogenetic analysis (37). PCR amplifications were performed with a Techne PHC-3 temperature cycler (Techne, Cambridge, United Kingdom) as follows. Target DNA (10 to 20 ng) or 1 μl of cDNA diluted 1:1,000 was mixed with 25 pmol of each of the appropriate primers, 5 μl of 10× PCR buffer (i.e., 100 mM Tris-HCl [pH 9], 15 mM $MgCl_2$, 500 mM KCl, 0.1% [wt/vol] gelatin, 1% [vol/vol] Triton X-100), 4 μl of 2.5 mM deoxyribonucleoside triphosphate solution, and sterile water in a final volume of 50 μl and was overlaid with 2 drops of mineral oil (Sigma Chemicals Co. Ltd.) in a 0.5-ml vial. To minimize the nonspecific annealing of the primers to nontarget DNA, 0.5 U of SuperTaq DNA polymerase (HT Biotechnology Ltd.) at a temperature of 80°C was added to the reaction mixture after the initial denaturing step (94°C for 5 min). In addition, to increase the specificity of the amplification and to reduce the formation of spurious by-products, a "touchdown" PCR (9) in which the annealing temperature was set to 65°C, which is 10°C above the expected annealing temperature, and decreased by 1°C every second cycle until a touchdown of 55°C, at which temperature 10 additional cycles were carried out, was performed. The annealing temperature, T_m (in degrees centigrade), was estimated as $81.5 + 16.6(\log_{10}[cat^+]) + 0.41$ (% G+C) − (600/*N*) (46). Denaturation was carried out at 95°C for 1 min, the annealing time was 1 min, and primer extension was carried out at 72°C for 3 min. The ramp times were set to the fastest mode of the PHC3 Thermocycler. Aliquots (5 μl) of the amplification products were analyzed by electrophoresis in 2% (wt/vol) Nusieve agarose gels (FMC) containing 0.5 μg of ethidium bromide per ml.

DGGE analysis. PCR products obtained with primer combination GM5F-GC clamp and DS907R were analyzed by DGGE. DGGE was performed with a Bio-Rad Protean II system as described previously (35, 36). PCR products were prepared as follows. PCR product (300 μl) was precipitated with 30 μl of 5 M NaCl and 750 μl of ethanol at −80°C for 1 h. After centrifugation, the pellet was washed with 70% (vol/vol) ethanol, dried under a vacuum, dissolved in 15 μl of water, and applied to the DGGE gel.

DGGE was performed with 6% (wt/vol) polyacrylamide gels in 0.5× TAE (20 mM Tris-acetate [pH 7.4], 10 mM acetate, 0.5 mM Na_2 EDTA) with denaturing gradients ranging from 20 to 70% denaturant (100% denaturant corresponds to 7 M urea and 40% [vol/vol] formamide deionized with AG501-X8 mixed bed resin [Bio-Rad Laboratories, Inc.]). Gels were made with 6% (wt/vol) acrylamide stock solutions (5.694 g of acrylamide and 0.306 g of bis-acryloylcistamine [20:1]

TABLE 1. Primers and oligonucleotides used in this study

Primer	Positions	Sequence	Description
GM5F-GC-clamp[a]	341–357	5′-GC-clamp-CCTACGGGAGGCAGCAG-3′	General eubacterial primer for 16S rRNA gene DGGE fragment amplification (35, 36)
DS907-reverse[b]	907–928	5′-CCCCGTCAATTCCTTTGAGTTT-3′	General eubacterial primer for 16S rRNA gene DGGE fragment amplification (35, 36)
Probe 804	804–821	5′-CAACGTTTACTGCGTGGA-3′	rRNA oligonucleotide probe for the genera *Desulfobacterium* and *Desulfobacter* and for *D. multivorans*, *D. variabilis*, and *D. sapovorans* (7)
Probe 687	687–702	5′-TACGGATTTCACTCCT-3′	Probe for the genus *Desulfovibrio* (7)
Probe 385	385–402	5′-CGGCGTCGCTGCGTCAGG-3′	Probe for the genera *Desulfovibrio* and *Desulfobulbus* (1) and for other delta subdivision species and gram-positive bacteria (42)

[a] GC-clamp: 5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3′.
[b] There is a G/A mismatch at position 917 of the target site for *Desulfomicrobium escambium*, *Desulfovibrio desulfuricans*, *Desulfovibrio longreachii*, and members of the genus *Desulfobacterium*. In test PCRs, the primer mismatch did not affect amplification under the conditions employed.

per 100 ml of solution) of 20 and 70% denaturant with a Bio-Rad Econo pump and Bio-Rad model 385 gradient former. DGGE gels were allowed to polymerize for at least 12 h. Electrophoresis was performed for 4 h at a constant 200 V and a temperature of 60°C. After electrophoresis, the gels were incubated for 15 min in Milli-Q water containing ethidium bromide (0.5 mg/liter), rinsed for 10 min in Milli-Q water, and photographed on a UV transillumination table (302 nm) with a Cybertech CS1 digital camera (Cybertech, Berlin, Germany).

Small pieces of selected DGGE bands were punched from the DGGE gel before the blotting procedure. The PCR products of the cutouts were eluted and reamplified as described previously (36). The cutout positions are indicated in accompanying figures (see Fig. 2 and 5).

Blotting and hybridization analysis of DGGE gels. DGGE-separated PCR products were analyzed by blotting with rRNA-targeted probes (35, 36). The gels were equilibrated in 0.5× TBE (45 mM Tris-borate [pH 8], 45 mM boric acid, 1 mM Na_2 EDTA) for 15 min. The DGGE-separated PCR products were transferred to a nylon membrane (Hybond-N+; Amersham, Amersham, United Kingdom) with a Trans-Blot SD semidry transfer cell (Bio-Rad Laboratories, Inc.). Electrotransfer was performed for about 1 h at a constant 400 mA. The membrane was incubated for 15 min in 0.4 M NaOH–0.6 mM NaCl solution to denature the DNA, subsequently neutralized by two rinses in a large volume of 2.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and exposed for 45 s to 302-nm UV light to cross-link the DNA fragments to the membrane.

The 16S rRNA region amplified by primers GM5F and DS907R includes several target sites for general and genus-specific oligonucleotide probes for sulfate-reducing bacteria. Three such probes (Table 1) were used to analyze the DGGE patterns for 16S rRNA amplificates of sulfate-reducing bacteria. Probes were labeled with digoxigenin with the digoxigenin oligonucleotide labeling kit and protocols from the manufacturer, Boehringer, Mannheim, Germany. Digoxigenin-labeled probes were detected by an antibody coupled with alkaline phosphatase, which gives a chemiluminescent reaction with CSPD (Tropix, Bedford, Mass.). Hybridization was performed as described by Muyzer et al. (36). For probe 385, a hybridization and washing temperature of 50°C was used (35). Under these hybridization conditions, probe 385 did not discriminate among different sulfate-reducing populations on the basis of a possible single G/A mismatch in position 395 of the 16S rRNA sequence. (Members of the genera *Desulfovibrio* and *Desulfobulbus* have a G at this position, whereas members of the genera *Desulfomicrobium* and *Desulfobacter* have an A.) The probe was therefore targeted at the general delta subdivision of the sulfate-reducing population. Discrimination against two mismatches was, however, confirmed by test blots with appropriate reference strains. Probe 804 was hybridized at 40°C and washed at 45°C (7). Probe 687 was hybridized at 40°C and washed at 45°C (7). The probes were removed from the membrane for rehybridization as described by Muyzer et al. (36).

The holes punched out of the DGGE gel served as position markers for the alignment of the DGGE ethidium bromide and hybridization pictures. Aligning the ethidium bromide-stained and blotted DGGE patterns requires these position markers, since the blotting process distorts the proportions of the gel. The positions of the holes are marked in accompanying figures (see Fig. 2 and 5). The alignment was done with the software program Photoshop 3.0 (Adobe).

Sequencing of PCR products. Reamplified PCR products obtained from the excised DGGE bands were gel purified as follows. A small well was cut into the agarose gel in front of the selected PCR product. Electrophoresis was continued until the PCR product migrated into the buffer-filled well, where it was taken up with a pipette and transferred into an Eppendorf vial and subjected to ethanol precipitation. The purified PCR products were sequenced directly: 10 to 100 ng of purified PCR product was mixed with 2 μl of 5× concentrated sequencing buffer (200 mM Tris-HCl [pH 7.5], 100 mM $MgCl_2$, 250 mM NaCl), 1 μl of 5% Nonidet P-40 (Sigma) aqueous solution, 2 pmol of primer, and double-distilled water in a final volume of 10 μl. The double-stranded PCR product was denatured by heating the 10-μl volume to 95°C for two 5-min periods, with a short centrifugation in between to recollect the 10-μl volume. The sequencing reaction was started by adding 1.0 μl of 0.1 M dithiothreitol, 2.0 μl of dNTP solution (200 nM [each] dGTP, dATP, and dTTP), 0.5 μl of [α-^{33}P]dCTP (10 μCi/μl; 3,000 Ci/mmol), and 2 μl (1 U) of Sequenase 2.0 (U.S. Biochemicals). After a short centrifugation to collect and mix the sample volume (15.5 μl), the sample was incubated for 5 min at 37°C.

The sample volume was then divided into four 3.5-μl portions, and each portion was added to dideoxynucleotide termination solutions (2.5 μl each, with each containing 80 μM dGTP, dATP, dTTP, and dCTP and 8 μM dideoxynucleotide). After a 5-min incubation at 37°C, the reaction was terminated by adding 4 μl of a solution of 96% (vol/vol) formamide and 20 mM EDTA. The samples were heated for 5 min at 95°C, and 2.5 μl of the samples was loaded onto a 0.25- to 0.40-mm wedge sequencing gel (6% [vol/vol] acryl-bisacrylamide [30:1] and 7 M urea). After electrophoresis at a constant 60 W for 4 h, the gel was fixed, dried, and exposed with an X-ray film (Kodak XAR5).

Sequence alignment and phylogenetic tree inference. The 16S rRNA sequences were aligned with those of other bacteria obtained from the Ribosomal Database Project (RDP) (31). The SIMILARITY_RANK tool of the RDP was used to search for close evolutionary relatives. Sequence alignments were prepared with the sequence alignment editor SEQAPP (17). Distance matrixes were calculated with DNADIST as implemented with the software package PHYLIP (version 3.5) developed by Felsenstein (11), with the Jukes-Cantor model, which assumes independent change at all sites with equal probability, being used (24). Phylogenetic trees were constructed from evolutionary distances calculated with the algorithm of Fitch and Margoliash as implemented in the program FITCH with the software package PHYLIP (version 3.5).

Nucleotide sequence accession numbers. The sequences obtained in this study are available from GenBank under accession numbers L40767, L40783, L40789, L40784, L40785, L40786, and L40787.

RESULTS

Extracted nucleic acids. Genomic DNA and rRNA were isolated from different water layers of Mariager Fjord, and equal volumes (5 μl) of the isolated nucleic acids (dissolved in 50 μl) were analyzed on an ethidium bromide-stained agarose gel (Fig. 1). Genomic DNA, in the upper positions on the gel, and rRNAs, appearing as two distinct double bands, are the predominant nucleic acids visible in the agarose gel. Large-subunit rRNAs, eukaryotic 28S rRNA and prokaryotic 23S rRNA, form the upper double band. Small-subunit rRNAs, eukaryotic 18S rRNA and prokaryotic 16S rRNA, appear as the lower double band. Eukaryotic 28S rRNA and 18S rRNA are found only in fully or partially oxygenated water layers above and in the chemocline to a depth of 14.5 m, whereas prokaryotic 23S rRNA and 16S rRNA are found in both oxic and anoxic water layers.

Total DNA, 16S rRNA, and 18S rRNA were quantified by scanning and integrating the staining intensities of the DNA and rRNA bands. The 18S rRNA disappeared below the chemocline. 16S rRNA showed a maximum abundance in the chemocline at a depth of 14.5 m where the concentration was

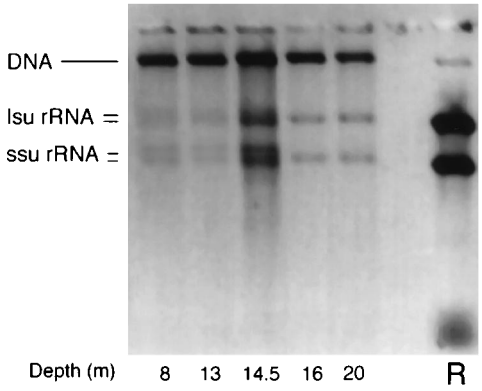

FIG. 1. Agarose gel of nucleic acids extracted from Mariager Fjord water samples obtained from depths of 8, 13, 14.5, 16, and 20 m. Nucleic acids (0.5 to 1.0 μg) of each sample were loaded on the gel. RNA (1 μg) of *Desulfovibrio baculatus* DSM 2555 was run as the standard in lane R. The amounts of total extracted nucleic acids were in the range of 5 to 10 μg per sample. lsu, large subunit; ssu, small subunit.

increased by approximately a factor of four compared with that of other layers of the water column (see Fig. 3A). The increased 16S rRNA content of cells in the chemocline was also evident from the in situ fluorescence hybridization intensities, which were determined with a general eubacterial 16S rRNA probe (see Fig. 6D of the accompanying paper [42]). The DNA concentrations remained almost constant in all water layers of Mariager Fjord, with a slight increase in the chemocline. The equal DNA concentrations correspond to uniform bacterial numbers obtained by general nucleic acid stains (see Fig. 6A of reference 42). Fluorescent in situ hybridizations and nucleic acid extractions were performed with bacteria sampled at the same time and location (42).

DGGE analysis of PCR-amplified rDNA fragments. 16S rDNA fragments, obtained after enzymatic amplification of nucleic acids from Mariager Fjord water column samples, were analyzed by DGGE. The resulting pattern of bands was visualized by ethidium bromide staining (Fig. 2A). Different bands of the DGGE profile correspond to different PCR-amplified 16S rRNA fragments obtained from different bacterial species or strains. The complex pattern of DGGE fragments is thus derived from the bacterial populations and their nucleic acids. Very small differences in the sequences of amplified 16S rRNA fragments, ranging from 1 to 10 nucleotides, are usually sufficient to separate similar PCR products (52), although positional overlap of bands from two different species cannot be excluded. Aware of this caveat, we view the DGGE band pattern as an approximate representation of the bacterial species and strain diversity in the water column of Mariager Fjord. The DGGE pattern can be interpreted in two different ways. First, specific bands emerge and disappear in different water layers (Fig. 2A). Some bands in the DGGE profile occur in all layers of the water column (Fig. 2A, band 1); others are found in the oxic water layer only (band 2), in the anoxic deeper water layers (band 3), or specifically in the chemocline (band 4). Supposing a constant PCR amplification bias for (or against) a specific bacterial rDNA or rcDNA sequence, the appearance and disappearance of a DGGE band reflect the increase and decrease of the corresponding bacterial population. Intensities of different DGGE bands, derived from different bacterial species, do not allow quantitative conclusions about the abundance of the different bacteria, because of a possible unknown PCR bias in the amplification of different templates.

The second way to interpret the DGGE pattern is to compare DGGE bands which were derived from reverse-transcribed 16S rRNA with those from the corresponding genes, 16S rDNA. Since metabolically active cells are generally presumed to have a higher rRNA content and rRNA/DNA ratio than inactive, dormant cells (29, 41, 45), rRNA-derived DGGE bands could conceptually correspond to the metabolically active bacterial populations of Mariager Fjord while rDNA-derived DGGE bands could reflect the numerically dominant populations. Limiting such comparisons to rcDNA- and rDNA-derived PCR products with identical mobilities (presumably from the same bacterial species) to rule out PCR bias due to different template sequences, it is likely that such patterns point to actual differences between the presence and expression of a particular 16S rRNA gene. DGGE patterns of PCR-amplified bacterial 16S rRNA genes differ markedly from those of reverse-transcribed, PCR-amplified 16S rRNA (Fig. 2A). Chemocline bands a and b appear dominant in the DGGE pattern obtained from transcribed rRNA but are only faintly visible in the DGGE pattern of amplified 16S rRNA genes (Fig. 2A). All clearly defined DGGE bands were excised as shown in Fig. 2A. However, only bands which hybridized with probes for sulfate-reducing bacteria in subsequent blotting experiments were reamplified and sequenced.

Hybridization analysis of DGGE patterns. To identify bands of the DGGE patterns which are possibly derived from sulfate-reducing bacteria, the DGGE patterns were blotted onto a nylon membrane and hybridized with digoxygenin-labeled 16S rRNA probe 385 or with probe 804 (Table 1). Probe 385 was designed for gram-negative sulfate-reducing bacteria of the delta subdivision of the proteobacteria (1). Several gram-positive non-sulfate-reducing bacteria also carry the target sequence of the probe (42). Acetate-oxidizing sulfate-reducing bacteria were monitored with probe 804, which is specific for the acetate-oxidizing sulfate-reducing genera *Desulfobacter* and *Desulfobacterium* and for *Desulfosarcina variabilis*, *Desulfococcus multivorans*, and *Desulfobotulus sapovorans* (7). Probe 687, specific for members of the genus *Desulfovibrio*, was also applied. In DGGE blotting and hybridization experiments, probe 687 gave only very weak hybridization signals with DGGE fragments from 16- and 20-m-deep and sediment samples. In repeated membrane hybridization experiments with probe 687, unusually low hybridization signals were also obtained in part with positive controls, and therefore the probe was not used further.

The results of consecutive hybridization of the DGGE pattern with probes 804 and 385 are shown in Fig. 2B and C. Probe 385 hybridized only weakly with bands of rRNA-derived amplificates from oxic water at depths of 8 and 13 m but gave an intense hybridization signal with two rRNA-derived bands from samples from the chemocline at depths of 14.5 and 16 m. Both bands, labeled a and b, show identical positions in the DGGE pattern (Fig. 2). The anoxic water sample from a depth of 20 m and the sediment sample from a depth of 26 m gave a strong but diffuse hybridization signal with probe 385 which was probably derived from a multitude of different organisms which are not properly resolved in the DGGE pattern. A similarly diffuse hybridization signal with probe 385 was found for rDNA-derived amplificates from anoxic water and sediment. The strong chemocline bands a and b are only faintly visible in the rDNA-derived DGGE pattern and give a low hybridization signal with probe 385 (Fig. 2B). This result might indicate that this chemocline bacterium is far more significant because of its activity than because of its numerical presence.

Hybridization signals with probe 804 were found only with samples from below the chemocline, with which it gave hybrid-

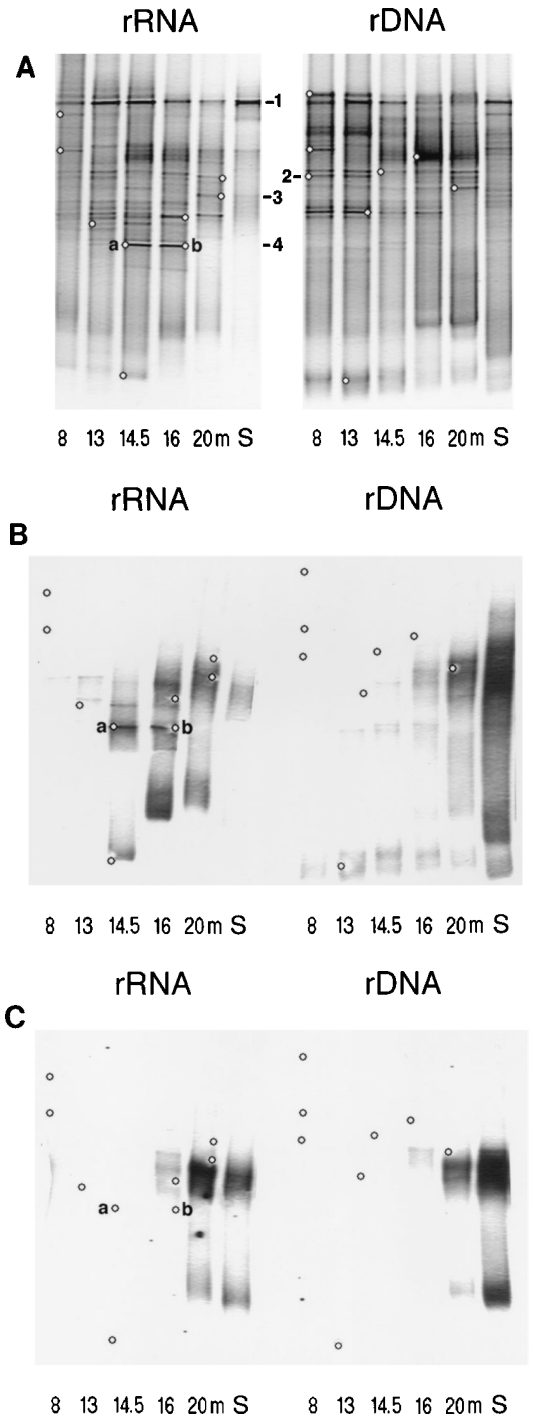

FIG. 2. (A) Ethidium bromide-stained DGGE pattern of PCR products derived from rDNA and rRNA extracted from the Mariager Fjord water column and sediment samples. rRNA-derived DGGE bands for water column samples from depths of 8, 13, 14.5, 16, and 20 m and for sediment samples (S) from a depth of 24.3 m are shown. rDNA-derived DGGE bands for the same samples are also shown. The small circles indicate gel portions that were excised for reamplification and sequencing. Numbers 1 to 4 refer to DGGE bands discussed in Results ("DGGE analysis of PCR-amplified rDNA fragments"). DGGE bands a and b are also discussed in Results ("Hybridization analysis of DGGE patterns"). (B) The same DGGE gel was membrane blotted and hybridized with rRNA probe 385 (1). The small circles indicate gel portions that were excised for reamplification and sequencing, which allowed a correlation to bands in the ethidium bromide-stained DGGE gel in panel A to be made. Bands a and b were sequenced. S, sediment sample. (C) The same DGGE gel was membrane blotted and hybridized with rRNA probe 804 specific for *Desulfobacter* spp., *Desulfobacterium* spp., *D. variabilis*, *D. multivorans*, and *D. sapovorans* (7). The small circles indicate gel portions that were excised for reamplification and sequencing, which allowed a correlation with bands in the ethidium bromide-stained DGGE gel in panel A to be made. S, sediment sample.

ization signals in both the rRNA- and the rDNA-derived DGGE patterns (Fig. 2C). Hybridization intensities of probes 385 and 804 were integrated for both rRNA- and rDNA-derived DGGE patterns. The integrated hybridization intensities of probes 385 and 804 for different water layers are given in Fig. 3B. The hybridization intensity of probe 385 generally increased with depth. In the rRNA-derived DGGE pattern, it reached a local maximum with amplificates from the chemocline at 16 m because of the high intensity of band a/b (Fig. 2). The hybridization intensity of probe 804 also increased with depth for both rDNA- and rRNA-derived DGGE patterns (Fig. 3B).

Interestingly, these results obtained by a qualitative method, PCR, reflect a trend which is defined by conceptually different, quantitative approaches. MPN counts indicate an increase of the sulfate-reducing bacterial population at the chemocline by a factor of approximately 10 (Fig. 3C). The integrated fluorescent hybridization intensities of whole cells obtained by direct hybridization of 16S rRNA with probe 385 and without PCR amplification also increased by 1 order of magnitude (see Fig. 7A of reference 42). These population shifts took place against a background of almost constant bacterial cell density (see Fig. 6A of reference 42) and nucleic acid concentration (Fig. 3A) in the water column.

Sequencing and identification of DGGE bands a and b. rRNA chemocline bands a and b (Fig. 2) were reamplified and sequenced. The sequences were compared with each other and analyzed for their phylogenetic affiliations with known sulfate-reducing and delta subdivision bacteria. Hybridization with probe 385 did not provide enough phylogenetic resolution, as it allowed a cross-reaction with the marine nitrifier *Nitrospina gracilis*, for instance. When the probe sequence was compared with the 16S rRNA reference sequences of the RDP (31), the sequence of probe 385 matched those of at least six known non-sulfate-reducing delta subdivision bacteria, *Bdellovibrio bacteriovorans*, four myxobacteria (*Angiococcus disciformis*, *Archangium gephyra*, *Cystobacter fuscus*, and *Corallococcus coralloides*), and *N. gracilis* (50). Myxobacteria, typical soil bacteria, are unlikely organisms in marine habitats. However, *N. gracilis* is a marine nitrifier which could occur in the Mariagerfjord chemocline and could cross-react with probe 385; furthermore, nitrate and nitrite concentrations were highest in the upper chemocline (see Fig. 3D of reference 42). The gel positions of the *N. gracilis*-derived DGGE fragment and of the dominant rRNA bands a and b (Fig. 2) were compared in separate DGGE experiments and found to be different (results not shown).

Bands a and b (Fig. 2) had identical sequences, consistent with their identical positions in the gel. The complete sequence of the DGGE fragment was determined and, by using the similarity check facilities of the RDP (31), identified as a delta subdivision sequence. This result was substantiated by checking the sequence for the signature nucleotides of the delta subdivision (58). The sequence was compared with sequences of sulfate-reducing and non-sulfate-reducing bacteria of the delta subdivision obtained from GenBank and the RDP, but it could not be assigned to a known phylogenetic branch of the delta subdivision, including recent molecular isolates (6, 8, 25), and was not related to sequences obtained from MPN cultures.

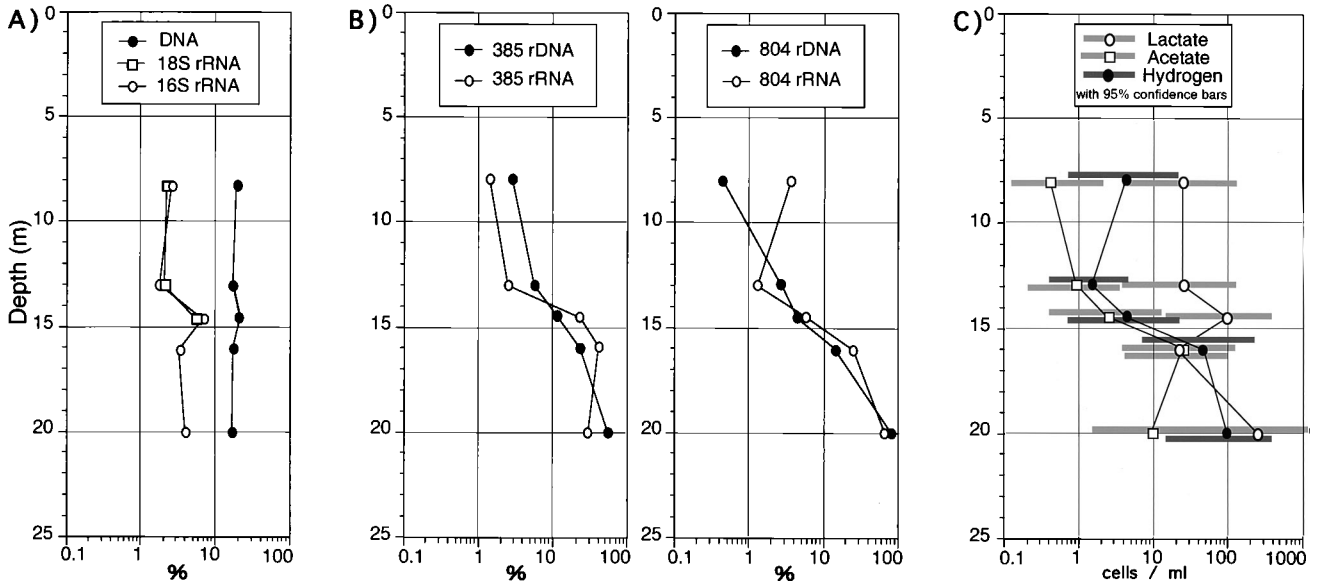

FIG. 3. (A) Integrated ethidium bromide staining intensities of extracted rRNAs (prokaryotic 16S rRNA and eukaryotic 18S rRNA) and total extracted DNA from water column samples from depths of 8, 13, 14.5, 16, and 20 m, plotted as percentages of the total integrated ethidium bromide staining intensity. (B) Integrated hybridization intensities of rRNA- and rDNA-derived DGGE bands with probes 385 and 804 for water column samples from depths of 8, 13, 14.5, 16, and 20 m, plotted as percentages of total integrated hybridization intensity. (C) MPN counts of cultivable sulfate-reducing bacteria (cells per milliliter) for water column samples from depths of 8, 13, 14.5, 16, and 20 m. The bars around the datum points indicate 95% confidence intervals (2).

Instead, it appeared to be a new, distinct phylogenetic lineage within the delta subdivision. This sequence positions this bacterium between the incompletely and the completely oxidizing sulfate-reducing bacteria and in relative proximity to non-sulfate-reducing members of the delta subdivision, such as *N. gracilis*, *Geobacter metallireducens*, *Syntrophobacter wolinii*, and members of the genera *Pelobacter*, *Desulfuromusa*, and *Desulfuromonas* (Fig. 4). The chemical conditions of the chemocline, especially the peak concentrations of elemental sulfur, oxidized iron and manganese, and nitrate (see Fig. 3 of reference 42), allow several possible metabolic modes, i.e., iron and manganese reduction (*G. metallireducens* and members of the genera *Pelobacter* and *Desulfuromonas*), fermentation of organic compounds (members of the genus *Pelobacter*), sulfur reduction (members of the genera *Desulfuromusa* and *Desulfuromonas*), nitrification (member of the genus *Nitrospina*), sulfur disproportionation, and sulfate reduction (delta subdivision sulfate reducers). A definite answer would require isolation of this bacterium in pure culture.

MPN counts of sulfate-reducing bacteria. Cultivable sulfate-reducing bacteria in the Mariager Fjord water column were counted by MPN serial dilution. Lactate, acetate, and hydrogen were used as substrates (Fig. 3C). Acetate-utilizing bacteria were found in low numbers (10 to 30 cells per ml) in anoxic water at depths of 16 and 20 m. Even in the sediment, we found only low numbers (10^3 to 10^4 cells per ml). This result might be the consequence of cell clumping, as is often observed for nonmotile, marine, acetate-oxidizing sulfate reducers, i.e., members of the genera *Desulfobacter* and *Desulfosarcina* (56, 57). MPN counts with lactate gave significantly higher numbers. Approximately 25 cultivable cells of sulfate-reducing bacteria per ml were counted in oxic water layers above the chemocline. Cell numbers below the chemocline increased to approximately 250 cells in the anoxic water layer at a depth of 20 m and to 10^5 to 10^6 cells per ml in the bottom sediment. A similar trend was found when hydrogen was used as the substrate.

MPN cultures from high dilutions were PCR amplified and analyzed by DGGE. The DGGE patterns were blotted and hybridized with probes 385 and 804. As an example, Fig. 5A shows the ethidium bromide-stained DGGE pattern of selected positive MPN dilutions which used lactate as the substrate. The hybridization of this DGGE gel with probe 385 is shown in Fig. 5B. After hybridization with probe 385, the DGGE pattern of the MPN cultures was compared with the DGGE patterns of 16S rDNA fragments obtained from the extracted nucleic acids, i.e., DNA and rRNA, to identify bands with equal or similar gel positions and hybridization signals. Such identical or similar DGGE bands found in both DGGE patterns could represent bacteria which were recovered both as molecular isolates and as MPN cultures. Whenever appropriate, similar MPN bands and water column bands were run on DGGE gels side by side to facilitate comparison before being reamplified and sequenced. Upon this closer examination, the investigated MPN bands and water column molecular isolates differed from each other.

Sulfate-reducing bacteria in the oxic layer of the water column. The existence of a specific physiological and phylogenetic group of oxygen-tolerant sulfate-reducing bacteria was considered, since high sulfate reduction rates and high counts of sulfate-reducing bacteria have repeatedly been reported for oxic marine sediments and microbial mats (4, 14, 21, 23, 43). To test this assumption, sulfate-reducing bacteria growing in MPN cultures inoculated with oxic water samples from depths of 8 and 13 m were identified by partial 16S rRNA sequences. Four DGGE bands of the lactate MPN series which were obtained in the dilution corresponding to 10 cells per ml and which hybridized with probe 385 were sequenced: band c from 8 m, band d from 13 m, band e from 20 m, and band f from 13 m (Fig. 5B). Band e was obtained from a MPN sample from the anoxic zone at a depth of 20 m but was included in the sequencing, since its position in the DGGE gel was similar to those of bands c and d (Fig. 5B). DGGE bands c, d, and e (Fig. 5B) turned out to be *Desulfovibrio* sequences that were most

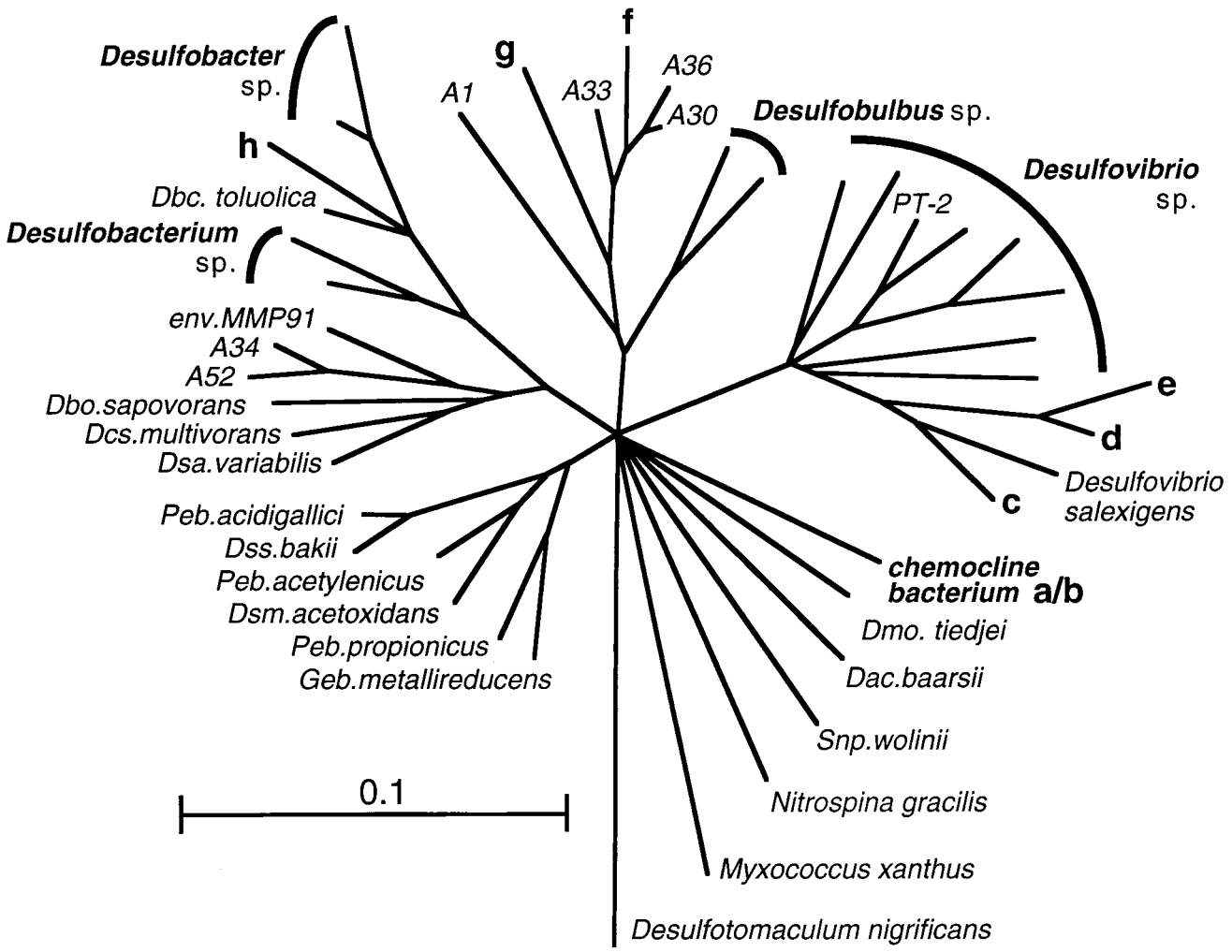

FIG. 4. This 16S rRNA distance tree for delta subdivision bacteria and sulfate reducers functions as an identification scheme and overview for MPN isolates c, d, e, f, g, and h from oxic waters and rRNA amplificate a/b from the chemocline of Mariager Fjord. The tree is based on 16S rRNA sequence positions 341 to 907 (*Escherichia coli* numbering). The scale bar corresponds to 0.1 substitutions per nucleotide position. The tree was rooted with the gram-positive sulfate reducer *Desulfotomaculum nigrificans* serving as the outgroup. Multifurcations indicate lines of descent for which the relative branching order cannot be unambiguously determined, considering the different treeing methods and the limited amount of sequence information in a DGGE fragment. The sequences used to prepare this figure were derived from the RDP with the anonymous ftp server at the University of Illinois in Urbana, Ill. The RDP was updated on 19 June 1994 (31). The new sequences from the Mariager Fjord samples as determined by DGGE and MPN analysis are available from GenBank under accession numbers L40767, L40783, L40789, L40784, L40785, L40786, and L40787 (sequences a/b, c, d, e, f, g, and h, respectively). Relevant molecular isolates from other sources include A1, A30, A33, A34, A36, and A52 (8), env.MMP91 (6), and PT-2 (25). Dbc, *Desulfobacula*; Peb, *Pelobacter*; Dss, *Desulfuromusa*; Dsm, *Desulfuromonas*; Dmo, *Desulfomonile*; dac, *Desulfoarculus*.

closely related to that of the marine, salt-requiring species *Desulfovibrio salexigens* (Fig. 4). Sequence f (Fig. 5B) was distantly related to those of members of the genus *Desulfobulbus*. Another *Desulfobulbus*-related sequence, g, was obtained from a depth of 8 m in the first dilution step of the hydrogen MPN series. Sequences f (Fig. 5B) and g clustered with four molecular isolates obtained from Florida marine sediments, A1, A30, A33, and A36 (8), indicating a coherent, *Desulfobulbus*-related phylogenetic group of presumably sulfate-reducing bacteria. Sequence g closely resembled the sequence of a psychrotrophic, vacuolated, sulfate-reducing bacterium recently isolated from sediments from Kysing Fjord in Denmark (19a). A *Desulfobacter-Desulfobacula*-like sequence h was obtained from a depth of 14.5 m, the central chemocline, in the second dilution step of the hydrogen MPN series corresponding to 10 cells per ml. This sequence did not form a coherent phylogenetic group with related molecular isolates. Two of the Florida molecular isolates, A34 and A55 (8), and an uncultivable magnetotactic multicelled prokaryote (6) belonged to the *Desulfococcus-Desulfosarcina-Desulfobotulus* group (Fig. 4).

The investigated sulfate-reducing bacteria obtained by MPN series from the oxic water layers of Mariager Fjord are phylogenetically affiliated with known sulfate-reducing bacteria. *Desulfovibrio* spp. and *Desulfobulbus*-related strains are feasible inhabitants of the oxic water layers of Mariager Fjord, since several species of these genera are capable of respiring various substrates aerobically. *Desulfovibrio* strains respire a wide variety of substrates: H_2, lactate, pyruvate, formate, ethanol, SO_3^{2-}, $S_2O_3^{2-}$, and H_2S (5). *Desulfobulbus propionicus* respires propionate, SO_3^{2-}, and H_2S (5). Interestingly, the *Desulfovibrio* sequences from Mariager Fjord were phylogenetically most closely related to that of the marine species *D. salexigens*, which showed almost no aerobic respiration at all with the substrates tested (5), and to that of a recently isolated

marine *Desulfovibrio* sp. from Solar Lake (Egypt), which respired aerobically with various substrates (28a). The Mariager Fjord molecular isolates were not related to a cultured *Desulfovibrio* molecular isolate, PT-2, from an anaerobic, sulfidogenic bioreactor (25). Aerobic growth of oxygen-respiring sulfate-reducing bacteria, defined as more than one doubling of protein, has not been observed so far (32). It therefore seems unlikely that sulfate-reducing bacteria from Mariager Fjord actually grow as free-living aerobes in the oxic water column; rather they are more likely to persevere in this environment by particle association or as components of marine snow (16, 48).

DISCUSSION

Integration of molecular, microbiological, and biogeochemical approaches. Different approaches in the investigation of the Mariager Fjord bacterial ecosystem, such as fluorescent in situ hybridization and chemical analysis, DGGE analysis of PCR-amplified 16S rDNA fragments, hybridization analysis with specific probes, DNA sequencing, and MPN counts, result in different data sets which have to be cross-checked and reevaluated for a valid description of the bacterial populations within their natural habitat.

We have used DGGE analysis of PCR-amplified 16S rDNA fragments to infer an overall picture of the bacterial populations in different water layers of Mariager Fjord. DGGE analysis of DNA fragments obtained by amplification of rDNA and reverse-transcribed rRNA visualized the different patterns of bacterial activity and bacterial presence in the water column. DGGE patterns are of a qualitative nature and importance and thus have to be treated with caution when quantitative conclusions are desired.

The PCR, which predates the DGGE analysis, may selectively amplify specific members of a given nucleic acid population, resulting in a quantitatively distorted representation of the natural bacterial populations (35). Comparisons of rDNA- and rcDNA-derived DGGE bands with identical mobilities and the ensuing qualitative conclusions about rRNA content and possibly metabolic activity of the corresponding bacterial population rely on three assumptions. First, rcRNA yield by reverse transcription increases with rRNA content. Second, PCR amplifications of genomic DNA sequences and the corresponding reverse-transcribed hexanucleotide-primed cDNAs are roughly equally efficient. Third, the amplification of a particular template should be largely unaffected by the presence of other templates. These assumptions are implied when the chemocline bacterium with rcDNA-derived DGGE band a/b is referred to as highly active. Supporting evidence is provided by pure culture and coculture studies. PCR amplifications of *Desulfovibrio* [Ni Fe]-hydrogenase cDNA give the highest yield in the early logarithmic growth phase of bacterial cultures (53a). PCR amplification of hexanucleotide-primed 16S rcDNA and genomic 16S rDNA sequences of a defined *Desulfovibrio-Marinobacter* coculture yielded the same DGGE patterns and presumably the same set of 16S rRNA sequences (33a) without chimeric artifacts. PCR amplification of genomic DNA sequences and corresponding cDNAs thus did not miss a member of the model community.

The use of universal PCR primers in the amplification step combined with the effective separation of different PCR products enables DGGE to resolve a wide range of microbial diversity in a given microbial ecosystem without the constraints of specific media or the laborious sequencing of clone libraries. The novel, highly active chemocline organism representing a new phylogenetic lineage within the delta proteobacterial subdivision would probably not have been identified by any other molecular method. Cloning of PCR products could have yielded the bare sequence but would have given little information about the high activity of this organism in the chemocline.

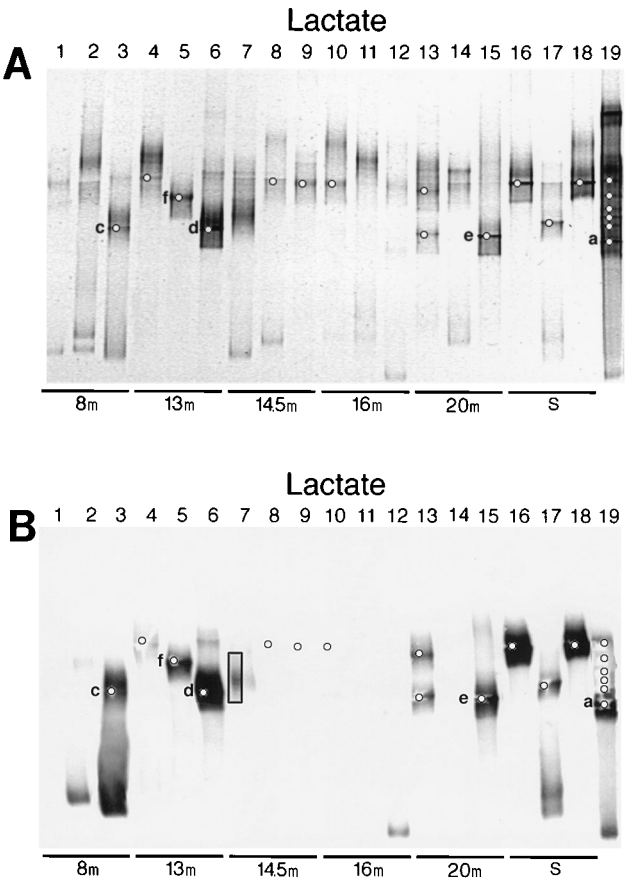

FIG. 5. (A) Ethidium bromide-stained DGGE pattern of PCR products derived from MPN cultures using lactate as the substrate. MPN counts were done in triplicate. Each depth is represented by three samples, numbered consecutively, from high-positive MPN dilutions. Lanes 1 to 3, MPN cultures from the oxic zone at a depth of 8 m (dilutions of 10^0, 10^{-1}, and 10^{-1}); lanes 4 to 6: MPN cultures from the upper chemocline at a depth of 13 m (dilutions of 10^0, 10^{-1}, and 10^{-1}); lanes 7 to 9, MPN cultures from the central chemocline at a depth of 14.5 m (dilutions of 10^{-1}, 10^{-1}, and 10^{-1}); lanes 10 to 12, MPN cultures from the lower chemocline at a depth of 16 m (dilutions of 10^0, 10^{-1}, and 10^{-1}); lanes 13 to 15, MPN cultures from the anoxic water layer at a depth of 20 m (dilutions of 10^{-2}, 10^{-2}, and 10^{-1}); lanes 16 to 18, MPN cultures from anoxic sediment samples (S) at a depth of 24.3 m (dilutions of 10^{-2}, 10^{-5}, and 10^{-3}); lane 19, DGGE bands derived from extracted rRNA from a sample from the central chemocline at a depth of 14.5 m, with DGGE band a (cf. Fig. 2B) so that this predominant chemocline band can be compared with the MPN bands. The small circles indicate portions of the gel at DGGE bands c, d, e, and f which were punched out, reamplified, and sequenced. (B) Blotted DGGE pattern from panel A hybridized with rRNA probe 385. The small circles indicate portions of the gel at DGGE bands c, d, e, and f which were punched out, reamplified, and sequenced. All other information is as for panel A. The box in lane 7 shows the only positive hybridization signal with probe 804.

Although we have discussed DGGE of rDNA fragments as a predominantly qualitative method to describe the structure of microbial populations, it reflects quantitative trends to some degree and is, in this investigation, congruent with explicitly quantitative methods. The DGGE pattern of the delta subdivision of sulfate-reducing bacteria, as obtained from membrane hybridizations with probes 385 and 804 (Fig. 2B and C), is congruent with the MPN counts of sulfate-reducing bacteria (Fig. 3C). Both approaches indicate that the most sulfate-reducing bacteria can be found in the anoxic water layer and in

the bottom sediment of Mariager Fjord. MPN counts indicate an increase in the numbers of sulfate-reducing bacteria from above to below the chemocline by approximately a factor of 10. The integrated intensity of the DGGE blot hybridization signals of probes 385 and 804, as shown in Fig. 3B, shows a similar increase of at least 1 order of magnitude. Interestingly, the same trend is reflected by whole-cell fluorescent hybridization intensities, as shown in Fig. 7D of the accompanying study (42), although the methods differ considerably. In situ hybridization of 16S rRNA with fluorophor-labeled oligonucleotides does not involve a PCR amplification step, which is a prerequisite of DGGE, and thus does not involve the possible amplification bias of the PCR.

In principle, MPN counts give a more stringent quantitative picture of the distribution of sulfate-reducing bacteria in the water column, since they count only viable, cultivable, actually sulfate-reducing cells, no matter how small this population might be in comparison with other bacterial populations. The concentration of cultivable sulfate-reducing bacteria in the water column of Mariager Fjord, as determined by MPN counts, remained below 1,300 cells per ml, which was the upper limit of the 95% confidence interval for the highest MPN count (lactate, 20-m depth) (Fig. 3C). This selectivity of MPN counts is reflected by the finding that MPN isolates did not match major bands of the DGGE pattern. The DGGE pattern of PCR-amplified nucleic acids conceptually represented the general bacterial population, since the PCR primers chosen for the reamplification step were targeted to universally conserved sites of the 16S rRNA sequence. The physiological selectivity of MPN counts is often regarded as an obstacle to the complete analysis of a microbial community but should be viewed as a specific advantage and an important complement for rRNA-based molecular methods, which can, by their nature, give no direct information on the physiological capabilities of bacterial populations. This information can sometimes be inferred from the 16S rRNA sequence if a molecular isolate falls into a well-known, phylogenetically and physiologically coherent bacterial group. On the other hand, molecular isolates without clear affinities to known bacterial genera remain, at best, ecophysiologically ambiguous. The sequencing and phylogenetic placement of DGGE bands a and b derived from an uncultured chemocline bacterium thus gave no clear indication of the potential physiological capabilities of the organism.

Direct cell counts reveal other specific limitations of culture-dependent approaches. Cultivable sulfate-reducing bacteria in the water column, estimated by MPN counts as 2×10^1 to 3×10^2 cells per ml, represent only a minor fraction of the total bacterial number (Fig. 3C). Ethidium bromide staining indicated 2×10^6 to 4×10^6 cells per ml and fluorescent in situ hybridization with a general eubacterial probe resulted in 1×10^6 to 2×10^6 cells per ml, as shown in Fig. 6A and B of the accompanying study (42). Fluorescent in situ hybridization counts with probe 385 indicated cell numbers in the range of 10^4 to 10^5 cells per ml, 3 orders of magnitude higher than the MPN counts of cultivable sulfate-reducing bacteria, as shown in Fig. 7A and B of the accompanying study (42). This discrepancy between total and cultivable cell numbers, the "Great Plate Count Anomaly" (49), is particularly severe in marine habitats. In oligotrophic and mesotrophic marine habitats, only 0.1% or less of the general bacterial population is generally recovered by cultivation-dependent approaches (12, 20, 27, 28, 49), which contrasts with the higher values, (5 to 15%) found in such extremely eutrophic environments as activated sludge (53). This discrepancy is usually attributed to the inadequacy of the media used in cultivation-based approaches but may also have other, more profound reasons. Populations of viable but nonculturable cells account for much of the total bacterial population and activity in situ (19, 40). Such cells could still be viable in a given medium but do not grow beyond a few cell divisions and are therefore not recognized in plate or MPN counts. In a soil model system inoculated with a *Pseudomonas fluorescens* strain, only 0.21% of the starting population could be recovered as cultivable cells after 40 days but approximately 20% of the starting population was found as viable but nonculturable cells (3). These cells were able to form microcolonies but did not grow beyond a few cell divisions and were only detectable by a combined microcolony-epifluorescence assay (3). Thus, the viable, cultivable population of the *Pseudomonas* strain corresponded to only 1% of the viable but nonculturable population. A similar ratio of viable and culturable and viable but nonculturable cells could apply to the Mariager Fjord sulfate reducers. Culturable sulfate-reducing bacteria in the anoxic water layers, estimated by MPN as being 200 to 300 cells per ml, correspond to 0.2 to 0.3% of the 10^5 cells per ml found by in situ hybridization with probe 385 (42).

The chemical conditions in the water column, the position of the chemocline, and the extent and overlap of oxidized and reduced water layers (42) are consistent with the results obtained in this molecular and microbiological investigation. The continuous gradients of chemical conditions in the water column are mirrored by highly differentiated bacterial populations in the water column, as shown by DGGE (Fig. 2A). The availability of a wide range of electron donors and acceptors in the chemocline, such as oxygen, sulfide, elemental sulfur, sulfate, nitrate, ammonia, and oxidized and reduced forms of manganese and iron (42), is connected with high bacterial activity and rRNA yield in the chemocline (Fig. 1). The numbers of cultivable sulfate reducers increased within but not below the chemocline, as was evidenced by MPN counts and congruent with fluorescent in situ hybridization of individual cells and membrane hybridization of PCR-amplified 16S rRNA and rDNA fragments.

This result suggests the potential for sulfate reducers to utilize alternative electron acceptors in the chemocline (5, 30, 44, 47). Sulfur disproportionation, which has been demonstrated for enrichments, pure cultures, and the sulfate-reducing bacterium *D. propionicus*, is another possible process in the chemocline. Elemental sulfur, together with oxidized iron and manganese for the concomitant reoxidation of sulfide produced by disproportionation, was found in concentrations of 0.5 μM in the chemocline (30, 51). These reactions might be attributed to certain highly active delta subdivision chemocline bacteria, such as the molecular isolate found by DGGE and sequencing in this study, and the long slim rod-shaped bacterium apparent by fluorescent in situ hybridization with probe 385 shown in the 16-m sample in Fig. 5 of the accompanying paper (42). Isolation of new biogeochemically significant bacterial types, combined with systematic probe development, is necessary to link microbiological and biogeochemical processes.

Summary. Different approaches in this polyphasic study allowed us to infer this overall picture of the sulfate-reducing bacterial populations of Mariager Fjord. Amounts of total bacterial nucleic acids remained almost constant throughout the water column of Mariager Fjord, with the exception of the chemocline. The bacterial population was continuously changing in composition but not in total mass throughout the water column and showed a highly differentiated pattern of active versus dormant subpopulations, as was revealed by DGGE analysis of 16S rRNA- and 16S rDNA-derived PCR amplificates. An rRNA-rich and therefore probably highly active subpopulation of novel delta subdivision bacteria was found in the

chemocline of Mariager Fjord by DGGE analysis and subsequent sequencing of the individual bands. Delta subdivision sulfate-reducing bacteria have their maximum cell density in the anaerobic and anoxic water layers within and below the chemocline, a result substantiated by MPN counts, hybridization analysis of the DGGE patterns, and fluorescent in situ hybridization of individual cells (42). Cultivation-dependent MPN counts of sulfate-reducing bacteria were consistently 3 orders of magnitude lower than direct counts by fluorescent in situ hybridization, but both approaches indicated an increase in the numbers of sulfate-reducing bacteria in the chemocline by approximately 1 order of magnitude. This ratio also resulted from an analysis of the integrated hybridization intensities of PCR-amplified rRNA and rDNA-derived DGGE bands with probes 385 and 804 (Fig. 3B). Although the maximum number of sulfate-reducing bacteria occurred in and below the chemocline, cultivable sulfate-reducing bacteria were also quantified in the oxic part of the water column by MPN. Cultivable sulfate-reducing bacteria of the oxic water layers were shown by 16S rRNA sequencing to be affiliated to the genera *Desulfovibrio* and, although more distantly, *Desulfobulbus*.

Biogeochemical, molecular, and microbiological approaches contributed to this study of the sulfate-reducing bacteria and the microbial ecosystem of Mariager Fjord. The methodological limitations of each approach constrain the range over which valid conclusions can be drawn and require a careful integration of these different perspectives to perceive the whole picture of a natural habitat. Unexpected mismatches between different data sets were found, such as the clearly different DGGE patterns derived from rRNA and rDNA, the nonidentity of molecular and culture isolates, and the gap between fluorescent and culture-dependent bacterial counts. Nevertheless, these discrepancies are in fact highly valuable indicators of real phenomena: the highly differentiated pattern of bacterial activity, the abundance of uncultured bacteria, and the selectivity of our own approaches for the study of a microbial ecosystem.

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