Applied and Environmental Microbiology, Feb. 2008, p. 624–632 Vol. 74, No. 3
0099-2240/08/$08.00+0 doi:10.1128/AEM.02137-07

Subfossil 16S rRNA Gene Sequences of Green Sulfur Bacteria in the Black Sea and Their Implications for Past Photic Zone Anoxia▽

Ann K. Manske, Uta Henßge, Jens Glaeser,† and Jörg Overmann*

Department Biologie I, Bereich Mikrobiologie, Ludwig-Maximilians-Universität München, Maria-Ward-Str. 1a, D-80638 München, Germany

Received 18 September 2007/Accepted 13 November 2007

The Black Sea is the largest extant anoxic water body on Earth. Its oxic-anoxic boundary is located at a depth of 100 m and is populated by a single phylotype of marine green sulfur bacteria. This organism, *Chlorobium* sp. strain BS-1, is extraordinarily low light adapted and can therefore serve as an indicator of deep photic zone anoxia (A. K. Manske, J. Glaeser, M. M. M. Kuypers, and J. Overmann, Appl. Environ. Microbiol. 71:8049–8060, 2005). In the present study, two sediment cores were retrieved from the bottom of the Black Sea at depths of 2,006 and 2,162 m and were analyzed for the presence of subfossil DNA sequences of BS-1 using ancient-DNA methodology. Using optimized cultivation media, viable cells of the BS-1 phylotype were detected only at the sediment surface and not in deeper layers. In contrast, green sulfur bacterial 16S rRNA gene fragments were amplified from all the sediment layers investigated, including turbidites. After separation by denaturing gradient gel electrophoresis and sequencing, 14 different sequence types were distinguished. The sequence of BS-1 represented only a minor fraction of the amplification products and was found in 6 of 22 and 4 of 26 samples from the 2,006- and 2,162-m stations, respectively. Besides the sequences of BS-1, three additional phylotypes of the marine clade of green sulfur bacteria were detected. However, the majority of sequences clustered with groups from freshwater habitats. Our results suggest that a considerable fraction of green sulfur bacterial chemofossils did not originate in a low-light marine chemocline environment and therefore were likely to have an allochthonous origin. Thus, analysis of subfossil DNA sequences permits a more differentiated interpretation and reconstruction of past environmental conditions if specific chemofossils of stenoec species, like *Chlorobium* sp. strain BS-1, are employed.

At present, the Black Sea represents the largest anoxic water body on Earth, and 87 to 92% of the Black Sea volume is permanently anoxic (3, 21, 39). In the center of the Black Sea, sulfide-containing water layers extend upward to 82 to 110 m below the sea surface (24, 35, 39). In the chemocline, green sulfur bacteria form a 20-m-thick layer persisting at in situ light intensities of 0.0022 to 0.0008 μmol quanta m^{-2} s^{-1} (24). 16S rRNA gene sequence analysis of these bacteria revealed the presence of a single phylotype. After selective enrichment, *Chlorobium* sp. strain BS-1 was found to be halophilic and to exhibit extraordinary low-light adaptation commensurate with the environmental conditions in situ (24). At higher light intensities, this Black Sea strain is rapidly outcompeted by other green sulfur bacteria due to its significantly lower maximum growth rate (24, 31). Its unusual physiological properties thus make strain BS-1 a specific indicator for deep photic zone anoxia in marine environments.

The Black Sea has been used as a contemporary analogue of the much larger sulfidic oceans postulated for the Proterozoic period (1, 8, 30). Based on geochemical evidence, an initial freshwater period lasted from 22,000 years before the present until about 9,000 years before the present (7) and was followed by inundation of the Black Sea basin by salt water after glacial melting and a rise in the level of the Mediterranean Sea (18). This converted the Black Sea into an anoxic marine basin and resulted in deposition of organic carbon-rich sapropel layers (Unit II) on top of the older limnic sediments (Unit III) (33, 43). The presence of pigment biomarkers of obligately anoxygenic phototrophic green sulfur bacteria in Unit II sediments indicates that water column anoxia reached the photic zone about 7,200 to 6,200 years ago (33, 38). Initially, sediments with higher organic carbon contents (Unit IIb) were deposited, and this was followed by a period of lower productivity and increased input of terrigenous material (Unit IIa) (17, 18). The most recent layer, Unit I, is a finely laminated coccolith layer (18) which originated from the invasion of the marine coccolithophorid *Emiliana huxleyi* between 3,500 and 1,600 years before the present, which was initiated by an increase in salinity.

Isorenieratene/β-isorenieratene and chlorobactene occur almost exclusively in the obligately anaerobic phototrophic green sulfur bacteria (38, 43) and therefore have served as indicators for stable stratification of the water column and sulfidic conditions during deposition of Units I and IIb (33, 38, 43). However, since the ~80 known phylotypes of green sulfur bacteria contain only these two types of characteristic carotenoids, these compounds alone do not permit differentiation between species occupying different niches, such as freshwater and marine environments or low- and high-light-intensity environments (5). In order to obtain a more detailed reconstruction of the ancient Black Sea, we ana-

* Corresponding author. Mailing address: Department Biologie I, Bereich Mikrobiologie, Ludwig-Maximilians-Universität München, Maria-Ward-Str. 1a, D-80638 München, Germany. Phone: 49 89 2180 6123. Fax: 49 89 2180 6125. E-mail: j.overmann@lrz.uni-muenchen.de.
† Present address: Justus-Liebig-Universität Gießen, Institut für Mikro- und Molekularbiologie, Heinrich-Buff-Ring 26-32, 35392 Gießen, Germany.
▽ Published ahead of print on 26 November 2007.

lyzed subfossil 16S rRNA gene sequences of green sulfur bacteria in deep-sea sediments of the Black Sea. Specifically, we searched for sequences of *Chlorobium* sp. strain BS-1 as indicators for deep photic zone anoxia.

MATERIALS AND METHODS

Study sites. Sediment samples were obtained during cruise M51 leg 4 of the R/V *Meteor* between 12 and 28 December 2001. The sampling sites were located on the northwestern continental slope of the Black Sea. Core 7620 was recovered at 42°56.2′N, 30°01.9′E and a water depth of 2,006 m. Core 7605 was recovered at 42°30.7′N, 30°14.7′E and a water depth of 2,162 m. In both cases, a gravity corer was employed. Surface sediments were obtained at the same locations using a multicorer. A third sediment core, MD04-2762, was retrieved with a piston corer on 12 May 2004 at 42°38′89N, 32°45′94E and a water depth of 2,210 m during cruise MD139-ASSEMBLAGE of the R/V *Marion Dufresne*. The latter location is also in the western deep basin but is 150 to 200 km east of the previous two sampling stations.

Sample preparation and precautions to prevent contamination. Sediment samples were prepared immediately after retrieval of the sediment cores in a laboratory on the research vessel. Aseptic DNA techniques were employed throughout the procedure (4, 5, 6). The cores were split longitudinally, and the exposed surface of each sediment core was rapidly frozen with powdered dry ice and subsequently lifted off. This generated an uncontaminated sediment surface through which individual samples were obtained with sterile plastic syringes whose ends were cut off. The samples were stored frozen at −20°C in sterile 50-ml plastic tubes until processing. Green sulfur bacteria had never been introduced or worked with in the laboratory. Further precautions against contamination of the samples with foreign DNA included the use of a security level 2 laminar flow hood dedicated to low-template-number samples. Prior to each use, the hood was UV sterilized for 4 h, and all surfaces were subsequently sterilized with sodium hypochlorite (3%, vol/vol). Certified nucleic acid-free disposable plastics were used throughout the procedures and were routinely autoclaved before use. All solutions were prepared in fresh double-quartz-distilled water, sterile filtered, and autoclaved. As a control for contamination during DNA extraction, two procedural blanks without sediment were subjected to the whole extraction and purification protocol along with the sediment samples. One microliter of each of these extraction controls (corresponding to the average volume of sediment extracts used for amplification) was included in subsequent PCR amplifications. As additional controls, each amplification experiment included reaction mixtures without a DNA template to independently control for contamination of the PCR reagents.

DNA extraction. Genomic DNA was extracted from 5- to 10-g subsamples employing an UltraClean Mega soil DNA kit (Mo Bio Laboratories, Inc., Solana Beach, CA) according to the instructions of the manufacturer. An additional sonication step was used in order to improve bacterial cell lysis and mixing (level 6 for 2 min on ice; Sonifier B 15 cell disruptor; Branson, Danbury, CT). DNA extracts were passed through Genomic tip-20 columns (Qiagen, Hilden, Germany) after the ionic strength of the sample was adjusted with 3-*N*-(morpholino)-propanesulfonic acid (MOPS)—KOH (pH 7.0) and 5 M NaCl.

Cells from the Black Sea chemocline at station 7620 at a depth of 100 m were collected from 120 liters of water by tangential flow (Pellicon 2 fitted with a 0.1-μm-pore-size VVPP-C Durapore filter membrane; Millipore, Bedford, MA). The resulting cell suspension was subsequently concentrated on polycarbonate filters (diameter, 47 mm; pore size, 0.1 μm; Millipore), and the genomic DNA was extracted using the phenol-chloroform/isoamyl alcohol protocol (11).

The DNA extracts were concentrated in Centricon YM-50 ultrafiltration devices (Millipore) used according to the instructions of the manufacturer. DNA concentrations were determined fluorometrically by dye binding with PicoGreen (Molecular Probes, Eugene, OR).

PCR and DGGE. 16S rRNA gene fragments of green sulfur bacteria were selectively amplified using primers GC341f (25) and GSB822r (32), which target all known marine and most freshwater green sulfur bacteria (5). The PCR products were separated by denaturing gradient gel electrophoresis (DGGE) (26) using an Ingeny phorU system (Ingeny International BV, Goes, The Netherlands). Two or three amplification reaction mixtures were combined, concentrated to a volume of 15 μl with a SpeedVac (Savant), and loaded onto 6% (wt/vol) polyacrylamide gels in 1× 40 mM Tris-acetate–1 mM EDTA (pH 7.4) containing a linear 30 to 70% denaturant gradient (100% denaturant was 7 M urea plus 40% [vol/vol] formamide). After electrophoresis, the gels were stained with SYBR gold (Molecular Probes) for 45 min. Gel images were captured with a digital camera (Spot RT color; Diagnostic Instruments, MI) and processed with the Spot RT software (version 3.1). Individual DNA bands were excised from the gel and eluted in 40 μl of 10 mM Tris-HCl (pH 8.5) by incubation at 65°C for 45 min.

Sequencing and phylogenetic analysis. One microliter of the eluted DNA was reamplified with primers 341f and GSB822r. In a few cases, nested PCR was necessary for efficient reamplification. In these cases, primers GSB532f (32) and GSB822r were used and the annealing temperatures used were 57 and 52°C. For sequencing, the reamplification products were purified with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and eluted in 50 μl of 1 mM Tris-HCl (pH 8.5). Cycle sequencing was performed with an AmpliTaq FS BigDye terminator cycle sequencing kit (Applied Biosystems) by following the protocol supplied by the manufacturer. Samples were run on an ABI Prism377 capillary sequencer (Applied Biosystems).

In a few cases the analysis of reamplification products revealed the presence of multiple sequences. Such amplificates were ligated into the vector pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and subsequently cloned into TOP10 chemically competent cells according to the instructions supplied by the manufacturer. The selective agent in LB agar plates was kanamycin (50 $\mu g\ ml^{-1}$). Plasmids were isolated from overnight cultures of picked clones in liquid LB medium supplemented with kanamycin. For isolation and purification of plasmids a QIAprep spin miniprep kit (Qiagen, Hilden, Germany) was employed. Plasmids were checked by restriction analysis using RsaI (Fermentas, St. Leon-Rot, Germany).

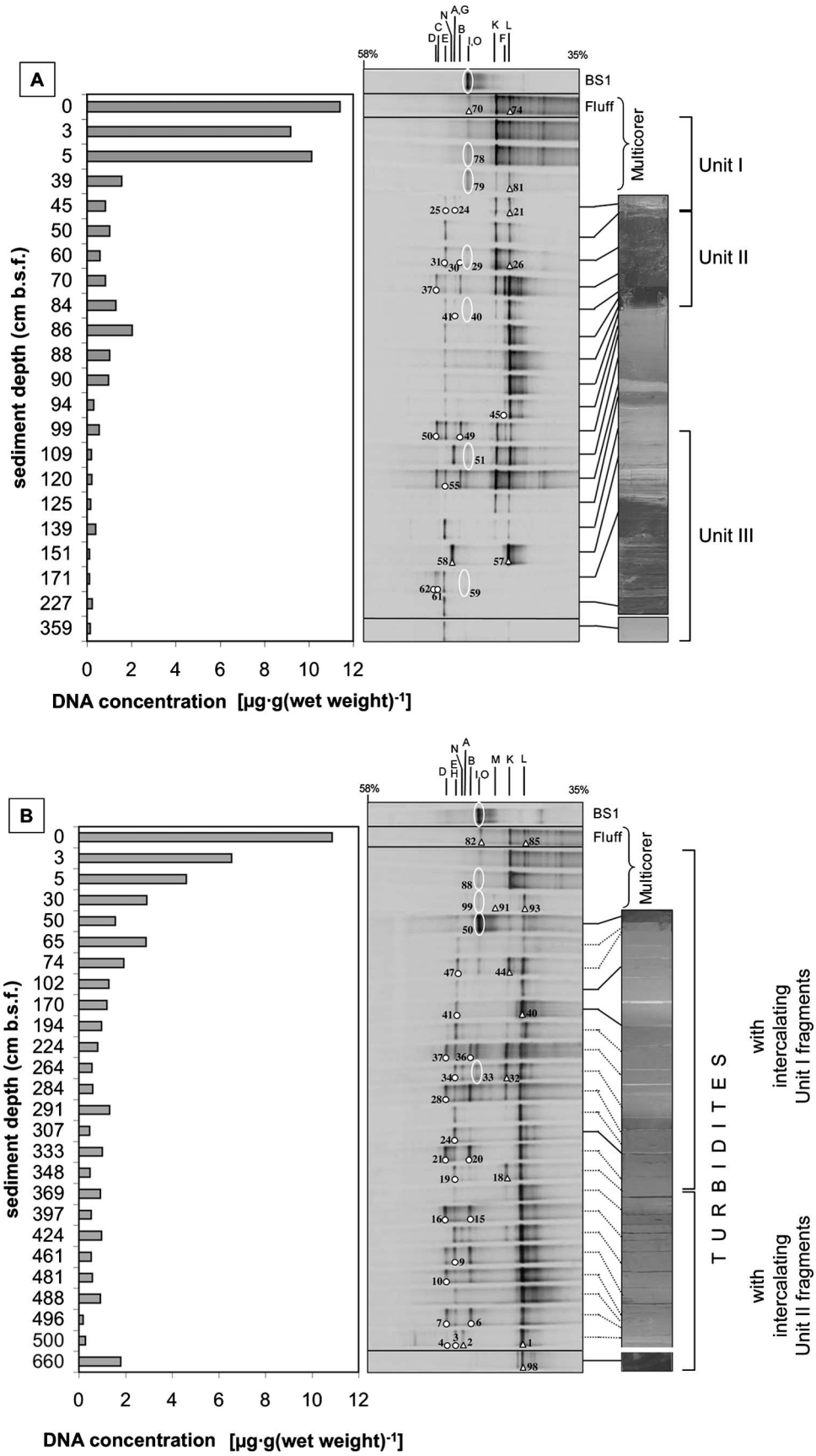
All 16S rRNA gene sequences obtained in the present study were checked for possible chimeras using the CHIMERA-CHECK online analysis program of the RDP-II database (23).

16S rRNA gene sequences were phylogenetically analyzed using the ARB package (22). For automated alignment the Fast Aligner V1.03 was used. Alignments were corrected by using the 16S rRNA secondary structure information for *Chlorobium vibrioforme* DSMZ 260^T, available at http://www.rna.ccbb.utexas.edu/. Phylogenetic trees that included 16S rRNA gene sequences of available strains and environmental sequences of green sulfur bacteria, as well as closely related sequences of the deeply branching green sulfur bacteria, were constructed. First, sequences that were longer than 1,200 bp were used for calculation employing the maximum likelihood algorithm (Fast DNA_ML). Shorter sequences were inserted after this by using the Parsimony Interactive Tool implemented in the ARB software package, without changing the overall tree topology (12). The resulting tree topology was checked for correct distances with a PHYLIP distance matrix using Jukes-Cantor correction. No filter was used, and only the alignment positions which were present in all sequences were used.

Viable cells in Black Sea sediments. Most-probable-number (MPN) series were set up using 1:10 dilution steps. In the first round of measurements, we used two types of cultivation media established for cultivation of green sulfur bacteria and of the Black Sea chemocline bacterium strain BS-1 in particular. Initially, standard medium supplemented with 2.5 mM sulfide and 3 mM acetate was employed (28). In the second MPN approach, a synthetic marine mineral medium adjusted to the Black Sea chemocline salinity and supplemented with 2 mM sulfide, 0.5 mM sodium 2-oxoglutarate, 0.5 mM pyruvate, and 0.5 mM glutamate was employed. Eight parallel MPN series were prepared in Chromacol 96-well microtiter plates with preinserted 500-μl glass vials; the plates were sealed with a polytetrafluoroethylene-coated silicon WebSeal mat (Kupfer Chromatographie Handel GmbH, Pfungstadt, Germany). Cultures were set up immediately after sampling on the ship. Distribution of the anoxic media and inoculation were conducted in an anaerobic chamber under a nitrogen atmosphere. MPN series were inoculated with sediments from station 7605 obtained from the sediment surface, as well as from depths of 13, 14 to 26, and 75 cm.

Based on our failure to detect viable cells of green sulfur bacteria in any of the samples from station 7605 (see Results and Discussion), we repeated the growth assays using optimized media and additional sediment samples which became available during the subsequent cruise of the R/V *Marion Dufresne*. The medium was optimized further for cultivation of the Black Sea chemocline phylotype BS-1 and consisted of artificial seawater (pH 7.2), adjusted to the ionic strength of the Black Sea chemocline, supplemented with 1% (vol/vol) freshly prepared yeast extract, 2 mM sodium acetate, and 200 μM dithionite (24). Portions (22.5 ml) of sterile medium in screw-cap tubes were inoculated with 0.5 cm^3 of sediment from core MD04-2762, and 10 consecutive 1:10 dilution steps were prepared in three parallel experiments. The series were then incubated at 15°C with 5 μmol quanta $m^{-2}\ s^{-1}$ for 4 months. As a negative control, the growth medium without an inoculum was incubated under the same conditions.

Nucleotide sequence accession numbers. The 16S rRNA gene sequences obtained in this study have been deposited in the EMBL database under accession numbers AM779890 to AM779903.



RESULTS AND DISCUSSION

Sediment cores. At station 7620, at total of 7.38 m of mostly laminated sediments was recovered by gravity coring. The upper 10 cm of the core consisted of finely laminated coccolith ooze representing Unit I (Fig. 1A, right panel). A multicorer was deployed at the same site to sample the flocculent surface (fluff) layer, the sediment surface, and the upper portion of Unit I. A comparison of the vertical concentration profiles for total carbon, organic carbon, and total sulfur between the sediments from the multicorer and the gravity core revealed that the coccolith ooze of Unit I extended from the sediment surface down to 50 cm below the sea floor (bsf). Below this, 39 cm of dark, organic carbon-rich sapropels of Unit II was detected (Fig. 1A). Then 40 cm of dark gray clay followed, which could not be assigned to an established unit (Fig. 1A, right panel). The bottom part of the gravity core contained lacustrine sediments of Unit III and included numerous black, iron sulfide-rich layers.

The 7.32-m-long gravity core obtained at station 7605 contained a 15-cm-thick sapropel layer on top. Below this, there was 6 m of thick turbidite layers. The turbidites originated in the upper continental slope at the margin of the Black Sea basin and were interspersed with thin layers of Units I and II (Fig. 1B, right panel). In order to investigate the mobility of biomarker 16S rRNA gene sequences of *Chlorobium* sp. strain BS-1 in the Black Sea, in our analyses of core 7605 we focused on the turbidite layers.

Genomic DNA was extracted from 22 depths of core 7620 and 26 different depths of core 7605, including samples of the fluff layer from both deep-water stations. At station 7620, significantly larger amounts of genomic DNA were recovered from sediments from the upper 5 cm bsf than from below this area (Fig. 1A). At station 7605, the DNA content decreased almost exponentially in the upper 1 m of sediment (Fig. 1B).

Vertical distribution of green sulfur bacterial 16S rRNA gene fingerprints and sequences. 16S rRNA gene fragments of green sulfur bacteria (*Chlorobi*) were amplified with primers which target 84% of all available green sulfur bacterial sequences and all known marine phylotypes (5). Each amplification reaction experiment was rigorously checked for contamination with extraneous DNA by routinely including a set of procedural blanks and PCR controls. These controls never yielded amplification products.

Separation of the amplification products by DGGE yielded 11 different melting types for the different sediments (Fig. 1A and B). Seven of the phylotypes (phylotypes B, D, E, I, L, N, and O) were detected at both stations, whereas two rare phylotypes (phylotypes C and F) were detected only in sediments from station 7620 and one phylotype (phylotype M) occurred only in sediments from station 7605. The most frequent phylotype, phylotype L, was found in almost all sediment layers (Fig. 1). The second most frequent phylotype, phylotype E, occurred in 36 of the samples, and phylotype K was detected in one-half of the 48 samples. Interestingly, phylotypes D and B invariably cooccurred in the same 10 samples. Our results indicate that the vertical and horizontal differences in the composition of 16S rRNA gene sequences of green sulfur bacteria are rather limited in Black Sea sediments.

Seventy-nine of the DNA bands representing all the different melting types were selected for reamplification and sequencing. Frequent melting types were analyzed for different depths. This analysis revealed that two of the melting types each represented two different 16S rRNA gene sequences (phylotypes E and H and phylotypes I and O) (Fig. 1). Two other melting types each mostly contained a single 16S rRNA gene sequence (i.e., 8 of 10 bands were phylotype E, and 2 of 12 bands were phylotype I) (compare Fig. 1 and 2). The four remaining major melting types (Fig. 1) always yielded only a single phylotype (phylotypes B, D, K, and L). Some of the DNA fragments did not yield unambiguous results even if a cloning step was introduced before sequencing. This observation is in line with the results of a previous study of subfossil green sulfur bacteria sequences in eastern Mediterranean sapropels, where a similar fraction of the 16S rRNA gene fragments recovered was found to contain ambiguous sequences (5). Overall, our analysis yielded 58 reliable 16S rRNA gene sequences (numbered bands in Fig. 1).

The BS-1 phylotype (phylotype I) was detected more rarely than most other sequences. At station 7620, it was detected at 5 and 39 cm bsf within Unit I, at 60 and 84 cm bsf within Unit II, in the dark gray layer below Unit II (109 cm bsf), and at a depth of 171 cm (Fig. 1A). At station 7605, BS-1 was detected in the upper sediment layers of Unit I, but only in one turbidite layer below this (at 244 cm bsf) (Fig. 1B). Furthermore, the very low signal intensity of the BS-1 phylotype in most sediment layers suggests that the level of this sequence was low.

The presence of isorenieratene in sediments of Units I and IIb has been taken as evidence for photic zone anoxia in the ancient Black Sea (20). *Chlorobium* sp. strain BS-1 is the only green sulfur bacterium presently occurring in the Black Sea chemocline. BS-1 has been shown to persist over decades in this habitat and is well adapted to the low-light conditions in situ. Therefore, the rare detection and low abundance of DNA from *Chlorobium* sp. strain BS-1 in the subfossil sediment layers were rather unexpected. Our findings suggest that (i) either the in situ conditions (in particular, underwater light

FIG. 1. Vertical concentration profiles of genomic DNA extracted from Black Sea sediments (left panels), separation of 16S rRNA gene fragments of green sulfur bacteria by DGGE (middle panels), and images of gravity cores (right panels). (A) Sediments from station 7620. The fluff layer and samples from 3, 5, and 39 cm bsf were obtained with the multicorer; all deeper sediment samples originated from the gravity core. Sediment layers from the multicorer and the gravity cores were correlated based on vertical profiles of total carbon, organic carbon, and total sulfur (see text). (B) Sediment core from station 7605 containing mostly turbidites. The solid lines connecting DGGE lanes and sediment core images show the depths of intercalating Units I and II; the dotted lines show the depths of turbidites. The DGGE images are negative images of SYBR gold-stained gels. The fingerprint of strain BS-1 from the chemocline of the Black Sea is shown for comparison. Circles indicate sequences of *Chlorobiaceae*, and triangles indicate sequences of deeply branching members of the green sulfur bacterial phylum. Fingerprints of phylotype BS-1 are circled. Lines above the DGGE gels indicate the different melting types; the corresponding 16S rRNA gene sequence types are indicated by letters. The percentages are the denaturant concentrations in the DGGE gels.

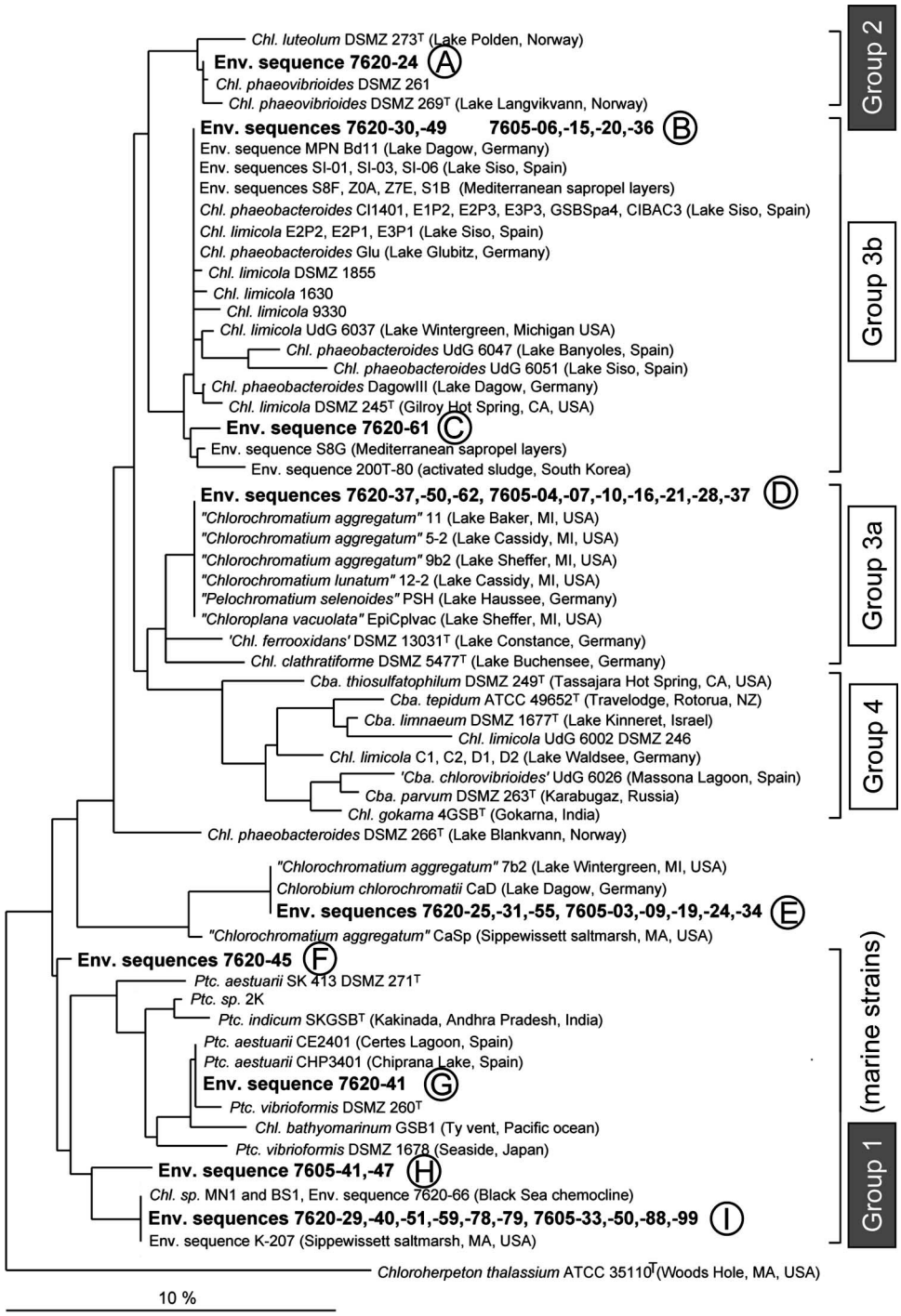


FIG. 2. Phylogenetic affiliation of 16S rRNA gene sequences of *Chlorobiaceae* obtained from cores 7620 and 7605. The numbers indicate the core (7620 or 7605), followed by the band number (Fig. 1). Phylotypes A through I are indicated by bold type. Currently recognized groups of *Chlorobiaceae* (19) are shown in rectangles. Groups comprising salt-tolerant or salt-requiring strains are shown in black rectangles. Bar = 0.1 fixed point mutation per nucleotide. Env., environmental; *Chl*., *Chlorobium*; *Cba*., *Chlorobaculum*; *Ptc*., *Prosthecochloris*.

intensities) differed significantly during past periods of alleged water column anoxia or (ii) some of the 16S rRNA gene sequence types did not originate from the overlying water column. The following phylogenetic analyses provide novel information on the origin of green sulfur bacterial biofossils in the Black Sea sediments.

Phylogenetic analysis of subfossil sequences and implications for the paleomicrobiology of the Black Sea. The green sulfur bacterial phylum (*Chlorobi*) consists of a crown group of closely related green sulfur bacteria sensu stricto (the family *Chlorobiaceae*), as well as an increasing number of deeply branching phylotypes which so far have not been cultured and

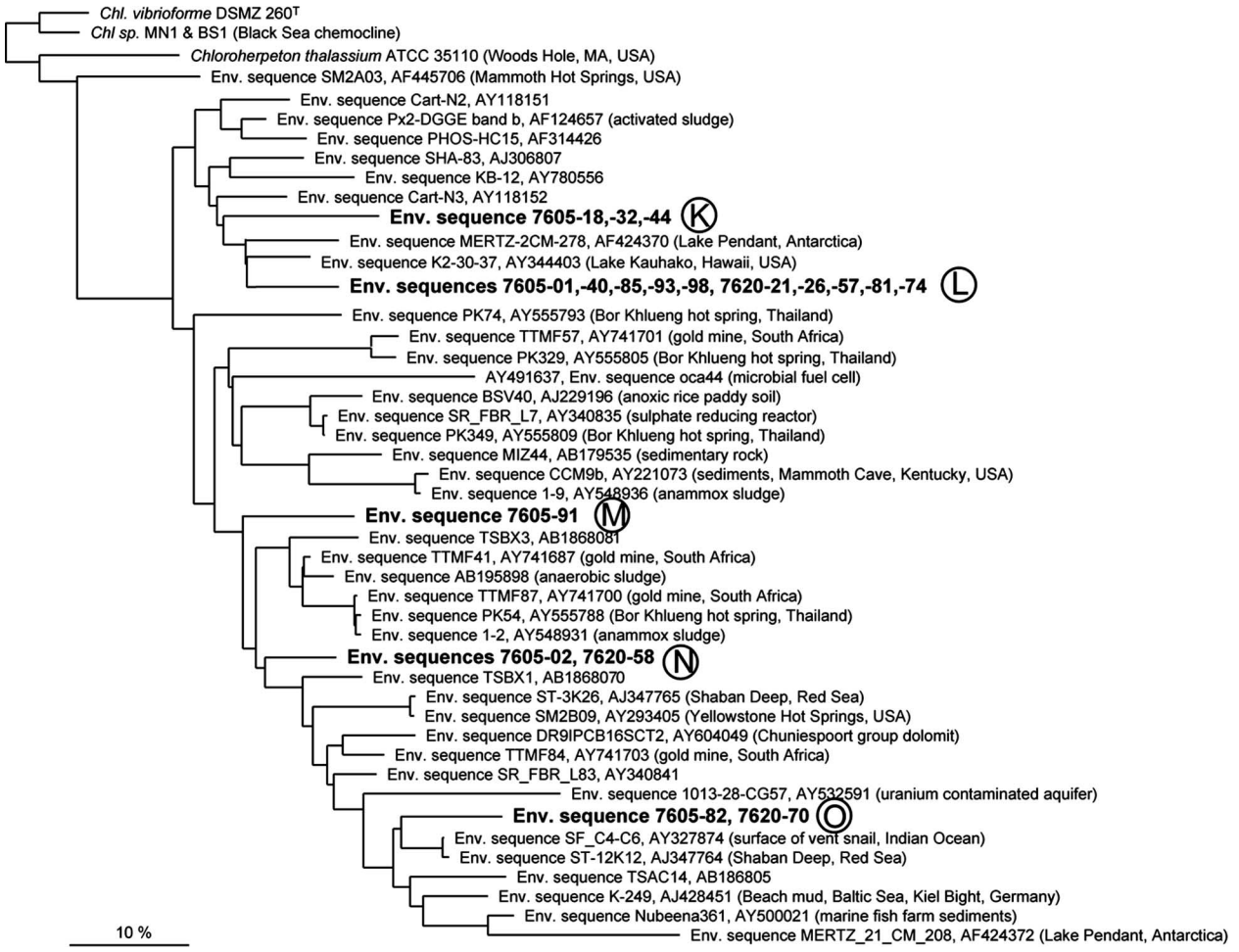


FIG. 3. Phylogenetic affiliation of 16S rRNA gene sequences of deeply branching members of the green sulfur bacterial phylum obtained from cores 7620 and 7605. The numbers indicate the core (7620 or 7605), followed by the band number (Fig. 1). Phylotypes K through O are indicated by bold type. Bar = 0.1 fixed point mutation per nucleotide. Env., environmental; *Chl*., *Chlorobium*.

hence have unknown physiology (5, 29). *Chloroherpeton thalassium* ATCC 35110^T is located at the base of the *Chlorobiaceae* crown group and separates this group from the deeply branching sequences. Nine different 16S rRNA gene sequence types (types A to I) grouped with the *Chlorobiaceae* crown group (Fig. 2), whereas five other phylotypes (phylotypes K to O) branched more deeply within the phylum (Fig. 3). According to our analyses, four of the nine sequence types of the *Chlorobiaceae* (types A, C, F, and H) and all five sequence types of the less-well-studied more deeply branching *Chlorobi* were novel. This observation, together with the fact that our extraction and PCR controls consistently yielded negative results (see above), strongly indicates that the 16S rRNA gene fragments recovered in the present study originated from the sediments and did not represent contamination. The 14 different phylotypes of green sulfur bacteria recovered from Black Sea sediments surpass the 4 sequence types detected in a previous study of eastern Mediterranean sediments (5). In aquatic sediments, subfossil DNA has been shown to be largely degraded over a few thousand years (4). The higher diversity of subfossil sequences which persisted in the Black Sea sediments may therefore be attributed to the fact that they are younger (<10,000 years) than the Eastern Mediterranean sediments (up to 217,000 years).

The phylogeny of green sulfur bacteria suggests that the nine *Chlorobiaceae* sequences from the Black Sea sediments originated from obligately anaerobic photolithoautotrophs. First, all cultured representatives are members of the *Chlorobiaceae* crown group and require the simultaneous presence of light, sulfide, and CO_2 for growth. Second, the uncultured *Chlorobiaceae* have also typically been detected in illuminated sulfidic environments, like the chemoclines of lakes, lagoons, or benthic microbial mats (5, 29, 32). The *Chlorobiaceae* sequences most likely represent different types of green sulfur bacteria based on the following reasoning. Of the 10 available genome sequences of *Chlorobiaceae* (http://genome.jgi-psf.org/mic_home.html), 7 harbor a single *rrn* operon, whereas 3 genomes (the genomes of *Chlorobium tepidum* ATCC 49652^T, *Chlorobium phaeobacteroides* DSMZ 266^T, and *Chlorobium luteolum* DSMZ 273^T) contain two operons. The 16S rRNA gene sequences of *C. tepidum* and *C. phaeobacteroides* are identical, while the sequence of *C. luteolum* differs by only two deletions (based on bioinformatic analyses, these deletions may actually represent sequencing artifacts). Furthermore, all

Chlorobiaceae tested so far have single fingerprints on DGGE gels (13, 29, 32).

Further information on the origin of the subfossil *Chlorobiaceae* sequences can be derived from a more detailed phylogenetic analysis. Within the radiation of the *Chlorobiaceae*, the marine species can be reliably identified since they constitute a distinct phylogenetic cluster ("group 1") which is well separated from the other lineages (19) (Fig. 2). Besides the 16S rRNA gene sequence of BS-1, which was detected in 10 different sediment layers (sequence type I) (Fig. 2), three additional sequences clustered with marine group 1. One sequence (7620-41) was identical to that of *Prosthecochloris aestuarii* CE 2401 and was detected only once as a very faint signal at the bottom of the sapropel of Unit II. Sequence type F was observed as a faint signal in six consecutive sediment layers at the bottom of the Unit II sapropel and below these layers (Fig. 1A). The fourth marine sequence type, type H, was recovered from a turbidite and from a Unit I layer at site 7605 (Fig. 1B). Sequence 7620-24 (phylotype A) was detected only once and fell into *Chlorobiaceae* group 2, which comprises the salt-tolerant members (5, 19).

The comparatively low abundance of the sequence of *Chlorobium* sp. strain BS-1 in the sediments most likely should be attributed to the very low sedimentation rate of the BS-1 cells based on the following evidence. In contrast to other oxic-anoxic environments with accumulations of phototrophic sulfur bacteria (27), the vertical concentration profile of bacteriochlorophyll *e* pigments in the Black Sea showed a remarkably sharp decline below the chemocline and dropped below the detection limit at a depth of 120 m (24, 34, 35). Second, chlorophyll *a*, but not bacteriochlorophyll *e*, was detected in the sedimenting matter in the Black Sea water column (34), although the total amount of bacteriochlorophyll *e* detected in the chemocline was greater than the total amount of chlorophyll *a* in the upper oxygenated water layers (35). Third, the DNA sequence of chemocline strain BS-1 could not be detected in the fluff layers of both cores, indicating that the recent rate of sedimentation of this sequence toward the bottom was very low. This cumulative evidence suggests that only a very small fraction of the green sulfur bacteria in the Black Sea chemocline in fact reaches the deep-sea sediments, leading to the observed relative enrichment of the other phylotypes.

Whereas the marine and salt-tolerant phylotypes of the *Chlorobiaceae* were detected mostly as faint bands, most members of the nonmarine subgroups of the *Chlorobiaceae* yielded stronger signals on DGGE gels and occurred at many sediment depths. This is particularly true for phylotypes B, D, and E, which correspond to *Chlorobiaceae* lineages that are found exclusively in freshwater habitats and represent the most frequently detected lacustrine sequences worldwide (Fig. 2). Our results thus suggest that the assemblage of subfossil DNA of *Chlorobiaceae* in Black Sea sediments is dominated by sequences of cosmopolitan freshwater species. At least the DNA of typical freshwater *Chlorobiaceae* may have originated from environments like lakes or coastal lagoons, where these bacteria frequently form dense blooms. Long-distance transport of green sulfur bacterial biomarkers has previously been shown for North Atlantic deep-sea sediments (36). Evidence for the horizontal transport of green sulfur bacteria through oxygenated surface water of the Black Sea and their survival in this water comes from the enrichment of *Chlorobiaceae* from oxygenated Black Sea sediments sampled at a shallow depth, 64 m (15), and from the recent discovery of a green sulfur bacterium with unexpected oxygen tolerance (2). At the margins of the Black Sea, cyclonic gyres displace the chemocline downward, which prevents the buildup of a population of *Chlorobiaceae* in the water column in these regions (24). Yet strong fingerprints of *Chlorobiaceae* phylotypes B, D, and E were detected in the turbidites originating from the upper shelf. Phylotypes B and D were detected even more often in the turbidites than in the typical deep-sea sediments of core 7620, suggesting that phylotypes B and D are allochthonous.

No long-term survival of BS-1 cells in Black Sea sediments. Previous studies reported the enrichment of *C. phaeobacteroides* from 660- and 2,240-m-deep sediment layers (16). In order to determine the numbers of viable cells in the deep-sea sediments, we used four different sediment layers from station 7605 as inocula and set up MPN series with two different growth media. However, none of the cultures yielded enrichment cultures of green sulfur bacteria. Therefore, additional MPN series were established during a subsequent cruise with samples from the surface of the deep-sea sediment. This time, the medium employed was further optimized for growth of phylotype BS-1 and was supplemented with freshly prepared yeast extract and sodium acetate, as well as 200 μM dithionite as an additional reductant (24). This second round of MPN series yielded cultures of green sulfur bacteria that were brown; however, they developed only in the first two dilution steps.

Analysis of the green sulfur bacteria with group-specific PCR-DGGE yielded a single melting type (Fig. 4A). Sequencing of the 16S rRNA fragments confirmed that the cultures contained only the *Chlorobium* sp. strain BS-1 phylotype. Screening of the MPN cultures with eubacterial primers yielded only one green sulfur bacterial 16S rRNA gene fragment, which again was identical to that of BS-1 (Fig. 4B). All additional fingerprints detected (Fig. 4B) were excised and sequenced, and they were found to originate from groups of chemotrophic bacteria (*Deltaproteobacteria*, *Bacteroidetes*, and *Fusobacteria*). The corresponding value for the MPN as calculated from the liquid dilution series was 2.2 cells g (wet weight) sediment^{-1}, indicating that only very few viable cells of *Chlorobium* sp. strain BS-1 persisted even in the surface sediments of the Black Sea. Based on these data, we concluded that the 16S rRNA gene sequences of *Chlorobium* sp. strain BS-1 represent subfossil biomarkers and not living cells.

Deeply branching members of the green sulfur bacteria. The specific primers employed in the present study retrieved five sequences (phylotypes K to O) of deeply branching members of the green sulfur bacteria. This group is only distantly related to cultivated anoxygenic phototrophic species, does not include any cultured species, and is highly diverse and phylogenetically highly divergent, with up to 33% sequence variability in the 16S rRNA gene. Two of the deeply branching members of the green sulfur bacteria (phylotypes K and L) occurred in almost all deep-sea sediment layers of the Black Sea and thus were ubiquitous, in contrast to the *Chlorobiaceae* fingerprints. To date, the ecological role and physiology of these bacteria remain unclear. Representatives have been detected in soil, in mud (29), in anoxic freshwater lake sediments (37), on the

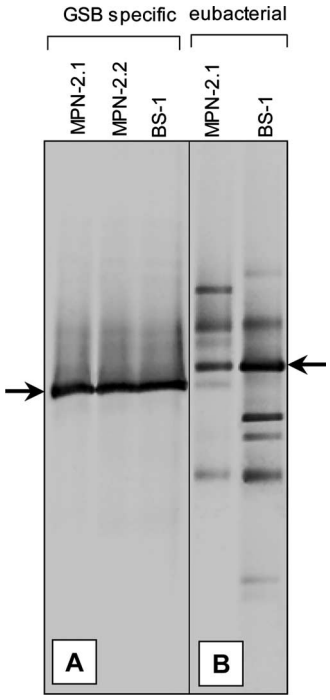


FIG. 4. DGGE of 16S rRNA gene sequences in MPN cultures from Black Sea sediments. (A) Amplification products generated with primers specific for green sulfur bacteria (GSB). (B) Amplification products generated with primers for eubacteria. BS-1, enrichment culture from the chemocline (24); MPN-2.1 and MPN-2.2, different MPN cultures from the Black Sea sediments. The arrows indicate the melting position of BS-1.

surface of a marine gastropod, and in the vicinity of a hot spring in the Indian Ocean (14). They occur in bacterial assemblages that degrade aromatic compounds (41, 42) or in the halocline of the Red Sea (9), as well as in other aphotic environments, and therefore may represent chemotrophs. Our findings are in contrast to recent results of a study of Mediterranean sapropels (5) in which deeply branching sequence types were not recovered.

Conclusions. Previously, subfossil isorenieratene (33) and sulfurized isorenieratane have been found in Unit I and Unit II (10, 38, 40, 43) and have been considered indicators of past photic zone anoxia in the Black Sea. The current study was the first analysis of subfossil 16S rRNA gene sequences in these sediments. As all known members of the *Chlorobiaceae* are obligate photoautotrophs and as no viable cells of green sulfur bacteria were detected in deeper sediment layers, the sequences obtained in the present study are highly unlikely to have originated from bacteria growing within the sediments and hence are subfossils. The phylotypes recovered mostly represent freshwater *Chlorobiaceae*, indicating that autochthonous DNA biomarkers, such as sequences of *Chlorobium* sp. strain BS-1, can be diluted significantly by allochthonous biomarkers deposited in the deep-sea sediments. Our results cast doubt on the general applicability of green sulfur bacterial carotenoids as sole biomarkers for photic zone anoxia in the overlying water column. Analysis of subfossil DNA sequences permits a more differentiated interpretation and reconstruction of past environmental conditions in euxinic environments if specific chemofossils of stenoec species, like *Chlorobium* sp. strain BS-1, are employed. Such sequences may also serve as biomarkers for photic zone anoxia in other parts of the ancient oceans.

ACKNOWLEDGMENTS

We thank the chief scientist of the cruise, Bo Barker Jørgensen, and the master and crew of the R/V *Meteor* for support during collection of the sediment cores and the master and crew of the R/V *Marion Dufresne* for their help during collection of the sediment cores in May 2004. Gilles Lericolais, the chief scientist of the ASSEMBLAGE cruise, provided the opportunity to sample the cores on the vessel. The technical assistance of Martina Mayer is gratefully acknowledged. We especially thank Helge Arz, Frank Lamy, and André Bahr for the core descriptions provided during the R/V *Meteor* cruise.

This work was supported by grants Ov 20/7-1 and Ov 20/8-1 to Ov 20/8-3 from the Deutsche Forschungsgemeinschaft to J. Overmann.

REFERENCES

1. **Anbar, A. D., and A. H. Knoll.** 2002. Proterozoic ocean chemistry and evolution: a bioinorganic bridge? Science **297:**1137–1142.
2. **Beatty, J. T., J. Overmann, M. T. Lince, A. K. Manske, A. S. Lang, R. E. Blankenship, C. L. Van Dover, T. A. Martinson, and F. G. Plumley.** 2005. An obligately photosynthetic bacterial anaerobe from a deep-sea hydrothermal vent. Proc. Natl. Acad. Sci. USA **102:**9306–9310.
3. **Codispoti, L. A., G. E. Friederich, J. W. Murray, and C. M. Sakamoto.** 1991. Chemical variability in the Black Sea: implications of continuous vertical profiles that penetrated the oxic/anoxic interface. Deep-Sea Res. **38**(Suppl. 2)**:**S691–S710.
4. **Coolen, M. J. L., and J. Overmann.** 1998. Analysis of subfossil molecular remains of purple sulfur bacteria in a lake sediment. Appl. Environ. Microbiol. **64:**4513–4521.
5. **Coolen, M. J. L., and J. Overmann.** 2007. 217,000 year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. Environ. Microbiol. **9:**238–249.
6. **Coolen, M. J. L., H. Cypionka, A. Smock, H. Sass, and J. Overmann.** 2002. Ongoing modification of Mediterranean Pleistocene sapropels mediated by prokaryotes. Science **296:**2407–2410.
7. **Degens, E. T., and D. A. Ross.** 1972. Chronology of the Black Sea over the last 25,000 years. Chem. Geol. **10:**1–16.
8. **Degens, E. T., and P. Stoffers.** 1976. Stratified waters as a key to the past. Nature **263:**22–27.
9. **Eder, W., M. Schmidt, M. Koch, D. Garbe-Schönberg, and R. Huber.** 2002. Prokaryotic phylogenetic diversity and corresponding geochemical data of the brine-seawater interface of the Shaban Deep, Red Sea. Environ. Microbiol. **4:**758–763.
10. **Freeman, K. H., S. G. Wakeham, and J. M. Hayes.** 1994. Predictive isotopic biogeochemistry: hydrocarbons from anoxic marine basins. Org. Geochem. **21:**629–644.
11. **Fuhrman, J. A., D. E. Comeau, Å. Hagström, and A. M. Chan.** 1988. Extraction from natural planktonic microorganisms of DNA suitable for molecular biological studies. Appl. Environ. Microbiol. **54:**1426–1429.
12. **Gich, F., J. Garcia-Gil, and J. Overmann.** 2001. Previously unknown and phylogenetically diverse members of the green nonsulfur bacteria are indigenous to freshwater lakes. Arch. Microbiol. **177:**1–10.
13. **Glaeser, J., and J. Overmann.** 2004. Biogeography, evolution, and diversity of epibionts in phototrophic consortia. Appl. Environ. Microbiol. **70:**4821–4830.
14. **Goffredi, S. K., A. Warén, V. J. Orphan, C. van Dover, and R. C. Vrijenhoek.** 2004. Novel forms of structural integration between microbes and a hydrothermal vent gastropod from the Indian Ocean. Appl. Environ. Microbiol. **70:**3082–3290.
15. **Gorlenko, V. M., P. V. Mikheev, I. I. Rusanov, N. V. Pimenov, and M. V. Ivanov.** 2005. 2005. Ecophysiological properties of photosynthetic bacteria from the Black Sea chemocline zone. Microbiology (New York) **74:**201–209.
16. **Hashwa, F. A., and H. G. Trüper.** 1978. Viable phototrophic bacteria from the Black-Sea bottom. Helgol. Wiss. Meeresunters. **31:**249–253.
17. **Hay, B. J.** 1988. Sediment accumulation in the central western Black Sea over the past 5,100 years. Paleoceanography **3:**491–508.
18. **Hay, B. J., and M. A. Arthur.** 1991. Sediment deposition in the Holocene Black Sea. Deep-Sea Res. **38**(Suppl. 2)**:**1211–1235.
19. **Imhoff, J. F.** 2003. Phylogenetic taxonomy of the family *Chlorobiaceae* on the basis of 16S rRNA and *fmo* (Fenna-Matthews-Olson protein) gene sequences. Int. J. Syst. Evol. Microbiol. **53:**941–951.
20. **Kohnen, M. E. L., J. S. Sinninghe Damsté, and J. W. DeLeeuw.** 1991. Biases from natural sulphurization in palaeoenvironmental reconstruction based on hydrocarbon biomarker distribution. Nature **349:**775–778.

21. **Konovalov, S. K., L. I. Ivanov, and A. S. Samodurov.** 2001. Fluxes and budgets of sulphide and ammonia in the Black Sea anoxic layer. J. Mar. Syst. **31:**203–216.
22. **Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumann, M., Bachleitner, and K. H. Schleifer.** 1998. Bacterial phylogeny based on comparative sequence analysis. Electrophoresis **19:**554–568.
23. **Maidak, B. L., J. R. Cole, T. G. Lilburn, C. T. Parker, P. R. Saxman, R. J. Farris, G. M. Garrity, G. J. Olsen, T. M. Schmidt, and J. M. Tiedje.** 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res. **29:**173–174.
24. **Manske, A. K., J. Glaeser, M. M. M. Kuypers, and J. Overmann.** 2005. Physiology and phylogeny of green sulfur bacteria forming a monospecific phototrophic assemblage at a depth of 100 meters in the Black Sea. Appl. Environ. Microbiol. **71:**8049–8060.
25. **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
26. **Muyzer, G., S. Hottenträger, A. Teske, and C. Waver.** 1995. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA—a new molecular approach to analyse the genetic diversity of mixed microbial communities, p. 3.4.4.1–3.4.4.22. *In* A. D. L. Akkermans, J. D. Van Elsas, and F. J. De Bruijn (ed.), Molecular microbial ecology manual, 2nd ed. Kluwer, Dordrecht, The Netherlands.
27. **Overmann, J.** 1991. Standortspezifische Anpassung bei phototrophen Schwefelbakterien. Konstanzer Dissertationen Bd. 321. Hartung-Gorre, Konstanz, Germany.
28. **Overmann, J., and N. Pfennig.** 1989. *Pelodictyon phaeoclathratiforme* sp. nov., a new brown-colored member of the *Chlorobiaceae* forming net-like colonies. Arch. Microbiol. **152:**401–406.
29. **Overmann, J., and C. Tuschak.** 1997. Phylogeny and molecular fingerprinting of green sulfur bacteria. Arch. Microbiol. **167:**302–309.
30. **Overmann, J., and A. K. Manske.** 2006. Anoxygenic phototrophic bacteria in the Black Sea, p. 543–561. *In* L. Neretin (ed.), Past and present water column anoxia. NATO ASI series. Springer-Verlag, Berlin, Germany.
31. **Overmann, J., H. Cypionka, and N. Pfennig.** 1992. An extremely low-light-adapted green sulfur bacterium from the Black Sea. Limnol. Oceanogr. **37:**150–155.
32. **Overmann, J., M. J. L. Coolen, and C. Tuschak.** 1999. Specific detection of different phylogenetic groups of chemocline bacteria based on PCR and denaturing gradient gel electrophoresis of 16S rRNA gene fragments. Arch. Microbiol. **172:**83–94.
33. **Repeta, D. J.** 1993. A high resolution historical record of Holocene anoxygenic primary production in the Black Sea. Geochim. Cosmochim. Acta **57:**4337–4342.
34. **Repeta, D. J., and D. J. Simpson.** 1991. The distribution and recycling of chlorophyll, bacteriochlorophyll and carotenoids in the Black Sea. Deep-Sea Res. **38:**969–984.
35. **Repeta, D. J., D. J. Simpson, B. B. Jørgensen, and H. W. Jannasch.** 1989. Evidence for anoxygenic photosynthesis from the distribution of bacteriochlorophylls in the Black Sea. Nature **342:**69–72.
36. **Rosell-Melé, A., M. A. Maslin, J. R. Maxwell, and P. Schaefer.** 1997. Biomarker evidence for "Heinrich" events. Geochim. Cosmochim. Acta **61:** 1671–1678.
37. **Schwarz, J. I. K., W. Eckert, and R. Conrad.** 2007. Community structure of *Archaea* and *Bacteria* in a profundal lake sediment Lake Kinneret (Israel). Syst. Appl. Microbiol. **30:**239–254.
38. **Sinninghe Damsté, J. S., S. G. Wakeham, M. E. L. Kohnen, J. M. Hayes, and J. W. de Leeuw.** 1993. A 6,000-year sedimentary molecular record of chemocline excursions in the Black Sea. Nature **362:**827–829.
39. **Sorokin, Y. I.** 2002. The Black Sea. Ecology and oceanography. Backhuys Publishers, Leiden, The Netherlands.
40. **Summons, R. E., and T. G. Powell.** 1987. Identification of aryl isoprenoids in source rocks and crude oils: biological markers for the green sulfur bacteria. Geochim. Cosmochim. Acta **51:**557–566.
41. **Ulrich, A. C., and E. A. Edwards.** 2003. Physiological and molecular characterization of anaerobic benzene-degrading mixed cultures. Environ. Microbiol. **5:**92–102.
42. **von Wintzingerode, F., B. Selent, W. Hegemann, and U. B. Göbel.** 1999. Phylogenetic analysis of an anaerobic, trichlorobenzene-transforming microbial consortium. Appl. Environ. Microbiol. **65:**283–286.
43. **Wakeham, S. G., J. S. Sinninghe Damsté, M. E. L. Kohnen, and J. W. DeLeeuw.** 1995. Organic sulfur compounds formed during early diagenesis in Black Sea sediments. Geochim. Cosmochim. Acta **59:**521–533.