

## Microbial Diversity of Hydrothermal Sediments in the Guaymas Basin: Evidence for Anaerobic Methanotrophic Communities†

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Microbial communities in hydrothermally active sediments of the Guaymas Basin (Gulf of California, Mexico) were studied by using 16S rRNA sequencing and carbon isotopic analysis of archaeal and bacterial lipids. The Guaymas sediments harbored uncultured euryarchaeota of two distinct phylogenetic lineages within the anaerobic methane oxidation 1 (ANME-1) group, ANME-1a and ANME-1b, and of the ANME-2c lineage within the *Methanosarcinales*, both previously assigned to the methanotrophic archaea. The archaeal lipids in the Guaymas Basin sediments included archaeol, diagnostic for nonthermophilic euryarchaeota, and *sn*-2-hydroxyarchaeol, with the latter compound being particularly abundant in cultured members of the *Methanosarcinales*. The concentrations of these compounds were among the highest observed so far in studies of methane seep environments. The  $\delta$ -<sup>13</sup>C values of these lipids ( $\delta$ -<sup>13</sup>C = -89 to -58‰) indicate an origin from anaerobic methanotrophic archaea. This molecular-isotopic signature was found not only in samples that yielded predominantly ANME-2 clones but also in samples that yielded exclusively ANME-1 clones. ANME-1 archaea therefore remain strong candidates for mediation of the anaerobic oxidation of methane. Based on 16S rRNA data, the Guaymas sediments harbor phylogenetically diverse bacterial populations, which show considerable overlap with bacterial populations of geothermal habitats and natural or anthropogenic hydrocarbon-rich sites. Consistent with earlier observations, our combined evidence from bacterial phylogeny and molecular-isotopic data indicates an important role of some novel deeply branching bacteria in anaerobic methanotrophy. Anaerobic methane oxidation likely represents a significant and widely occurring process in the trophic ecology of methane-rich hydrothermal vents. This study stresses a high diversity among communities capable of anaerobic oxidation of methane.

Deep-sea hydrothermal vent sites encompass very diverse environments for microbial life, such as black smoker chimney walls and orifices, warm vents and seeps, symbiont-harboring invertebrates, surface-attached microbial mats, and floating particles in vent plumes. Hydrothermally heated sediments characterize the Guaymas Basin hydrothermal vent site in the Gulf of California. The basin has a very high sedimentation rate (1 to 2 mm/year) due to high biological productivity in surface waters and a large terrigenous input. Consequently, the Guaymas Basin vent sites are covered with, on average, 100-m- and up to 500-m-thick layers of organic-rich, diatomaceous sediments (11, 73). Chemical interactions between hydrothermal vent fluids and sediment accelerate diagenetic processes such as metal sulfide precipitation and high-temperature pyrolysis of organic material (43) and create chemical conditions that differ significantly from those at the more common bare lava vent sites. The metal content of the vent fluids is reduced by several orders of magnitude (85). Diffuse venting through the sediments at temperatures up to 200°C releases vast quantities of petroleum hydrocarbons, short-chain organic acids,

and ammonia via pyrolysis of complex organic substrates (4, 52, 86). The methane content in the vent fluids is in the range of 12 to 16 mM (270 to 370 ml kg<sup>-1</sup> at standard temperature and pressure), which is approximately 2 orders of magnitude higher than those of most bare lava vent sites (87). The carbon isotopic composition ( $\delta$ -<sup>13</sup>C) of the methane ranges from -51 to -43‰, suggesting a thermocatalytic origin from sedimentary organic material (87). The high concentration and relatively low isotopic composition of methane differ from those of most other mid-ocean ridge systems, where methane originates from high-temperature reactions involving H<sub>2</sub> and CO<sub>2</sub>, and from mantle outgassing (17).

This metabolic menu of Guaymas vent fluids and sediments supports unusually rich microbial diversity in all three domains of life. A large variety of unusual bacteria, archaea, and eukaryotes have been isolated or described from the Guaymas Basin. The archaea include hyperthermophilic archaeal sulfate reducers of the genus *Archaeoglobus* (10) and hyperthermophilic methanogens of the genera *Methanopyrus* and *Methanococcus* (36, 40). Many bacterial populations that were found at the Guaymas Basin have adapted to the petroleum hydrocarbon-rich environment: for example, anaerobic sulfate-reducing bacteria that oxidize alkanes, aromatic compounds, and fatty acids (69) and aerobic, aromatic hydrocarbon-degrading bacteria with a preference for aromatic carboxylic acids (26). The sulfidic vent fluids and high sulfate reduction rates fuel massive bacterial mats of autotrophic, sulfur-oxidizing, filamentous

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*Beggiatoa* spp. (30, 57). The first comparative survey of culturable eukaryotic protists at Guaymas and other Pacific Ocean vent sites (2) was complemented by a eukaryotic 18S rRNA census of Guaymas that revealed novel, in part deeply branching eukaryotic lineages (21). However, the possibility that methane could sustain extensive microbial communities at Guaymas Basin vent sediments has not yet been investigated.

In this study, we have combined bacterial and archaeal 16S rDNA sequence surveys with structural and isotopic analyses of archaeal and bacterial lipids to constrain structural and metabolic features of the microbial community.

#### MATERIALS AND METHODS

**Sampling sites and characteristics.** Sediment cores were retrieved during dives with the research submersible *Alvin* (Woods Hole Oceanographic Institution) on a cruise to the Guaymas Basin in April and May 1998. Two sediment cores were investigated in detail. Core A (*Alvin* dive 3203, 26 April 1998; diameter = 15 cm, length = 35 cm) from the Everest Mound area in the southern Guaymas vent field (27°1'388"N, 111°24'112"W) was covered with an orange *Beggiatoa* spp. mat, indicating a flow of sulfide from the sediment that sustains these chemolithoautotrophic sulfide oxidizers. The sediment consisted of very soft olive-green ooze in the surface layers and, below a depth of 7 cm, became grayish and developed a petroleum-like sheen. Below 10 cm, gas bubbles were noted after recovery. During *Alvin* dive 3207 (30 April 1998) at the Orpheus site (27°0'435"N, 111°24'612"W), core C, a 6-inch-diameter core without a *Beggiatoa* mat, was selected as a contrasting sample to core A. The upper 6 cm consisted of a black layer of liquid petroleum ooze; below 7 cm in depth, the sediment turned olive and became thicker. Gas bubbles in the sediment appeared at a 15-cm depth. In situ temperature profiles of both sediments were determined before coring by using *Alvin's* thermoprobe; the uppermost 5 cm of each core had a temperature range from cold ambient seawater to moderately hot vent fluids (ca. 2°C to 57°C, core C, and 2°C to 74°C, core A). Both cores came from sediments with high rates of sulfate reduction and organic carbon turnover (A. Weber and B. B. Jørgensen, submitted for publication). The cores were brought to the surface no more than 6 h after sampling and were sliced in 1-cm intervals within an additional 6 h after retrieval. Multiple 2-ml portions of sediment were taken from each centimeter layer and immediately frozen at -80°C for nucleic acid extraction and lipid analysis onshore. A sediment core covered with a *Beggiatoa* mat, without a temperature profile (core T), was obtained from the Rebecca's Roost area (27°1'131"N, 111°40'687"W) in the southern Guaymas vent field by the remotely operated vehicle *JASON*. From core T, sample material for phylogenetic analyses was only available from the 2.5- to 5-cm layer. Lipid analyses of an aliquot of this layer revealed a high degree of similarity of the biomarker distribution to the overlying 0- to 2.5-cm layer. Isotope data of individual compounds were not determined for the 2.5- to 5-cm layer.

**Nucleic acid isolation.** A bead-beating procedure combined with hot phenol extraction was used to isolate high-molecular-weight DNA and rRNA. The following protocol is a modification of the method used by Stahl and coworkers (74). Sediment (0.5 g) was mixed with 895  $\mu$ l of lysis buffer (50 mM Tris [pH 8], 25% sucrose), 62.5  $\mu$ l of 20% (wt/vol) sodium dodecyl sulfate solution, and 0.5 g of baked zirconium beads (0.1-mm diameter). The sample was subjected to 15 s of bead-beating by using a Mini-beadbeater (BioSpec Products, Bartlesville, Okla.). After the bead-beating step, 42  $\mu$ l of a solution consisting of 20 mg of proteinase K/ml was added, followed by 60 min of incubation at 55°C under constant agitation. The sample (total volume, approximately 1.6 ml) was divided into two equal aliquots, mixed with equal volumes of a phenol-chloroform-isoamyl alcohol mixture (25:24:1 [vol/vol/vol]; pH 7.9), and incubated at 55°C for 10 min. After centrifugation, the aqueous phases (volume, approximately 0.8 ml each) were transferred to two new vials. The two organic phases were mixed with 200  $\mu$ l of Tris-EDTA buffer each (pH 7.5), incubated at 55°C for 10 min, and centrifuged, and the aqueous extracts were combined with the previous aqueous phases. Both combined aqueous phases were extracted two to three times more with a phenol-chloroform-isoamyl alcohol mixture as described above. The two samples were recombined in a 15-ml Falcon tube (total volume, approximately 4 ml) and mixed with 0.4 ml of a 3 M sodium acetate solution and 8 ml of ethanol. Nucleic acids were precipitated overnight at -20°C and centrifuged at 8,000 rpm in a Sorvall RC2-B centrifuge for 40 min at 4°C in baked 50-ml Corex glass tubes. The pellet was washed with 70% ethanol, dried, taken up in 100  $\mu$ l of Tris-EDTA (pH 7.5), transferred to a microcentrifuge tube, and stored at -80°C.

**16S rRNA gene amplification, cloning, and sequencing.** Near-complete 16S rRNA genes were PCR amplified on a Gene-Amp PCR Cycler 9700 (Perkin-Elmer) by using archaeal primers ARC-8F (5'-TCGGTTGATCCTGCC-3') and ARC-1492R (5'-GGCTACCTTGTACGACTT-3') and bacterial primers BAC-8F (5'-AGRGTGTTGATCCTGGCTCAG-3') and BAC-1492R (5'-CGGC TACCTTGTACGACTT-3'). The archaeal PCR primers were checked on the basis of a comprehensive Ribosomal Database Project archaeal alignment to make them as unbiased as possible for use in multitemplate PCR. The 8F archaeal primer was terminated at position 23, thus avoiding ambiguities in archaeal 16S rRNA sequences (positions 24 to 27) that would otherwise form a source of PCR bias (66). This primer was used together with the reverse archaeal primer, 1492R, which also did not contain ambiguities. The bacterial primers were checked in the same way, with a general bacterial alignment of near-complete bacterial 16S sequences that included 3' and 5' regions. Each PCR cycle (30 total) consisted of a 1-min denaturation step at 94°C, a 1-min annealing step at 55°C, and a 2-min elongation step at 72°C. PCR products were checked on an agarose gel, the bands were cut out, and PCR products were extracted with the Prep-A-Gene DNA purification kit (Bio-Rad, Hercules, Calif.), following the manufacturer's protocols. The PCR product was resuspended in 10  $\mu$ l of 10 mM Tris (pH 7.5). The purified PCR products were a-tailed as follows, to improve cloning efficiency: approximately 50 ng of purified PCR product was mixed with 5  $\mu$ l of 10 $\times$  PCR buffer [200 mM Tris (pH 8.55), 100 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM MgCl<sub>2</sub>, 300 mM KCl, 0.1% (wt/vol PCR buffer) gelatin, 0.5% (vol/vol PCR buffer) NP-40], 5  $\mu$ l of deoxynucleoside triphosphate (2 mM), and 1 U of Promega *Taq* DNA polymerase in a 50- $\mu$ l total volume. The mixture was incubated at 72°C for 10 min, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and centrifuged for 5 min. The aqueous top layer was then transferred to a fresh microcentrifuge tube. The a-tailed PCR product was precipitated with 10% (vol/vol aqueous layer) 3 M sodium acetate and 2 volumes of 100% ethanol, washed with 70% ethanol, and resuspended in 4  $\mu$ l of distilled water. The PCR products were cloned by using the TOPO XL PCR cloning kit (Invitrogen Corp., Carlsbad, Calif.) and transformation by electroporation according to the manufacturer's specifications.

Sequence data were gathered on a LI-COR 4200L automated sequencing apparatus by using infrared dye-labeled T7F and M13R primers (LI-COR, Lincoln, Nebr.) and a cycle sequencing protocol (Sequiterm; Epicenter Inc., Madison, Wis.) according to the manufacturers' instructions. This system stores the sequence results as image files for subsequent base determination and sequence assembly. The 3' and 5' ends of each PCR product were covered by one sequence read each; the central 0.6- to 0.9-kb portion was covered double-stranded. Base calls and PHD quality values were determined by using e.Seq (Build 1.0.52) DNA sequence analysis software from LI-COR. For each individual clone, forward and reverse reads were assembled by using Phrap version 0.960731 (28).

**Phylogenetic analysis.** Sequence data were compiled by using ARB software ([www.arb-home.de](http://www.arb-home.de)) and aligned with sequences obtained from the GenBank database by using the ARB FastAligner utility. Resulting alignments were manually verified against known secondary structure regions. Representative clones (determined by neighbor-joining clustering patterns) were selected for further analyses. We limited comparisons to 1,353, 1,352, and 1,432 sites that could be unambiguously aligned according to conservation of primary and secondary structures for the nonproteobacterial, proteobacterial, and archaeal data sets, respectively. Alignments and sites included in our analyses are available upon request.

Analyses were performed by using minimum evolution and parsimony methods implemented in PAUP\* version 4.08b (76). Heuristic searches under minimum evolution criteria used 1,000 random-addition replicates per data set, each followed by tree bisection-reconnection topological rearrangements. Models for use in minimum evolution analyses of the archaeal, proteobacterial, and non-proteobacterial data sets were evaluated using the likelihood ratio test implemented in Modeltest v.3.0 (67). For all three data sets, all models tested that were simpler than the general time reversible (GTR) + invariant sites (I) + gamma model had a significantly poorer fit to the data for the reference topology (based on a neighbor-joining tree according to Jukes-Cantor). Therefore, minimum evolution distance trees were inferred by using a GTR model with six classes of substitutions, unequal base frequencies, proportion of invariant sites, and the evolutionary rate of the remaining portions of sites differing according to a gamma distribution (with shape parameter  $\alpha$ ). Model parameters estimated for minimum evolution analyses for the archaeal, proteobacterial, and nonproteobacterial datasets were  $\alpha$  = 0.7636, 0.6718, and 0.8071 and I = 0.1902, 0.3858, and 0.1997, respectively. Bootstrapping for minimum evolution and parsimony was done with 1,000 replicates per data set.

**Analysis of sedimentary microbial lipids.** Lipid analyses were carried out by following a procedure reported earlier (32, 59). Sediment samples (~0.6 to 0.7 g

TABLE 1. Stable carbon isotopic compositions and concentrations of selected biomarkers from archaea, bacteria, and eukaryotes

Compound <sup>a</sup>	Symbol (Fig. 1) <sup>c</sup>	Source	$\delta^{13}\text{C}$ /abundance ( $\mu\text{g/g}$ dry sediment)			
			Core A (layer 0–1 cm)	Core A (layer 1–2 cm)	Core C (layer 0–1 cm) <sup>b</sup>	Core T (layer 0–2.5 cm) <sup>b</sup>
Archaeol	A	Archaea	–58‰/22	–60‰/20	–81‰/1.4	–72‰/18
<i>sn</i> -2-Hydroxyarchaeol	B	Archaea	–70‰/6	nd/1	–81‰/1.5	–89‰/14
Bicyclic diphyanediol	C	Archaea	–68‰/54	–68‰/18	Not present	–66‰/31
MAGE-C <sub>16</sub>	D	Bacteria	–41‰/26	–41‰/3	Not present	UCM/2
MAGE-C <sub>18:1</sub>	E	Bacteria	–38‰/34	–37‰/3	Not present	UCM/5
DAGE-C <sub>30:1</sub>	F	Bacteria	–28‰/21	–33‰/3	Not present	UCM/1
DAGE-C <sub>16/17</sub>	G	Bacteria	–40‰/7	–40‰/7	Not present	–34‰/3
DAGE-C <sub>16/17</sub>	H	Bacteria	–45‰/12	–47‰/10	Not present	–44‰/3
DAGE-C <sub>17/17</sub>	I	Bacteria	–46‰/19	–51‰/13	Not present	–53‰/2
DAGE-C <sub>17/17</sub>	J	Bacteria	–45‰/23	–49‰/26	Not present	–43‰/2
DAGE-C <sub>17/18</sub>	K	Bacteria	–44‰/18	–49‰/27	Not present	nd/3
Hopan-29-ol	L	Bacteria	–29‰/18	–32‰/20	nd/1.4	–35‰/8
Hopan-22-ol	M	Bacteria	–37‰/7	–36‰/5	nd/0.5	–34‰/6
Cholesterol	N	Eukarya	–21‰/64	–28‰/73	–25‰/6	UCM/17

<sup>a</sup> C<sub>16</sub> designates the carbon number of the alkyl moiety. Carbon numbers of ether-bound alkyl rests are assigned as follows: C<sub>30:1</sub> indicates the total carbon number of the two alkyl groups, in this example with a total of one degree of unsaturation, i.e., a double bond or a ring; C<sub>16/17</sub> indicates a hexadecylether moiety bound to the *sn*-1 glycerol carbon and a heptadecylether moiety at *sn*-2.

<sup>b</sup> nd, not determined; UCM, not determined due to unresolved complex mixture.

<sup>c</sup> Symbols refer to Fig. 1.

dry weight, 22 g for core T, 0- to 2.5-cm layer) were sterilized by ultrasonication (ultrasonic bath) in ~20 ml of a mixture of dichloromethane and methane (9:1 [vol/vol]) for ~30 min. After evaporation of the residual solvent, the sediments were oven dried at ~50°C. Free lipids were extracted from dried and homogenized sediments by using a DIONEX Accelerated Solvent Extraction 200 system at 100°C and 1,000 lb/in<sup>2</sup>, with dichloromethane (DCM)-methanol (90:10 [vol/vol]) as the solvent. Extracts were separated into four fractions of increasing polarity by using SUPELCO LC-NH<sub>2</sub> glass cartridges (500 mg of sorbent) and a sequence of solvent mixtures of increasing polarity (hydrocarbons, 4 ml of *n*-hexane; ketone-esters, 6 ml of 3:1 [vol/vol] *n*-hexane-DCM; alcohols, 7 ml of 9:1 [vol/vol] DCM-acetone; carboxylic acids, 8 ml of 2% formic acid in DCM). Individual compounds were quantified and identified by using an HP 6890 gas chromatograph (GC) equipped with a J&W DB-5 capillary column (60-m length, 0.32-mm inner diameter, 0.25- $\mu\text{m}$  film thickness) and coupled to an HP 5973 mass-selective detector and a flame ionization detector. Stable carbon isotopic compositions of individual compounds were determined by using a Finnigan Delta Plus mass spectrometer coupled to an HP 6890 GC and equipped with a CP-Sil 5 column (60-m length, 0.25-mm inner diameter, 0.25- $\mu\text{m}$  film thickness). Column temperatures of both GC systems were programmed to increase from 40°C (1 min, isothermal) to 130°C at a rate of 20°/min and then to 320°C (60 min, isothermal) at a rate of 4°C/min. Alcohols were analyzed for their trimethylsilyl ethers and esters, respectively, after reaction with a *N,O*-bis(trimethylsilyl)trifluoroacetamide reagent (BSTFA + 1% trimethylchlorosilane) in a 1:1 mixture of DCM and pyridine. Reported stable carbon isotopic values are means of at least two analyses and have been corrected for the presence of carbon atoms added during derivatization. Differences between individual analyses were mostly less than 1‰, with the exception of core T, which was strongly affected by an unresolved complex mixture (UCM) related to hydrothermally generated petroleum. Quantification of individual compounds was based on their flame ionization detector signal relative to that of known amounts of internal standards.

**Nucleotide sequence accession numbers.** The Guaymas 16S rRNA gene sequences have the following GenBank accession numbers: archaea, AF419624 to AF419656; proteobacteria, AF420334 to AF420370; all other bacteria, AF419657 to AF419699. Sequences were checked for chimera formation by using the Chimera check program of the Ribosomal Database Project II database; three chimeric bacterial sequences were found and removed from the analysis (51).

## RESULTS

**Archaeal and bacterial lipids and biomarkers.** The archaeal lipids archaeol, diagnostic for nonthermophilic members of the *Euryarchaeota*, and *sn*-2-hydroxyarchaeol, predominantly occurring in cultured members of the *Methanosarcinales*, were

found in very high abundance in the surface layers of cores A and T (Table 1). To date, observations of similarly high accumulations of these two compounds have been limited to mud volcano sites in the Mediterranean (61). In contrast, cold seep sites from the California and Oregon continental margin exhibited lower concentrations (7, 32), i.e., in a range similar to those observed in core C. The stable carbon isotopic compositions of these compounds ( $\delta^{13}\text{C}$ , –89 to –58‰) are indicative of an origin from oxidation and assimilation of <sup>13</sup>C-depleted methane (Fig. 1). In addition, cores A and T contain very significant amounts of an additional compound of archaeal origin, a biphytanediol with two pentacyclic rings (<http://docserver.bis.uni-oldenburg.de/publikationen/dissertation/2001/ruetra01/ruetra01.html>) (70) with a <sup>13</sup>C depletion similar to those of archaeol and hydroxyarchaeol (Table 1 and Fig. 1).

Both cores A and T contain abundant bacterial lipid biomarkers, namely, monoalkylglycerol ethers (MAGE) and dialkylglycerol ethers (DAGE) (Fig. 1 and Table 1). Related and identical compounds had been observed earlier in sediments hosting active methanotrophic communities (32, 62). Structural analogs of the latter group are known only from the most deeply branching bacteria, *Thermodesulfobacterium commune* (45) and the *Aquificales* (37). In both cores, their isotopic compositions ( $\delta^{13}\text{C}$  values in the range of –50 to –35‰; with the sole exception, that of DAGE-C<sub>30:1</sub>, at around –30‰) are significantly lower than those of lipids from marine eukaryotic algae (e.g., cholesterol) (Table 1) and bulk organic matter (~–27‰), a pattern consistent with previous observations from cold methane seeps. In contrast, these compounds were not present in core C.

**Archaeal 16S rRNA gene sequence profiles.** Cores A, T, and C yielded predominantly archaeal 16S rRNA clones of two phylogenetic lineages of uncultured archaea, presumed anaerobic methane-oxidizing archaeal group 1 (ANME-1) and ANME-2 (31, 59). The clones from cores A and T were dominated by members of the ANME-1 cluster, which constituted 95% and 87% of all archaeal clones, respectively. These eur-



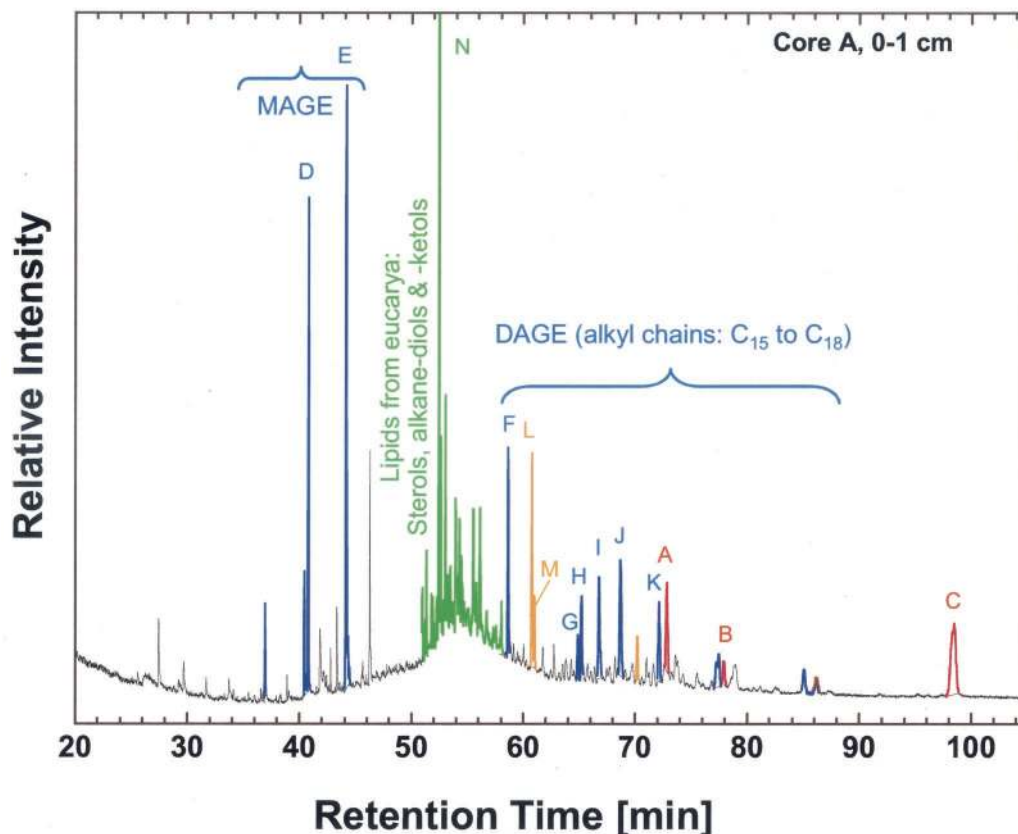


FIG. 1. Reconstructed ion chromatogram of alcohol fraction from core A sample, 0 to 1 cm. Colors of peaks designate their biological sources: red, methanotrophic archaea; blue, presumed bacterial members of methanotrophic community; orange, aerobic bacteria; green, eucaryotes, e.g., marine algae. Refer to Table 1 for further details.

yarchaeota were originally suggested as likely mediators of anaerobic methanotrophy (31), but in the past few years, evidence has accumulated mostly for an involvement of the ANME-2 cluster (7, 59, 60). The ANME-1 group clones obtained from the Guaymas Basin showed considerable phylogenetic diversity and formed two major lineages. The ANME-1a group, which corresponds to the previously defined ANME-1 cluster (31, 59), has a sister group, ANME-1b, that is so far represented by sequences from the Guaymas Basin only. Although the two groups are not closely related to each other (83 to 87% sequence similarity between ANME-1a and ANME-1b, compared to >89% within ANME-1a members alone), their sister-group relationship is always supported with 98 to 100% bootstrap support. The ANME-1 archaea have no close cultured relatives; in all analyses, they formed an independent phylogenetic cluster among the *Euryarchaeota* that could not be subsumed under another archaeal group, for example, the methanogenic lineages of *Thermoplasma*, *Thermococcus*, or *Archaeoglobus* (Fig. 2). In contrast to cores A and T, which yielded mostly ANME-1 and ANME-1b archaeal clones (96 and 87% of all archaeal clones obtained from cores A and T), core C predominantly yielded clones of the ANME-2 group of archaea (Fig. 2). In core C, they constitute 76% (52 clones) of all archaeal clones sequenced. The ANME-2 lineages form distinct branches among the cultured members of the *Meth-*

*anosarcinales*, of comparable phylogenetic depth to those of the genera *Methanosaeta*, *Methanosarcina*, and *Methanolobus* (Fig. 2). All Guaymas Basin ANME-2 clones fell into the subgroup ANME-2c of the ANME-2 cluster (59), with mutual sequence similarity greater than 95%. The Guaymas sequences showed lower sequence similarities (87.2 to 89.2%) to the ANME subgroups 2a and 2b, here represented by the Santa Barbara and Eel River sequence clones 24a1f10 and 2E36a2A4. The ancestral node of the ANME-2c subgroup includes not only the ANME-2a and 2b subgroups but also cultured members of the *Methanosarcinales*, for example, the acetoclastic and methyl-disproportionating genera *Methanosarcina* and *Methanolobus*. In other words, the ANME-2 lineages are paraphyletic within the *Methanosarcinales* (Fig. 2).

Besides ANME-2 clones within the *Methanosarcinales*, the only members of other methane-metabolizing families that were found in the Guaymas sediments belonged to the mesophilic or moderately thermophilic family *Methanomicrobiales*. Three clones, represented by CSR002, were related to the  $H_2$ -/ $CO_2$ - and formate-utilizing genera *Methanogenium* and *Methanomicrobium*, and the single clone C1R002 was closely related to the species *Methanocorpusculum parvum*, which uses  $H_2$ , formate, and secondary alcohols as electron donors (Fig. 2).

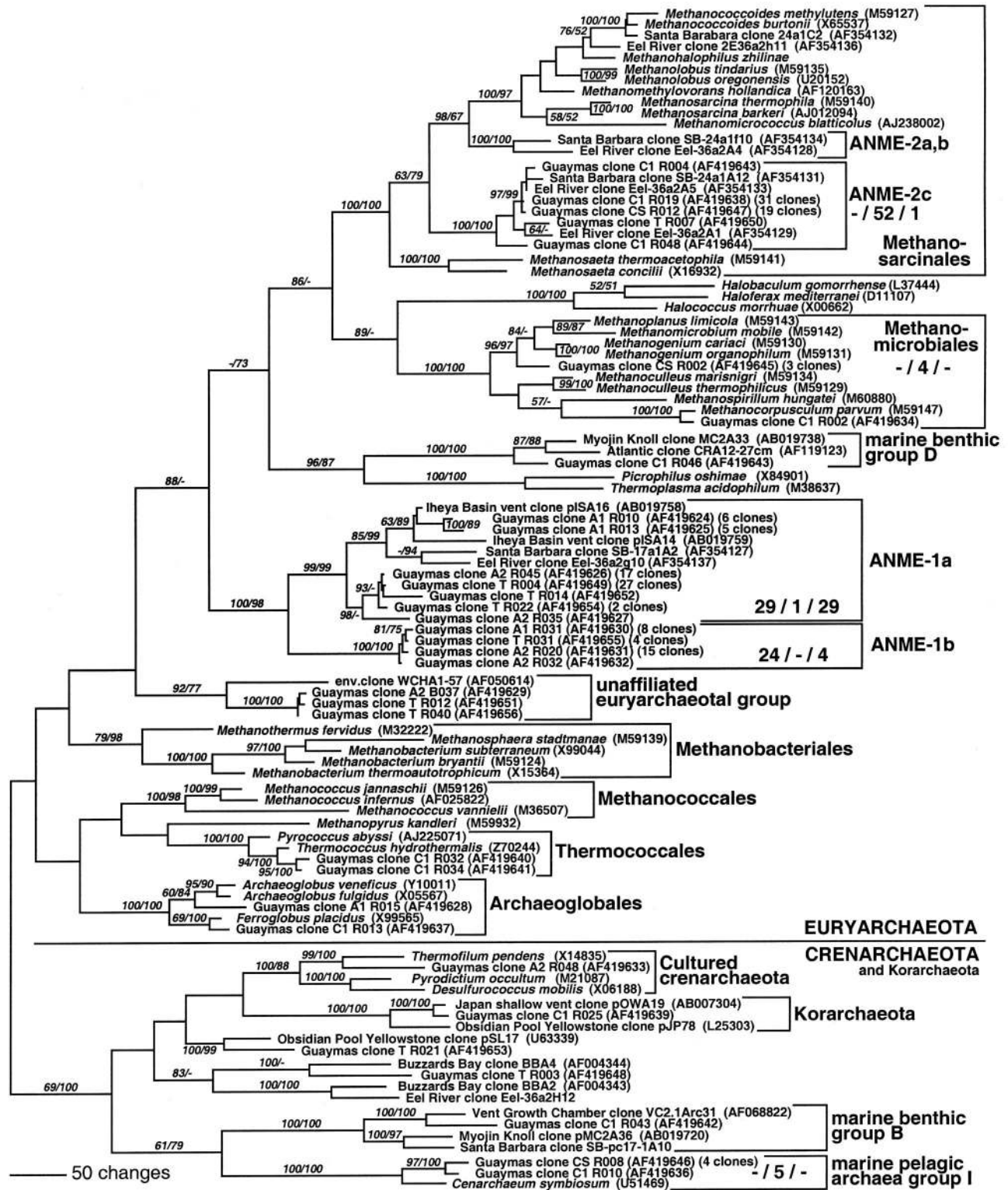


FIG. 2. Distance tree of archaeal clones from Guaymas sediment cores A, C, and T, based on near-complete 16S rDNA sequences. Bootstrap values (in percent) are based on 1,000 replicates each (distance and parsimony) and are shown for branches with more than 50% bootstrap support. The sequence for *Methanohalophilus zhilinae* was obtained from the ARB sequence server (version May 2001). Crenarchaeotal sequences SB-pc17-1A10 and Eel-36a2H12 were provided by Victoria Orphan. For the major phylogenetic divisions, the numbers of core A, core C, and core T clones are given in that order. For multiple near-identical clones (>99% identity), the number of multiples is given after the GenBank number of a representative clone. Some sequence types occurred in several sediment layers: Guaymas clone A1 R010 was found four times in the 0- to 1-cm layer of core A and two times in the 1- to 2-cm layer of core A. Guaymas clone A2 R045 was found 15 times in the 0- to 1-cm layer of core A and 2 times in the 1- to 2-cm layer of core A. Guaymas clone T R022 was found one time in the 0- to 1-cm layer of core C and one time in core T.

**Rarely retrieved archaeal clones.** In addition to the ANME groups, diverse archaeal lineages and relatives of hyperthermophilic cultured archaea were found in the Guaymas sediments, each represented by a few or single clones. Four clones were closely related to the hyperthermophilic, sulfur-reducing thermococcales and the sulfate- and metal-reducing archaeoglobales that have both been cultured previously from Guaymas sediments (10, 39). Two crenarchaeotal clones were related to uncultured hyperthermophilic lineages. Clone TR021 was affiliated to clone pSL17 from the hot, sulfidic spring Obsidian Pool in Yellowstone Park (3). A single clone (C1R025) was a member of the *Korarchaeota*; its closest, nearly identical relative was clone pOWA19 from a shallow marine hydrothermal vent in Japan (78). The temperatures observed in the studied sediments are below those optimal for hyperthermophiles, which explains the rare occurrences of hyperthermophile-related clones.

Five core C clones, four of them from supernatant seawater, belonged to the pelagic marine crenarchaeal group I that includes *Cenarchaeum symbiosum*. In this tree, they are represented by clones C1R001 and CSR008 (Fig. 2). These archaea most likely have their origin in the water column, their natural habitat. Other archaea occur in a wide variety of sedimentary habitats that includes vent sites but is not limited to them. Core C harbored members (clones C1R043 and C1R046) of euryarchaeotal lineages that were originally described in a study of deep Atlantic nonhydrothermal sediment, namely, the marine benthic groups B and D (84).

The core A clone A2B037, which was obtained with bacterial primers, and the core T clones TR012 and TR040 formed a phylogenetic lineage among the *Euryarchaeota* that also included the euryarchaeotal clone WCHA1-57 from the methanogenic zone of an oil- and chlorinated solvent-contaminated aquifer (20). The natural petroleum deposits at Guaymas and the aquifer with anthropogenic pollution both harbor members of this euryarchaeotal lineage that at present cannot be subsumed under any other cultured or uncultured archaeal lineage.

**Bacterial 16S rRNA gene sequences.** The bacterial clone libraries obtained are phylogenetically highly diversified and include numerous cultured as well as uncultured bacterial lineages (Fig. 3 and 4). In contrast to the archaeal sequences, no single phylogenetic group of bacteria dominated the clone collection. Instead, the four most common bacterial phylotypes that occurred in both cores A and C included members of the epsilon and delta *Proteobacteria*, the green nonsulfur bacterial phylum, and the candidate division OP11 (Fig. 3 and 4).

Delta-proteobacterial clones constituted 13% of the bacterial clone libraries of cores A and C (eight and nine clones, respectively); they included members of the propionate-oxidizing family *Desulfobulbaceae*, members of *Myxobacteria*, several deeply branching delta-*Proteobacteria*, and a member of the sulfur-reducing genus *Desulfuromusa* (Fig. 3). Representatives of the Eel-2 group, an uncultured group of delta-*Proteobacteria* from Eel River methane seep sediments (59), were also found. In the Eel River as well as the Guaymas Basin sediments, these Eel-2 group delta-proteobacterial clones came from samples that yielded predominantly ANME-1 archaeal clones.

Epsilon-proteobacterial clones constituted the largest segment (28 to 31%) of the bacterial clone library (22 clones in

core A and 15 clones in core C). Most epsilon-proteobacterial clones branched with the epsilon 1 group of uncultured epsilon-*Proteobacteria* (1) from a wide variety of hydrothermal and nonhydrothermal deep-sea sediments (clones NKB8, -9, -10, and -12 in cold seeps of the Nankai Trough [47]; Japanese deep-sea clones BD1-4, BD 2-1, BD2-5, and BD7-6 [48]; and cold seep deep-sea clone JTB315 [49]) and with sulfide-oxidizing exosymbionts of the vent shrimp *Rimicaris exoculata* (65) and the hydrothermal vent annelid *Alvinella pompejana* (12). A phylogenetically distinct group of epsilon-*Proteobacteria* (epsilon group II) (1) contained four Guaymas clones, the nitrate-reducing sulfur oxidizer *Thiomicrospira denitrificans*, and several clones from Pele's vents near Hawaii (55).

The green nonsulfur bacterial phylum accounted for 6 to 14% of the clones in cores A and C. This bacterial phylum has been named for its anaerobic and thermophilic cultured representatives, the green nonsulfur phototrophic bacteria from terrestrial hot springs, such as *Chloroflexus aurantiacus* and *Herpetosiphon geysericola*. The Guaymas clones branched among the uncultivated members of this phylum, clones from Obsidian Pool (38), from subsurface palaeosol (15), from a methanogenic aquifer contaminated with jet fuel waste and aromatic and chlorinated hydrocarbons (20), and from enrichments growing on chlorinated aromatic compounds (89).

Members of the uncultured candidate division OP11 and OP11-affiliated lineages constituted 9% of the clones from cores A and C. The OP11 division was found originally in Obsidian Pool (38), in the methanogenic zone of a jet fuel-contaminated aquifer (20), and in cold deep-sea sediments (48); cultured representatives are not known at present.

Not all bacterial phyla were equally represented in the clone libraries of cores A and C. In particular, core C harbored two specific bacterial populations that were entirely missing from the core A clone library, gamma-*Proteobacteria* (22%) and members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum (9%). Most gamma-proteobacterial clones were related to sulfur-oxidizing bacterial endosymbionts of bivalves at hydrothermal vents and sulfidic mud flats (13). The clones of the CFB phylum, ubiquitous degraders of carbohydrate polymers in the marine environment, were mostly obtained from the supernatant water portion of core C, consistent with their aerobic physiology and their abundance in the marine water column (18).

Core A yielded several clones that belonged to a deeply branching bacterial phylotype (13%), which could not be subsumed under another bacterial group. Depending on the method of phylogenetic analysis, a marginal affiliation with the thermophilic sulfate reducers of the genus *Thermodesulfobacterium* (uncorrected sequence similarity to *Thermodesulfobacterium*, ca. 85%) was obtained, but a sister-group relationship, suggested by preliminary ARB parsimony trees, could not be substantiated by bootstrap. No related sequences were available in GenBank. So far, this cluster of sequences has been obtained from Guaymas sediments only (Fig. 4).

Clones affiliated to other bacterial lineages were retrieved in small members, including members of the *Verrucomicrobium* lineage and *Planctomycetales*, the high-G+C gram-positive bacteria, the *Chloroplasts*, and the uncultured OP5, OP3, and OP1 candidate divisions. Four clones from core A and one clone from core C fall into the OP8 candidate subdivision;



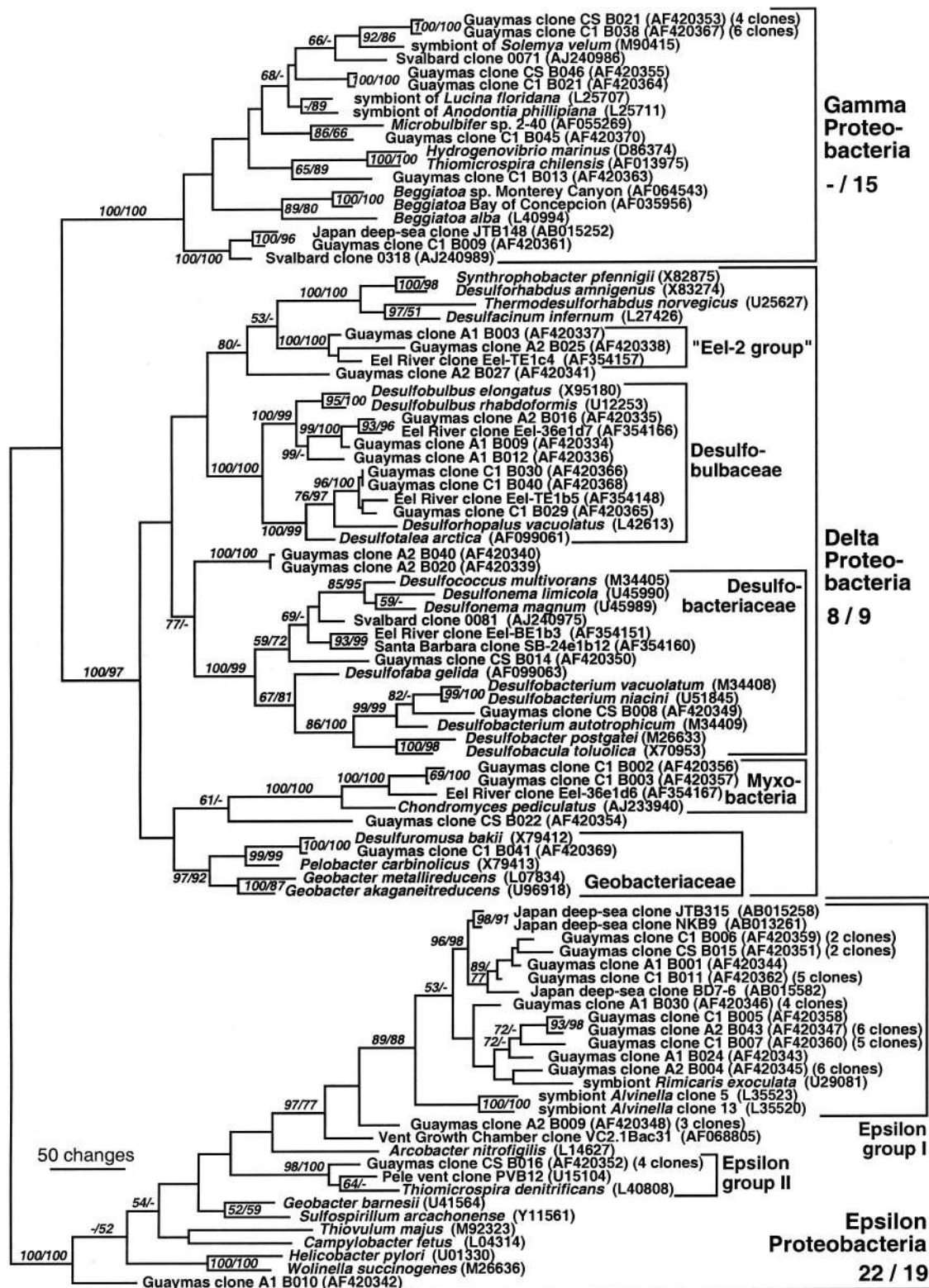


FIG. 3. Distance tree of proteobacterial clones from Guaymas sediment cores A and C, based on near-complete 16S rDNA sequences. Bootstrap values (in percent) are based on 1,000 replicates each (distance and parsimony) and are shown for branches with more than 50% bootstrap support. For the major phylogenetic divisions, the numbers of core A and core C clones are given in that order. For multiple near-identical clones (>99% identity), the number of multiples is given after the GenBank number of a representative clone. Some sequence types occurred in several sediment layers: Guaymas clone A1 B030 was found three times in the 0- to 1-cm layer of core A and one time in the 1- to 2-cm layer of core A, Guaymas clone A2 B043 was found one time in the 0- to 1-cm layer of core A and five times in the 1- to 2-cm layer of core A, and Guaymas clone A2 B004 was found three times in the 0- to 1-cm layer of core A and three times in the 1- to 2-cm layer of core A.

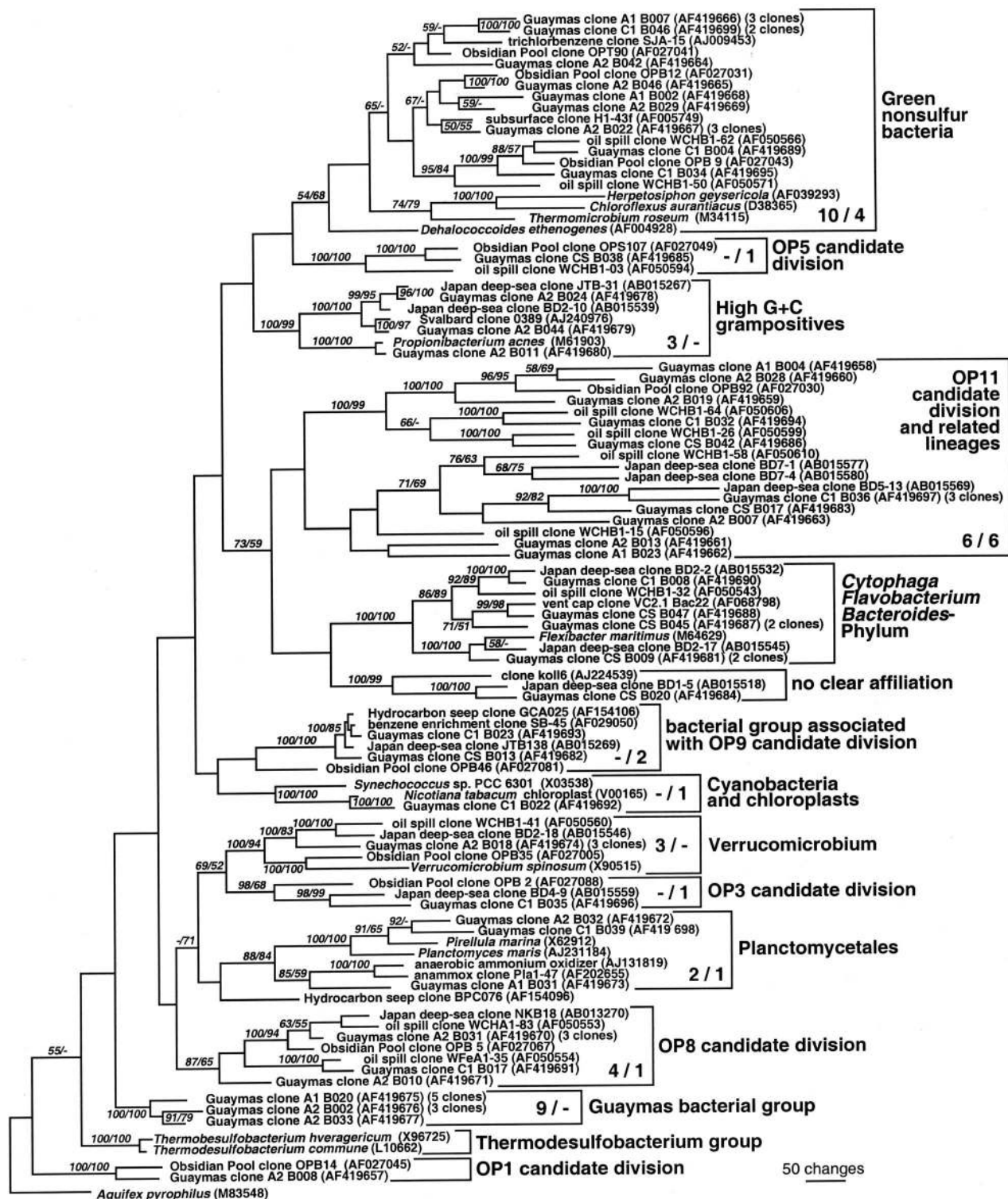


FIG. 4. Distance tree of bacterial clones, excluding proteobacteria, from Guaymas sediment cores A and C, based on near-complete 16S rDNA sequences. Bootstrap values (in percent) are based on 1,000 replicates each (distance and parsimony) and are shown for branches with more than 50% bootstrap support. For the major phylogenetic divisions, the numbers of core A and core C clones are given in that order. For multiple near-identical clones (>99% identity), the number of multiples is given after the GenBank number of a representative clone. Some sequence types occurred in several sediment layers: Guaymas clone A1 B007 was found two times in the 0- to 1-cm layer and one time in the 1- to 2-cm layer of core A, Guaymas clone A2 B031 was found two times in the 0- to 1-cm layer and one time in the 1- to 2-cm layer of core A, Guaymas clone A1 B020 was found three times in the 0- to 1-cm layer and two times in the 1- to 2-cm layer of core A, and Guaymas clone A2 B002 was found one time in the 0- to 1-cm layer and two times in the 1- to 2-cm layer of core A.



their closest relatives included clones from deep-sea cold seep sediments (the Nankai Trough clone NKB18 [47]), Obsidian Pool (38), and the jet-fuel-contaminated aquifer (20).

Two clones from core C formed a well-supported cluster with clones SB-45 (included here) and SB-15, both from a benzene-mineralizing, sulfate-reducing mixed culture (64); with clones JTB138 (included here) and JTB243 from a deep-sea cold seep area in the Japan Trench (49); and with clone GCA025 (58) from hydrocarbon seep sediment. Minimum evolution trees suggested an affiliation of this group with a representative clone of the candidate division OP9, clone OPB46, but this grouping remains so far unsupported by bootstrap analysis (Fig. 4).

## DISCUSSION

**Prokaryotic lipids and their isotopic composition as evidence for anaerobic methanotrophy.** Stable carbon isotopic signatures of diagnostic prokaryotic membrane lipid products indicate the presence and activity of anaerobic methanotrophic consortia in hydrothermally active Guaymas sediments. Moreover, the high abundance of prokaryotic biomarkers with distinctly low carbon isotopic compositions (Table 1) suggests that methanotrophy is an important mode of microbial carbon assimilation in these sediments. It has to be noted that these biomarkers likely are degradation products of their biologically produced polar lipid counterparts that constitute the cell membrane. Although in the strictest sense these lipid biomarkers are presumed to be more stable than the 16S rRNA SSU sequence biomarkers to which they were compared, a series of studies suggests that these and other isotopically depleted archaeal lipids are particularly abundant in environments that host active anaerobic methanotrophic communities (7, 31, 32, 33, 59, 60, 61, 72, 80). Complementary to the lipid biomarker isotopic signatures, 16S rRNA sequences have identified the uncultured archaeal ANME-1 and ANME-2 lineages in the Guaymas sediments that are characteristic for methane-oxidizing microbial communities; the sequence data sets are broadly congruent with the biomarker evidence. However, substantial differences in both the distribution and isotopic compositions of prokaryotic lipids and in the composition of 16S rRNA clone libraries were observed between the cores.

The membrane lipid products of the Guaymas Basin are derived from present and active microbial populations, for several reasons. In surface sediments of Guaymas Basin, the concentration of the analyzed glycerol ethers diminished rapidly; in core T, their concentration was near the detection limit below a depth of 2.5 cm. Unresolved organic compounds that could not be resolved by gas chromatography predominated in deeper sediment layers. Sedimentary conditions do not favor long-term burial for neutral lipids; they are limited to the surface layers and thus show the same vertical distribution as phospholipid ester-linked fatty acids that occur only in the upper few centimeters of the Guaymas sediments (29). These observations are consistent with very high microbial activity in these sediment surface layers (5, 86a) and with the complete oxidation of fatty acids that has been demonstrated for pure cultures of sulfate-reducing bacteria from the Guaymas Basin (69). Therefore, it is unlikely that the neutral lipids in the Guaymas sediment surface layer reflect fossil remnants of past

microbial activity. The alternative, import of lipids from the aerobic water column, is also extremely unlikely. Producers of archaeol and hydroxyarchaeol commonly inhabit anoxic sediments or anoxic, methane-rich water columns in anoxic basins such as the Black Sea (72). Compounds related to the bicyclic biphytandiol in Guaymas occur in oceanic water columns and sediments, but they have isotopic compositions around  $-20\%$  (71) and are derived from cosmopolitan, cold-water planktonic archaea (19, 34).

The occurrence of isotopically highly fractionated archaeol and *sn*-2-hydroxyarchaeol in the Guaymas sediments is strong evidence for anaerobic methane oxidation by archaea. The isotopic composition of these archaeal compounds in the Guaymas sediments is compatible with an incorporation of carbon derived from isotopically depleted methane. Archaeol and *sn*-2-hydroxyarchaeol have been found in a variety of methane-rich, sedimentary environments with isotopic compositions from  $-133$  to  $-41\%$  (33). Such low isotopic compositions indicate a partial to exclusive biosynthesis from methane-derived carbon (31, 33, 59, 61, 79). The relatively broad range of isotopic compositions is related to differences in isotopic composition of methane and presumably to other poorly constrained environmental and physiological factors (33). Another biomarker of the Guaymas sediments that is conspicuously tied to anaerobic methane oxidation is a biphytandiol with two pentacyclic rings with an isotopic signature similar to those of archaeol and *sn*-2-hydroxyarchaeol (Table 1). This and other biphytanyl derivatives were originally only found in cultured, extremely thermophilic crenarchaeota but more recently have also been identified in planktonic coldwater archaea of the marine water column (34), specifically in group I cold-water crenarchaeota related to *Crenarchaeum symbiosum* (19). Also, biphytanyl derivatives occur in sediments at methane seeps with isotopic signatures indicative of methane assimilation (61, 71). Notably, the biphytanyl derivative is absent in the ANME-2-dominated core C.

**The ANME-1 archaea as candidate for anaerobic methane oxidation.** The molecular, isotopic, and phylogenetic evidence in this study suggests that ANME-1 archaea are a source of these  $^{13}\text{C}$ -depleted lipids, as implied by earlier observations of ANME-1 archaea at sites of active anaerobic methanotrophy (31, 79). Specifically, the Guaymas data set argues for an active role of ANME-1 archaea in anaerobic methane oxidation, since the ANME-I clones were obtained from Guaymas sediment samples that consistently showed archaeal lipid signatures for methane oxidation. Cores A and T have yielded exclusively ANME-1 clones, combined with abundant,  $^{13}\text{C}$ -depleted *sn*-2-hydroxyarchaeol, archaeol, and biphytandiol.

An important argument for the involvement of ANME-1 archaea in anaerobic methane oxidation is their consistent occurrence pattern in methane-rich sediments and cold methane seeps. These sites include methane seeps in Eel River Basin (31), Santa Barbara Basin (59), the methane oxidation zone in near-shore sediments in Aarhus Bay, Denmark (80), and hydrothermal sediments in the Iheya Basin in the Okinawa Trough, Japan (77). Recent observations by Thomsen et al. (80) lend additional support for the involvement of ANME-1 archaea in anaerobic methanotrophy, showing a predominance of ANME-1 archaea in the sulfate reduction-methanogenesis transition layer together with unusual dissimilatory sulfite re-

ductase genes. These findings are consistent with a coupling of anaerobic methanotrophy with sulfate reduction (35, 82).

In the Guaymas samples, ANME-1 archaea were found associated with  $^{13}\text{C}$ -depleted cyclic biphytanyldiol biomarkers. The cyclic,  $^{13}\text{C}$ -depleted biphytanediol, commonly considered characteristic for cold-water crenarchaeota (e.g., see reference 71), occurred only in cores A and T, which yielded ANME-1 clones, whereas it was absent in the ANME-2-dominated core C. The association of biphytanediol derivatives with ANME-1 archaea could explain previous observations of  $^{13}\text{C}$ -depleted, cyclic  $\text{C}_{40}$  biphytanyl derivatives in Mediterranean cold seep sediments (61) (no phylogenetic data shown) and in Black Sea sediments (79) but not in others such as ANME-2 dominated sediments at methane seeps in Santa Barbara Basin and Eel River Basin (32, 59). Biphytanyl derivatives found at those seep sites have isotopic compositions ( $\sim -20\text{‰}$ ) indicative of an origin from archaeal plankton. The sequence data and the temperature range of the surface samples do not suggest a high abundance of hyperthermophilic crenarchaeota, a possible alternative source of cyclic biphytanedols, in the Guaymas sediment surface samples. A precise quantification of both ANME groups in environmental samples would clarify the question of whether either ANME group could be solely responsible for anaerobic methane oxidation when the other group is absent.

**ANME-2 archaea in Guaymas.** Previous studies have left no doubt that archaea affiliated with the ANME-2 lineage, together with sulfate-reducing bacteria affiliated with the *Desulfosarcinales*, oxidize methane in several sedimentary environments (7, 59, 60). Sequences of the ANME-2 group were always found in sampling sites that either showed anaerobic methane oxidation or were compatible with this process, such as gas hydrates in the Gulf of Mexico (46) and at cold methane seeps in the Eel River Basin (the Eel River clones BA2H11 and TA1a4 [31] and other close affiliates [59]) and in salt marsh sediments in England (clones 2MT7 and 2C83) (56), sediments of the Santa Barbara Basin (59), and methane hydrate sediments at the Cascadia Margin (7).

With fluorescent in situ hybridization (FISH) of methane-laden sediments, it was shown that ANME-2 archaea occurred in syntrophic association with sulfate-reducing bacteria of the *Desulfosarcina* lineage (7, 59). The stable carbon isotopic signatures of these syntrophic bacterial-archaeal associations, revealed by a new combination of FISH and secondary ion mass spectrometry, clearly indicated isotopically depleted methane as the carbon substrate of these archaea and their associated sulfate reducers (60). In the Guaymas samples, ANME-2 clones have been obtained only from core C, together with highly  $^{13}\text{C}$ -depleted archaeol and *sn*-2-hydroxyarchaeol (Table 1). Interestingly, only clones of the ANME-2c subgroup have been obtained from Guaymas; clones of the ANME-2a and 2b subgroups are missing. The existing phylogenetic diversity among ANME-2 lineages suggests some degree of physiological and biochemical divergence, similar to the divergence among the cultured lineages within members of the *Methanosarcinales* lineage that contain acetoclastic and methyl-disproportionating lineages (8). Ecophysiological differences and distinct occurrence patterns can set the different ANME-2 subgroups apart from each other; a particular habitat or methane-oxidizing microbial community may harbor only one of the ANME-2 groups but not all of them simultaneously.

**Diverse archaea at sites of anaerobic methanotrophy.** Many archaea exist in the clone libraries with no evidence for their involvement in anaerobic methanotrophy. Group B and D benthic archaea were found in the Guaymas sediments; they are widespread in methane-oxidizing sediments and vents but are not limited to these habitats. Group D benthic archaea, a sister group of the *Thermoplasmatales*, include Japanese hydrothermal vent clones pMC2A203 and pMC2A33, from a black smoker chimney at the Myojin Knoll on the Izu-Ogasawara arc (78); the Eel River methane seep clones TA1f2, TA1e6, and TA1c9 (31); and clones ANME1, ANME7, ANME17, ANME19, and ANME23 from Baltic sediments (80). Orphan and co-workers noted the wide environmental distribution of this group, the "*Thermoplasmatales* relatives," in nonhydrothermal marine sediments as well as methane seeps (59). Similarly, archaea of the marine benthic group B have been found at Japanese hydrothermal vents (clone pMC2A36) (77), in hydrothermal vent in situ growth chambers at the Mid-Atlantic Ridge (clone VC2.1Arc31) (68), in sediments of the Santa Barbara Basin (clone sbpc17-1A) (59), and in near-shore and deep Atlantic sediments (83, 84).

**Sulfate-reducing bacteria involved in anaerobic methanotrophy.** The bacterial isotopic signatures of the Guaymas sediment samples strongly argue for an involvement of bacteria in anaerobic methane oxidation. Isotopically light,  $^{13}\text{C}$ -depleted bacterial MAGE and DAGE lipids with a presumed origin from unknown methane-consuming bacterial syntrophs (32) cooccurred with ANME-1 archaeal clones in cores A and T (Table 1), in contrast to the Eel River sediments, where they mostly coincided with ANME-2 clones (59). These compounds had been observed earlier in sediments at methane seeps in the Eel River and Santa Barbara basins (32, 59) and at mud volcanoes in the Mediterranean Sea (61), where they have been interpreted as being associated with bacterial syntrophic partners of methanotrophic archaea. Their  $\delta\text{-}^{13}\text{C}$  signature of mostly  $-50$  to  $-35\text{‰}$  is intermediate between the isotopic signatures of archaeal methane-derived biomarkers and eukaryotic photosynthetic biomasses, suggesting that these bacteria assimilate carbon of mixed origin. In particular, the dialkyl ethers are not known from proteobacterial sulfate-reducing bacteria, which have been previously identified as syntrophic symbionts within methane-oxidizing consortia at cold gas hydrate sites (7). The monoalkyl ethers could have a more general bacterial source as products of the relatively widespread alkyl-acyl glycerol derivatives with mixed ether- and ester-linked alkyl moieties (27). Notably, a simple distribution of monoalkyl ethers with  $\text{C}_{16}$  and  $\text{C}_{18}$  alkyl moieties has been observed recently in members of *Desulfosarcinales* (<http://docserver.bis.uni-oldenburg.de/publikationen/dissertation/2001/ruetra01/ruetra01.html>) (70).

As long as bacteria are capable of syntrophic interaction with methane-oxidizing archaea and of consuming reaction products of anaerobic methane oxidation, syntrophic candidates are not necessarily limited to classical delta-proteobacterial sulfate-reducing bacteria. The highly diverse members of the uncultured bacterial phyla that were detected in cores A and C could include alternative sulfate-reducing bacteria that could fill this niche. The bacterial sequence profiles in samples from the Guaymas Basin show some broad overlap with those of other methane-rich sediments and seeps. A comparison of

different methane-rich sediments and seeps has shown that gamma-, delta-, and epsilon-*Proteobacteria*, members of the CFB phylum, members of the gram-positive, fermentative genus *Propionigenium*, and uncultured members of the OP9 candidate subdivision and of the green nonsulfur phylum were found in at least half of these environments (59). Since anaerobic methane oxidation is coupled to sulfate reduction (82), it is possible that members of uncultured bacterial lineages that cooccur with anaerobic methane-oxidizing archaea could be novel sulfate reducers. Since DAGE lipids have so far been found in phylogenetically deeply branching bacteria, the new, deeply branching bacterial phylotype in core A could represent a candidate group (represented by clones A1B020, A2B002, and A2B033) (Fig. 4). This phylotype is present in core A but absent in core C and thus matches the distribution of the  $^{13}\text{C}$ -depleted bacterial DAGE lipids.

Interestingly, an autonomous character for some methanotrophic archaea—at least under some specific conditions—cannot be excluded. Clumps of ANME-2 archaea have been found repeatedly by FISH analysis which appear not to form consortia with any bacterial syntrophs (60). A systematic survey of ANME archaea and their syntrophs in natural environments, optimally by FISH-secondary ion mass spectrometry, is needed to clarify which bacterial syntrophs are required for anaerobic methane oxidation and to what extents (V. J. Orphan, C. H. House, K.-U. Hinrichs, K. D. McKeegan, C. Paull, W. Ussler, and E. F. DeLong, Eos. Trans. AGU Fall Meet. Suppl. 82[47], abstr. B12B-0127, 2001).

Although the question of their role as methane-oxidizing syntrophs remains open, sulfate-reducing bacteria are highly active in the Guaymas sediments. The sequencing survey identified mesophilic sulfate-reducing bacteria (mostly members of the mesophilic *Desulfobulbaceae*) as well as hyperthermophilic sulfate-reducing archaea (members of *Archaeoglobales*) in the Guaymas surface sediments (Fig. 2 and 3). Sulfate reduction rate measurements in Guaymas sediments have consistently shown high activities for mesophilic, thermophilic, and hyperthermophilic sulfate reducers (22, 41, 42, 86a). Cores taken in the vicinity of core A on the same dive (no. 3203) showed high sulfate reduction maxima of 300 to 2,000  $\text{nmol cm}^{-3} \text{ day}^{-1}$  for the temperature range of 12 to 35°C near the sediment surface, with the highest rate invariably occurring in the uppermost-centimeter layer (86a). Also, thermophilic sulfate-reducing activity at 70 and 80°C peaked in this layer, with a second peak for 70°C between the 5- and 10-cm depths (86a). Carbon substrates for sulfate reduction are available in the Guaymas sediments. Propionate, the signature substrate of the *Desulfobulbaceae*, and acetate, used by many sulfate-reducing bacteria, including members of the *Archaeoglobales* (88), are abundant in the Guaymas sediments. Their concentrations decrease towards the sediment surface, where sulfate-reducing bacteria are most active (52).

**Bacterial diversity in the Guaymas sediments.** Numerous bacterial lineages in the Guaymas sediments were not linked to anaerobic methane oxidation. Several phylogenetic groups have been typically recovered from sulfidic, oxic-anoxic interfaces. The epsilon-*Proteobacteria* of epsilon group I (1) have been found in a wide variety of microoxic or anoxic aquatic habitats, including a sulfidic cave river with white filamentous microbial mats (1), the anoxic portion of the chemocline of the

Cariaco Basin (50), and anoxic or microoxic deltaic muds in the Gulf of Papua (81). Analogous to the epsilon-*Proteobacteria*, gamma-proteobacterial clones in Guaymas that are related to sulfur-oxidizing bivalve symbionts (13) could be involved in sulfur oxidation.

Clones of the green nonsulfur bacterial phylum in Guaymas Basin have their closest relatives in geothermal springs, subsurface soil, petroleum hydrocarbon-rich sites, and enrichment cultures on aromatic hydrocarbons (15, 20, 38, 89). The only cultured representative of the nonphotosynthetic members of the green nonsulfur phylum, the anaerobic dechlorinating bacterium "*Dehalococcoides dehalogenes*," can grow by oxidation of hydrogen and concomitant dechlorination of tetrachloroethene to ethene; mixed microbial extracts are also required for growth (53). These habitat preferences of nonphotosynthetic green-nonsulfur bacteria are compatible with the geochemical features of the Guaymas hydrothermal sediments. In this natural reactor, organic biomass undergoes pyrolysis and thermal alteration to a wide variety of petroleum hydrocarbons, including unbranched alkanes, cycloalkanes, triterpanes, steranes and diasteranes, and aromatic hydrocarbons (4).

Similar to those of the green nonsulfur bacteria, the habitat characteristics of members of the OP11 candidate division (20, 38, 48) suggest an anaerobic phenotype, with thermophilic as well as nonthermophilic representatives. OP11 bacteria also occur in cold, nonhydrothermal deep-sea sediments without obvious petroleum hydrocarbon contamination, where green nonsulfur bacteria have not been found (for example, clones BD2-3, BD5-13, BD7-1 and BD7-4) (48). No cultured representative of this group is known at present.

Like the clones of the nonphotosynthetic green nonsulfur bacteria and OP11 candidate division, members of the OP8 cluster and the OP9-affiliated clusters that occurred at Guaymas were generally found at cold seeps and in hydrocarbon-rich environments and enrichments (49, 58, 64). Although their environmental distribution suggests an anaerobic metabolism and involvement in degradation of aromatic hydrocarbons and petroleum compounds, the physiology and environmental activity of these diverse bacterial groups remain to be determined by pure culture studies.

**Alternative causes of carbon isotopic depletions.** The question arises whether low  $\delta$ - $^{13}\text{C}$  values in prokaryotic lipids could also be produced by other pathways than methane oxidation, for example, by autotrophic archaeal methanogenesis.  $\delta$ - $^{13}\text{C}$  values in the Guaymas sediments ranged from  $-13.3$  to  $-8.5\text{‰}$  for  $\text{CO}_2$  and  $-14$  to  $-9.6\text{‰}$  for calcite (63). Data on  $\Delta\delta$ - $^{13}\text{C}$  between carbon source and biomass exist for a few autotrophic methanogenic species in pure culture and allow estimation of projected  $\delta$ - $^{13}\text{C}$  values of lipids in the Guaymas environment. The biomass of the obligate autotroph *Methanobacterium thermoautotrophicum* was depleted in  $^{13}\text{C}$  relative to  $\text{CO}_2$  by 24‰ (24). The  $\Delta\delta$ - $^{13}\text{C}$  towards lipids should be higher, as suggested by results with the facultative autotroph *Methanosarcina barkeri*. Here, cell biomass was depleted by ca. 20‰ after growth on trimethylamine, whereas lipid phytanyl chains showed a larger  $\Delta\delta$ - $^{13}\text{C}$  of  $-37\text{‰}$  (75). Assuming a  $\Delta\delta$ - $^{13}\text{C}$  of  $-40\text{‰}$  between Guaymas  $\text{CO}_2$  and lipid phytanyl chains of autotrophic methanogens, projected  $\delta$ - $^{13}\text{C}$  values for lipids in the sediment would reach  $-50$  to  $-55\text{‰}$ . Actual minimum values in archaeal lipids, however, reach  $-89\text{‰}$



(Table 1). For other significant chemolithoautotrophic CO<sub>2</sub> assimilation pathways, namely, the Calvin cycle and the reverse citric acid cycle, the  $\Delta\delta^{13}\text{C}$  values between CO<sub>2</sub> and cell biomass amount to  $-25\text{‰}$  and  $-8$  to  $-12\text{‰}$ , respectively, leading to smaller fractionation effects (25). As in other environments where archaeal lipid  $\delta^{13}\text{C}$  values reach minima of  $\sim 130\text{‰}$  (33), it is inconceivable those in the Guaymas Basin are derived from autotrophic methanogens or other autotrophic archaea. The relatively high (in comparison to other sites)  $\delta$  values of the archaeal lipids likely result from the relatively high  $\delta$  value of the substrate methane in Guaymas Basin sediments (87).

**Anaerobic methane oxidation and trophic ecology of vent sites.** Anaerobic oxidation of methane provides a notable source of energy and biomass at hydrothermal vents and cold hydrocarbon seeps, in addition to the better known oxidation of sulfide by free-living and symbiotic bacteria and the aerobic oxidation of methane by symbiotic bacteria of mussels and pogonophorans (9). Anaerobic oxidation of methane is a significant process in coastal marine sediments and in particular at cold methane seeps (33). Methane seeps that harbored conspicuous populations of vent animals in the Oregon subduction zone showed anaerobic oxidation of methane (44); these findings implied that anaerobic methane oxidation should be considered as part of the trophic ecology of vent ecosystems. Based on results at a cold methane seep with dense giant clam communities in Sagami Bay, Japan, Muzusawa and coworkers (54) suggested that a mixture of methane of biogenic origin ( $\delta^{13}\text{C} = -85$  to  $-60\text{‰}$ ) and thermogenic origin ( $\delta^{13}\text{C} = -50$  to  $-35\text{‰}$ ) is oxidized in the sediment column beneath the cold seep clam community (*Calyptogena soyoeae*). The resulting dissolved inorganic carbon of the pore water with an isotopic composition of  $-45$  to  $-40\text{‰}$  is incorporated into the soft tissue of the symbiont-harboring clams via sulfur-oxidizing bacterial symbionts. The clam tissue has a  $\delta^{13}\text{C}$  value of around  $-35\text{‰}$  (54). This  $^{13}\text{C}$  depletion is at the upper margin of isotopic signatures in animals with sulfur-oxidizing bacterial symbionts (including calyptogena) that fix carbon via the Calvin-Benson-Bassham cycle, also named the  $-30\text{‰}$  group for the typical isotopic signature (16). A comparison of  $\delta^{13}\text{C}$  values in bivalve mollusks from different sites showed generally lighter  $^{13}\text{C}$  signatures ( $-40$  to  $-35\text{‰}$ ) for vent bivalves at methane-rich cold seeps and hydrocarbon seeps than for bivalves from sulfide-dominated hydrothermal vent sites (23), suggesting a contribution of sedimentary methane oxidation to the CO<sub>2</sub> pool that is subsequently incorporated into bivalve biomass via their autotrophic, sulfur-oxidizing symbionts. Aerobic, methylotrophic symbionts of mytilid clams and pogonophorans produce distinct, significantly lighter isotopic signatures in the tissue of their host animals, in the range of approximately  $-80\text{‰}$  to  $-40\text{‰}$  (14). Interestingly, contribution of CO<sub>2</sub> from methane oxidation has been observed in sulfide-oxidizing, autotrophic *Beggiatoa* spp. from methane seeps, which showed unusually low isotopic signatures (approximately  $-50\text{‰}$ ) in their biomass (Orphan et al., Eos. Trans. AGU Fall Meet. Suppl. 82[47], 2001).

The highly diverse bacterial and archaeal communities of the Guaymas basin sediments suggest a wide diversity of microbial physiologies and an untapped potential for novel microorganisms. The uncultured OP subdivision bacteria, the

green nonsulfur bacteria, several proteobacterial lineages, and the ANME archaea lineages in Guaymas have evolutionary cousins in natural and anthropogenic hydrocarbon-rich sites, methane seeps and sediments, geothermal environments, and sulfide gradients. The Guaymas hydrothermal vent sites combine features of these habitats, which is reflected in their complex, multifaceted microbial communities. The enrichment and isolation of microbial key species, and the concomitant analysis of large genomic fragments from environmental DNA (6), will lead towards a better understanding of metabolic potential and activities of these microbial ecosystems.

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