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Methanogen Diversity Evidenced by Molecular Characterization of Methyl Coenzyme M Reductase A (*mcrA*) Genes in Hydrothermal Sediments of the Guaymas Basin

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The methanogenic community in hydrothermally active sediments of Guaymas Basin (Gulf of California, Mexico) was analyzed by PCR amplification, cloning, and sequencing of methyl coenzyme M reductase (*mcrA*) and 16S rRNA genes. Members of the *Methanomicrobiales* and *Methanosarcinales* dominated the *mcrA* and 16S rRNA clone libraries from the upper 15 cm of the sediments. Within the H_2/CO_2- and formate-utilizing family *Methanomicrobiales*, two *mcrA* and 16S rRNA lineages were closely affiliated with cultured species of the genera *Methanoculleus* and *Methanocorpusculum*. The most frequently recovered *mcrA* PCR amplicons within the *Methanomicrobiales* did not branch with any cultured genera. Within the nutritionally versatile family *Methanosarcinales*, one 16S rRNA amplicon and most of the *mcrA* PCR amplicons were affiliated with the obligately acetate utilizing species *Methanosaeta concilii*. The *mcrA* clone libraries also included phylotypes related to the methyl-disproportionating genus *Methanococcoides*. However, two *mcrA* and two 16S rRNA lineages within the *Methanosarcinales* were unrelated to any cultured genus. Overall, the clone libraries indicate a diversified methanogen community that uses H_2/CO_2, formate, acetate, and methylated substrates. Phylogenetic affiliations of *mcrA* and 16S rRNA clones with thermophilic and nonthermophilic cultured isolates indicate a mixed mesophilic and thermophilic methanogen community in the surficial Guaymas sediments.

The hydrothermally active sediments of the Guaymas Basin (Gulf of California, Mexico) have an active methane cycle, with two major sources and several potential sinks of methane. Diffuse venting through the Guaymas sediments at temperatures up to 200°C leads to pyrolysis of organic material in the organic-rich sediments (maximal total organic carbon concentration, 3 to 12% [wt/wt] near the surface [25]). Pyrolysis products include significant amounts of aliphatic and aromatic petroleum hydrocarbons, short-chain fatty acids, ammonia, and methane (2, 30, 57). The methane content in the Guaymas hydrothermal fluids ranges from 12 to 16 mM (270 to 370 ml kg^{-1} at standard temperature and pressure), which is approximately 2 orders of magnitude higher than those of most bare lava vent sites (57). The carbon isotopic composition ($\delta^{13}C$) of Guaymas methane ranges from −51 to −41‰, suggesting a predominantly thermocatalytic origin from sedimentary organic material (37, 57).

Diverse communities of methanogenic archaea produce methane in the Guaymas sediments. Several hyperthermophilic methanogens, including members of the genus *Methanococcus* (recently renamed *Methanocaldococcus*) and the most thermophilic methanogen identified to date, *Methanopyrus kandleri*, have been isolated from the Guaymas sediments (19, 21, 22, 23, 24). Based on initial 16S rRNA gene surveys, the methanogen communities of Guaymas also include relatives of mesophilic species and genera within the families *Methanomicrobiales* and *Methanosarcinales* (50). In some localities near the sediment surface, abundant methanotrophic archaeal communities reoxidize methane and incorporate it into their cellular biomass. Their taxonomically informative membrane lipids assume the characteristic, highly ^{13}C depleted carbon isotopic signature that combines the source methane signal and the isotopic fractionation of the methanotrophic organisms (50). With methanogenic as well as methane-oxidizing communities, the Guaymas Basin sediments provide a model system for studying anaerobic methane cycling, with applicability to diverse hydrothermal and nonhydrothermal marine sediments and subsurface environments (51).

To expand our understanding of the methanogenic communities of the Guaymas Basin, we carried out molecular surveys using 16S rRNA gene sequencing in combination with sequencing a key gene of methanogenesis, methyl coenzyme M reductase. The holoenzyme consists of two alpha (*mcrA*), two beta (*mcrB*), and two gamma (*mcrG*) subunits. It catalyzes heterodisulfide formation between methyl coenzyme M and coenzyme B, with the subsequent release of methane (13). Methyl coenzyme M reductase is a diagnostic indicator of methanogenesis and methanogenic archaea (14, 52). There are no reports of lateral gene transfer for methyl coenzyme M reductase from anaerobic to aerobic methylotrophic bacteria that have appropriated C_1 transfer enzymes and coenzymes of methanogenic pathways (7). The subunits of the methyl coenzyme M reductase holoenzyme are phylogenetically conserved and informative (18). Because the phylogeny of the alpha subunit closely parallels the 16S rRNA phylogeny of methanogens,

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mcrA provides a target for PCR-based molecular analyses of methanogenic communities in different environmental settings (17, 27, 28, 46). The methyl coenzyme M reductase operon and the *mcrA* gene are functionally linked and phylogenetically conserved in methanogenic and methanotrophic archaea; thus, PCR assays for the *mcrA* gene also detect the methane-oxidizing version of the methyl coenzyme M methyl reductase gene (18). This study focuses on methanogen diversity and novel lineages of *mcrA* genes in the methanogen community of the Guaymas Basin sediment samples that we previously characterized by sequence analyses of PCR amplicons for coding sequences for rRNAs and key enzymes of dissimilatory (*dsr*) sulfur metabolism (10, 11).

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 1998. Core B (*Alvin* dive 3205, 28 April 1998; diameter, 6 in; length, 25.3 cm) was obtained from the Everest Mound area in the Southern Guaymas vent field (27°00.888′N, 111°24.734′W). A second core from the same site was used for shipboard measurements of sulfate reduction rates under different temperature regimes (station 3 in reference 55). No *Beggiatoa* mats were present at the sampling sites. In situ temperature profiles of the sediment using *Alvin*'s thermoprobe were determined prior to coring. The seawater temperature at the sediment surface was 3°C; the sediment had a temperature of 16°C at a depth of 5 cm, 48 to 55°C at 10 cm, 93 to 96°C at 15 cm, 112 to 113°C at 20 cm, and 136 to 140°C at 25 cm. A similar temperature profile was measured half a meter away (16 to 26°C at 5 cm, 50 to 54°C at 10 cm, 71 to 78°C at 15 cm, 93°C at 20 cm, and 105 to 110°C at 25 cm). The cores were brought to the surface within 6 h of sampling and were sliced at 1-cm intervals within 6 h after retrieval. The upper 6 cm consisted of a black layer of liquid petroleum ooze; the ooze turned more viscous and less fluid below 7 cm. Gas bubbles appeared in the sediment at 7 cm depth. Multiple 2-ml portions of the sediments were taken from each centimeter layer and immediately frozen at −80°C for nucleic acid extraction.

Porewater concentration analyses. Porewater was obtained by spinning down approximately 1 to 2 ml of sediment samples and harvesting the supernatant (ca. 0.2 to 0.5 ml). Dissolved organic acids were analyzed by high-performance liquid chromatography as described previously (1); sulfate was analyzed using standard ion chromatographic techniques.

Nucleic acid extraction and *mcrA* and 16S rRNA gene amplification, cloning, and sequencing. Total genomic DNA was extracted from 30 mg of sediment using the UltraClean Soil DNA kit (MO BIO Labs. Inc., Solana Beach, CA). DNA was extracted from a total of eight sediment layers between 1 and 15 cm depth (1, 3, 5, 7, 9, 11, 13, and 15 cm). The archaeal primers 8F and 1492R (50) were used for the 16S rRNA PCRs. DNA samples of two adjacent sediment layers were pooled (depths of 1 and 3, 5 and 7, 9 and 11, and 13 and 15 cm) before PCR amplification of *mcrA* genes with primers *mcr* F and *mcr* R (46). Each PCR mixture for *mcrA* contained 5 μl of each primer solution (10 pmol/μl), 1 μl of DNA, 5 μl of 10× PCR buffer, 1 μl of 10× bovine serum albumin (10 mg/ml), 5 μl of 10× deoxynucleoside triphosphates (2 mM each dATP, dCTP, dGTP, and dTTP), and 0.3 μl of *Taq* DNA at 5 U/μl in a final reaction volume of 50 μl. Amplification was carried out in a gradient PCR cycler (Eppendorf). After initial denaturation for 2 min at 94°C, 30 cycles were performed, with each cycle consisting of 1 min at 94°C, 1 min at 49°C, and 3 min at 72°C. A final cycle of 1 min at 94°C, 3 min at 49°C, and 10 min at 72°C completed each amplification. Reamplification of *mcrA* genes was performed with all samples by using the same reaction mixture and PCR protocol but adding 1 μl of the product from the first amplification instead of 1 μl of fresh DNA extract. PCR products were purified on an 0.8% agarose gel, then poly(A) tailed, and cloned using the TOPOXL PCR cloning kit before being transformed into *Escherichia coli* according to the manufacturer's protocols (Invitrogen, San Diego, CA). PCR amplification of 16S rRNA genes was performed from pooled DNAs of all sediment layers, as described previously (10).

The *mcrA* gene and 16S rRNA gene sequences were determined on an ABI 3730 sequencer using the M13 universal primers SP010 and SP030 (http://www.operon.com). The 16S rRNA was also sequenced using the universal internal primer 1055R to obtain full-length sequences. The sequences were assembled by using the Sequencher software package (http://www.genecodes.com) and aligned with sequences from GenBank using the ARB software (www.arb-home.de) fast aligner utility.

Phylogenetic analysis. The *mcrA* sequences were translated using MacClade (http://macclade.org) and aligned using ClustalX (53). The *mcrA* amino acid alignment was visually checked for ambiguities and discontinuous alignment portions, Based on low BLAST score similarities and aberrations in alignments, we excluded four of the putative *mcrA* sequences from further phylogenetic analysis. Bayesian analyses were performed using well-established methods with the MRBAYES 3.0B4 program (20). The *mcrA* subunit gene sequence analysis incorporated approximately 160 amino acid positions. We used the fixed (Poisson) amino acid model for protein substitution. The chain length for all analyses was 2×10^6 generations, with trees sampled every 100 generations. Parameters did not change significantly after several thousand sampled trees. Only 10^6 generations were used to compute tree topology and posterior probability. The 16S rRNA phylogeny considered 1,265 positions that could be unambiguously aligned. The CHIMERA-CHECK online analysis program of the RDP-II database (29) did not detect any chimeras in the 16S rRNA amplicons. Using the neighbor-joining algorithm and the ARB database, we generated a dendrogram under likelihood settings that employed the model GTR+I+G, as selected by Model Test, version 3.06 (39). Bootstrap resampling with minimum evolution and parsimony methods in PAUP*, version 4.08b (47), tested the robustness of inferred topologies.

Nucleotide sequence accession numbers. GenBank accession numbers for archaeal 16S rRNA gene sequences are as follows: clone 4A10, AY835407; 4C08, AY835408; 7H07, AY835409; 4H08, AY835410; 7B08, AY835411; 7H12, AY835412; 4A12, AY835413; 4E11, AY835414; 4F12, AY835415; 7A07, AY835416; 7F07, AY835417; 1E10, AY835418; 4A08, AY835419; 7A08, AY835420; 4G12, AY835421; 4H11, AY835422; 7C08, AY835423; 1E11, AY835424; 4D08, AY835425; 4B09, AY835426; 4E09, AY835427; 7A09, AY835428. GenBank accession numbers for the *mcrA* gene sequences are as follows: clone D08, AY837763; H07, AY837764; C10, AY837765; B12, AY837766; C01, AY837767; B06, AY837768; C05, AY837769; D11, AY837770; D06, AY837771; D07, AY837772; A07, AY837773; B09, AY837774; D03, AY837775.

RESULTS

Diversity of methanogens based on *mcrA* genes. Of the *mcrA* clones, 73% were related to the *Methanomicrobiales* and 26% to the *Methanosarcinales*. A single clone (D03) was similar to the *Methanococcales*, but we detected no PCR amplicons that were related to either the *Methanobacteriales* or the *Methanopyrales* (Fig. 1). Within the *Methanomicrobiales*, a cluster of 15 near-identical clones (represented by clone D08) formed a sister group to the genus *Methanocorpusculum*. Five near-identical clones (represented by H07) and a single clone (C10) were affiliated with *Methanoculleus thermophilus*; a single *mcrA* clone (B12) branched with *Methanomicrobium mobile*. A cluster of 39 *mcrA* clones within the *Methanomicrobiales* (represented by the two most divergent clones, C01 and B06) was most closely related to the *mcrA* clone OS77 from landfill material enrichments. It showed no specific affiliation with cultured species or genera (28).

Within the *Methanosarcinales*, a major group of 20 *mcrA* clones (represented here by clones C05, D11, D7, and D06) formed several sister lineages to the acetoclastic methanogen *Methanosaeta concilii*. Of these, clone D06 branched in a basal position to *mcrA* phylotypes that have been linked to the methane-oxidizing ANME-2 group based on phylogenetic congruence (18). Single clones represented the other two *mcrA* phylotypes within the *Methanosarcinales*. Clone B09 was closely related to the methyl-disproportionating species *Methanococcoides burtonii* and *Methanococcoides methylutens*. Clone A07 had no specific relatives and was basal to the *mcrA* gene of *Methanosaeta concilii* and the ANME-2-associated *mcrA* genes and related phylotypes from Guaymas. Within the *Methano-*

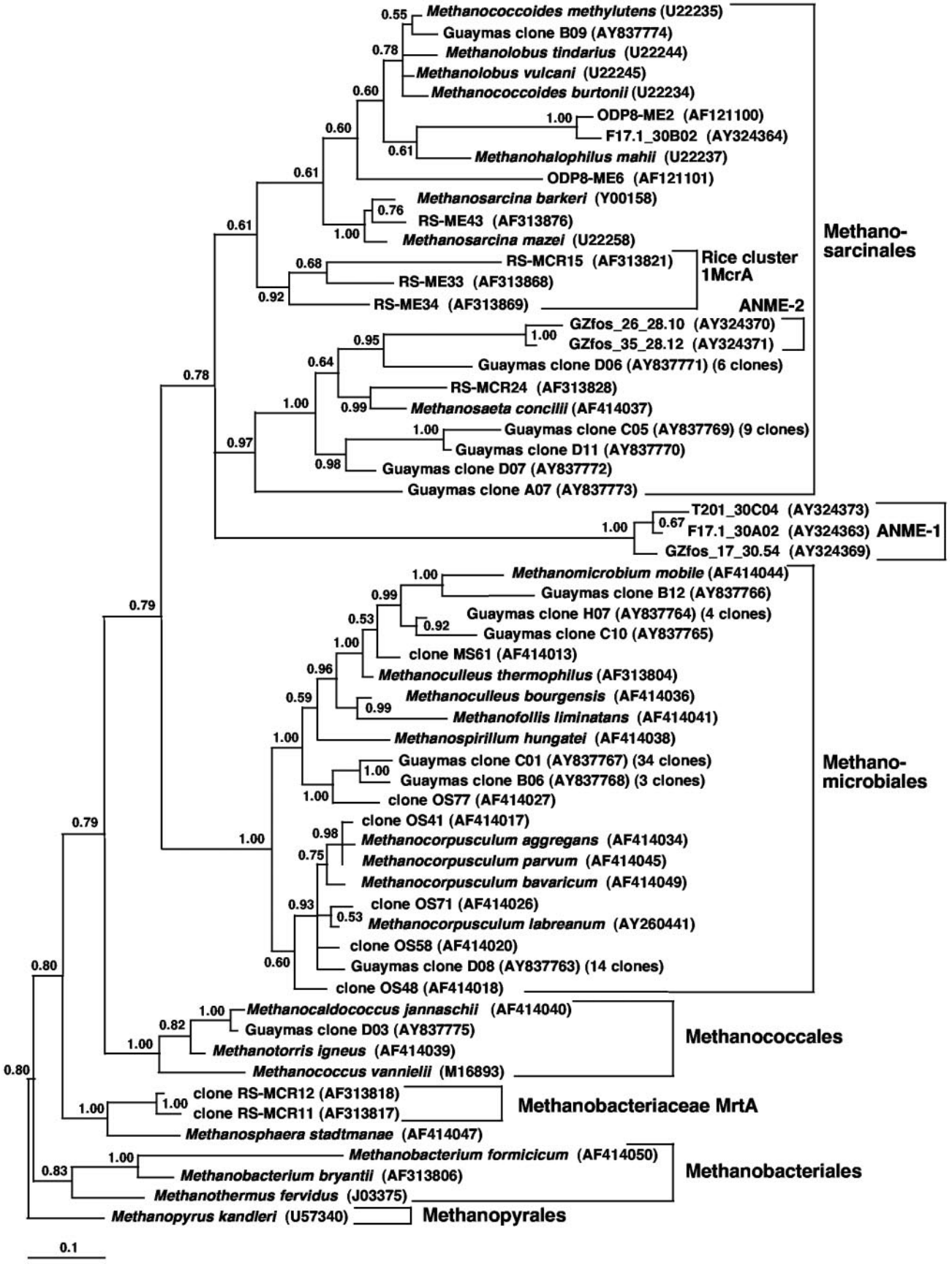

FIG. 1. Phylogenetic tree based on translated, partial amino acid sequences of *mcrA* genes from methanogenic archaea. Sequences were retrieved from Guaymas core B sediment layers (1 to 15 cm). The tree topology and posterior probability were constructed using Bayesian analyses. For sequences representing multiple, near-identical clones, the number of additional clones is given in parentheses.

coccales, a single clone (D03) was closely related to the obligately chemolithoautotrophic, thermophilic species *Methanocaldococcus jannaschii* (22).

Archaeal diversity based on 16S rRNA. The 16S rRNA survey of Guaymas sediments revealed a highly diverse archaeal community, with clones affiliated with diverse, mutually exclusive, monophyletic groups of cultured and uncultured archaea (Fig. 2). The Guaymas archaeal clones belong to the methanogen orders *Methanomicrobiales* and *Methanosarcinales*, a novel lineage of uncultured *Euryarchaeota*, several uncultured sister lineages of the *Thermoplasmatales*, and uncultured *Crenarchaeota* of marine benthic group B and marine pelagic group I (Fig. 2). Most of the 16S rRNA clones (54%) grouped within the *Methanomicrobiales* (Fig. 2). Of these, nine clones were very closely related to cultured species of the genus *Methanocorpusculum*, which use H_2, formate, and secondary alcohols as electron donors (8). Twenty-two identical clones formed a branch that is basal to the H_2/CO_2- and formate-utilizing chemolithoautotrophic species *Methanoculleus bourgensis* and *Methanoculleus thermophilus* (Fig. 2). Two clones were nearly identical to the previously obtained Guaymas clone CSR002 and formed a monophyletic branch with the H_2/CO_2- and formate-utilizing genera *Methanomicrobium* and *Methanogenium*.

Amplicon sequences within the *Methanosarcinales* constituted 8% of the 16S rRNA clone library (Fig. 2). A single clone (7F07) converged upon a monophyletic group that includes the cultured species *Methanosaeta concilii* and *Methanosaeta thermophila*. Three clones within the *Methanosarcinales* were not related to cultured species but formed a monophyletic group with clone OF23FC from a high-temperature petroleum reservoir (33). A single clone (4G12) formed a deep branch within the *Methanosarcinales* without affiliation to cultured genera and species or to other phylotypes (Fig. 2).

Other archaeal 16S rRNA clones from Guaymas belonged to novel lineages without cultured members, similar to those found in previous Guaymas studies (50), in hydrocarbon-contaminated soils (12), and in diverse hydrothermal vents (31, 42, 48). Major euryarchaeotal lineages are the Guaymas euryarchaeotal group (50), the deep-sea hydrothermal vent euryarchaeotal 2 cluster (DHVE2) as a sister lineage to the *Thermoplasmatales* (31), and the SAGMEG-1 cluster from subsurface water in South African gold mines (48) (Fig. 2). The only crenarchaeotal clones found in this survey belonged to marine benthic group B, whose members have been found in marine sediments and hydrothermal vent samples (42, 50) (Fig. 2).

Porewater and temperature gradients. Sulfate concentrations in porewater ranged from 18 to 19 mM near the sediment surface to 0.2 to 0.3 mM at a depth of 11 cm and deeper; the background is probably due to sulfide reoxidation (Fig. 3A). Most organic acids in the porewater remained undetectable (*n*-butyrate, isobutyrate, isovalerate) or below 20 μM (propionate) in the upper 11 cm of the sediment and appeared at significantly higher concentrations below a depth of 11 cm (Fig. 3A and B). Acetate concentrations were an order of magnitude higher than concentrations of other organic acids; they remained between 100 and 200 μM within the 5- to 10-cm depth range and reached 900 μM at a depth of 15 cm (Fig. 3A). Formate was the only organic acid that showed a peak (60 μM) near the surface, while below 10 cm depth, formate concentrations decreased to less than 10 μm (Fig. 3A). The elevated levels of most organic acids below 11 cm depth coincided with increasing in situ temperatures of at least 50 to 60°C at this depth (Fig. 3B).

DISCUSSION

Comparison of 16S rRNA and *mcrA*. In general, the phylogenetic patterns of 16S rRNA amplicons are consistent with those for *mcrA* clone libraries, although quantitative representation of phylogenetic groups in 16S rRNA and *mcrA* clone libraries shows significant differences. Arguing on the basis of similar topologies, within the *Methanobacteriales*, several 16S rRNA as well as *mcrA* clones represent members of the genus *Methanocorpusculum*. The large cluster of identical 16S rRNA clones (22 clones, represented by clone 7H12) and the OS77-related cluster of 39 *mcrA* clones display corresponding phylogenetic positions that are basal to the genera *Methanogenium*, *Methanoculleus*, and *Methanomicrobium*. The *mcrA* clone B12 may correspond to the 16S rRNA clones 4A12 and 4E11; both genes branch with *Methanomicrobium mobile*. However, there is no clear 16S rRNA counterpart for the five *mcrA* clones related to *Methanoculleus thermophilus*.

Within the *Methanosarcinales*, the *Methanosaeta*-associated 16S rRNA clone 7F07 may correspond to one of the multiple *mcrA* clone lineages that branch near *Methanosaeta concilii* (Fig. 1); however, it is not possible to make a definite identification. There is no 16S rRNA counterpart to *mcrA* clone B09 within the genus *Methanococcoides*. Interestingly, one deeply branching lineage each of *mcrA* clones (A07) and 16S rRNA clones (4G12) occurs within the *Methanosarcinales*; however, these lineages are not related to known cultured species that might serve as phylogenetic anchors.

The lack of *Methanococcales* (except for clone D03) and *Methanobacteriales mcrA* genes in the clone libraries is consistent with the absence of a coding region for the isoenzyme *mrtA*. The operon encoding the classical methyl coenzyme M reductase type I, or MCR-I (*mcrBDCGA*), which includes the *mcrA* subunit, is present in all methanogens (41). Members of the *Methanobacteriales* and *Methanococcales* also contain an isoenzyme, methyl coenzyme M reductase type II (MCR-II), encoded by the *mrtBDGA* operon (26, 38) or *mrtBGA* (6). The *mrtA* sequences RSMC R12T and RSMC R11T and the *mrtA* gene of *Methanosphaera stadtmanae* represent the *mrtA* cluster of the *Methanobacteriales* and *Methanococcales* (27). The Guaymas *mcrA* lineages are unrelated to other, recently discovered *mcrA* lineages, including Rice cluster I *mcrA* (27), deeply branching *mcrA* lineages from terrestrial sites OS55 and OS18 (28), and the d/e cluster, which is phylogenetically congruent to ANME-1 (18).

Metabolic potential and substrate spectrum. The composition of the 16S rRNA and *mcrA* clone libraries and the phylogenetic affiliation of the Guaymas clones to cultured species with known physiology, in particular hydrogenotrophic, methylotrophic, and acetoclastic members of the *Methanosarcinales* and the *Methanomicrobiales* (3, 4), suggest a likely substrate spectrum for the Guaymas methanogen community.

Acetate utilization by methanogens. The *mcrA* and 16S rRNA clone libraries contain close relatives of the acetoclastic

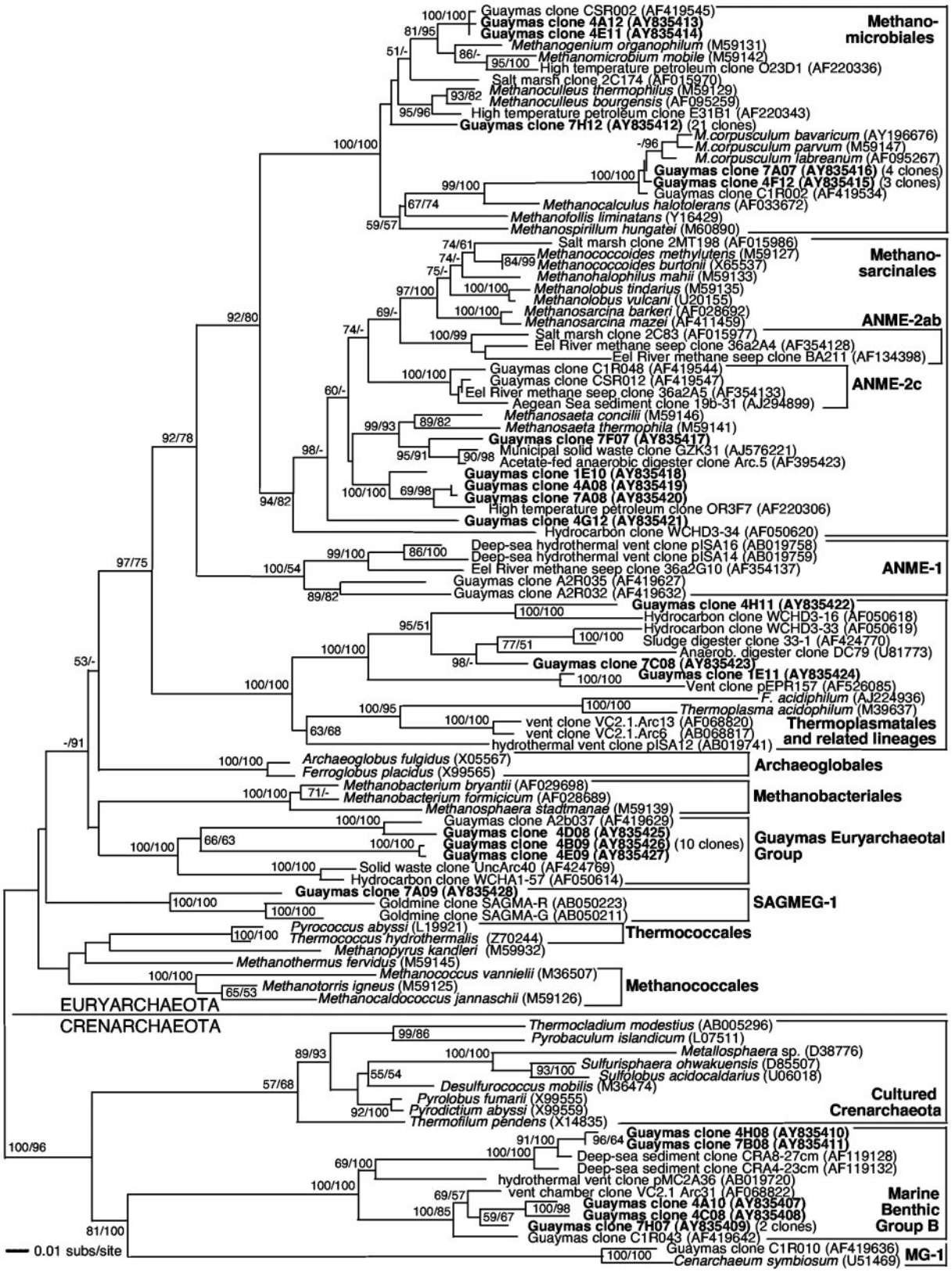

FIG. 2. Phylogenetic tree based on 16S rRNA sequences of archaeal clones from Guaymas core B sediment layers (1 to 15 cm). The tree was constructed with PAUP*. Bootstrap values based on 500 replicates for each distance and parsimony are shown for branches with more than 50% bootstrap support. The labeled brackets define order- or phylum-level lineages of cultured and uncultured archaea and specific lineages of methane-oxidizing archaea (ANME-1, ANME-2).

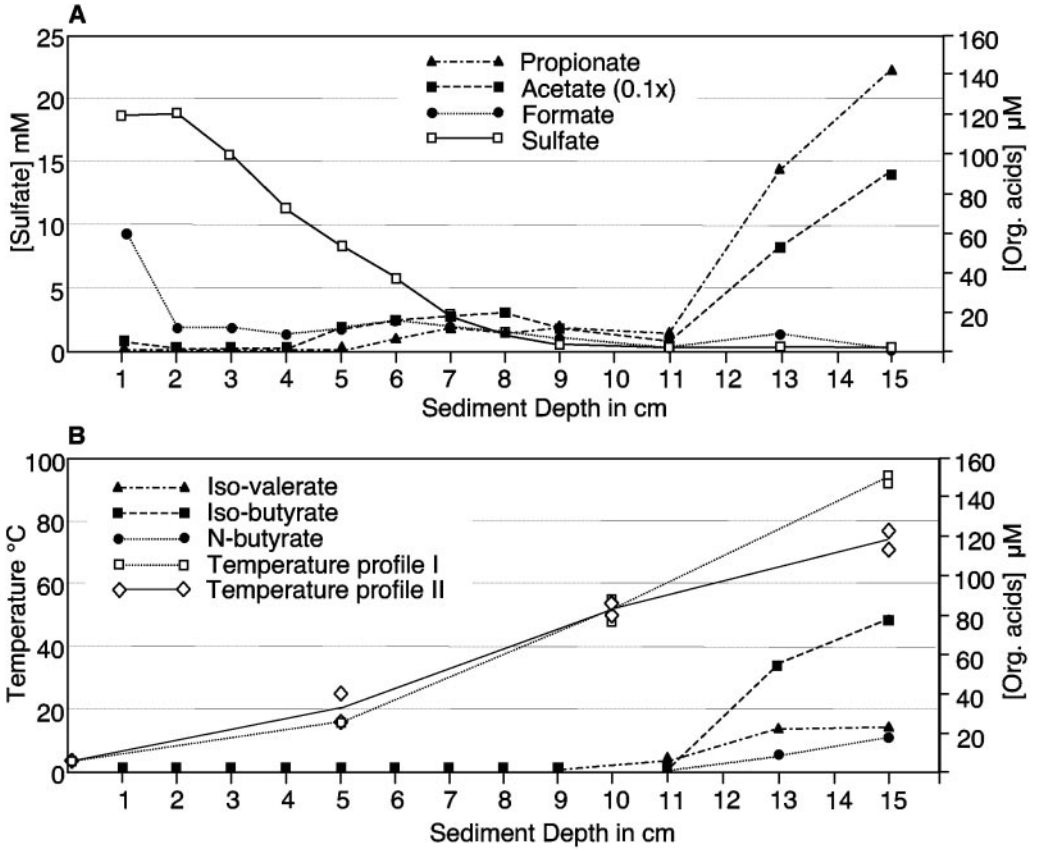

FIG. 3. Concentration profiles of sulfate, acetate, propionate, and formate (A) and of *n*-butyrate, isobutyrate, and isovalerate (B) and in situ temperature profiles (B) in Guaymas sediments. Acetate concentrations in panel A are scaled down by a factor of 10 to match the scale of the other low-molecular-weight compounds. See Materials and Methods for details on temperature profiles.

species *Methanosaeta concilii*, indicating that acetoclastic methanogens represent significant community members in the Guaymas sediments. Acetate is also required by several hydrogenotrophic and formate-utilizing methanogenic species and genera (*Methanocorpusculum*, *Methanoculleus*, *Methanomicrobium*) among the *Methanomicrobiales* that are represented in 16S rRNA and *mcrA* clone libraries. Cultured members of these genera generally utilize hydrogen or formate, but most strains require acetate or complex organic substrates as an additional carbon source for biomass synthesis. They do not catabolize acetate to methane (3).

Previously measured acetate profiles in Guaymas sediments from diverse locations are compatible with microbial acetate consumption in the upper sediment layers. Acetate and other diverse aliphatic acid anions are produced via thermocatalytic breakdown of complex organic substrates in the deeper sediment layers (30). The lowest acetate concentrations occur near the sediment surface; higher concentrations of 10 to 30 μm are found in the upper sediment layers (15 to 20 cm); maximal acetate concentrations, in one case above 1 mM, are observed in the deepest sediment layers (30). A similar acetate profile was found in this study, with the highest acetate concentrations near 1 mM in the deepest, hottest sediment horizon (13 to 15 cm) analyzed. The broad sediment layers with lower acetate concentrations (100 to 200 μM between 5 and 10 cm; below 100 μM between 0 and 5 cm) are unlikely to be the exclusive result of diffusion loss; acetate-consuming microbial processes, including acetate oxidation to CO_2 by sulfate-reducing bacteria, are active in these sediment layers. In a parallel molecular study of the sulfate-reducing bacterial communities in Guaymas in the same sediment core, PCR amplification, cloning, and sequencing of the phylogenetically informative key gene encoding dissimilatory sulfate reductase (*dsrAB*) identified a major cluster of sulfate-reducing bacteria closely related to the obligately acetate oxidizing *Desulfobacter* group (10). These sulfate-reducing bacterial clones co-occur with methanogen clones of acetate-utilizing genera (and families) within the same sediment samples, suggesting that acetate-utilizing methanogens coexist with sulfate reducers. This is unusual for sulfate-containing marine sediments, where acetate concentrations are generally limiting and sulfate-reducing bacteria outcompete methanogens for acetate (40). Here, in situ acetate concentrations in the 100 μM range can support coexisting acetate-utilizing methanogens and sulfate reducers; sulfate and acetate coexist in the 5- to 10-cm sediment horizon (Fig. 3A).

Methyl disproportionation. The *mcrA* gene clone libraries indicate that C_1 organic compounds serve as substrates for Guaymas methanogen communities. Members of the genera *Methanolobus* and *Methanococcoides* within the *Methanosarcinales* generate methane by disproportionation of C_1 compounds, such as methanol, methylamine, and trimethylamine (4). Since competing sulfate-reducing bacteria do not utilize

these C_1 substrates, genera of methanogens utilizing such C_1 compounds usually dominate marine methanogen communities within the zone of sulfate reduction (40, 54). The occurrence of *mcrA* genes closely related to *Methanococcoides* species (clone B09) indicates that disproportionation of methanol or methylated substrates contributes to methanogenesis in the Guaymas sediments.

Hydrogenotrophic methanogenesis. Members of all three methanogen orders detected here (*Methanosarcinales*, *Methanomicrobiales*, *Methanococcales*) can contribute to hydrogenotrophic methanogenesis in the Guaymas sediments. Three processes can supply hydrogen, including the upwelling of hydrogen- and CO_2-rich hydrothermal fluid through the sediment layer, fermentation of diverse organic substrates in organic-rich sediments, and syntrophic acetate degradation. Hydrogen is a major component of the vent fluids in the Guaymas Basin (56). Hydrogenotrophic methanogens can utilize H_2 that is produced during syntrophic acetate oxidation by specialized acetate-oxidizing bacterial communities; it has been noted that the H_2-consuming methanogens that drive syntrophic acetate oxidation are different from those that perform acetoclastic methanogenesis (32).

Several *mcrA* clone groups are not closely related to cultured species, and therefore it is difficult to infer their metabolic potential. For example, within the *Methanomicrobiales*, the major *mcrA* cluster branching with the environmental clone OS77 may represent a novel *Methanomicrobiales* lineage or a yet unsequenced *mcrA* gene from a recognized species (28). If the general characteristics of other genera within the *Methanomicrobiales* can be applied, this lineage may represent hydrogenotrophic and formate-utilizing methanogens.

Methanogenesis and microbial alkane cracking. The diversified methanogenic community in the Guaymas Basin hydrothermal sediments includes 16S rRNA gene and *mcrA* phylotypes representing mesophilic and thermophilic, acetoclastic, C_1 compound-disproportionating, and CO_2/H_2-autotrophic methanogens. The substrates for this methanogenic community can be derived from thermal and microbial degradation of recalcitrant hydrothermal petroleum compounds, including aliphatic and aromatic hydrocarbons that are abundant in Guaymas sediments (2, 45). The depth profiles for low-molecular-weight organic acids and their increasing concentrations in hot, deep sediment layers (Fig. 3) strongly support a thermocatalytic origin of these substrates, as suggested previously for Guaymas (30) and in similar form for nonhydrothermal deep-subsurface sediments (58). However, selective microbial utilization of recalcitrant precursor molecules appears to be co-occurring with thermal degradation, as indicated by the selective depletion of specific *n*-alkanes and aliphatic components in Guaymas surface sediments (2). Enrichment studies demonstrated that methane production involves unbranched long-chain alkanes, in a strictly anaerobic "microbial alkane-cracking" process that most likely proceeds via anaerobic alkane degradation to acetate and hydrogen, coupled to acetoclastic and CO_2/H_2 methanogenesis (59). Although the alkane-activating process and its specific microbial catalysts remain to be identified, methanogenic enrichment cultures growing in the laboratory on hexadecane show a community composition similar to that of the Guaymas sediments and contain *Methanosaeta*- and *Methanoculleus*-related 16S rRNA phylotypes (59). As demonstrated by laboratory enrichments at 28°C, this microbial process does not require high temperatures (59) and could therefore take place in the relatively cool surface layers of the Guaymas sediments. Previous physiological studies of novel sulfate-reducing bacteria in Guaymas sediments have demonstrated the unusual versatility of the Guaymas anaerobic microbial communities in degrading highly inert hydrocarbons such as alkanes and long-chain fatty acids (43). The methanogenic communities are probably performing the terminal remineralization steps in these complex degradation pathways.

Mesophilic and thermophilic methanogens. The 16S rRNA and *mcrA* gene clone libraries indicate a mixed mesophilic and thermophilic methanogen community in the Guaymas sediments. Methanogens span a wide range of growth temperatures, from moderate psychrophiles that grow optimally at temperatures below 20°C to hyperthermophiles that grow at temperatures up to 110°C (5). The sediment layers that were analyzed here by 16S rRNA and *mcrA* sequencing represent a temperature range of approximately 3 to 94°C, approximately the complete methanogen growth temperature spectrum. In some cases, it is possible to infer temperature characteristics for Guaymas methanogenic populations based on closely related cultured species, and related phylotypes in thermal habitats.

The *mcrA* clone D03 most likely represents a hyperthermophilic methanogen that is closely related to the hyperthermophilic, obligately H_2/CO_2 utilizing chemolithoautotroph *Methanocaldococcus jannaschii* (22). Members of the genus *Methanocaldococcus* have optimal growth temperatures between 80 and 90°C and have frequently been enriched and isolated from the Guaymas sediments (21, 22, 23). However, this *mcrA* clone was retrieved from the cool upper 3 cm layers of the Guaymas sediments (Table 1). Five *mcrA* clones (represented by clone H07) are the closest relatives of the thermophilic species *Methanoculleus thermophilus*, which grows between 37 and 65°C and has a temperature optimum of 55°C (9) (Fig. 1). These *Methanoculleus*-related *mcrA* clones were isolated from intermediate layers of the sediment, with a mesophilic to moderately thermophilic temperature regime (Table 1). In general, *mcrA* clone groups that occur most frequently in this data set (the *Methanocorpusculum* group, the *Methanosaeta*-related phylotypes, and the OS77-related *Methanomicrobiales* clones) occur in all four or in three out of four depth layers (Table 1). Sediment horizon in situ temperatures do not correlate with methanogenic community composition, based on nonquantitative *mcrA* clone libraries. For example, thermophile-related clones do not cluster in the deeper, hot sediment layers. Possibly, these clone distributions are influenced by homogenization effects; downcoring may carry surface clones into deeper sediment layers while outgassing during transport to the surface may carry subsurface clones to the sediment surface.

The 16S rRNA clone library (from homogenized sediment of all depths) also includes some phylotypes that are closely related to cultured thermophilic species and to clones from hot petroleum fields. Guaymas clones 1E10, 4A08, and 7A08 form a tight cluster with clone OR3F7 from a hot petroleum reservoir, within the *Methanosarcinales* (21). Clone 7F07 and related environmental clones from other sites branched with two

TABLE 1. Pattern of occurrence of *mcrA* clones in different sediment depths and in situ temperature regimes

Order	Cultured genus or uncultured phylogenetic lineage or cluster (no. of *mcrA* clones)	No. of redundant clones[a] (name of representative clone) at the following sediment depths (temp):				Inferred methanogenic substrate[b]
		1 and 3 cm (~3–10°C)	5 and 7 cm (~15–30°C)	9 and 11 cm (~40–60°C)	13 and 15 cm (~70–95°C)	
Methanomicrobiales	*Methanocorpusculum* (15)	1		7 (D08)	7	H_2 + CO_2, formate, sometimes alcohols
	Methanoculleus (6)		3 (C10)	3 (H07)		H_2 + CO_2, formate, sometimes alcohols
	Methanomicrobium (1)				1 (B12)	H_2 + CO_2, formate
	Uncultured OS77 (39)	17 (C01)	11 (B06)	8	3	Possibly H_2 + CO_2, formate
Methanosarcinales	Basal to ANME-2: D06 type (8 total)	4 (D06)	4			Possibly acetate
	Basal to *Methanosaeta* and ANME-2: C05 type (12 total)	2	5 (C05)	2 (D11, D07)	3	Possibly acetate
	Methanococcoides (1)			1 (B09)		Methanol methylamines
	Basal to ANME-2 and *Methanosaeta* (1)			1 (A07)		Unknown
Methanococcales	*Methanocaldococcus jannaschii* (1)	1 (D03)				H_2 + CO_2

[a] Groups of near-identical clones, represented by selected clones listed here and in Fig. 1.
[b] Based on the general characteristics of the phylogenetically most closely related methanogen genera and orders.

Methanosaeta species, the mesophile *Methanoseaeta concilii* and the thermophile *Methanosaeta thermophila*, with a temperature range of 55 to 60°C (36).

A parallel molecular study of sulfate-reducing prokaryotic communities in the same Guaymas sediments (in the surface layer [1 to 7 cm]) indicated that most sulfate-reducing bacterial phylotypes were not related to thermophilic lineages; this is consistent with the in situ temperature range of ca. 3 to 30°C (Fig. 3). These inferences about phenotypic characteristics were constrained by the absence of cultured relatives (10). Sulfate reduction rates in sediment cores from the same sampling location peaked in the mesophilic and thermophilic temperature range (40 to 70°C) in the upper 12 cm. Only the deepest sediment layers (12 to 24 cm) were dominated by hyperthermophilic sulfate-reducing communities with temperature optima between 80 and 90°C (55). If these sulfate reduction rate measurements are taken as a guideline for temperature preferences of other bacteria and archaea, a predominantly hyperthermophilic methanogen community would be expected in the 12- to 24-cm depth range.

Physical and chemical controls. All markers or proxies for microbial activity reach a few centimeters into the hydrothermally active sediments, with considerable variability between sites and parameters. Depth ranges include 10 to 15 cm for bacterial phospholipid fatty acids (15), 8 cm for cultivable thermophilic archaeal isolates of the genus *Thermococcus* (49), 7 cm for *dsrAB* genes of sulfate-reducing bacteria (10), ca. 20 to 30 cm for sulfate-reducing activity measured by radioactive tracers (55), 15 cm for dibiphytanyl archaeal lipids (44), 1 to 5 cm for archaeal 16S rRNA genes including ANME-1 and ANME-2 (50), and 15 cm for 16S rRNA and *mcrA* genes (this study). With a single exception (15), in situ temperature measurements are available and indicate that the extreme temperatures of the vent fluids in the sediment column limit the downward extent of active microbial populations and compress the Guaymas microbiota into the surface layers of the hydrothermally active sediments. Microbial life may occupy a greater depth range in less active and less hot sediment regions, on the periphery of hydrothermally active sediment areas.

Physical and chemical controls must be studied in more detail in order to understand the occurrence pattern and population density of methanogenic and methane-oxidizing archaea. A previous Guaymas survey based on 16S rRNA sequences and compound-specific carbon isotopic analyses of taxonomically informative lipid biomarkers detected abundant populations of presumed anaerobic methane-oxidizing archaea in Guaymas, in different cores, always near the sediment surface in the upper 1 to 5 cm (50). These sediments had steep, concave temperature profiles compatible with upwelling of hot, methane-rich vent fluids (50). Interestingly, no *mcrA* genes that are phylogenetically congruent to anaerobic methanotrophs of the ANME-1 and ANME-2 groups were obtained in this study (18, 34, 35). The sediment samples analyzed here come from a cooler site with a more gradual and linear temperature gradient (Fig. 3), which indicates conductive heat transfer and lesser upwelling of methane-rich vent fluids. As a working hypothesis, the occurrence patterns of methanogenic and methanotrophic communities in the Guaymas sediments could be controlled by hydrothermal flow patterns in the Guaymas sediment; their patchiness results from the dynamic, temporally and spatially changing hydrothermal flow and circulation patterns within the sediments. The distribution of sulfide-oxidizing *Beggiatoa* mats on the Guaymas sediment surface supports this working hypothesis. Sulfide-oxidizing *Beggiatoa* mats cover the sediment and its methanotrophic communities. This population structure requires dynamic, alternating flow patterns of sulfide and oxygenated seawater that result from hydrothermal fluid upwelling and lateral seawater entrainment; diffuse flow is apparently not sufficient (16). The sediments analyzed here lacked *Beggiatoa* mats, which is consistent with low or no vent fluid upwelling to the sediment surface.

The functional gene and rRNA surveys highlight the diversity and functional versatility of microbial populations in the

Guaymas Basin sediments. The small-scale mosaic of hot and cold sediments, of different temperatures and vent fluid flow regimes, combined with high microbial density and diversity in this unique habitat, provides a natural laboratory to test the impact of different physical and chemical controls on microbial community composition and density. Future integrated geochemical and microbiological studies are required to show in greater detail how geochemical parameters shape the microbial ecosystem of the Guaymas vents.

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