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Evidence of Active Methanogen Communities in Shallow Sediments of the Sonora Margin Cold Seeps

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In the Sonora Margin cold seep ecosystems (Gulf of California), sediments underlying microbial mats harbor high biogenic methane concentrations, fueling various microbial communities, such as abundant lineages of anaerobic methanotrophs (ANME). However, the biodiversity, distribution, and metabolism of the microorganisms producing this methane remain poorly understood. In this study, measurements of methanogenesis using radiolabeled dimethylamine, bicarbonate, and acetate showed that biogenic methane production in these sediments was mainly dominated by methylotrophic methanogenesis, while the proportion of autotrophic methanogenesis increased with depth. Congruently, methane production and methanogenic *Archaea* were detected in culture enrichments amended with trimethylamine and bicarbonate. Analyses of denaturing gradient gel electrophoresis (DGGE) fingerprinting and reverse-transcribed PCR-amplified 16S rRNA sequences retrieved from these enrichments revealed the presence of active methylotrophic *Methanococcoides burtonii* relatives and several new autotrophic *Methanogenium* lineages, confirming the cooccurrence of *Methanosarcinales* and *Methanomicrobiales* methanogens with abundant ANME populations in the sediments of the Sonora Margin cold seeps.

In cold seep ecosystems, sediments are colonized by various dense microbial and sometimes macrofaunal populations, forming a mosaic of patchy habitats on the seafloor (1, 2). The metabolism of these organisms, based on chemosynthesis, is mainly fueled by seep fluids, rich in reduced compounds and hydrocarbons, such as methane (3). Most of the methane is consumed microbiologically by anaerobic and aerobic methanotrophic communities before reaching the water column, forming an efficient biofilter (4). In marine sediments and typically in cold seep ecosystems, methanogenesis driven by archaeal communities accumulates large amounts of methane, which can be trapped in gas hydrates. Microbial populations involved in methane production (methanogens) are phylogenetically affiliated with 7 orders within the phylum *Euryarchaeota*, which comprises the *Methanosarcinales*, *Methanocellales*, *Methanomicrobiales*, *Methanococcales*, *Methanopyrales*, *Methanobacteriales* (5), and the recently described *Methanoplasmatales* (6), also known as *Methanomassiliicoccales* (7). Furthermore, deeply branching uncharacterized orders have been recently detected (8). Enrichment cultures from methane-rich environments, such as marine sediments, mangroves, animal guts, or wastewater bioreactors, previously showed that methanogens could use different substrates for methane production under anaerobic conditions (9–18). In marine sediments, methylated compounds (e.g., methylamine, dimethylamine, trimethylamine [TMA], methanol, dimethylamine-sulfate, and dimethylsulfide), volatile fatty acids (formate and acetate), bicarbonate, and, more recently, choline and glycine betaine (19, 20) have been identified as primary carbon substrates for methanogenesis. These compounds can be metabolized through three different specific methanogenic pathways: methanogenesis from H_2-CO_2, aceticlastic methanogenesis, and methylotrophic methanogenesis (21).

Located in the Guaymas Basin (Gulf of California), the Sonora Margin cold seep ecosystem is composed of various visible faunal assemblages and white microbial mats (22–24). Sediments underlying the microbial mats and their periphery are characterized by high methane concentrations (around 900 μM and 500 μM, respectively) (23). The carbon isotopic signature of this methane ($\delta\ ^{13}C\text{-}CH_4 = -63‰$ to $-90‰$) suggests that significant amounts of biogenic methane are produced by methanogenic populations. However, in contrast to the adjacent high-temperature hydrothermal sediments of the Guaymas Basin, from which several hyperthermophilic methanogens were isolated (25–27), no methanogens were detected by microscopic and rRNA sequence surveys of recent Sonora Margin sediments (23). However, 16S rRNA gene sequences related to known methanogenic lineages were detected only rarely in previous studies using clone libraries (23) and 454 pyrosequencing (24). Furthermore, quantitative measurements using real-time quantitative PCR (qPCR) with 16S rRNA gene primer sets specifically targeting putative methanogenic groups suggested that *Methanomicrobiales* and *Methanococcales* represented only a minority of the microbial community (0.1 to 1% of the total archaeal 16S rRNA gene copy

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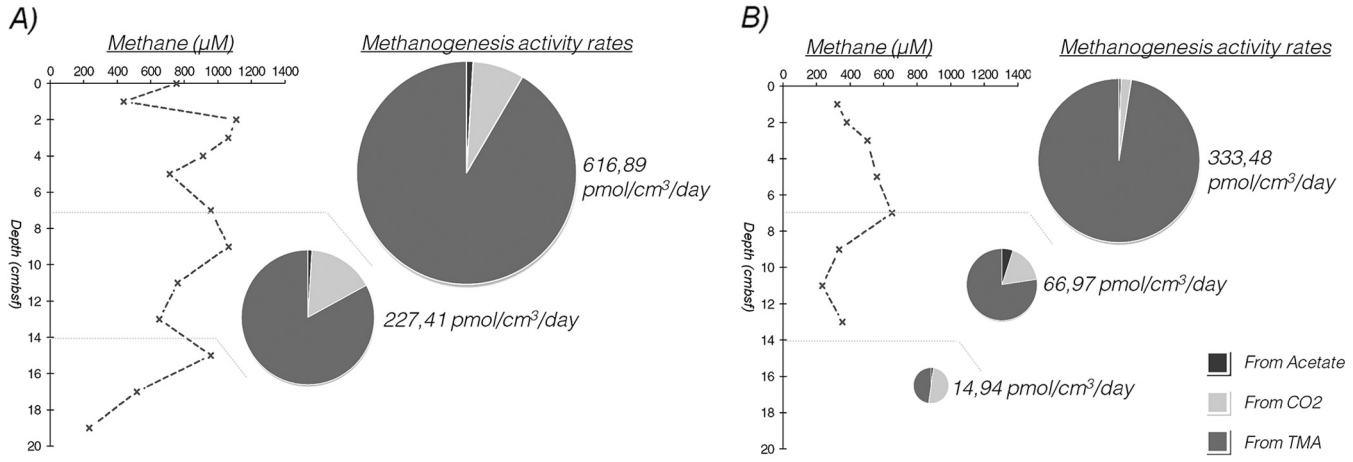

FIG 1 Sediment depth profiles of methane concentrations and methanogenesis rates. (A) White Mat 14. (B) Edge of White Mat 14. The relative proportions of acetate, bicarbonate, and dimethylamine methanogenesis rates are represented in pie charts for each sediment section. The size of the pie chart is proportional to the total methanogenesis rate. The methane concentrations are from Vigneron et al. (23).

number) in the shallow sediment layers (0 to 17 cm below seafloor [cmbsf]) (23). In contrast, *Methanosarcinales* related to anaerobic methanotrophs (ANME), previously found to be active and abundant in these sediments (23), dominated throughout the shallow sediments of the Sonora Margin (30 to 92% of the total archaeal 16S rRNA gene copy number). Thus, the activity and biodiversity of methanogenic microbial populations remained unclear.

In this study, we investigated the production of biogenic methane in the Sonora Margin cold seeps by analyzing major metabolic pathways for methane production in marine environments. The phylogenetic and metabolic diversity of methanogenic communities was explored using enrichment cultures and activity measurements designed to target acetotrophic, hydrogenotrophic, and methylotrophic methanogens.

MATERIALS AND METHODS

Sediment samples. Sediment samples were collected from Sonora Margin cold seeps during the oceanographic cruise BIG (Ifremer) with the R.V. *L'Atalante* and the D.S.V. *Nautile* in June 2010. Two different habitats from the Vasconcelos site (27°35.577′N, 111°28.984′W), sampled in triplicate using 20-cm-long push cores (PC), were selected for enrichment cultures and activity measurements: (i) an extended white microbial mat (White Mat 14 [WM14]; PC1, PC2, and PC3), characterized by an average methane concentration of 900 μM throughout the core (Fig. 1), and (ii) the surrounding macrofauna (Edge of White Mat 14 [EWM14]; PC6, PC8, and PC11), characterized by an average methane concentration of 500 μM (Fig. 1) (23). Before each sampling, autonomous temperature sensors (T-Rov; NKE Electronics, Hennebont, France) recorded *in situ* temperatures around 3°C from the sediment surface down to 40 cmbsf on each habitat. On board, the sediment cores were transferred in a cold room immediately after retrieval and sectioned aseptically in 2-cm-thick layers. For enrichment cultures, 6 cm^3 of each sediment layer was transferred into 50-ml vials containing 10 ml of sterile and anoxic artificial seawater (DSMZ medium 246a). The vials were crimp sealed with butyl rubber septum stoppers and aluminum crimp tops (Bellco Glass Inc., Vineland, NJ, USA) and then flushed with N_2 and stored at 4°C under a 200-kPa N_2-CO_2 (80:20) gas atmosphere. For activity measurements, PC2 and PC6 were subsampled using triplicate mini-push cores. These mini-push cores were hermetically sealed under an N_2 gas atmosphere in aluminum bags (Grüber-Folien, Germany) and stored at 4°C for processing in the laboratory.

Culture media for enrichment of methanogens. Two sediment cores from each habitat (WM14 PC1 and PC3; EWM14 PC8 and PC11) were used to inoculate independent duplicate enrichments. Methanogenic enrichments were performed anaerobically in 50-ml vials. Medium 141 from DSMZ was used with slight modifications: organic substrates were omitted, except yeast extract with a final concentration of 0.2 g $liter^{-1}$. The medium was prepared and sterilized under an 80% N_2 and 20% CO_2 gas atmosphere. In order to enrich CO_2-reducing, aceticlastic, and methylotrophic methanogens, four separate enrichment media supplemented with H_2-CO_2 (80:20; 200 kPa), acetate (10 mM) under an H_2-CO_2 or N_2-CO_2 gas atmosphere, and TMA (20 mM) were used. One milliliter of sediment suspension from different sections (0 to 6 cmbsf, 6 to 10 cmbsf, and 10 to 15 cmbsf) of each core was inoculated into 9 ml of medium (pH 7). The slurries were mixed and serially diluted to 10^{-3}. A total of 136 cultures were prepared, including uninoculated medium used as a negative control for each condition (Table 1). In order to enhance microbial growth, all the cultures were incubated at 12°C, higher than the average *in situ* temperature (3°C). The cultures were periodically checked (every month) for methane production for 2 years. The methane concentrations in the vial headspaces of the cultures were determined by using a micro MTI M200 gas chromatograph (SpectraLab, Markham, Canada) equipped with an MS-5A capillary column and a Poraplot U capillary column (Agilent Technologies, Santa Clara, CA, USA) via sterile needle. The presence of putative methanogenic communities from methane-producing enrichments was confirmed by epifluorescence microscopy (model BX60, equipped with a U-RFL-T UV supply unit; Olympus, USA). Enrichments were stopped when more than 50 UV-autofluorescent cells per microscope field (magnification, ×1,000) were detected. Renewal of carbon and energy sources (200 kPa of H_2-CO_2 or 10 mM acetate) was anaerobically and sterilely carried out after 1 year of incubation.

Methanogenic-activity measurements. Potential rates of methanogenesis were monitored on anaerobically stored subsamples using ^{14}C-radiolabeled substrates, 3 months postcruise, at Cardiff University, United Kingdom. Subsamples were pooled in 42 cm^3 of sediment slurries, corresponding to 7-cm-thick sediment layers (0 to 7 cm and 7 to 14 cm for WM14 and 0 to 7 cm, 7 to 14 cm, and 14 to 21 cm for EWM14), and then dispensed into 20-ml vials before injection of labeled substrates. Triplicate vials were monitored with addition of radiotracers ([^{14}C]bicarbonate [19 μl containing 74 kBq], [^{14}C]acetate [19 μl containing 397 kBq], and [^{14}C]dimethylamine [19 μl containing 176 kBq]) for each sediment section. Additional vials were monitored without radiotracers as negative controls. The vials were incubated at close to *in situ* temperatures (4°C)

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TABLE 1 Enrichment conditions and levels of dilution applied to samples

Enrichment conditions	Enrichment[a]											Dilution
	WM14						EWM14					
	PC3			PC4			PC8			PC11		
	Z1	Z2	Z3	Z1	Z2	Z3	Z1	Z2	Z3	Z1	Z2	
Trimethylamines		+	+		+	+	+	+	+	+	+	10^{-1}
	+	+	+	+	+	+	+	+	+	+	+	10^{-2}
	+	+	+	+	+	+	+	+	+	+	+	10^{-3}
Acetate												10^{-1}
												10^{-2}
												10^{-3}
H_2-CO_2	+	+	+	+	+							10^{-1}
	+	+	+	+	+	+	+	+	+	+	+	10^{-2}
	+	+	+	+	+	+	+	+	+	+	+	10^{-3}
H_2-CO_2 with acetate	+	+	+	+	+	+	+	+	+	+		10^{-1}
	+	+	+	+	+	+	+	+	+	+	+	10^{-2}
	+	+	+	+	+	+	+	+	+	+	+	10^{-3}

[a] +, positive enrichment (methane production and detection of UV autofluorescent cells); empty cells, no methane accumulation in the headspace; Z1, 0 to 6 cmbsf; Z2, 6 to 10 cmbsf; Z3, 10 to 15 cmbsf.

with magnetic agitation. Activity measurements were terminated by addition of 1 M NaOH, and the vials were processed as described previously (28). Methanogenesis rates were calculated based on the proportion of labeled gas produced from the ^{14}C substrate, the incubation period, an assumed sediment porosity of 70%, and the measured cold pool size of the substrate. Because the incubation conditions were not identical to the original sediment conditions, the measured rates might differ from those *in situ*.

RNA extraction, purification, and reverse transcription. Total RNA from methane-producing enrichments was extracted and purified from 2 ml of enrichment culture using a Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's recommendations. Absence of residual DNA was checked by PCRs before reverse transcription. Total RNA was reverse transcribed using a Quanta qScript kit (Quanta Bioscience, Gaithersburg, MD, USA) according to the manufacturer's protocol.

PCR-DGGE of 16S rRNA. PCR-denaturing gradient gel electrophoresis (DGGE) was used to monitor the archaeal diversity in positive enrichments. Archaeal reverse-transcribed 16S rRNA was amplified by PCR using the archaeal primers A8F (5′-CGG TTG ATC CTG CCG GA-3′) and A1492R (5′-GGC TAC CTT GTT ACG ACT T-3′) (29). All PCRs were carried out in a final volume of 25 μl using the GoTaq polymerase kit (Promega, Madison, WI, USA) according to the manufacturer's recommendations. The PCR conditions were as follows: denaturation at 94°C for 1 min, annealing at 49°C for 1 min 30 s, and extension at 72°C for 2 min for 30 cycles. PCR amplicons were checked on agarose gels, and then, the PCR products were reamplified with primers 340F (5′-CCC TAC GGG GYG CAS CAG-3′), containing a GC clamp at the 5′ end (30), and 519R (5′-TTA CCG CGG CKG CTG-3′) (31). PCRs were carried out as described previously (14). Positive and negative controls were used in all PCR amplifications.

DGGE was carried out as described previously (14). DGGE profiles were analyzed using PyElph 1.4 software (32). At least one enrichment per DGGE fingerprint pattern was selected for amplification, cloning, and sequencing of the reverse-transcribed archaeal 16S rRNA.

Methanogenic diversity based on 16S rRNA. 16S rRNA sequences from reverse-transcribed RNA of positive enrichments selected after DGGE were amplified using the A8F-A915R primers (33, 34). The PCR conditions were as follows: 30 cycles of a denaturation step at 94°C for 40 s, annealing at 57°C for 1 min 30 s, and extension at 72°C for 3 min. The PCR products were purified on agarose gels and then cloned using a TOPO XL PCR cloning kit (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocols. Sequencing of the inserts was carried out by GATC Biotech (Constance, Germany) using the M13 universal primers (M13f, 5′-GTA AAA CGA CGG CCA GTG-3′; M13r, 5′-GGA AAC AGC TAT GAC CAT G-3′). Sequences were analyzed using the NCBI BLAST search program in GenBank (35) and aligned with the closest representative sequences using the Mafft program (36). The sequence data were analyzed with the MEGA 4.0.2 program (37). Phylogenetic trees were estimated by maximum-likelihood and neighbor-joining methods using RAxML 7.2.8 (38) with GTRCAT approximation of the model and the Kimura two-parameter correction matrix coupled to pairwise deletion parameters, respectively. The robustness of the inferred topology was tested by bootstrap resampling (1,000 replicates).

Nucleotide sequence accession numbers. The sequences from the study were deposited in the EMBL database under the following accession numbers: HG973458 to HG973475.

RESULTS

Potential activity measurements. Methanogenic activities were 54 to 29% higher in WM14 sediments than in EWM14 sediments. In both sediment cores, the total methanogenesis rate decreased with depth (Fig. 1), but more rapidly throughout EWM14 sediments (80% decrease) than WM14 sediments (63% decrease). In WM14 sediments, although methylotrophic methanogenesis significantly decreased with depth (560 to 180 pmol cm^{-3} day^{-1}; *t* test *P* value, 0.04), it consistently represented the major methanogenesis processes (91 to 83% of the total methanogenesis). In contrast, although hydrogenotrophic methanogenesis was relatively steady (36 to 49 pmol cm^{-3} day^{-1}; *t* test *P* value, 0.58) throughout WM14, it represented a higher proportion of the total methanogenesis at depth (0 to 7 cmbsf, 8%; 7 to 14 cmbsf, 16%). However, aceticlastic methanogenesis remained low (5 pmol cm^{-3} day^{-1}) throughout WM14 sediments, representing 1% of the total methanogenesis. In EWM14 sediments, although methylotrophic methanogenesis also dominated methanogenic processes in the

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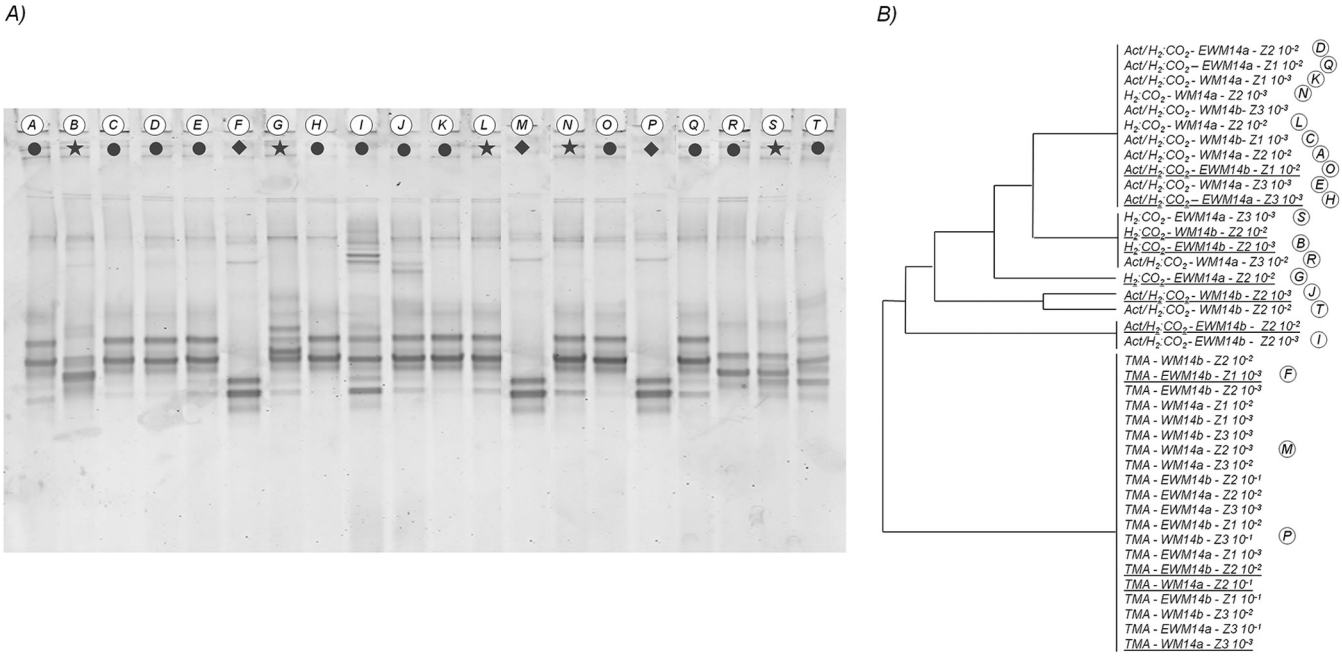

FIG 2 DGGE analysis of archaeal 16S rRNA diversity. (A) DGGE profiles for 20 samples represented by letters (A to T) in the dendrogram in panel B. The different media are represented by symbols: dots, acetate/H_2-CO_2; stars, H_2-CO_2; diamonds, trimethylamines. (B) Dendrograms from cluster analysis of DGGE profiles. The underlined samples were selected for analysis of the phylogenetic diversity of methanogens. Act, acetate; WM14a, WM14 PC3; WM14b, WM14 PC4; EWM14a, EWM14 PC8; EWM14b, EWM14 PC11; Z1, 0 to 6 cmbsf; Z2, 6 to 10 cmbsf; Z3, 10 to 15 cmbsf.

upper sediment section (98% of the total methanogenesis), it decreased markedly (analysis of variance [ANOVA] *P* value, 0.001) with depth, representing only 48% of the total methanogenesis (7 pmol cm^{-3} day^{-1}) in sediments below 14 cmbsf. In contrast, hydrogenotrophic methanogenesis in EWM14 sediments remained relatively constant (~8 pmol cm^{-3} day^{-1}; ANOVA *P* value, 0.87), representing the major methanogenesis processes (50%) at the bottom of the core. Aceticlastic methanogenesis was consistently low throughout EWM14 sediments, with rates around 2 pmol cm^{-3} day^{-1} (2% of the total methanogenesis).

Methanogenic enrichments. After 2 years of incubation, positive methane production and growth of methanogens were recorded in 90 enrichments, representing 33 different substrate and sample combinations (Table 1). Methane production or cell growth was not detected in the negative controls. TMA, acetate with H_2-CO_2, and H_2-CO_2 were found to stimulate growth of methanogenic communities from all WM14 (microbial mat) and EWM14 (macrofauna) sediment layers. Methane production was not detected with acetate as the sole carbon and electron donor for both WM14 and EWM14 sediments. Methanogens were detected by epifluorescence microscopy by targeting the fluorescent coenzyme F420 (39). F420 is not restricted to methanogens, as it has also been detected in anaerobic methanotrophic communities and archaeal Marine Group 1 (40). However, UV autofluorescent cells were detected only in enrichments where methane production occurred, strongly suggesting that these UV-autofluorescent cells were methanogens. UV-autofluorescent free coccoid-shaped cells were widespread in the samples regardless of the enrichment conditions. Unusual cell morphologies, such as long and thick spiral UV-fluorescent cells, were detected only occasionally at the beginning of the enrichment procedure in cultures amended with H_2-CO_2 (data not shown).

Archaeal community structure. Forty representative enrichments from all 33 different positive culture conditions were analyzed using RT-PCR–DGGE (Fig. 2A). The archaeal community structures grouped in seven different clusters, mainly correlated with the carbon substrates used (Fig. 2). In order to phylogenetically identify the active methanogens, 16S rRNA clone libraries from 11 positive enrichment cultures were analyzed (underlined in Fig. 2B). As the RT-PCR–DGGE profiles showed relatively limited archaeal diversity within the enrichments, only 10 clones per library were sequenced. For each clone library, all 10 sequences were highly similar to each other (97% sequence similarity). 16S rRNA sequences, amplified from enrichment cultures with TMA, were closely related to *Methanococcoides burtonii* and *Methanococcoides alaskense* (98% sequence similarity), within the order *Methanosarcinales*. In contrast, sequences obtained from enrichments with H_2-CO_2 and acetate or with H_2-CO_2 as carbon and energy sources were mainly distantly related to *Methanogenium cariaci* (96% sequence similarity) and could therefore represent a new species of *Methanogenium* (*Methanogenium* group 1) (Fig. 3). Sequences obtained from enrichment culture from WM14 (6 to 10 cmbsf) amended with acetate and H_2-CO_2 were closely related to *M. cariaci* (98% sequence similarity) (*Methanogenium* group 2) (Fig. 3). However, enrichment culture from EWM14 (6 to 10 cmbsf), also amended with H_2-CO_2, harbored a different methanogenic population, composed of sequences only very distantly related to *Methanogenium marinum* (93% sequence similarity; *Methanogenium* group 3) (Fig. 3).

DISCUSSION

Methanogenic populations in the Sonora Margin sediments. The methane isotopic ratio measured previously in these samples suggested that methane in the Sonora Margin shallow sediments

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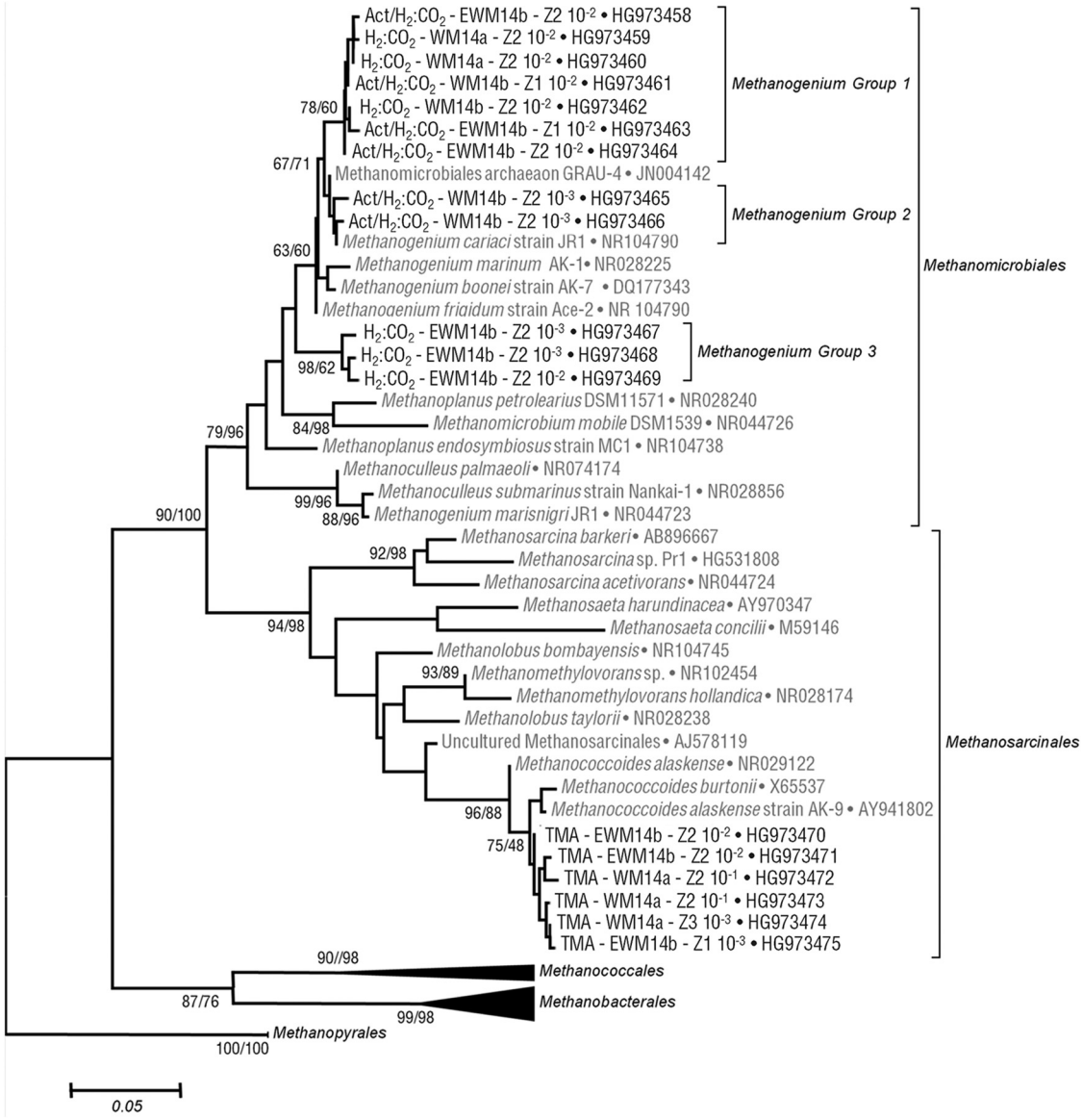

FIG 3 Neighbor-joining (NJ) phylogenetic tree of the archaeal 16S cDNA sequences amplified from selected enrichment cultures. Maximum-likelihood (ML) topologies were similar. Bootstrap support values obtained for NJ/ML analyses are reported at the nodes (1,000 replicates). Sequences from this study are in boldface. Highly similar sequences (>97% identical) from the same sample were clustered, but only one representative sequence is shown. The scale bar indicates five substitutions per 100 nucleotides.

was mainly of biogenic origin (41). Furthermore, our results show that at least 91% of the biogenic methane in surface (0 to 7 cmbsf) sediments was produced by methylotrophic methanogenesis, suggesting that among the tested substrates, methylated amines were the main methane precursors in these sediments. Occurrence of methylotrophic methanogenesis throughout the sediments was supported by detection of 16S rRNA sequences related to *M. burtonii* and *M. alaskense* in enrichment cultures amended with trimethylamine. These methylotrophic methanogens that can generate methane by disproportionation of methylated amines appear to be widespread in cold seep environments (14, 42). However, these environments might harbor only low abundances of *Methanococcoidetes* lineages, as related sequences were rarely directly detected without previous methanogenic enrichments (43–45) or specific functional-gene amplifications (46–49). Enrichment steps are generally required for the detection and identification of *Methanococcoides* lineages in cold seep sediments (13, 14, 42, 50, 51). The presence and activity of these methanogens in these sulfate-rich sediments (22 to 5 mM sulfate) (23), as observed previously in other marine sediments (14, 47, 50, 52, 53), were probably a consequence of utilization of noncompetitive methanogenic substrates, such as methylamines (17, 19, 54). Methylated amines were presumably available in the surface sediments of WM14 and EWM14, as marine invertebrates, observed in high densities over these sediments, can accumulate large amounts of osmolytes (e.g., betaine and trimethylamine *N*-oxide) and choline (widespread in cell membranes) in their tissues that can be subsequently released in the sediments and degraded to smaller methylated amines (e.g.,

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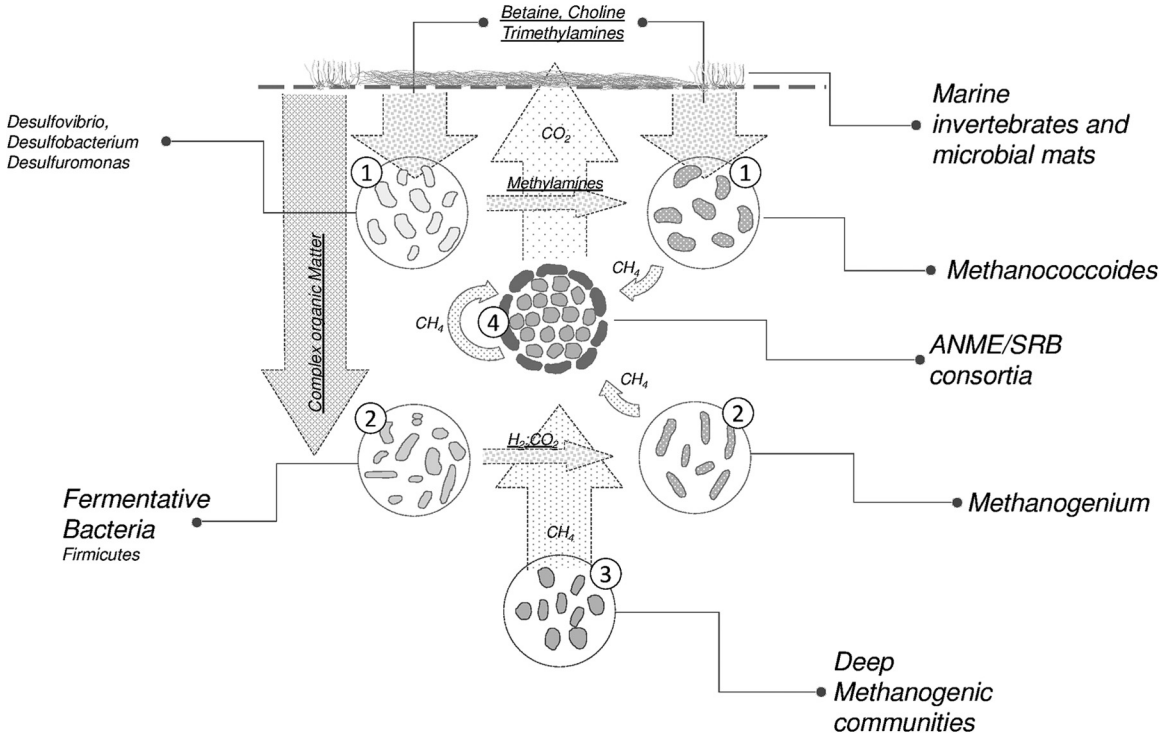

FIG 4 Hypothetical model (not to scale) of microbial methane cycling in the Sonora Margin cold seep sediments. Each microbial group is characterized by a specific function: group 1, methylotrophic methanogenesis by *Methanococcoides* lineages directly from surface organism inputs or after primary degradation by various bacteria (*Desulfovibrio*, *Desulfobacterium*, and *Desulfuromonas*) previously detected in environmental samples (60); group 2, hydrogenotrophic methanogenesis by *Methanogenium* lineages after organic-matter degradation by fermentative bacteria (*Firmicutes*) previously detected in environmental samples (24, 60); group 3, methane production by the deepest methanogenic communities detected in the deepest (1 to 9 mbsf) sediments of the Sonora Margin cold seeps (46) and other, potentially unidentified methanogens; group 4, potential methanogenesis activity of ANME communities (68, 70). SRB, sulfate-reducing bacterium.

TMA, *N*,*N*-dimethylglycine, and *N*,*N*-dimethylethanolamine) (Fig. 4) (55). For example, TMA concentrations in marine sediments were previously shown to be related to the abundance of benthic invertebrates (56). Furthermore, degradation of choline and betaine to TMA has been reported for the deltaproteobacterial lineages *Desulfovibrio* (57), *Desulfobacterium* (58), and *Desulfuromonas* (59), detected previously by a 16S rRNA survey in the Sonora Margin sediments (60). However, it has recently been demonstrated that *Methanococcoides* species can also directly utilize choline and betaine to produce methane and therefore bypass the need for the bacterial-degradation step (Fig. 4) (19, 61). Hence, the use of invertebrate-derived substrates might explain the widespread occurrence of *Methanococcoides* in organic-rich marine environments, such as cold seeps (14, 42, 44, 45), tidal flats (47, 53, 62), whale fall (63), and mangrove sediments (64), usually colonized by benthic invertebrates. These results also support studies showing cooccurrence of sulfate reduction and methylotrophic methanogenesis in marine sediments (17, 65).

In contrast to methylotrophic methanogenesis, hydrogenotrophic methanogenesis rates were below those measured previously in seep and nonseep marine sediments (<0.4 to 30 nmol cm^{-3} day^{-1} [28]) but were similar to hydrogenotrophic methanogenesis rates measured in the Amsterdam and Mercator mud volcanoes (42, 66). Although methylotrophic methanogenesis dominated in surface sediments, the proportion of hydrogenotrophic methanogenesis increased with depth, representing up to 50% of the methane production at the bottom of the EWM14 core. In these organic-rich sediments, hydrogen could be produced by fermentation of organic matter by heterotrophic bacteria (49), such as members of the phylum *Firmicutes* (Fig. 4), previously detected in significant proportions in these environmental samples (24, 60). The presence of active hydrogenotrophic methanogenesis in these sediments was also supported by the growth of methanogens in enrichment cultures amended with H_2-CO_2. All the 16S rRNA sequences detected in these enrichments were affiliated with the genus *Methanogenium* (order *Methanomicrobiales*) and were detected previously using qPCR in the original environmental samples (23). The characterized *Methanogenium* strains are psychrophilic to thermophilic methanogens (0 to 62°C), mainly isolated from marine sediments, and can use formate or H_2-CO_2 as a substrate. Three distinct lineages of *Methanogenium* were identified (groups 1, 2, and 3 [Fig. 3]) in these enrichment cultures of Sonora Margin cold seep sediments. Sequences affiliated with *Methanogenium* group 1 were detected from all enrichments amended with H_2-CO_2 and formed a distinct phylogenetic group that might represent a new lineage. A second group (*Methanogenium* group 2), closely related to *M. cariaci* (98% sequence similarity) strains previously identified in other cold seep sediments (13, 44, 67), was detected only in enrichments from sediments underlying the white mat amended with acetate and H_2-CO_2. A third group of sequences (*Methanogenium* group 3) distantly related to *M. marinum* (93% sequence

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similarity) was detected in only two enrichment cultures amended with acetate and H_2-CO_2 from EWM14 sediments (6 to 10 cmbsf) and could also represent a new genus within the order *Methanomicrobiales*. Similarly, different putative H_2-CO_2-utilizing *Methanomicrobiales* lineages related to *Methanocorpusculum*, *Methanoculleus*, and *Methanomicrobium* were also detected previously in the neighboring hot hydrothermal sediments of the Guaymas Basin (49). *Methanomicrobiales* were the only hydrogenotrophic methanogens detected in these shallow sediments, suggesting that members of this order could be responsible for most of the hydrogenotrophic methanogenesis in Sonora Margin sediments.

Acetate has been previously proposed as a significant substrate for methanogenesis in the hydrothermal sediments of the Guaymas Basin (49). However, rates of acetate methanogenesis in these cold seep sediments were very low (1/20 of H_2-CO_2 methanogenesis), as they were below the typical rates measured in these environments (28). Moreover, no methanogens were enriched with acetate as a sole carbon and energy source, although aceticlastic methanogens related to *Methanosarcina baltica* were previously detected in these sediments using different enrichment conditions (incubation temperature, 25°C) (20). Putative mesophilic aceticlastic methanogens were not detected, as opposed to the hydrothermal sediments of the basin (49), suggesting that aceticlastic methanogens in the Sonora Margin were at low abundance and therefore difficult to enrich. Aceticlastic methanogens in these sediments could also be outcompeted for acetate by sulfate-reducing communities detected previously (60) and associated with high sulfate concentrations (68).

Have we caught them all? In this study of shallow sediments of the Sonora Margin using culture-based approaches, four different methanogenic lineages were identified, whereas only one was detected from the same environmental samples, using culture-independent methods. This suggests that enrichment cultures can lower the detection limits of methanogens in these environments (14, 42). Moreover, detection of lineages affiliated with *Methanosarcinales* and *Methanomicrobiales* is consistent with previous culture-independent surveys of archaeal communities associated with the Sonora Margin shallow sediments (23, 24). Contrary to results from qPCR and pyrosequencing studies, the sizes of the amplicons in this culture-dependent study allowed phylogenetic identification and characterization of the methanogen community. However, members of the order *Methanococcales* were previously quantified in abundance similar to that of the *Methanomicrobiales* (21). Mesophilic species of the *Methanococcales* are known to be extremely sensitive to osmotic changes (26) and have also been detected in low proportion in the hydrothermal sediments of the Guaymas Basin (49). Hence, the lack of *Methanococcales* lineages in our enrichment cultures might be due to the sample depressurization during the core recovery or to unsuitable culture conditions (e.g., temperature and time of incubation). Thus, despite the identification of several methanogen lineages, all the lineages might not have been detected.

Several studies showed that Sonora Margin sediments harbor high concentrations of ANME lineages (1, 2, and 3) distributed throughout the upper 20 cm of sediments (23, 24, 60). Commonly proposed as methane oxidizers, some ANME lineages might also produce methane (68–70) and be physiologically versatile (23, 68). Despite their abundance in the environmental samples, ANME aggregates disappeared rapidly in the cultures, and no ANME sequences were detected from these methane-producing enrichments. This might suggest that ANME were not methane producers under our culture conditions. However, we could not exclude the possibility that ANME lineages could use alternative methanogenic substrates, such as methanol, as recently proposed (68).

Together, these results indicated that the high methane concentrations measured in the Sonora Margin cold seeps are partially produced in the shallow sediments by active methanogens dominated by methylotrophic *Methanococoidetes*, whereas the proportion of CO_2-reducing methanogens related to *Methanogenium* increased with sediment depth (Fig. 4). Aceticlastic methanogens represented a minority of the methanogen community. However, the methanogenic contributions of other shallow, uncultured microorganisms and ANME lineages using different substrates, as well as deeply buried microorganisms, remain to be explored.

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