AEM Accepted Manuscript Posted Online 13 March 2015
Appl. Environ. Microbiol. doi:10.1128/AEM.00147-15

Evidence of active methanogen communities in shallow sediments of the Sonora Margin cold seeps

Adrien Vigneron#[1235], Stéphane L'Haridon[23], Anne Godfroy[123], Erwan G. Roussel[1234], Barry A. Cragg[4], R. John Parkes[4] and Laurent Toffin[123]

[1]*Ifremer, Laboratoire de Microbiologie des Environnements Extrêmes, UMR6197, Technopôle Brest Iroise, BP70, Plouzané, France*

[2]*Université de Bretagne Occidentale, Laboratoire de Microbiologie des Environnements Extrêmes, UMR6197, Technopôle Brest Iroise, BP70, Plouzané, France*

[3]*CNRS, Laboratoire de Microbiologie des Environnements Extrêmes, UMR6197, Technopôle Brest Iroise, BP70, Plouzané, France*

[4]*School of Earth and Ocean Sciences, Cardiff University, Cardiff CF10 3AT, United Kingdom*

[5]*School of Civil Engineering and Geosciences, Newcastle University, Newcastle upon Tyne NE1 7RU, United Kingdom*

Running Title: Methanogens in the Sonora Margin sediments

Keywords: Archaea / methanogenesis / Guaymas Basin / Methanococcoides / *Methanogenium*

Contact of corresponding author: Dr. Adrien Vigneron ; avignero@gmail.com ; tel :+33 298 224 396 ; fax +33 298 224 557

Abstract

In the Sonora Margin cold seep ecosystems (Gulf of California), sediments underlying microbial mats harbor high biogenic methane concentrations, fuelling various microbial communities such as abundant lineages of Anaerobic Methanotrophs (ANME). However

biodiversity, distribution and metabolism of the microorganisms producing this methane remain poorly understood. In this study, measurements of methanogenesis using radiolabelled 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 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 co-occurrence of Methanosarcinales and Methanomicrobiales methanogens with abundant ANME populations in the sediments of the Sonora Margin cold seeps.

Introduction

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 fuelled 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 at 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 to 7 orders within the phylum *Euryarchaeota* which includes 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 in anaerobic conditions (9-18). In marine sediments, methylated compounds (e.g. methylamine, dimethylamine, trimethylamine, methanol, dimethylamine-sulfate, dimethylsulfide), volatile fatty acids (formate, 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 these 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 only detected rarely in previous studies using clone libraries (23) and 454 pyrosequencing (24). Furthermore, quantitative measurements using real-time 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 number) in the shallow sediment layers (0-17 cmbsf)(23). In contrast, Methanosarcinales related to anaerobic methanotrophs (ANME), previously found active and abundant in these sediments (23), dominated throughout the shallow sediments of the Sonora Margin (30 to 92% of the total

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78 archaeal 16S rRNA gene copy number). Thus activity and biodiversity of methanogenic
79 microbial populations remained unclear.

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

85 **Material and Methods**

86 *Sediment samples.*

87 Sediment samples were collected from Sonora Margin cold seeps during the oceanographic
88 cruise BIG (Ifremer) with the R.V. *L'Atalante* and the D.S.V. *Nautile* in June 2010. Two
89 different habitats from the site Vasconcelos (27°35.577'N; 111°28.984'W), sampled in
90 triplicate using 20 cm length push cores (PC), were selected for enrichment cultures and
91 activity measurements: i) an extended white microbial mat (White Mat 14 thereafter WM14 ;
92 PC1, PC2 and PC3) characterized by an average methane concentration of 900 µM
93 throughout the core (Figure 1) and ii) the surrounding macrofauna (Edge of White Mat 14
94 thereafter EWM14 ; PC6, PC8 and PC11) characterized by an average methane
95 concentration of 500 µM (Figure 1) (23). Before each sampling, autonomous temperature
96 sensors (T-Rov; NKE Electronics, Hennebont, France) recorded *in situ* temperatures around
97 3°C from the sediment surface down to 40 cmbsf on each habitat. On board, sediment cores
98 were transferred in a cold room immediately after retrieval and sectioned aseptically in 2 cm
99 thick layers. For enrichment cultures, 6 cm^3 of each sediment layer were transferred into 50
100 ml vials containing 10 ml of sterile and anoxic artificial sea water (composition in DSMZ
101 medium 246a). Vials were crimp sealed with butyl rubber septa stoppers and aluminum
102 crimp tops (Bellco Glass Inc, Vineland, NJ, USA), then flushed with N_2 and stored at 4°C
103 under 200 kPa N_2:CO_2 (80:20) gas atmosphere. For activity measurements, PC2 and PC6

were sub-sampled using triplicate mini push-cores. These mini push-cores were hermetically sealed under N_2 gas atmosphere in aluminum bags (Grüber-Folien, Germany) and stored at 4°C for processing back to 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 excepted yeast extract with a final concentration of 0.2 g l^{-1}. The medium was prepared and sterilized under 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 H_2:CO_2 or N_2:CO_2 gas atmosphere and trimethylamine (TMA, 20 mM) were used. One ml of sediment suspension from different sections (0-6 cmbsf, 6-10 cmbsf and 10-15 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 negative controls for each condition (Table 1). In order to enhance microbial growth, all cultures were incubated at 12°C, greater than the average *in situ* temperature (3°C). Cultures were periodically checked (every month) for methane production for two years. Methane concentrations in the vial headspace of culture were determined by using a micro MTI M200 Gas Chromatograph (SpectraLab, Markham, Canada) equipped with MS-5A capillary column and Poraplot U capillary column (Agilent Technologies, Santa Clara, CA, USA) via sterile needle. Presence of putative methanogenic communities from methane producing enrichments was confirmed by epifluorescent microscopy (BX60 equipped with U-RFL-T, Olympus, USA). Enrichments were stopped when more than 50 UV autofluorescent cells per microscopic field (X1000) were detected. Renewal of carbon and energy sources (200 kPa of H_2:CO_2 or 10 mM of acetate) was anaerobically and sterilely carried out after one year of incubation.

Methanogenic activity measurements.

Potential rates of methanogenesis were monitored on anaerobically stored subsamples using ^{14}C radiolabeled substrates, three months post-cruise, at Cardiff University, UK. Subsamples were pooled in 42 cm^3 of sediment slurries, corresponding to 7 cm thick sediment layers (0-7 cm and 7-14 cm for WM14 and 0-7 cm, 7-14 cm and 14-21cm for EWM14), then dispensed into 20 ml vials before injection of labelled substrates. Triplicate vials were monitored with addition of radiotracers (^{14}C-bicarbonate [19 µl containing 74 kBg], ^{14}C-acetate [19 µl containing 397 kBg], ^{14}C-di-methylamine [19 µl containing 176 kBg]) for each sediment section. Additional vials were monitored without radiotracers as negative controls. Vials were incubated at close to *in situ* temperatures (4°C) with magnetic agitation. Activity measurements were terminated by addition of 1 M NaOH then processed as described previously (28). Methanogenesis rates were calculated based on the proportion of labelled gas produced from the ^{14}C-substrate, the incubation time period, an assumed sediment porosity of 70% and the measured cold pool size of the substrate. Because incubation conditions were not identical to the original sediment conditions, 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 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 Quanta qScript® kit according to the manufacturer's protocol (Quanta Bioscience, Gaithersburg, MD, USA).

PCR-DGGE of 16S rRNA.

PCR-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 PCR reactions were carried out in a final volume of 25 µl using the GoTaq polymerase kit (Promega, Madison, WI, USA) according to manufacturer's recommendations. 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, then PCR products were re-amplified 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). PCR 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). PCR conditions were as follows: 30 cycles of denaturation step at 94°C for 40s, annealing at 57°C for 1 min 30s and extension at 72°C for 3 min. PCR products were purified on agarose gel then cloned using 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. Sequences were analyzed using NCBI BLAST search program GenBank (35) and aligned with the closest representative sequences using Mafft program (36). Sequences data were analyzed with the MEGA 4.0.2 program (37). Phylogenetic trees were estimated with maximum likelihood and neighbor-joining methods using RAxML 7.2.8 (38) with GTRCAT approximation of model and the Kimura two parameters correction matrix coupled to pairwise deletion parameters respectively. The robustness of inferred topology was tested by bootstrap resampling (1000). Sequences were

deposited in the EMBL database under the following accession numbers: HG973458-HG973475.

Results

Potential activity measurements.

Methanogenic activities were 54-29% higher in WM14 sediments compared to EWM14 sediments. In both sediments cores, total methanogenesis rate decreased with depth (Figure 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} d^{-1}, t-test p-value : 0.04), it consistantly represented the major methanogenesis processes (91-83% of the total methanogenesis). In contrast, although hydrogenotrophic methanogenesis was relatively steady (36-49 pmol cm^{-3} d^{-1}, t-test p-value : 0.58) throughout WM14, it represented a higher proportion of the total methanogenesis at depth (0-7 cmbsf: 8%; 7-14 cmbsf: 16%). However, aceticlastic methanogenesis remained low (5 pmol cm^{-3} d^{-1}) throughout WM14 sediments, representing 1% of the total methanogenesis. In EWM14 sediments, although methylotrophic methanogenesis also dominated methanogenic processes in the upper sediment section (98% of the total methanogenesis), it decreased markedly (ANOVA p-value : 0.001) with depth only representing 48% of the total methanogenesis (7 pmol cm^{-3} d^{-1}) in sediments below 14 cmbsf. In contrast, hydrogenotrophic methanogenesis in EWM14 sediments remained relatively constant (≈8 pmol cm^{-3} d^{-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} d^{-1} (2% of the total methanogenesis).

Methanogenic enrichments.

After two 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 growths were not detected in 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 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 only restricted to methanogens as it has also been detected in anaerobic methanotrophic communities and archaeal Marine Group 1 (40). However, UV autofluorescent cells were only detected 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 positive 33 different culture conditions were analyzed using RT-PCR-DGGE (Figure 2A). The archaeal community structures grouped in seven different clusters mainly correlated to the carbon substrates used (Figure 2). In order to phylogenetically identify the active methanogens, 16S rRNA clone libraries from eleven positive enrichment cultures were analyzed (underlined in Figure 2B). As RT-PCR-DGGE profiles showed relatively limited archaeal diversity within the enrichments, only ten clones per library were sequenced. For each clone library all ten 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 Methanosarcinales order. 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; Figure 3). Sequences obtained from enrichment culture from WM14 (6-10 cmbsf) amended with acetate and H_2:CO_2 were closely related to *Methanogenium cariaci* (98% sequence similarity) (*Methanogenium* group 2; Figure 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; Figure 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 was mainly from biogenic origin (41). Furthermore, our results show that at least 91% of the biogenic methane in surface (0-7 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 these 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 *Methanoccocoidetes* 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). Presence and activity of these methanogens in these sulfate-rich sediments (22-5 mM of sulfate) (23), as observed previously in other marine sediments (14, 47, 50, 52, 53), were probably a consequence of utilization of non-competitive methanogenic substrates such as

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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, 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. TMA, N,N-dimethylglycine, N,N-dimethylethanolamine; Figure 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 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 need for the bacterial degradation step (Figure 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 co-occurrence 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 non-seep marine sediments (<0.4-30 nmol cm^{-3} d^{-1} (28)) but were similar to hydrogenotrophic methanogenesis rates measured in 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 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 *Firmicutes* phylum (Figure 4), previously detected in significant proportions in these environmental samples (24,

60). Presence of active hydrogenotrophic methanogenesis in these sediments was also supported by growth of methanogens in enrichment cultures amended with H_2:CO_2. All 16S rRNA sequences detected in these enrichments were affiliated to the genus *Methanogenium*, (order Methanomicrobiales) and detected previously using Q-PCR in the original environmental samples (23). Characterized *Methanogenium* strains are psychrophilic to thermophilic methanogens (0-62°C) mainly isolated from marine sediments and can use formate or H_2:CO_2 as substrates. Three distinct lineages of *Methanogenium* were identified (Groups 1, 2 and 3; Figure 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 which 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 only detected 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 similarity) was only detected in 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).

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Moreover, no methanogens were enriched with acetate as 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 in 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 detection limits of methanogens in these environments (14, 42). Moreover, detection of lineages affiliated to 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, size of the amplicons in this culture-dependent study allowed phylogenetic identification and characterization of the methanogen community. However, members of the Methanococcales order were previously quantified in similar abundance to Methanomicrobiales (21). Mesophilic species of the Methanococcales are known to be extremely sensitive to osmotic changes (26) and have been also 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, time of incubation). Thus, despite the identification of several methanogenic lineages, all methanogen lineages might not have been detected.

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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 also 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 with our culture conditions. However we could not exclude 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 (Figure 4). Aceticlastic methanogens represented a minority of the methanogen community. However the methanogenic contribution of other shallow uncultured microorganisms and ANME lineages using different substrates as well as deeply buried microorganisms remains to be explored.

Acknowledgments

We are indebted to the crews of the research vessel *L'Atalante* and the submersible *Nautile* of the cruise "BIG" and the scientific team for their help on board. This cruise was funded by IFREMER (France) and has benefited from a work permit in Mexican waters (DAPA/2/28100*/3803, 28 October 2009). This study was supported by an IFREMER PhD grant.

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Legends for Figures

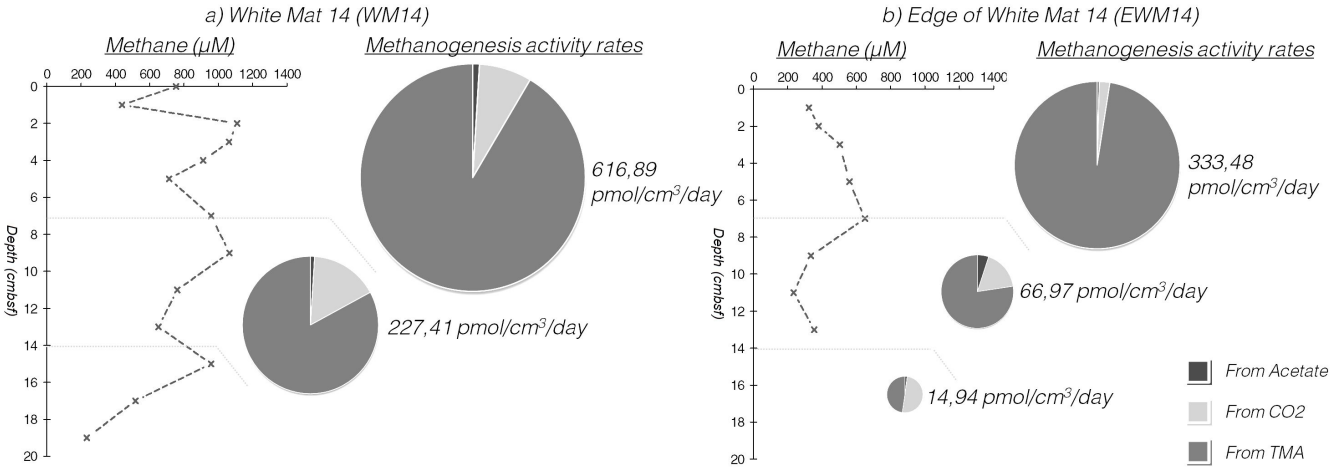
Figure 1: Sediment depth profiles of methane concentrations and methanogenesis rates. (A) White Mat 14. (B) Edge of White Mat 14. Relative proportions of acetate, bicarbonate and dimethylamine methanogenesis rates ($pmol.cm^3.d^{-1}$) are represented in pie charts for each sediment section. The size of the pie chart is proportional to the total methanogenesis rate. Methane concentrations were from Vigneron et al. 2013.

Table 1: Table summarizing enrichment conditions (trimethylamine, acetate, H_2:CO_2, H_2:CO_2 and acetate) and level of dilution (10^{-1}, 10^{-2}, 10^{-3}) applied to each sample. + indicate positive enrichment (methane production and detection of UV autofluorescent cells). Empty cells indicate no methane accumulation in the headspace. Z1: 0-6 cmbsf; Z2: 6-10 cmbsf; Z3: 10-15 cmbsf.

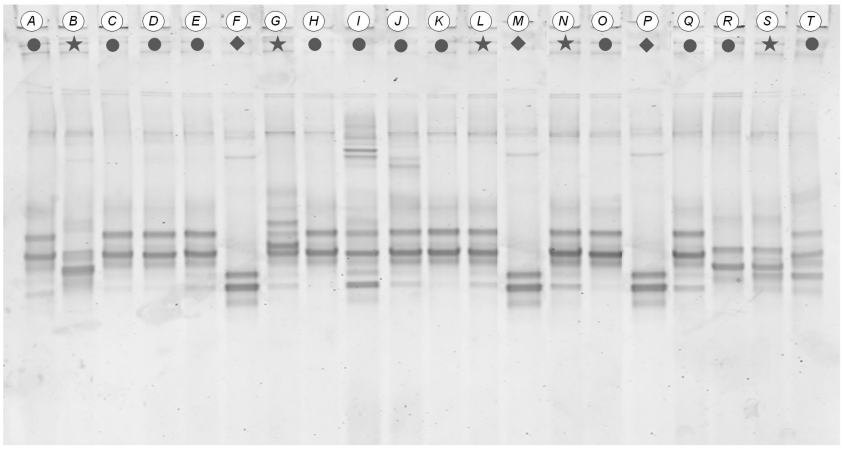
Figure 2: DGGE analysis of the 16S rRNA archaeal diversity. A) DGGE profiles for 20 samples represented by a letter (A-T) in the dendrogram. The different Media were represented by symbols: Black dot : Acetate/H_2:CO_2, Black Star : H_2:CO_2, Black Diamond : Trimethylamines. B) Dendrograms from cluster analysis of DGGE profiles. Underlined samples indicate samples selected for analysis of the phylogenetic diversity of methanogens. Act: Acetate; TMA: trimethylamines; WM14a: White Mat 14 PC 3; WM14b: White Mat 14 PC 4; EWM14a: Edge of White Mat 14 PC 8; EWM14b: Edge of White Mat 14 PC 11; Z1: 0-6 cmbsf; Z2: 6-10 cmbsf and Z3: 10-15 cmbsf.

Figure 3: Neighbor-Joining phylogenetic tree of the archaeal 16S cDNA sequences amplified from selected enrichment cultures. Maximum Likelihood (ML) topology was similar. Bootstrap supports obtained for NJ/ML analyses are reported at nodes (1000 replicates). Sequences from this study are highlighted in black. Only one representative sequence (>97% identical) is shown. Act: Acetate; TMA: tri-methylamines; WM14a: White Mat 14 PC 3; WM14b: White Mat 14 PC 4; EWM14a: Edge of White Mat 14 PC 8; EWM14b: Edge of White Mat 14 PC 11; Z1: 0-6 cmbsf ; Z2: 6-10 cmbsf and Z3: 10-15 cmbsf.

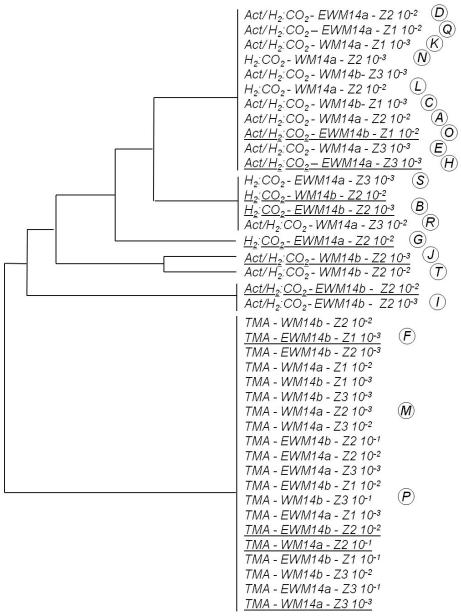
Figure 4: Hypothetical model (not to scale) of microbial methane cycling in the Sonora Margin cold seep sediments. Each microbial group was characterized by a specific function: 1). Methylotrophic methanogenesis by *Methanococcoides* lineages directly from surface organism inputs or after primary degradation by various bacteria (*Desulfovibrio*, *Desulfobacterium*, *Desulfuromonas*) previously detected in environmental samples (22). 2). Hydrogenotrophic methanogenesis by *Methanogenium* lineages after organic matter degradation by fermentative bacteria (Firmicutes) previously detected in environmental samples (24-60). 3). Methane production by deepest methanogenic communities detected in deepest (1-9 mbsf) sediments of the Sonora Margin cold seeps (46) and potentially unidentified other methanogens. 4). Potential methanogenesis activity of ANME communities (68, 70).

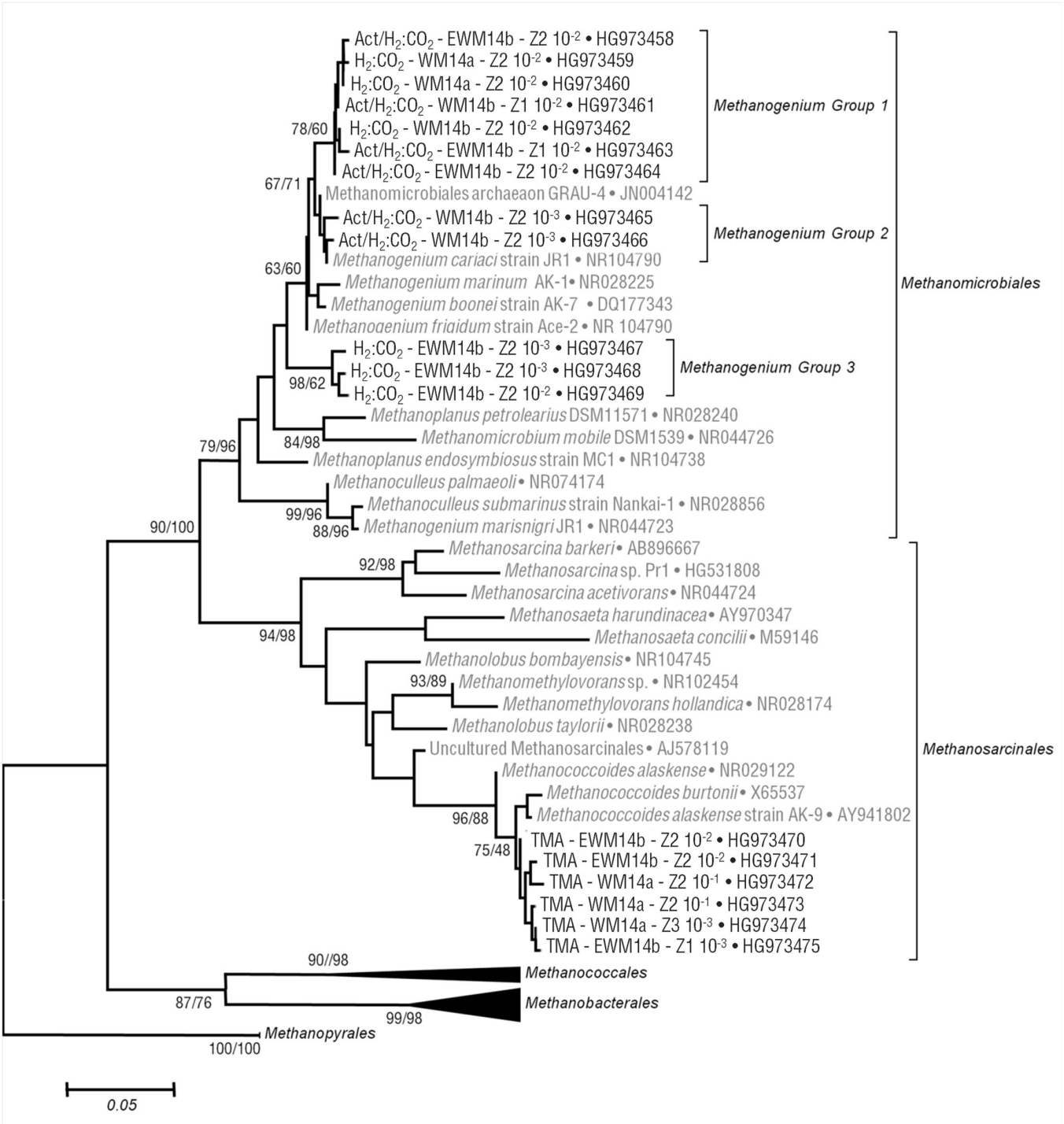


A) DGGE fingerprinting profiles



B) Cluster analysis of DGGE fingerprintings





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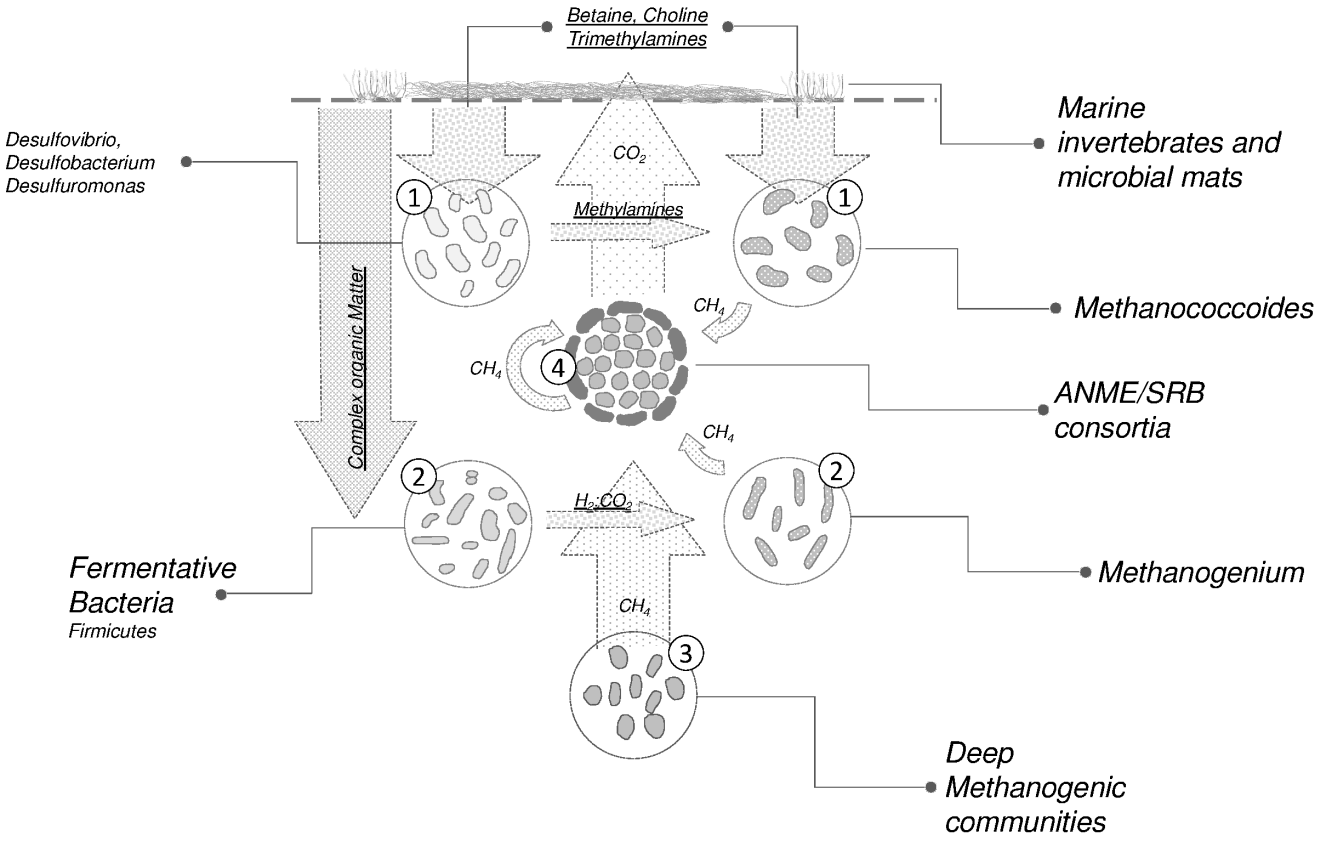

Table 1: Table summarizing enrichment conditions (trimethylamine, acetate, H_2:CO_2, H_2:CO_2 and acetate) and level of dilution (10^{-1}, 10^{-2}, 10^{-3}) applied to each sample. + indicate positive enrichment (methane production and detection of UV autofluorescent cells). Empty cells indicate no methane accumulation in the headspace. Z1: 0-6 cmbsf; Z2: 6-10 cmbsf; Z3: 10-15 cmbsf.

White Mat 14 (WM14)						*Edge of White Mat 14 (EWM14)*						
PC3			*PC4*			*PC8*			*PC11*			
Z1	*Z2*	*Z3*	*Z1*	*Z2*	*Z3*	*Z1*	*Z2*	*Z3*	*Z1*	*Z2*		
	+	+		+	+	+	+	+	+	+	10^{-1}	*Trimethylamines*
+	+	+	+	+	+	+	+	+	+	+	10^{-2}	
+	+	+	+	+	+	+	+	+	+	+	10^{-3}	
											10^{-1}	*Acetate*
											10^{-2}	
											10^{-3}	
+	+	+	+	+							10^{-1}	*H_2:CO_2*
+	+	+	+	+	+	+	+	+	+	+	10^{-2}	
+	+	+	+	+	+	+	+	+	+	+	10^{-3}	
+	+	+	+	+	+	+	+	+	+		10^{-1}	*H_2:CO_2 Acetate*
+	+	+	+	+	+	+	+	+	+	+	10^{-2}	
+	+	+	+	+	+	+	+	+	+	+	10^{-3}	