

A phylogenetic analysis of microbial communities associated with methane hydrate containing marine fluids and sediments in the Cascadia margin (ODP site 892B)

Kelly A. Bidle^a, Miriam Kastner^b, Douglas H. Bartlett^{a,*}

^a Marine Biology Research Division, Scripps Institution of Oceanography, 8602 La Jolla Shores Dr., La Jolla, CA 92093-0202, USA

^b Geosciences Research Division, Scripps Institution of Oceanography, La Jolla, CA 92093-0202, USA

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Abstract

Methane hydrates represent an enormous carbon and energy source in many low temperature deep marine sediments. However, little information is available concerning the nature of the microbial communities associated with these structures. Here, we describe a phylogenetic analysis based on ribosomal DNA (rDNA) sequences obtained from sediment and fluid samples present in a region of gas hydrate formation in shallow sediments within the Cascadia margin in and around Ocean Drilling Program (ODP) Site 892B. Our studies detected diverse sulfur-utilizing microbes, methanogens, methanotrophs, and non-thermophilic members of the kingdom Crenarchaeota. This is the first culture-independent phylogenetic analysis of a gas hydrate habitat. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Gas hydrates, also called gas clathrates, are crystalline compounds composed of water molecules that form rigid cage-like lattice structures. Most of these structures are occupied by gas molecules in nature, primarily methane [1]. Much of the current interest in gas hydrates stems from the fact that they are estimated to represent the largest hydrocarbon fuel reservoir on earth, pose a potential geohazard in

submarine slopes, and may be a source of atmospheric greenhouse methane gas. The stability of gas hydrates depends on low temperature, elevated pressure, and the appropriate chemical environment, therefore, the distribution of gas hydrates is primarily restricted to polar and deep oceanic regions. The gas hydrate occurrences in the shallow geosphere are associated with subduction zones in the uppermost few hundred meters [2].

The limited information available on the microbial ecology of gas hydrate regions suggests that microbes associated with C-1 metabolism and the sulfur cycle are dominant members of this environment. Carbon and hydrogen isotope ratios indicate that the

* Corresponding author. Tel.: +1 (619) 534-5233; Fax: +1 (619) 534-7313; E-mail: dbartlett@ucsd.edu

methane in most gas hydrate regions is primarily of microbial rather than thermogenic origin [1]. Anaerobic methane oxidation has been detected in numerous methane-containing sediments [3–8], but the isolation of an anaerobic microorganism capable of extensive methane oxidation has proved unsuccessful despite numerous attempts (reviewed in [9]). Thus far, only methanogens and sulfate reducers have been found capable of oxidizing methane, but only in very small amounts [10,11]. Several reports have observed a coincidence of methane oxidation and sulfate reduction in diverse marine environments [4–6,12], suggesting a possible role for sulfate reducers in the anaerobic oxidation of methane. For example, Zehnder and Brock [13] have speculated that increased methane oxidation by methanogens could occur if the products of methane oxidation were consumed by a microbial consortium including sulfate-reducing bacteria. However, inhibitor studies do not indicate a direct link between the two processes [3].

This study addresses the microbial diversity associated with high methane levels in and around Ocean Drilling Program (ODP) site 892B located within the Cascadia margin off the central Oregon coast. This site is characterized by active venting of methane-enriched pore fluids along a fault zone and surface sediment communities of clams and tube worms [4,14,15]. The top 20 m of this site have high hydrogen sulfide levels (>19 000 ppmv) and disseminated CH₄-H₂S hydrate [15,16]. Elsewhere within the Cascadia margin sediments off Vancouver Island increased bacterial abundance, sulfate reduction, methane oxidation, and culturable sulfate-reducing bacteria are present in deep sediment gas hydrate zones [4,17].

In recent years, it has become increasingly popular to utilize the techniques of the polymerase chain reaction (PCR) and DNA sequencing to assess the makeup of an environmental microbial population, thereby removing the need for and bias associated with culturing (reviewed in [18]). These techniques exploit the differences found at the level of rDNA molecules to identify the microbial members of a particular niche or community. Here we have used this cultivation-independent approach to provide the first general phylogenetic description of gas hydrate microbial communities, including analyses of both the Bacterial and Archaeal domains.

2. Materials and methods

2.1. Site description and collection of samples

All samples for this study were collected on the RV Atlantis/Alvin during a cruise in June 1998. Sediment samples for prokaryotic community analysis were collected from push cores taken at two adjacent sites (water depth ~675 m) designated Bioherm 'Dead Clam' (44°40.51'N 125°07.41'W) and 'Champagne Site' (44°40.45'N 125°07.374'W), which were determined to possess high levels of methane (Table 1 and Section 3). Fluids (~1 l) from the borehole at ODP site 892B on the Cascadia margin (44°40.537'N 125°07.086'W) were collected and passed through a 0.22-µm Sterivex-GS filter (Millipore) connected to a peristaltic pump. Sterivex filters were immediately frozen at -20°C on board ship and processed in our laboratory at SIO prior to community analysis.

2.2. Isolation and manipulation of DNA

Total DNA was isolated from ~5 g of sediment according to the methods of Zhou et al. [19]. All samples were taken ~20 cm below the top surface of the sediment from the centers of the push cores using sterile syringes, thus eliminating the possibility of introducing contaminants. Samples from 'Champagne Site' were processed within 1 h of the time they were brought on board ship, while samples from the 'Dead Clam' site were processed 6 weeks after the cruise, following storage at 4°C. Further purification of all sediment samples was necessary prior to PCR and was accomplished using Elutip-D columns (Schleicher and Schuell). Total DNA was isolated from borehole fluid samples from the Sterivex filters according to the protocols of Sommerville et al. [20]. No further DNA purification of fluid samples was necessary prior to PCR.

2.3. PCR, cloning, and RFLP analysis

Domain-specific primers (Bacterial, Archaeal, and Eukaryal [21]) were used in PCR, as well as primer sets specific for sulfate reducers [22] and methanogens [23]. PCR conditions in all cases were as follows: 1 min 92°C, 1 min 48°C, 1 min 72°C, for 25 cycles. Amplification products were cloned into the

pCR2.1-TOPO vector (Invitrogen). Primer sets were used to re-amplify insert DNA from individual clones to generate template DNA to be used in restriction fragment length polymorphism (RFLP) analysis. Template DNA was subjected to restriction digest using *Hae*III and *Msp*I (Promega) and subsequently electrophoresed through a 1.5% agarose gel. Plasmid DNA was isolated from all clones displaying unique RFLP patterns using the QIAprep plasmid purification kit (Qiagen). Duplicate clones were not further analyzed.

2.4. Sequence and phylogenetic analysis

DNA sequencing was performed using PCR cycle sequencing with either Prism or BigDye Terminator Ready Reaction mix (Applied Biosystems). Insert-specific, M13 reverse, and T7 oligonucleotide primers were used to determine partial nucleotide sequences of individual clones. The approximate numbers of base pairs of DNA generated from sequencing analysis were as follows: 950 bp of Archaeal 16S rDNA, 1.2 kb of Bacterial 16S rDNA, 550 bp of sulfate reducer 16S rDNA, and 750 bp of the methanogen *mcrA* gene. Phylogenetic analysis of sequences from this study as compared to the sequences of closely related organisms was performed using the Clustal W program [24] in conjunction with Neighbor Joining Plot [25] for tree construction.

2.5. Nucleotide sequence accession numbers

The GenBank accession numbers for the sequences used in the phylogenetic analyses are as follows: *T. denitrificans* (L40808); *D. toluolica* (X70953); *D. rhabdoformis* (U12253); *Desulfobulbus* sp. BG25 (U85473); *D. sulfoexigens* (Y13672); *D. magnum* (U45989); *T. tenax* (M35966); *P. occultum* (M21087); *D. mobilis* (M36474); *T. pendens* (X14835); *S. solfataricus* (D26490); *C. symbiosum*

(U51469); SB95-53 (U78197); TS10C299 (O52946); PVA-OTA-4 (U46680); PVA-OTA-2 (U46678); SB95-57 (U78199); JTA47 (O15277); ANTARCTIC 12 (U11043); SB95-8 (U78195); SB95-54 (U78198); SBAR5 (M88075).

Partial sequences of the 10 Bacterial and seven Archaeal 16S rRNA sequences used to generate the phylogenetic trees used in these studies and the three methanogen *mcrA* genes were submitted to GenBank and have the following accession numbers: ODPB-U4 (AF121082); ODPB-U9 (AF121083); ODP8-U1 (AF121084); ODP8-U3 (AF121085); ODP8-U6 (AF121086); ODP8-U10 (AF121087); ODPB-B3 (AF121088); ODPB-B4 (AF121089); ODPB-B7 (AF121090); ODPB-B9 (AF121091); ODPB-A2 (AF121092); ODPB-A3 (AF121093); ODPB-A6 (AF121094); ODPB-A7 (AF121095); ODPB-A9 (AF121096); ODPB-A12 (AF121097); ODPB-A18 (AF121098); ODP8-ME1 (AF121099); ODP8-ME2 (AF121100); ODP8-ME6 (AF121101).

3. Results and discussion

Pore water chemistry was performed on sediment samples and borehole fluids [26]. Chemical analysis revealed elevated levels (32 μ M–1.96 mM) of methane present in the Champagne Hill and Dead Clam sediment pore fluid, with slightly lower levels in the borehole fluid (Table 1). For comparison, CH₄ levels in shallow marine environments approach levels of \sim 10 nM [26]. Examination of sulfate levels from these sites showed that the SO₄ concentrations at Champagne Hill and ODP site 892 (\sim 28 mM) were comparable to those found in seawater [27]. The Dead Clam site was noticed to contain significantly lower SO₄ levels (\sim 17.4 mM) with increased amounts of H₂S, indicating that active sulfate reduction was occurring at this site.

The biological makeup of these sites was next ex-

Table 1
Methane and sulfur chemistry from the three different sites examined

Location	Type	SO ₄ (mM)	CH ₄ (mM) ^a	H ₂ S present
Champagne Hill (44°40.45'N 125°07.374'W)	push core	30.2	1.96	trace
Dead Clam (44°40.51'N 125°07.41'W)	push core	17.4	1.82	yes
Site 892 (44°40.537'N 125°07.086'W)	borehole fluid	28.3	0.032	trace

^aAssuming 85% porosity.

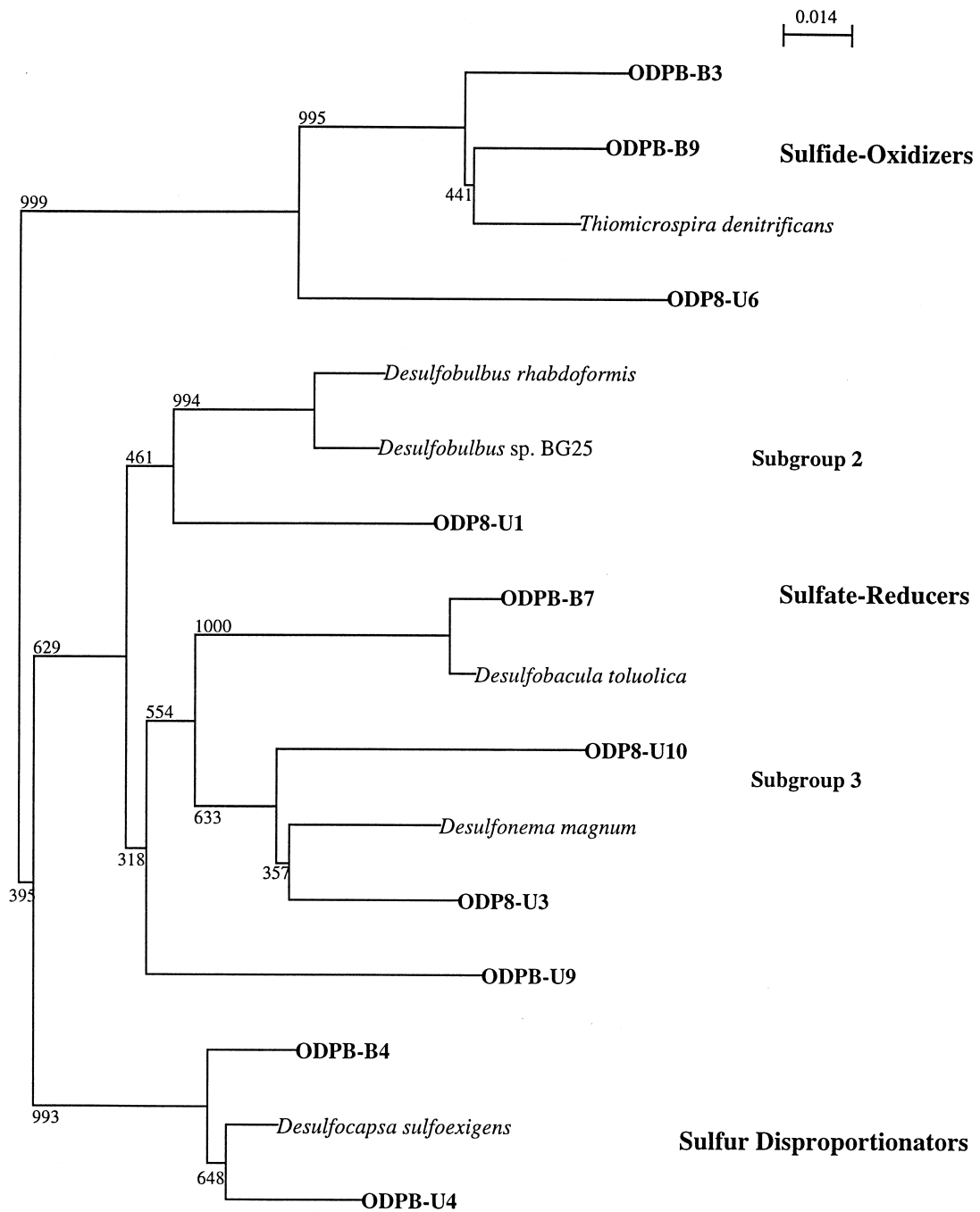


Fig. 1. Phylogenetic analysis of bacteria that utilize sulfur compounds, using ~550 bp of internal 16S sequence. ODPB, borehole fluid clones; ODB8, Bioherm clones. *E. coli* was used as the outgroup. Bootstrap values were derived from 1000 analyses. Scale bar, 0.014 estimated substitutions per nucleotide.

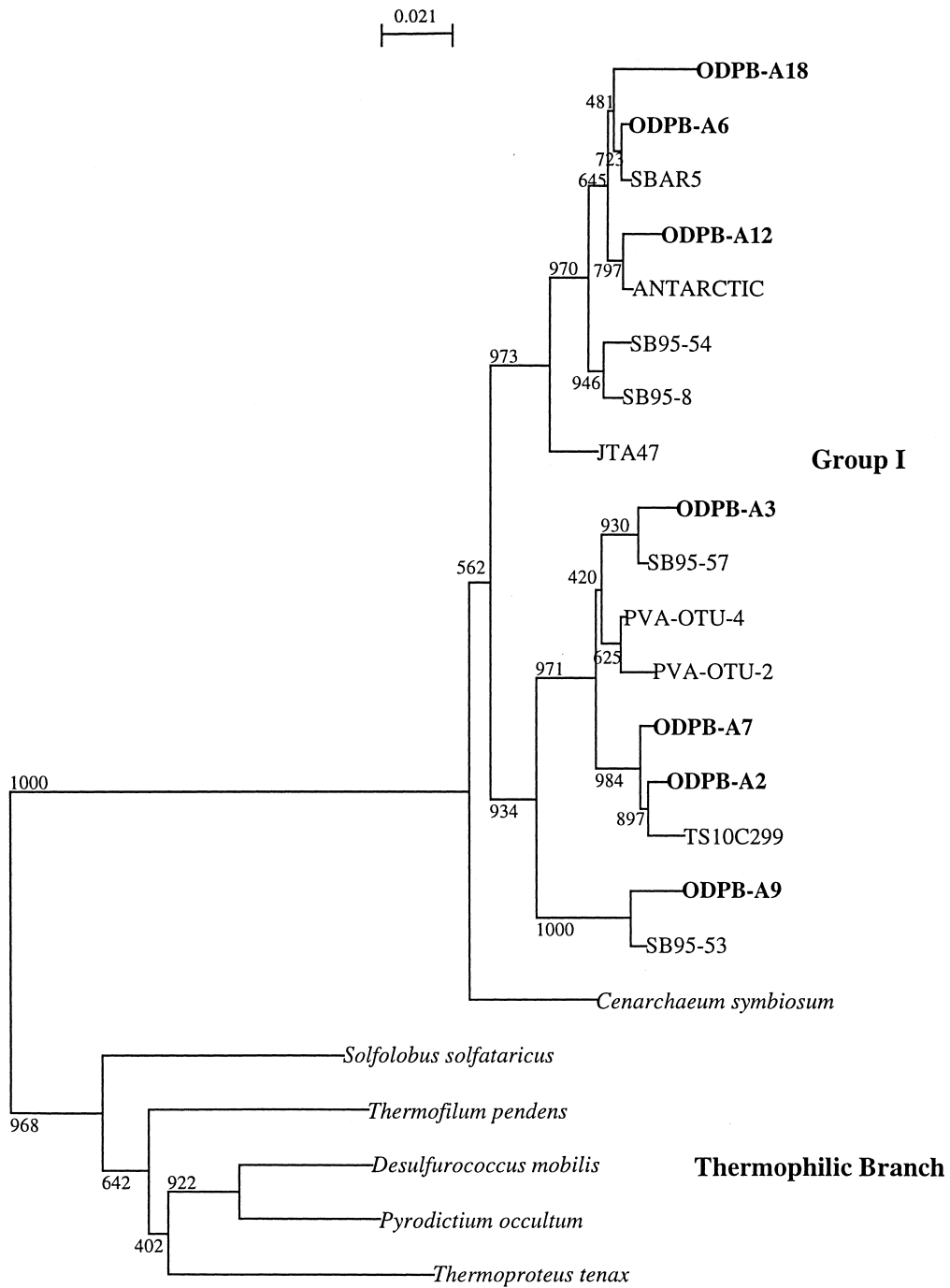


Fig. 2. Phylogenetic analysis of group I marine crenarchaeotes recovered from the borehole fluids, using ~900 bp of internal 16S sequence. *Methanococcus jannaschii* was used as the outgroup. Bootstrap values were derived from 1000 analyses. Scale bar, 0.021 estimated substitutions per nucleotide.

aminated. Following DNA extractions of both water and sediment samples, PCR amplification, cloning, and RFLP analysis, a total of 33 unique clones were subjected to sequencing analysis and phylogenetic tree reconstruction. Of these, seven were derived from Archaeal primers, 23 from Bacterial or sulfate-reducing primers, and three from methanogen-specific primers. No Eukaryal sequences were amplified.

Prior studies examining the diversity of bacteria from both shallow and deep marine sediments revealed that community composition is complex and encompasses several major groups within the Bacterial domain [28,29]. In this study, we found that sulfur-utilizing bacteria dominated the sediments and fluids of gas hydrate zones. Approximately half of the Bacterial 16S sequences corresponded to organisms which reduce, oxidize and/or disproportionate sulfur compounds. These results are qualitatively similar to those of Barnes et al. [17] who cultured sulfate-reducing bacteria from a deep subsurface gas hydrate zone also located within the Cascadia margin. However, whereas these investigators cultured exclusively *Desulfovibrio*-related bacteria, the sulfate reducers identified by our methods were more diverse, being related to both those bacteria that oxidize organic substrates incompletely to acetate (subgroup 2) and those that oxidize organic substrates completely to CO₂ (subgroup 3). More specifically, of these clones, five of the sequences were found to group with a variety of sulfate reducers, two grouped with *Desulfocapsa sulfoexigens*, a novel bacterium capable of inorganic sulfur disproportionation [30], and three were found to group with the sulfide-oxidizing, facultative anaerobe *Thiomicrospira denitrificans* (Fig. 1). The 13 remaining bacterial clones grouped to a range of different facultative anaerobes and anaerobic respirers, including species of *Shewanella*, *Cytophaga*, *Pseudoalteromonas*, and *Cobwellia*, in addition to iron reducers (*Geobacter* spp.) and a methanotroph (*Methylocaldum* sp.). It is noteworthy that while no sulfur specialists per se were identified from the Champagne Hill sediments, a number of 16S sequences were identified from these DNA samples that were homologous to sequences in the database that were obtained from environmental samples capable of hydrocarbon degradation and benzene mineralization.

Of the seven unique clones obtained using Archaea universal primers, all were from DNA obtained from the borehole fluid, and all were found to belong to the group I branch of marine crenarchaeotes (Fig. 2). These are the non-thermophilic, marine crenarchaeotes that predominate in non-surface waters (below 100 m [31]). To date there is no cultured organism from this group, thus all of the sequence comparisons are with uncultured isolates. Since the original seawater introduced into the borehole during its construction would have been expelled because of the overpressure in this well [32], our results suggest that these crenarchaeotes are autochthonous members of the marine sediment pore fluid or seawater within the fault zone.

In addition to identifying members of the Archaea with 16S primers, a primer set targeting the α subunit of the methyl coenzyme M reductase gene (*mcrA*), a key enzyme in the process of methanogenesis, was used. This primer set yielded three unique methanogen-specific sequences, all grouping within the family Methanosarcinaceae, from the DNA isolated from the Dead Clam sediments. Curiously, this DNA sample did not produce an amplification product with the Archaeal universal primers despite the fact that these primers routinely amplify both Crenarchaeal and Euryarchaeal (including methanogen) sequences ([21]; our unpublished results). The reason for this apparent discrepancy is unknown, but it could reflect differences in the proportion of methanogen and other Archaeal rDNA present in the DNA preparations. It may also be noteworthy that among the three gas hydrate DNA samples examined the Dead Clam site DNA preparation was not processed until several weeks after the initial collection trip. Thus, the microbial population in this sample could have changed during sample handling and incubation in a 4°C cold room. The fact that two out of three of the gas hydrate DNA samples did not contain detectable levels of methanogen sequences is not surprising as typical continental margin sediments contain an insufficient amount of organic carbon (~0.5–1.0%) available for conversion to the high levels of methane present in gas hydrate zones. Thus, it has been postulated that the site of methane production is most likely at greater burial depths and/or far from the gas hydrate layers in which the methane eventually concentrates [2].

Taken together, the results from the chemical and biological analyses are noteworthy in several regards. First, numerous sulfur-utilizing bacterial 16S sequences were found in these gas hydrate regions, which substantiates the culturing results of Barnes et al. [17] and supports previous work that suggests a possible role for methane oxidation by sulfate reducers [13]. Second, active sulfate reduction appears to be occurring in at least one of the three sites examined, based on the presence of H₂S and may be attributed to the sulfate-reducing bacteria identified in these studies. Third, elevated methane levels, particularly in the sediment pore fluids, appear to support communities of methanotrophs and bacterial species related to those found in other hydrocarbon environments. Fourth, methanogens do not appear to be highly abundant in two-thirds of the methane hydrate environments examined. It will now be possible to design probes for in situ quantitation of the abundance of particular phylotypes in gas hydrate regions, as well as to consider culturing techniques appropriate to the microbes identified here.

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