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Bacteria and *Archaea* Physically Associated with Gulf of Mexico Gas Hydrates

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Although there is significant interest in the potential interactions of microbes with gas hydrate, no direct physical association between them has been demonstrated. We examined several intact samples of naturally occurring gas hydrate from the Gulf of Mexico for evidence of microbes. All samples were collected from anaerobic hemipelagic mud within the gas hydrate stability zone, at water depths in the ca. 540- to 2,000-m range. The $\delta^{13}C$ of hydrate-bound methane varied from −45.1‰ Peedee belemnite (PDB) to −74.7‰ PDB, reflecting different gas origins. Stable isotope composition data indicated microbial consumption of methane or propane in some of the samples. Evidence of the presence of microbes was initially determined by 4,6-diamidino 2-phenylindole dihydrochloride (DAPI) total direct counts of hydrate-associated sediments (mean = 1.5×10^9 cells g^{-1}) and gas hydrate (mean = 1.0×10^6 cells ml^{-1}). Small-subunit rRNA phylogenetic characterization was performed to assess the composition of the microbial community in one gas hydrate sample (AT425) that had no detectable associated sediment and showed evidence of microbial methane consumption. *Bacteria* were moderately diverse within AT425 and were dominated by gene sequences related to several groups of *Proteobacteria*, as well as *Actinobacteria* and low-G + C *Firmicutes*. In contrast, there was low diversity of *Archaea*, nearly all of which were related to methanogenic *Archaea*, with the majority specifically related to *Methanosaeta* spp. The results of this study suggest that there is a direct association between microbes and gas hydrate, a finding that may have significance for hydrocarbon flux into the Gulf of Mexico and for life in extreme environments.

Gas hydrate is an ice-like mineral that crystallizes under conditions of high pressure, low temperature, and high gas concentration (9). It is composed of hydrocarbon and nonhydrocarbon gases held in cages of water molecules. Marine gas hydrate is thought to comprise an extremely large reservoir of reduced carbon, with energy content exceeding that of all conventional subsurface reserves of oil, gas, and coal combined (25). There has been significant interest in gas hydrate as a future energy resource, as a positive feedback mechanism for global warming, and as an agent of catastrophic sediment failure (24). It has been implicated in transient greenhouse warming at the Paleocene/Eocene transition (19, 32) and in a Jurassic oceanic anoxic event (13). Gas hydrate is also found in permanently frozen soils and glacial ices at high latitudes on Earth and is thought to be a component of icy planets and satellites, comets, and the Mars polar ice caps (reviewed in reference 9).

Microbial communities physically associated with gas hydrates and related sediments are potentially critical for gas hydrate stability, composition, and crystal structure. Via methanogenesis, microbes are indirectly involved in the formation of the most common form of gas hydrate on Earth, biogenic methane hydrate (51). There are indications that microbes anaerobically oxidize methane in the seep environment (6, 30, 46, 48) and within gas hydrate after crystallization (39).

The Gulf of Mexico (GOM) is a natural laboratory for studying gas hydrate dynamics and microbiology for several reasons. Gas hydrate is often found in sediments associated with natural gas venting and at cold hydrocarbon seeps, both of which are abundant on the northern continental slope (8). In some cases, so much gas hydrate is present that massive gas hydrate mounds break through the sediment surface (29). The GOM is also one of the few sites globally where both thermogenic (i.e., composed primarily of hydrocarbon gases derived from thermal degradation of petroleum) and biogenic (i.e., composed primarily of methane from biological methanogenesis) gas hydrates have been recovered (43). Gas hydrate at seep sites hosts complex chemosynthetic communities, where primary production is based on microbial consumption of methane and hydrogen sulfide (40). Finally, authigenic carbonates with extremely light carbon isotope signatures, which have been linked to anaerobic biological oxidation of methane (36), as well as massive gas hydrates, have been recovered in sediment cores from this region.

Geochemical evidence has indirectly shown microbial consumption of methane within gas hydrate (39) and petroleum components within cold hydrocarbon seep regions (41) on the northern continental shelf of the GOM. Additionally, the microbial diversity of gas hydrate-containing sediments in other regions has been investigated in several previous studies (4, 15, 30, 33). However, no direct observation of microbes within massive gas hydrates has been reported.

This study is the first to characterize a microbial community directly associated with massive gas hydrate. We report geo-

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TABLE 1. Description of sampled sites

Sample[a]	Location	Water depth (m)	Gas hydrate	Associated features	Descriptors	Reference
AT425	27° 34.1′ N, 88° 29.7′ W	1,920–1,930	Structure II	Authigenic carbonate, H_2S	Part of Mississippi Fan Foldbelt, salt ridge	42
KC695	26° 18′ N, 92° 12′ W	2,000	Structure I	H_2S	Edge of Sigsbee escarpment	This study
MC853	28° 7.4′ N, 89° 8.2′ W	1,060–1,070	Structure II	Authigenic carbonate, H_2S, free gas	Large sea floor mound (1.5 km across, ca. 30–40 m relief)	43
AT98	27° 51.1′ N, 89° 28.1′ W	1,076	Structure I	Free gas, authigenic carbonate, H_2S	On the middle Gulf slope near a fault, shallow salt	This study
GC185	27° 45.7′ N, 91° 30.5′ W	540	ND[b]	Free gas, authigenic carbonate, H_2S	Control sediment with no associated gas hydrate	38, 39

[a] AT, Atwater Canyon; KC, Keathley Canyon; MC, Mississippi Canyon; GC, Green Canyon.
[b] ND, no data.

chemical, microscopy, and DNA-based data supporting such a direct physical association.

MATERIALS AND METHODS

Geologic setting. The GOM continental slope is affected by large sheet-like salt thrusts that extend from the shelf edge across the continental slope to the Sigsbee Escarpment, near the edge of the abyssal plain (54). The geology is conducive to hydrocarbon seepage to the sea floor from a deeply buried petroleum system (53). Fracture zones associated with moving salt sheets and active faults provide conduits for fluid flow to the sea floor. Massive hydrocarbon seepage manifests itself at the Gulf sea floor as gas hydrate, oil-stained sediments, authigenic carbonate depleted in ^{13}C, and chemosynthetic communities (1, 28, 36, 37). Seeps and gas hydrate are concentrated along salt-withdrawal basin margins, over salt ridges, and near the edge of the Sigsbee Escarpment (43).

Sample collection. Samples were collected during 1998 to 2000 research cruises. The sites are described in Table 1 and Fig. 1 and were selected on the basis of seismic indications of hydrocarbon seepage within the gas hydrate stability zone. Samples were collected with a 6-m piston coring device (7-cm interior diameter). A high-speed winch facilitated rapid core recovery, before extensive gas hydrate decomposition could occur. Intact white-to-orange gas hydrate was observed in oil-stained sediments as vein fillings and as subspherical nodules with a radial pattern of crystallization. The gas hydrate was preserved by immersion in liquid nitrogen within minutes of core recovery. All samples had significant amounts of associated crude oil (ca. 30% [vol/vol]).

The MC853 and KC695 samples were massive gas hydrate with attached sediment. MC853 was located ca. 0.4 to 0.6 m, and KC695 was located ca. 1.4 m deep in the sediment column. Samples AT425 and AT98 were massive gas hydrate with no detectable attached sediment, located in the upper 20 cm of the sediment column. The GC185 sample was control sediment with no visible associated gas hydrate; however, we cannot exclude the possibility that small amounts of vein-filling hydrate were present in the GC185 sample, but decomposed prior to retrieval.

Analysis of gas hydrate samples. Aliquots of intact gas hydrate were removed from liquid N_2 storage and were picked to remove sediment when necessary. Cleaned samples were allowed to decompose under a water-filled bell jar to obtain large volumes of free gas. Aliquots of gas samples were immediately transferred to preevacuated metal vacutainers with a 60-ml gas-tight syringe and held at −20°C until analysis. Detailed analytical procedures for C_1–C_5 gas chromatography and measurement of isotopic properties of hydrocarbon gases have been described elsewhere (38). Concentrations of each hydrocarbon were expressed in parts per million by sediment volume and normalized as a percentage of total C_1–C_5 hydrocarbons. The $\delta^{13}C$ values are reported as parts per thousand (‰) relative to the Peedee belemnite (PDB) standard (precision of ±0.2‰), and the δD values are reported as parts per thousand relative to standard mean ocean water (SMOW) (precision of ±5‰).

Direct microscopic counts of cells in hydrate. Samples were removed from liquid N_2 and allowed to melt (sediment) or decompose (gas hydrate) in sterile containers. The resulting liquid or liquid-sediment mix was centrifuged at approximately 400 × *g* to separate gas hydrate fluids, oil, and sediment. No sediment pellet was observed for samples AT425 and AT98. For decomposed gas hydrate with no attached sediment, 1 ml of the aqueous phase was transferred to a filtration tower with care taken to avoid the organic phase. The samples were stained with the DNA-staining dye 4,6-diamidino 2-phenylindole dihydrochloride (DAPI; Sigma), and cells were counted as previously described (22). Attached sediment from samples MC853 and KC695 and the control sample, GC185, was diluted 1,000-fold, stained with DAPI, and counted as previously described (7). Due to intrinsic autofluorescence, the hydrocarbons present in the samples led to high background fluorescence, therefore, the cell counts presented are a minimal estimate.

DNA isolation, PCR amplification, and cloning. Remaining liquid from sample AT425 (ca. 50 ml) was filtered on to a 0.2-μm-pore-size Supor filter (Pall, Ann Arbor, Mich.). The filter was frozen in the presence of lysis buffer (20 mM Na-EDTA, 400 mM NaCl, 0.75 M sucrose, 50 mM Tris-HCl [pH 9.0]) and stored at −80°C. Total nucleic acids were extracted from the filters and purified as described elsewhere (12). Bacterial small subunit (SSU) rRNA genes were PCR amplified with primers S-D-Bact-0008-a-A-19 and S-D-Bact-1492-a-A-21 (14), and archaeal SSU rRNA genes were PCR amplified with primers A20F (11) and A958R (10). The PCR conditions used were 1 min of 95°C denaturation, 2 min of 55°C annealing, and 3 min of 72°C elongation for 35 cycles in an MJ Research thermal cycler. After a final 10-min incubation at 72°C, the product was purified with a gel extraction kit (Qiagen, Chatsworth, Calif.). Amplification products were cloned into the plasmid vector pCR2.1 by TA cloning (Invitrogen, Carlsbad, Calif.).

RFLP analysis and sequencing of clone libraries. SSU rDNA inserts were PCR-amplified under the same conditions as above with M13R and T7 primers. The product was digested with *Hha*I restriction endonuclease (New England Biolabs, Beverly, Mass.) at 37°C for 2 h. The banding patterns were grouped according to similarity, and representative members of each pattern group were fully, bidirectionally sequenced with either an ABI 3700 (Applied Biosystems, Inc., Foster City, Calif.) or a Licor 4200 (Licor, Inc., Lincoln, Neb) automated DNA sequencer. Multiple representatives were sequenced for restriction fragment length polymorphism (RFLP) patterns that had more than five members.

Sequence analysis. Sequences were initially aligned to their nearest neighbor by using the program ARB (Ludwig and Strunk, Technische Universität München, Munich, Germany [http://www.mpi-bremen.de/molecol/arb/]). The sequences were further manually aligned to sequences obtained from the GenBank database by using the Genetic Data Environment (GDE) version 2.0 sequence analysis software package (Smith, Millipore Corporation, Bedford, Mass.), as described elsewhere (35). Phylogenetic inference and evolutionary distance calculation were performed as described previously (35). Phylogenetic trees were constructed by the neighbor-joining method with the Kimura two-parameter model for nucleotide change (21).

Nucleotide sequence accession numbers. The rDNA sequences were entered into the GenBank database and were assigned accession no. AY053466 to AY053496.

RESULTS

Gas hydrate molecular and isotopic composition. The gas hydrate samples of the present study included examples of the two major types of gas hydrate found in the Gulf (Table 1).

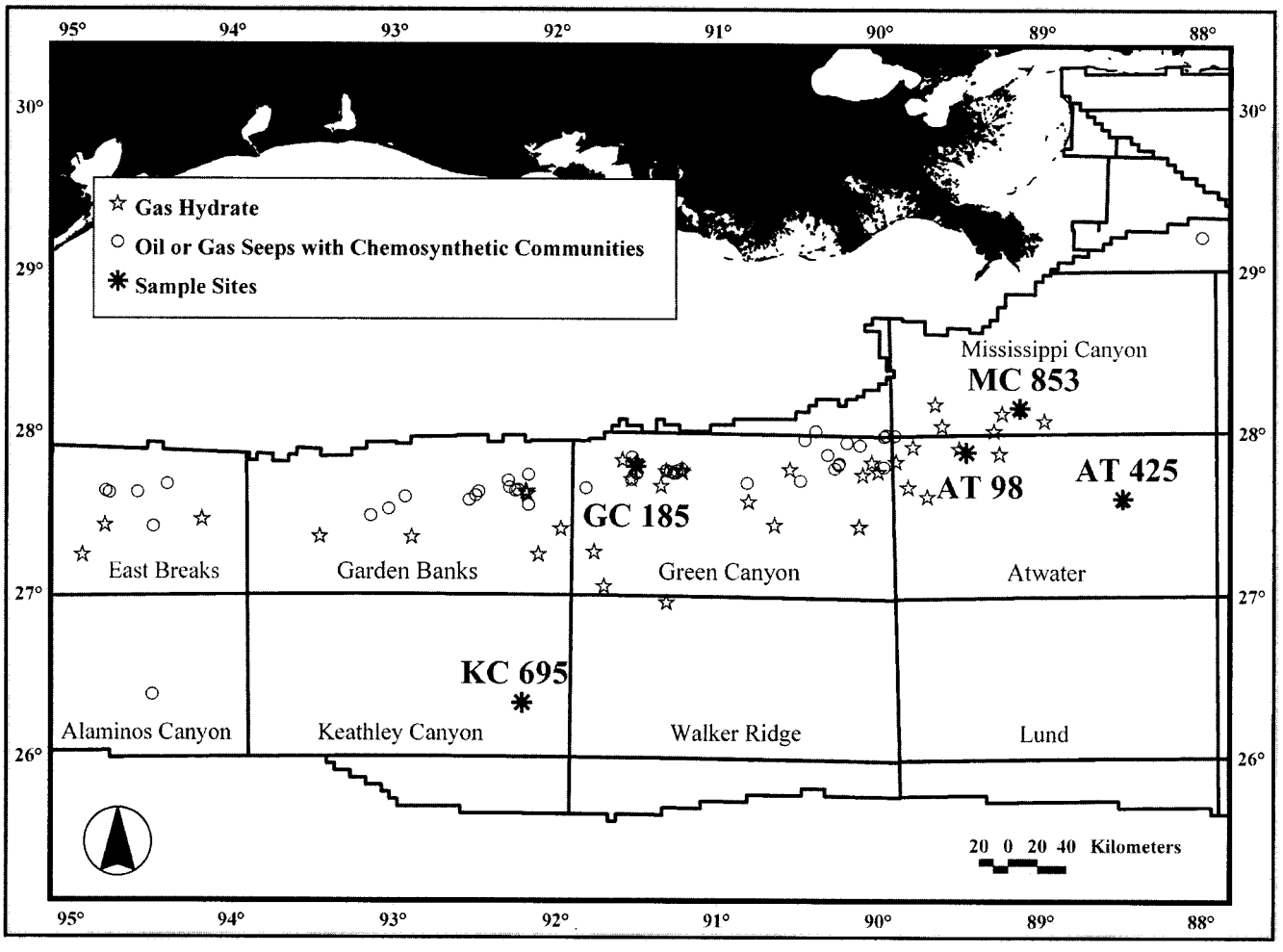

FIG. 1. Map of sampling sites. Locations of known shallow gas hydrates, major gas and oil seeps, and chemosynthetic communities across the northen continental slope of the GOM are also indicated (38).

Samples KC695 and AT98 were representatives of biogenic gas hydrate (structure I). Methane was the main hydrocarbon component, with $\delta^{13}C$ values of −70.1‰ PDB from KC695 and −74.7‰ PDB from AT98 (Table 2). The δD value of methane from AT98 was −155‰ SMOW. These $\delta^{13}C$ and the δD values were consistent with a microbial methane source (38). Small percentages of ethane were present in both samples, indicating minor mixing with thermogenic hydrocarbon gases.

Thermogenic gas hydrate (structure II) was represented by samples MC853 and AT425. Methane was the major hydrocarbon component, along with lesser percentages of thermogenic hydrocarbons in the order propane, ethane, isobutane, butane, and minor pentanes (Table 2). The $\delta^{13}C$ value of methane was −45.1‰ PDB from MC853 and −49.3‰ PDB from AT425. These values were enriched in ^{13}C relative to biogenic methane gas hydrate; therefore, the methane was likely thermogenic, from the deep subsurface hydrocarbon system of the Gulf (Table 2). However, in the seep environment,

TABLE 2. Normalized hydrocarbon concentrations and isotope properties of gasses from gas hydrate samples

Sample	% Methane concn ($\delta^{13}C$)[a]	δD Methane[b]	% Ethane concn ($\delta^{13}C$)	% Propane concn ($\delta^{13}C$)	% Isobutane concn ($\delta^{13}C$)	% *n*-Butane concn ($\delta^{13}C$)	% Isopentane concn ($\delta^{13}C$)	% *n*-Pentane concn ($\delta^{13}C$)	CO_2 $\delta^{13}C$
MC853	75.3 (−45.1)	−166	6.8 (−28.4)	11.3 (−24.5)	3.4 (−27.0)	0.7 (−23.4)	0.3 (ND)[c]	<0.1 (ND)	+11.6
AT425	77.3 (−49.3)	−148	5.0 (−38.2)	12.5 (−32.0)	3.0 (−31.6)	1.2 (−28.3)	0.9 (−28.8)	0.2 (−28.4)	+12.4
KC695	99.9 (−70.1)	ND	0.1 (ND)	0 (ND)	0 (ND)	0 (ND)	0 (ND)	0 (ND)	ND
AT98	99.9 (−74.7)	−155	0.1 (ND)	0 (ND)	0 (ND)	0 (ND)	0 (ND)	0 (ND)	ND
GC185	84.1 (ND)	ND	5.5 (ND)	5.7 (ND)	1.7 (ND)	0.8 (ND)	2.2 (ND)	0 (ND)	ND

[a] $\delta^{13}C$ values are reported in parts per thousand relative to the Peedee Belemnite standard.
[b] δD values are reported in parts per thousand relative to the SMOW standard.
[c] ND, no data.

TABLE 3. Direct counts of DAPI-stained cells from decomposed gas hydrate and sediment

Sample	Direct count of cells from[a]:	
	Hydrate (10^5 cells ml^{-1})	Sediment (10^8 cells/g^{-1} [wet wt])
AT425[b]	9.99 ± 4.72	
KC695	6.57 ± 1.68	11.7 ± 2.85
MC853	13.2 ± 2.96	10.1 ± 3.37
AT98[b]	10.5 ± 4.13	
GC185		8.18 ± 2.51

[a] Values are means ± standard deviations.
[b] Gas hydrate with no attached sediment.

we cannot exclude the possibility that a minor fraction of the methane was microbial in origin (38).

The δD value of methane was −166‰ SMOW from MC853 and −148‰ SMOW from AT425, and the $\delta^{13}C$ of propane was −24.5‰ PDB in MC853 (Table 2). These values are enriched in the heavy isotopes relative to unaltered vent gas (38, 39). Such enrichment is indicative of microbial consumption of these gaseous components from within the gas hydrate, an observation consistent with previous studies (38, 39). Isotope properties of other C_1–C_5 hydrocarbons and CO_2 (Table 2) were similar to those observed in unaltered vent gases.

Cell counts. Direct counts of DAPI-stained cells were ca. 10^6 cells ml^{-1} for the decomposed gas hydrate fluids and 10^9 cells g^{-1} (wet weight) for the sediments (Table 3). Cell counts for gas hydrate fluids with or without attached sediments were indistinguishable. The sediment with no intact associated gas hydrate (GC185) had similar cell counts to the sediments associated with gas hydrate. These direct counts are similar to those commonly obtained from standard marine systems (45). This result is unexpected, because other studies based on extractable lipid concentrations show up to 30-fold higher biomass, a value indirectly correlated to cellular abundance, in the Gulf seep system than in nearby marine sediments (C. Zhang, personal communication).

Microbial diversity. We focused on one of the samples, AT425, to characterize the microbial diversity associated with GOM gas hydrate. This gas hydrate was chosen because it had no associated sediment; therefore, all microbes in this sample were physically attached to or included within the gas hydrate. AT425 was also chosen because it showed signs of microbial oxidation of methane within the gas hydrate (see above).

Bacteria. There was fairly high bacterial diversity associated with the AT425 hydrate (Fig. 2). Of 127 rRNA clones analyzed, there were 21 different *Hha*I RFLP patterns (Table 4). Sequencing of representatives of these RFLP patterns confirmed that they were phylogenetically distinct. Diverse phylotypes related to *Actinobacteria* and low G + C (*Bacillus*, etc.) *Firmicutes*; β-, γ-, α-, and δ-*Proteobacteria*; and a group without clear affiliation with broad phylogenetic clades (AT425 EubC11) were the most frequently recovered sequences (Fig. 2 and Table 4). Overall, of 127 16S rDNA clones, there were 42 *Firmicutes*-related and 63 *Proteobacteria*-related clones. *Cytophaga*/*Flavobacterium*/*Bacteroides* (CFB)- and *Thermus*-related sequences were also obtained, but only at low frequency (Fig. 2 and Table 4).

Two of the *Firmicutes*-related clone groups were only distantly related to their nearest neighbor and appeared to be a distinct branch of the *Actinobacteria*. These are a group of 11 clones, represented by AT425 EubC5, and a related group of one clone (AT425 EubY10). Two other groups, totaling three clones, may also be affiliated with the *Firmicutes* (i.e., AT425 EubF1 and AT425 EubA5), although there is poor statistical (bootstrap) support for that affiliation. However, the majority of the 16S rDNA sequences clearly affiliated with the *Firmicutes* were related at the species level (>97% 16S rDNA sequence similarity) (44) to previously cultured organisms (Table 4). The closest relatives of these clones have heterotrophic metabolism, and most are aliphatic or aromatic hydrocarbon utilizers (Table 4).

A high similarity to previously cultured organisms also held for most *Proteobacteria* in this system (Fig. 2 and Table 4). The most frequently recovered sequence group, represented by AT425 EubC3, was specifically related to the fluorescent pseudomonad subgroup of the γ-*Proteobacteria*. This group is physiologically diverse, widespread, and abundant. The other γ-*proteobacterial* group of two clones, represented by AT425 EubD5, are specifically related to BD5-14 (98% similarity), an environmental 16S rDNA clone from deep sea sediments (27). Six 16S rDNAs were relatives of δ-proteobacterial sulfate-reducing bacteria (SRB), specifically the *Desulfosarcinales* and *Syntrophus* spp. AT425 EubD9, representing five clones, is specifically related (98% similarity) to a group of environmental 16S rRNA gene sequences that were originally found in a variety of sediments associated with the anaerobic oxidation of methane (33). The remaining δ-proteobacterial clone, AT425 EubF5, is related to *Syntrophus buswellii*, an organism that requires syntrophic H_2 shuttling for growth (50). The β- and α-*Proteobacteria*-affiliated 16S rDNA gene sequences, accounting for 19 clones, are related at the species level to previously cultured organisms with heterotrophic metabolism (Fig. 2 and Table 4).

AT425 EubC11, which represents 14 of 127 16S rDNA clones, is poorly affiliated with previously characterized 16S rDNA sequences in the public databases. This sequence showed a similarity of only 85% to its nearest neighbor, deep sea clone BD2-11 (Fig. 2 and Table 4). Other gene sequences that appear to affiliate with this group are primarily symbionts of marine sponges, such as UC51f (GenBank accession no. AF186416) and R11 (GenBank accession no. AF333520).

Only one clone related to the CFB group was recovered from this system. It is only distantly related to other members of the CFB group, being only 92% similar to its nearest neighbor (Table 4). The final group of four clones observed in this system, represented by AT425 EubA6, is related at the species level to *Thermus aquaticus* YT-1 (Fig. 2 and Table 4).

Archaea. In contrast to the bacterial diversity, the archaeal diversity in the AT425 sample was quite low (Fig. 3). Of 93 rRNA clones analyzed, only eight distinct *Hha*I RFLP patterns were observed, representing five phylogenetically distinct groups. This level of archaeal diversity is much lower than that observed in studies of standard marine sediments (31, 49), but it is similar to that observed in other hydrocarbon seep systems (15, 33).

Two of the archaeal groups, totaling 76 clones, were related to the methanogenic order *Methanosarcinales*. The most frequently recovered sequence group (73 clones) was specifically

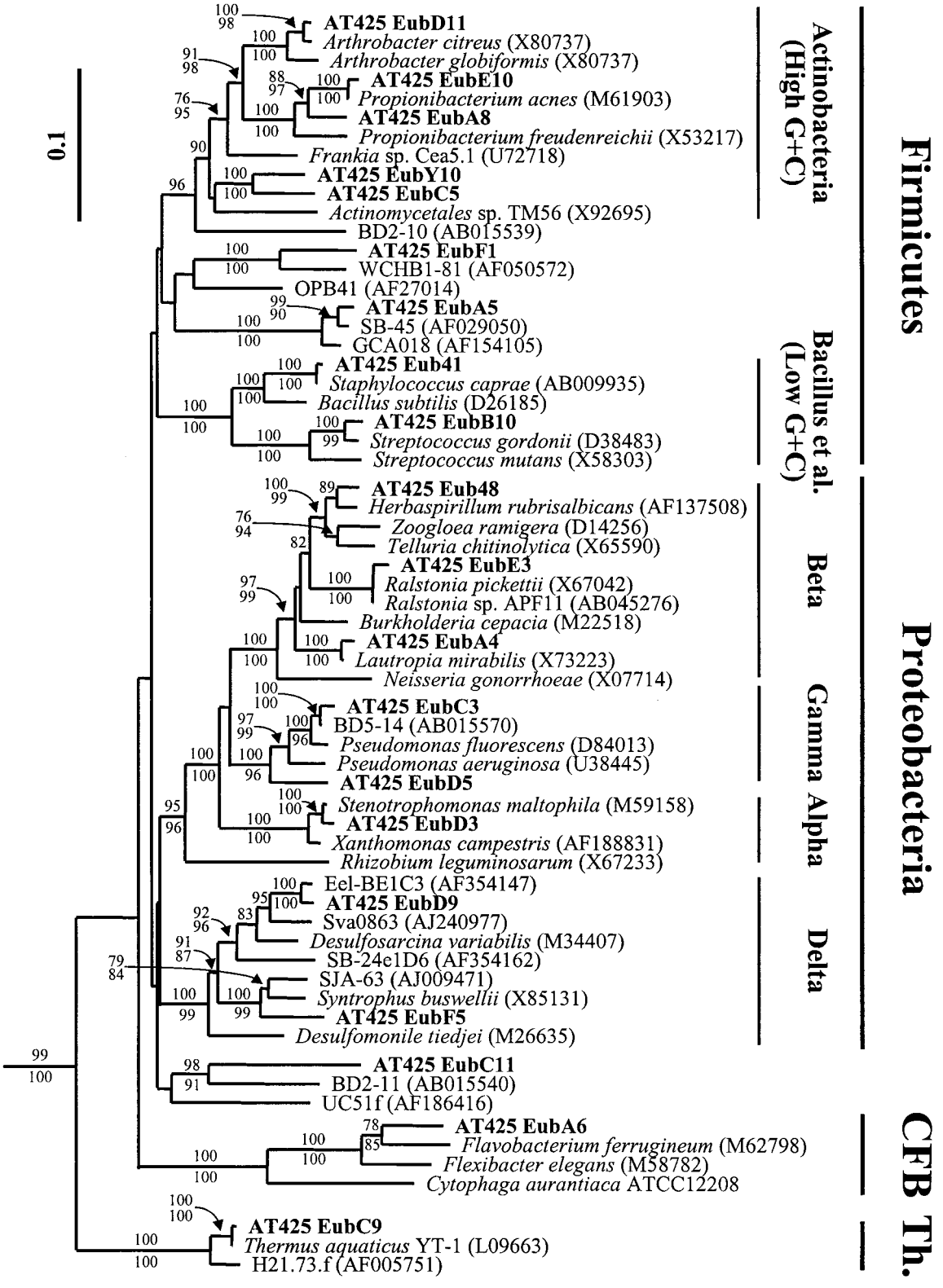

FIG. 2. Phylogenetic analysis of bacterial SSU rRNA gene clones from sample AT425. This phylogenetic tree was rooted with *Sulfolobus acidocaldarius*. A mask of 989 nucleotides, including all nonambiguously aligned positions, spanning nearly the full length of the SSU rRNA gene, was included. Bootstrap values (100 replications) generated by the neighbor-joining method are shown above relevant nodes, and those generated by maximum-parsimony analysis are shown below. Only bootstrap values above 70 are shown. Sequences from isolates are in italics, sequences from environmental gene clones are in plain text, and sequences from the AT425 sample are in boldface. GenBank accession numbers of the sequences from other studies are included. CFB, *Cytophaga*/*Bacteroides*/*Flavobacterium*. Th, *Thermus*.

TABLE 4. Grouping of microbes associated with sample AT425

Representative sequence	No. of clones	Nearest relative[a]	Phylogenetic group[b]	Similarity[c]
Bacteria (127 clones)				
AT425 EubE10	15	*Propionibacterium acnes*	*Actinobacteria*	0.99
AT425 EubC5	11	*Actinomycetales* sp. strain TM56	*Actinobacteria*	0.88
AT425 EubA8	3	*Propionibacterium acnes*	*Actinobacteria*	0.95
AT425 EubD11	3	*Arthrobacter citreus*	*Actinobacteria*	0.99
AT425 EubY10	1	*Frankia* sp. strain Cea5.1	*Actinobacteria*	0.89
AT425 EubB10	8	*Streptococcus gordonii*	Low G + C *Firmicutes*	0.98
AT425 Eub41	1	*Staphylococcus caprae*	Low G + C *Firmicutes*	0.99
AT425 EubF1	2	Hydrocarbon-contaminated aquifer clone WCHB1-81	*Firmicutes*?	0.91
AT425 EubA5	1	Benzene-mineralizing consortium clone SB-45	*Firmicutes*?	0.98
AT425 EubD3	8	*Stenotrophomonas maltophila*	α-*Proteobacteria*	0.99
AT425 EubE3	9	*Ralstonia pickettii*	β-*Proteobacteria*	0.99
AT425 Eub48	1	*Herbaspirillum rubrisalbicans*	β-*Proteobacteria*	0.97
AT425 EubA4	1	*Lautropia mirabilis*	β-*Proteobacteria*	0.99
AT425 EubC3	36	*Pseudomonas fluorescens*/deep sea clone BD5-14	γ-*Proteobacteria*	0.98
AT425 EubD5	2	Deep sea clone BD5-14	γ-*Proteobacteria*	0.94
AT425 EubD9	5	Hydrocarbon seep clone Eel-BE1C3	δ-*Proteobacteria*	0.98
AT425 EubF5	1	*Syntrophus buswellii*/trichlorobenzene-degrading consortium clone SJA-63	δ-*Proteobacteria*	0.93
AT425 EubA6	1	*Flavobacterium ferrugineum*	CFB	0.92
AT425 EubC9	4	*Thermus aquaticus* YT-1	*Thermus* and relatives	1.00
AT425 EubC11	14	Deep sea clone BD2-11	Unknown	0.83
Archaea (93 clones)				
AT425 ArC7	73	*Methanosaeta* sp. strain clone A1	*Methanosarcinales*	0.95
AT425 ArD2	2	Hydrocarbon seep clone Eel-TA1a4	ANME-2	0.95
AT425 ArE12	1	Hydrocarbon seep clone Eel-TA1a4	ANME-2	0.95
AT425 ArB7	8	Hydrocarbon seep clone Eel-BA2e8	ANME-1	0.99
AT425 ArB9	8	Hydrocarbon seep clone Eel-BA2e8	ANME-1	0.93
AT425 ArD10	1	Salt marsh clone 2C84	Salt marsh	0.94

[a] If more than one relative is listed, the similarities of each to the environmental gene clone are approximately equivalent.
[b] See Fig. 2 and 3 for more detail.
[c] Based on alignment of all nonambiguously aligned nucleotides. A sequence with a similarity of 1.00 is identical.

related to the genus *Methanosaeta*. The closest relative of these sequences, *Methanosaeta* sp. strain clone A1 (Fig. 3 and Table 4), is an rDNA gene sequence obtained from a consortium capable of degrading long-chain hydrocarbons (e.g., hexadecane) to methane, a process termed "microbial alkane cracking" (55). The remaining three clones within *Methanosarcinales* are specifically related (Fig. 3 and Table 4) to a group labeled ANME-2 (for anaerobic methane oxidizers 2) by Orphan and colleagues (33). ANME-2 has only been observed in sediments that exhibit anaerobic methane oxidation.

Two other groups totaled 16 clones and were related to the group ANME-1. This group has no previously cultured members and has only been described in sediments showing evidence of anaerobic methane oxidation (15, 33, 47). The final group, composed of one clone, was most closely related to a cluster of *Euryarchaeota* of unknown physiology found in marine sediments and also observed previously in anaerobic methane oxidation zones (15, 31, 33). No evidence of *Crenarchaeota* or other archaeal groups (e.g., *Korarchaeaota*) was observed.

One final group, comprised of a single clone (AT425 ArD10), was related to a group of environmental clones obtained from a salt marsh ecosystem (Fig. 3 and Table 4) (31). Related sequences were also obtained from the Eel River site and were associated with AOM (15). This group is composed entirely of environmental gene sequences, and no previously cultured organisms are associated.

DISCUSSION

Although of significant interest, little is known about gas hydrate crystallization or decomposition, or the role of gas hydrate in diagenetic processes. The presence of microbial cells directly associated with gas hydrate supports geochemical evidence that biology may have a significant effect on both the stability and composition of gas hydrate (38, 39). Because gas hydrate is estimated to be a larger reservoir of hydrocarbons than all oil, gas, and coal reserves combined (26), they could be an important, poorly understood carbon and/or energy source for microorganisms. Consumption of gas hydrate hydrocarbons by associated microbes could also play a significant role in global methane and carbon cycles and in diagenetic processes. This study was designed to find direct evidence of physical interactions between microbes and gas hydrates.

Molecular composition and isotope data. In contrast to simple biogenic methane, thermogenic hydrocarbons preserve complex information on their origin and alteration. For example, unaltered thermogenic methane from subsurface reservoirs and from unaltered sea floor vent gas will not often show large variation in isotopic properties (38, 39). Because there is no isotopic fractionation as a consequence of gas hydrate crystallization, the gas hydrate isotopic properties are generally very similar to that of the vent gas. Differences are attributed to bacterial oxidation after crystallization (38, 39). The δD observed for methane from sample AT425 (Table 2) was sim-

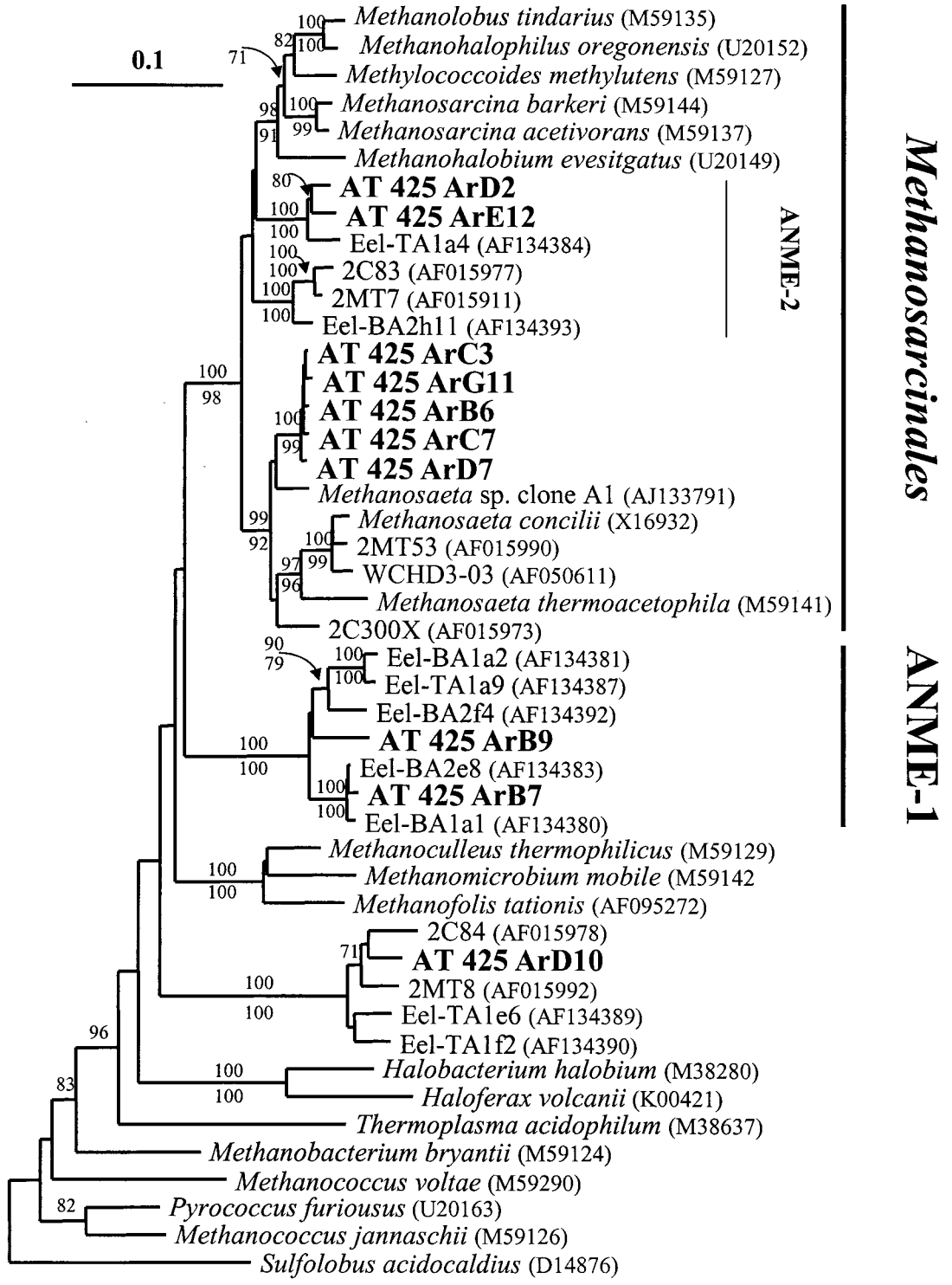

FIG. 3. Phylogenetic analysis of archaeal SSU rRNA gene clones from sample AT425. This unrooted phylogenetic tree was obtained by methods described in the legend to Fig. 2. A mask of 695 nucleotides, including all nonambiguously aligned positions, spanning approximately 900 nucleotides of the 5′ end of the SSU rRNA gene, was included in the analysis. Sequences from isolates are in italics, sequences from environmental gene clones are in plain text, and sequences from the AT425 sample are in boldface. Sequences labeled "Eel" are from a study of the Eel River area off the coast of northern California (15), and sequences labeled "2C" or "2M" are from a study of a coastal salt marsh (31). GenBank accession numbers of the sequences from other studies are included.

ilar to values previously observed for microbially altered gas hydrate from near the GC185 site and was enriched in D relative to unaltered vent gases (39, 43). Additionally, the propane from sample MC853 was enriched in ^{13}C relative to unaltered vent gas. Therefore, although we lack vent gas samples from the MC853 and AT425 sites for direct reference and comparison, we can infer that some components of the Gulf thermogenic gas hydrate have been impacted by microbial alteration after crystallization. These results support previous data indicating that microbes are directly consuming hydrocarbons within gas hydrates in the Gulf (39, 43).

Fluorescence microscopy data. We reasoned that microbes are likely to be physically associated with the gas hydrate, because direct consumption of gases would require a close physical interaction. Staining of hydrate-associated sediment and decomposed gas hydrate fluids with the DNA dye DAPI followed by fluorescence microscopy showed the presence of microbial cells within the gas hydrate samples. Three lines of evidence indicated that these cells were physically associated with the gas hydrate. First, all gas hydrate samples, including those that had no visible attached sediments, provided similar cell counts (Table 3). If the observed cells were due to contamination from sediment or seawater, cell counts lower than those normally observed for sediment or seawater would have been expected as a consequence of dilution. Second, the outer layers of the gas hydrate were lost upon retrieval by decomposition due to decreased pressure and increased temperature. Cells that were only peripherally associated with the gas hydrate would have been lost with the outer layers. Third, the isotopic evidence of methane and propane alteration within the gas hydrate requires the presence of microbes. Therefore, the microbes are most likely directly physically associated with the gas hydrates. At this time, we cannot determine the source of these microbes (i.e., seep sediments, oil associated with the gas hydrates, or an independent community specifically affiliated with the gas hydrate).

It is, perhaps, surprising that diverse (see below) groups of *Bacteria* and *Archaea* would be found in the highly crystalline environment of the gas hydrate. However, CH_4, Ar, N_2, and CO_2 can all form highly porous (as high as 40% porosity), "sponge-like" gas hydrates (23). Typical pore sizes are 100 to 400 nm for CH_4 hydrates, with occasional channels on the order of a few micrometers. Many *Bacteria* and *Archaea*, as well as their chemical substrates and waste products, would be able to freely move through pores of this size, allowing exchange with the external environment, which may be the primary source for microbes in this environment.

It is unclear why the gas hydrates had cell counts that were several orders of magnitude lower than for a similar amount of sediment. One possible explanation is that the gas hydrates have significantly higher cell counts than were observed here. Much of the surface of the gas hydrate samples was lost due to decomposition during retrieval. Additionally, due to differences in methodology for counting the sediment versus liquid samples, the concentration of autofluorescent hydrocarbons was much higher in the gas hydrate samples, which may have led to the underestimation of the number of cells in the gas hydrate samples. If this hypothesis is accurate, it might partially account for the difference between estimates of microbial biomass based on extractable lipids (C. Zhang, personal communication) and the direct microscopic counts reported here. However, this hypothesis must be tested further.

Despite these difficulties with the direct microscopic counting of microbial cells, this approach did show the presence of microbes within the gas hydrate structure. This is the first direct evidence of physical interactions between gas hydrates and microbes.

Phylogenetic analysis. We used rRNA phylogenetic analysis to determine the identity of the microbes associated with one sample of gas hydrate, AT425, a massive thermogenic hydrate with no associated sediment. Fairly high bacterial diversity and low archaeal diversity were associated with this sample (Fig. 2).

Bacteria. Several unusual features are apparent regarding the bacterial diversity in sample AT425. First, a large fraction (ca. 72%) of the recovered 16S rRNA gene sequences are related at the species level to previously cultured microbes (Fig. 2 and Table 4). A predominance of sequences that are nearly indistinguishable from previously cultured organisms is unusual in non-culture-based studies (17).

Second, the *Firmicutes* are more frequently recovered from this sample relative to other systems. Overall, 33 to 35% of the recovered gene sequences affiliated with the *Actinobacteria* or the low-G+C *Firmicutes* (Fig. 2 and Table 4). Although widespread in marine systems (17), to our knowledge, no other studies of marine systems, either planktonic or benthic, show such high recovery of *Firmicutes*-related gene sequences. None of the *Firmicutes* gene sequences in this sample appear to be affiliated with the so-called marine *Actinobacteria* (34) or other common, but previously uncultured, groups of *Actinobacteria*.

Third, a group of 11 clones, represented by AT425 EubC11, was recovered that is only poorly affiliated with 16S rRNA gene sequences in public databases (Fig. 2 and Table 4). Other gene sequences that may affiliate with this group, primarily symbionts of marine sponges such as UC51f (AF186416) and R11 (AF333520) (data not shown), were previously thought to be related to the *Actinobacteria* (52). However, our phylogenetic analysis does not support such a relationship (Fig. 2). Instead, this group appears to be a deep branch of the *Bacteria*. Further characterization is required to determine whether this group should be considered a candidate division of the *Bacteria* (See reference 17 for more information on candidate divisions.)

Fourth, four 16S rRNA gene clones, represented by AT425 EubC9, were recovered from this cold environment that are indistinguishable from *Thermus aquaticus* YT-1. It is unlikely that these sequences are contaminants from the *Taq* polymerase used for PCR amplification, because the brand used is recombinant and was purified from *Escherichia coli*. At this time, we are unable to provide an explanation for the recovery of multiple gene sequences nearly indistinguishable from a monophyletic group of obligately thermophilic and aerobic organisms in an anaerobic, cold environment. To our knowledge, there are no reports of *Thermus* spp. in nonthermophilic environments.

Archaea. There were two major groups of *Archaea* present in sample AT425: those related to 16S rRNA gene sequences recovered from sediments with active anaerobic oxidation of methane (ANME-1 and ANME-2 [33] and salt marsh clones [31]) and those specifically related to the genus *Methanosaeta* (Fig. 3). The level of diversity observed here is extremely low

compared to those in most other environmental, nonculture-based studies of *Archaeal* diversity (3), although it is similar to that observed in sediments that exhibit anaerobic methane oxidation (15, 33, 47).

It is intriguing that sequences specifically related to the genus *Methanosaeta* are so frequently recovered from sample AT425 (Fig. 3 and Table 4). This genus is a member of the *Methanosarcinales*, which are the only methanogenic *Archaea* capable of utilizing acetate (acetoclastic methanogenesis) or intermediate redox state C_1 compounds, such as methylamines or methanol (methylotrophic methanogenesis [5]). In fact, the only known energy-generating metabolism for *Methanosaeta* spp. is acetoclastic methanogenesis (5).

ANME-1 and ANME-2, also well represented in sample AT425 (Fig. 3 and Table 4), have no previously cultivated members. ANME-2-related sequences form a distinct branch within the *Methanosarcinales*. ANME-1, while clearly related to the methanogenic *Archaea*, is a distinct branch and is not specifically affiliated with any previously cultured methanogenic *Archaea*. We assume that both groups have methanogenic enzymes, because they are clearly related to the methanogenic *Archaea*, which are monophyletic and all have similar physiology (18). However, their specific role in this system is not known.

Predicted roles for microbial communities in GOM gas hydrate. We believe that it is likely that the microbial communities described here are active within the gas hydrates. High porosity of methane gas hydrate (pore sizes of 100 to 400 nm and pore volumes of approximately 25 to 40% [23]) allows potential substrates (e.g., sulfate) to enter and products of microbial metabolism (e.g., sulfide) to exit the gas hydrate structure without difficulty. Some pores may even be large enough for microbes to freely enter and leave the superstructure of the solid gas hydrate. Therefore, this microbial community may not be highly specialized and selected for life within a gas hydrate, but rather may freely exchange with the communities within the surrounding sediments.

Assuming that the microbial communities associated with the gas hydrates are active, two major metabolic activities are implied by the composition of the microbial community: anaerobic methane oxidation and nonmethane hydrocarbon oxidation.

AOM. Biological oxidation of methane in solid gas hydrate from the GOM has been observed previously, as indicated by both the isotope composition of methane and the molecular composition of gases held in the gas hydrate structure relative to unaltered vent gas (39). Additionally, these isotope composition shifts imply that solid gas hydrate can act as a substrate for microbial metabolism and growth, a process that has not been observed directly. Of hydrocarbon gases, methane is least tightly held in the crystal structure of gas hydrate, especially structure II. Therefore, it is the most accessible target of microbial consumption. Such activity can potentially change the composition of the gases held in the gas hydrate, hydrate stability, gas hydrate geochemistry, and sediment diagenesis (39).

No organism capable of net anaerobic oxidation of methane (AOM) has been isolated, however, geochemical evidence has indicated that microbially driven net oxidization of methane can occur under anaerobic conditions (reviewed in reference 48). Hoehler and colleagues have proposed a model wherein a methanogen (working in reverse) coupled to sulfate-reducing *Bacteria* (SRB) anaerobically oxidizes methane (16). This model has received support from several recent studies of compound-specific stable isotopes (reviewed in reference 48). Some of the *Archaea* in sample AT425, specifically those related to ANME-1 and ANME-2 (Fig. 3 and Table 4), are closely related to those previously shown to be associated with AOM (15, 33, 47). Additionally, one group of *Bacteria* (AT425 EubD9) in AT425 is closely related to a group of δ-*Proteobacteria* that have been found in these same AOM systems and may be important in the process of AOM (33). Furthermore, gas hydrate samples from the nearby Green Canyon area of the Gulf have previously been shown to be affected by AOM activity (38, 39), and sample AT425 shows isotopic evidence of microbial oxidation of methane (Table 2). Each of these lines of evidence implies that gas hydrate-associated microbial communities in this region are involved in anaerobic oxidation of the methane in gas hydrate.

Nonmethane hydrocarbon oxidation. We hypothesize that the large volume of hydrocarbons in the form of petroleum associated with the gas hydrate at this site may be a source of carbon and energy for many of the associated microbes. It has been noted that a significant amount of hydrocarbons enter the Gulf of Mexico through natural seeps, many of which also have associated gas hydrate (8, 20). Additionally, geochemical evidence based on comparisons of the isotopic composition of reservoir oils and those that enter the GOM indicates biological alteration of petroleum components (40). Aliphatic or aromatic hydrocarbon utilization is a widespread and common feature in the *Bacteria* (2). Therefore, it is possible that the bacterial community associated with natural gas hydrate may affect the flux of hydrocarbons into the GOM.

It is also possible that short-chain alkanes within thermogenic gas hydrate are a substrate for microbial activity. Propane $\delta^{13}C$ in sample MC853 appears to be affected by microbial consumption (described above and as shown in Table 2). However, other studies in the GOM have indicated that methane is the primary gas hydrate component oxidized by microbes and that short-chain alkanes, up to C_5, are relatively unaffected (39). Therefore, it is unclear whether consumption of short-chain alkanes in gas hydrates would be a significant carbon source for associated microbial communities.

Conclusions. In this study, we have shown that microbes are physically associated with methane hydrate and characterized one of the communities. This is the first study to show direct physical interaction between microbes and gas hydrate, a finding with important implications for gas hydrate stability, composition, and geochemistry. Our results are consistent with the notion that the microbes in this system likely consume liquid and/or volatile methane and nonmethane hydrocarbons both from the seep system and directly within gas hydrate. We plan to examine more communities to determine whether the results reported here are widely applicable to all gas hydrate or are specific to this study system. Also, more detailed community characterization with gas hydrates as well as the rest of the seep system, including seeking similar physical interactions between SRB and methanogenic *Archaea* as those observed by Boetius and colleagues (6), is necessary for a complete understanding of the GOM seep system. Future studies will also focus on the mechanisms of microbe-gas hydrate interactions,

anaerobic methane oxidation, and the significance of microbial consumption to the overall flux of hydrocarbons into the GOM.

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