

## Estimates of Biogenic Methane Production Rates in Deep Marine Sediments at Hydrate Ridge, Cascadia Margin<sup>∇†</sup>

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Received 17 September 2007/Accepted 2 March 2008

**Methane hydrate found in marine sediments is thought to contain gigaton quantities of methane and is considered an important potential fuel source and climate-forcing agent. Much of the methane in hydrates is biogenic, so models that predict the presence and distribution of hydrates require accurate rates of in situ methanogenesis. We estimated the in situ methanogenesis rates in Hydrate Ridge (HR) sediments by coupling experimentally derived minimal rates of methanogenesis to methanogen biomass determinations for discrete locations in the sediment column. When starved in a biomass recycle reactor, *Methanoculleus submarinus* produced ca. 0.017 fmol methane/cell/day. Quantitative PCR (QPCR) directed at the methyl coenzyme M reductase subunit A gene (*mcrA*) indicated that 75% of the HR sediments analyzed contained <1,000 methanogens/g. The highest numbers of methanogens were found mostly from sediments <10 m below seafloor. By considering methanogenesis rates for starved methanogens (adjusted to account for in situ temperatures) and the numbers of methanogens at selected depths, we derived an upper estimate of <4.25 fmol methane produced/g sediment/day for the samples with fewer methanogens than the QPCR method could detect. The actual rates could vary depending on the real number of methanogens and various seafloor parameters that influence microbial activity. However, our calculated rate is lower than rates previously reported for such sediments and close to the rate derived using geochemical modeling of the sediments. These data will help to improve models that predict microbial gas generation in marine sediments and determine the potential influence of this source of methane on the global carbon cycle.**

Subseafloor sediments near continental margins are often rich in dissolved methane as well as methane hydrates where pressures and temperatures are sufficient to maintain gases in this solid form (35, 53). Because this methane, whether dissolved in sediment pore waters or present as a hydrate, occupies a large volume globally and is inherently unstable, these formations are of considerable interest as a potential source of energy, a mechanism for climate change, and a factor in seafloor stability (21, 34).

Much of the methane in subseafloor hydrate formations and the surrounding sediments is biogenic (33), and therefore, the conceptual and computational models describing hydrate occurrence, distribution, and abundance benefit from the knowledge of accurate in situ methane formation rates. With information about the primary biological methane supply, models may be able to provide estimates of the timescale for hydrate accumulation (64) and the transport of methane into the hydrate stability zone, to confine where in the sediments methane production can occur (23), and to simulate the formation of hydrate deposits (17).

Determining accurate rates for microbial activities in sub-

surface environments, such as those that contain hydrates, is difficult. Subsurface microbial activities are believed to occur at exceedingly low rates, and most of the cells appear to be starved (20, 45). The direct measurement of in situ activities by using methods such as radiotracer-labeled substrate turnover is often used to assess microbial activity, but for cells that have been acquired from subsurface environments, this approach can lead to microbial rate estimates that are several orders of magnitude higher than values derived from geochemical modeling (49). Modeling the concentrations of biologically reactive chemical species in subsurface sediment pore water is an effective way of constraining in situ microbial activities in subsurface environments. Examples of this approach include estimates of in situ microbial activity along well-defined groundwater flow paths (42, 46) and in open-ocean and ocean margin sediments (20). Recently, a new rate law that incorporates thermodynamic and kinetic considerations for the electron-accepting and -donating half-reactions has been developed to predict the rate of microbial respiration in geological environments (29). This approach recognizes the importance of in situ conditions and requires concentration values for key pore water chemistry constituents.

Additional approaches for constraining the levels of microbial activity in environmental settings in order to complement traditional radiotracer methods and models that describe microbial respiration are desired. One approach uses biomass recycle reactors (BRRs) or retentostats that were developed to measure the metabolic rates of starved cells in laboratory set-

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

<sup>∇</sup> Published ahead of print on 14 March 2008.

tings (10, 31, 59). Because of the starvation conditions in these reactors, it was reasoned that such cells (which might be model microbes isolated from the environment of interest) could provide estimates of in situ microbial metabolic rates in the subsurface, where cells are also known to be starved (45). In the context of marine sediment systems where the methane is biogenic, we hypothesized that measures of methanogen activity in a BRR coupled with direct determinations of methanogen numbers in the sediments would yield reasonable estimates for in situ methanogenesis rates and, further, that these rates would be consistent with measured sediment parameters that constrain or dictate the rate of methanogenesis.

Our objective was to estimate rates of methanogenic activity in marine sediments where biogenic methane is an important component of the gas mix. Our method involved first determining the biomass of methanogens in samples from marine sediments from Hydrate Ridge (HR) in the northeastern Pacific Ocean, which we did by using quantitative PCR (QPCR) to target the methyl coenzyme M reductase I (MCR) gene (*mcrA*). *mcrA* is a key functional gene known to be conserved in methanogens from numerous environments (22, 39, 52). We then coupled these values for methanogen biomass at discrete locations in the sediments with estimates of the rate at which methane is made when methanogens are minimally metabolically active, an activity level proximate to that at which most microbes in the subsurface are believed to exist. Together, methanogen biomass in the sediments of HR and the specific rate of methanogenesis at a maintenance level of activity provided an estimate of the rate at which these microbial populations produce methane per unit volume of sediment. The estimated methanogenic rates were generally lower than many previously published experimental estimates for seafloor strata and other environments that are populated by methanogens. Our rate estimates may be useful in models that require a biogenic term for methane production in marine sediments.

#### MATERIALS AND METHODS

**Site description.** HR is a 25-km-long and 15-km-wide ridge off the coast of Oregon in the Cascadia accretionary complex, formed as the Juan de Fuca plate subducts obliquely beneath North America (56, 57). Substantial methane in the ridgetop sediments is present as free gas and as hydrate supplied by mixed thermogenic-microbial methane from deep in the system, where it exists in high-fluid-flow zones in the subsurface and immediately beneath the methane vents on the southern side of HR (41). Sites 1249 and 1250 were cored in these locations. There is also considerable biogenically produced methane created in situ in the sediments on the flanks and at the foot of HR (12, 41). Sites 1244, 1245, 1246, and 1251 were cored in these locations.

**Collection and handling of HR samples.** Forty-eight core samples were collected on Ocean Drilling Program leg 204 by using standard scientific core-drilling practices modified for microbiological studies (55a). Eight of these core samples were acquired from site 1249 or 1250 (near the southern summit of HR), and 40 samples were collected from sites 1244, 1245, 1246, and 1251 (on the flanks or foot of HR). Cores identified for microbiological characterization were tracked for quality assurance to estimate the potential for contamination by using fluorescent microspheres deployed within the core barrel and perfluorocarbon tracer in drill fluid (55b). Immediately after delivery to the deck of the ship, subsamples of cores were excised using a core cutter, the ends were sealed with core caps, and the samples were then carried to the refrigerated microbiology laboratory for additional processing. Samples were subdivided by cutting the Lexan core liner with the core cutter or a sterilized hacksaw into specified sizes (normally 5- or 10-cm lengths). The core liner ring was removed from the samples destined for QPCR analysis, the outer 1 to 2 mm of potentially contaminated sediment was pared away with a sterile spatula, and the samples were then double bagged, flushed with sterile ultrahigh-purity nitrogen, and finally

frozen at  $-80^{\circ}\text{C}$ . Samples were transferred to a liquid nitrogen-conditioned dry shipper (Minnesota Valley Equipment, Bloomington, MN), shipped overnight to the Idaho National Laboratory, and transferred on receipt to a  $-80^{\circ}\text{C}$  freezer.

DNA was extracted directly from 0.5- to 1.0-g HR sediments with a soil DNA isolation kit (MoBio Laboratories, Inc., Solana Beach, CA) following the manufacturer's suggested protocol for a maximum yield. Two subsamples from each sample were extracted independently.

**QPCR for methanogens in marine sediments.** In order to enumerate methanogens in marine sediments, we developed a QPCR protocol to detect the presence of the  $\alpha$  subunit of the MCR gene (*mcrA*), which is used as a functional attribute considered to be unique to methanogens (24, 39, 61). Previously designed PCR primers for this gene (24, 39) yielded 500-bp amplicons that were too long to be optimal for our application of QPCR. Therefore, using MCR sequences derived from GenBank entries for 24 cultivated and uncultivated methanogens (see Table S1 in the supplemental material), new primers capable of generating a shorter amplicon more amenable to QPCR were designed. Cultured representatives from each of the five methanogenic orders were used to test the primers that we designed (see the supplemental material).

**Cultivation-based determination of methanogen presence.** In order to determine whether methanogens were present in HR sediments by using a cultivation-based approach, time course incubations were initiated by using two radiolabeled substrates,  $[2\text{-}^{14}\text{C}]\text{acetate}$  and  $[^{14}\text{C}]\text{CO}_2$ . Evolutions of radiolabeled  $\text{CO}_2$  and methane were measured from duplicate tubes at eight time intervals from 2 to 90 days. These sediment preparations, including a duplicate set of enrichments without radiolabeled substrates, were established, incubated, and measured as previously described (16).

**Model methanogen and BRR operation.** To estimate the amount of methane generated by methanogens at a maintenance level of activity, *M. submarinus* (isolated from gas hydrate-bearing marine sediments in the northwestern Pacific Ocean [40]) was cultured in a BRR. All culture manipulations and incubations were performed in a glove bag (Coy Laboratory Products, Inc., Ann Arbor, MI) containing a  $\text{CO}_2\text{-H}_2\text{-N}_2$  atmosphere (5:5:90, by volume). The organism was grown to late log phase in a 1-liter screw-top glass medium bottle (Kimble/Kontes) sealed with a thick butyl rubber stopper and champagne bottle-type wire retainer. The cells were grown in MS medium (6) at  $21^{\circ}\text{C}$  with 50-kPa hydrogen provided in the headspace as the energy source.

The principles and operation of BRRs are explained elsewhere in detail (31, 59). In order to grow and sustain *M. submarinus*, a 350-ml BRR was operated inside the glove bag described above. Medium exchange in the BRR was accomplished by batch filtration through a mildly hydrophobic polypropylene vent filter (Whatman HEPA-Vent). Filtrations were performed at four-day intervals by removing half of the culture liquid and injecting fresh medium through the same filter to back-flush cells. After filtration, hydrogen was provided, initially at 101-kPa gauge pressure and then at decreasing levels of pressure as the culture reached stationary phase, as determined microscopically by acridine orange direct cell counts. The culture was periodically subcultured in fresh medium to confirm purity and viability.

Numbers of methanogens in the BRR were also determined using most-probable-number (MPN) enumerations as described previously (14).

**Methane production rate.** Once *M. submarinus* cells reached a prolonged stationary phase (cell density, ca.  $7.5 \times 10^7$  cells/ml), aliquots from the BRR were distributed into serum vials sealed with thick butyl rubber stoppers and stored at  $4^{\circ}\text{C}$  with 10.1-kPa hydrogen in the headspace. Residual dissolved gas (methane and hydrogen) was removed by five repeated 1-h evacuations and reequilibrations (1 h with shaking) of subculture headspace with oxygen-free  $\text{CO}_2\text{-N}_2$  (80:20). Headspace methane concentrations were measured by gas chromatography using a flame ionization detector and a reduced-gas detector (Trace Analytical, Menlo Park, CA). Once methane concentrations in the headspace had been reduced to ca. 0.3 to 0.6 Pa, 10-ml subsamples were distributed into serum vials so that the accumulation of methane (4 to 6 Pa) at a maintenance level of activity could be measured over short-term (<40-h) incubations. Negative controls were prepared by the addition of formaldehyde (final concentration, 3%) to kill the culture at the beginning of the incubations, and these controls were then analyzed in a fashion identical to that used for the live samples.

#### RESULTS

**QPCR to determine methanogen biomass in HR sediments.** Using DNA extracted from HR sediment amended with a known quantity of methanogen cells (see the supplemental material), we determined the minimum detection limit for the

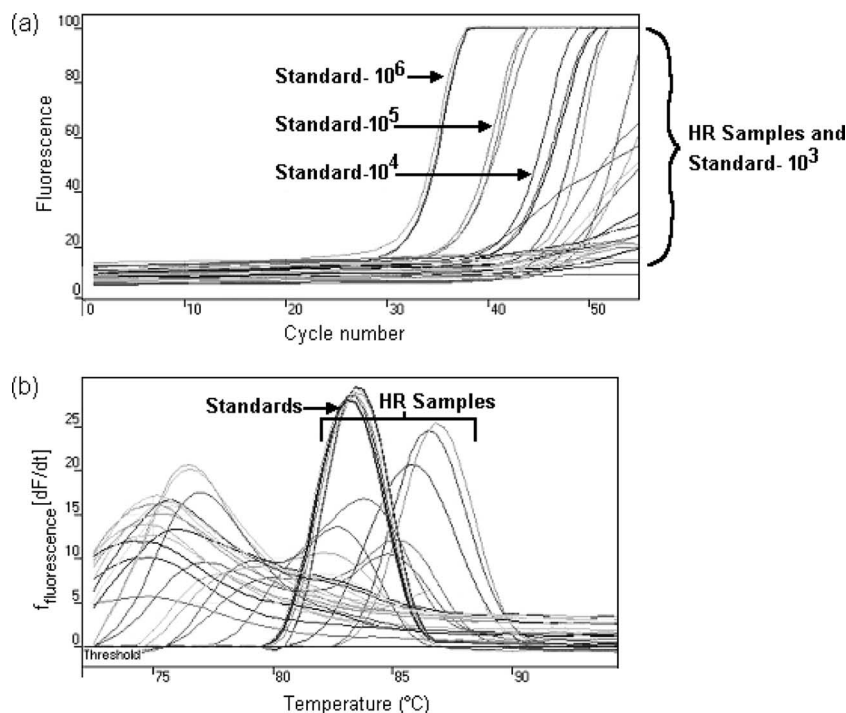


FIG. 1. (a) Representative QPCR plot comparing fluorescence to cycle number for *Methanocaldococcus jannaschii* standards and selected samples obtained from HR. (b) Melt curve analysis for the same standards and samples showing the comparison of changes in fluorescence (representative of denatured DNA due to sequence variability) over time as temperature was increased. Standards are amplified from sediments spiked with known concentrations of *M. jannaschii* cells and are expressed in terms of methanogen cells/g sediment. The analysis of 17 samples from depths of 1.0 to 5.0 mbsf are depicted, and eight of those samples yielded enough fluorescence in the QPCR reactions to be considered positive for the presence of the *mcrA* gene.

enumeration of cells with *mcrA*. Results at the level of 100 cells/g were only intermittently obtained (for about 38% of reactions). Therefore, the practical limit of detection for the assay was conservatively set at 1,000 methanogen cells/g sediment. We expect that our QPCR assay does not distinguish between methanogens and anaerobic methane-oxidizing archaea (ANME) that also possess the *mcrA* gene (see the supplemental material); however, we did not sample near the sulfate-methane interface where ANME cells would dominate. In using the term “methanogen cells/g,” we acknowledge that some of the detected genes may have come from ANME cells.

Most of the HR sediments that we analyzed yielded methanogen biomass estimates that were below the assay detection limits (Fig. 1). Only 25% of the samples could be confirmed as having any methanogens present by QPCR (Fig. 2). No increase in headspace methane was detected in any of the incubated sediment samples; thus, we could not detect cultivable methanogens in any of the samples by using conventional cultivation-based determinations. Also, most of the samples that had detectable levels of methanogens came from shallow sediments on HR. For example, of the 23 samples collected between 1 and 44 m below seafloor (mbsf), 35% of the QPCR analyses indicated the presence of methanogens. In contrast, of the 25 samples collected from areas deeper than 44 mbsf, only 8% showed evidence of more than 1,000 methanogens/g of sediment. Also, as shown in Fig. 2, practically all of the methanogen numbers estimated for the HR sediments were well

### Numbers of methanogens ( $\log_{10}^x$ ) per g sediment

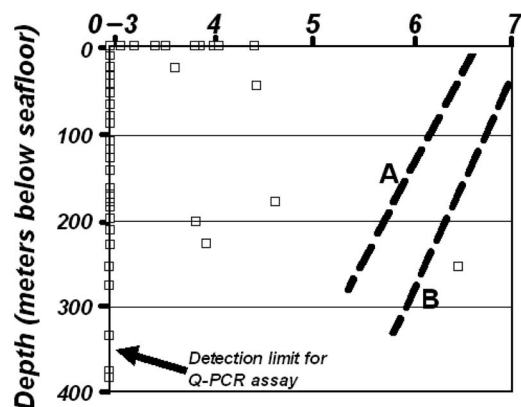


FIG. 2. Numbers of methanogens in HR sediments plotted against depth (mbsf). Methanogens were enumerated by QPCR using primers directed at the *mcrA* gene in DNA extracted from the sediments. Symbols shown are means of triplicate QPCR runs performed on a single DNA extract of sediment samples acquired from Ocean Drilling Program sites 1244, 1245, and 1251. Two such extractions were conducted on each sample. The y axis, at  $10^3$  methanogens/g, represents the limit of detection for the QPCR assay. Symbols do not distinguish between samples obtained from the different cores. Dashed lines represent the approximate numbers of total cells in the sediments for sites 1244 and 1245 (line A) and site 1251 (line B) (28).



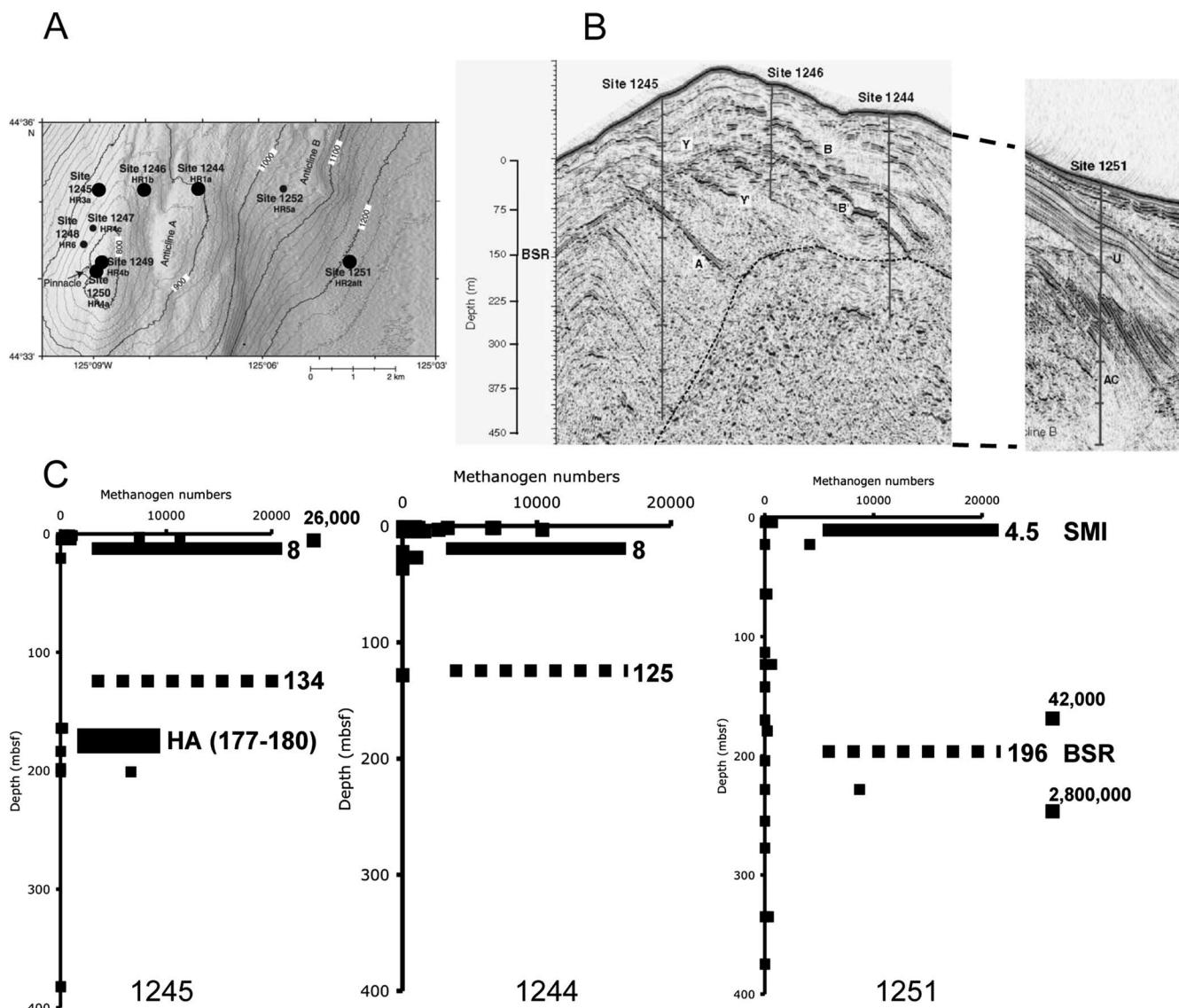


FIG. 3. Methanogen numbers (cells per g sediment) as determined by QPCR to detect the *mcrA* gene at three of the six sites on and near HR. A plan view (A) and a cross section derived by geophysical three-dimensional seismic survey (55) (B) show sites 1245, 1244 (both on the flank of HR), and 1251 (at the foot of HR), from which samples were acquired. Methanogen numbers for each of these three sites are plotted relative to depth in mbsf (C). One sample from site 1245 and two samples from site 1251 yielded higher *mcrA* gene numbers than all of the other samples, and the values for these genes are shown (converted to methanogen numbers). For each sample, three replicate QPCR analyses were performed for each of two subsamples. The depths of the sulfate-methane interface (SMI), the BSR, and the presence of horizon A (HA; only at site 1245) are marked at the appropriate depths and shown in red. At each location, the zone of hydrate stability extends from the seafloor to the depth of the BSR. Seismic reflections A, B, B', Y, Y', U, and AC in panel B are stratigraphic anomalies (55).

below (i.e., <1%) the reported total numbers of cells in the same sediments determined as a part of leg 204 (28).

In Fig. 3, methanogen numbers determined by QPCR are also shown with respect to three of the boreholes from which samples were obtained. These data indicate that no apparent difference with respect to numbers of methanogens in samples obtained from the flank of HR (sites 1244 and 1245) and those from the foot of HR, where hemipelagic sediments were sampled (site 1251) existed. Collectively, the samples from these three sites constituted 39 of the 48 samples that we characterized, and while most of these samples from areas deeper than 44 mbsf failed to show the presence of methanogens, a few

deeper sediments exhibited high numbers of methanogens. In some cases, the higher methanogen numbers that were found at considerable depth appeared within a few meters of striking geological features, such as the bottom-simulating reflector (BSR) (site 1251) and horizon A (site 1245). Among the eight samples collected from sites 1249 and 1250 at the southern summit of HR, where active methane venting occurs, only one sample (from 43.68 mbsf at site 1250) showed evidence of any methanogens (estimated at 41,000 methanogens/g).

As noted above, most often there was no detectable amplification of the *mcrA* gene in repeated measurements of a given HR sample. For these samples, we assumed that methanogens

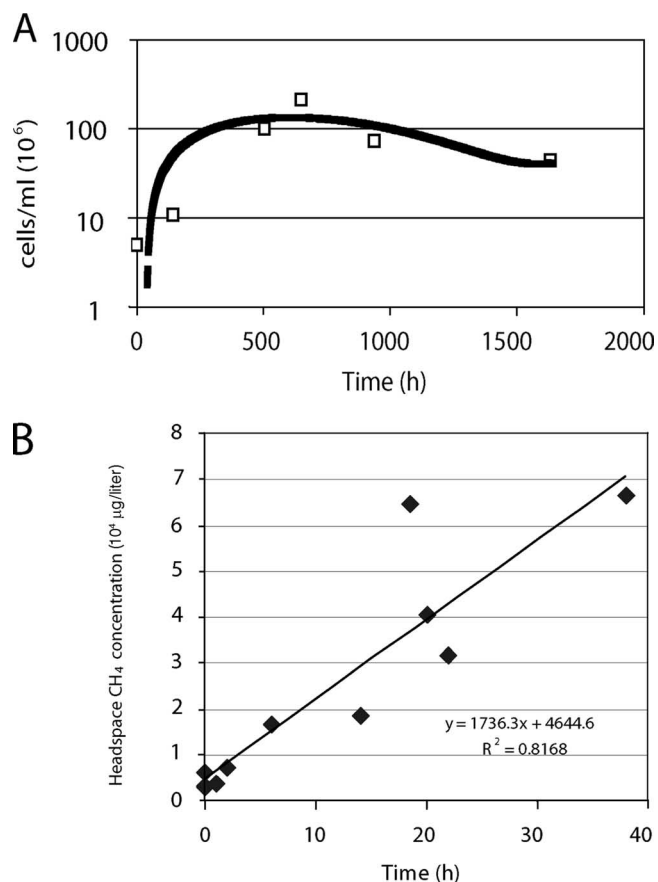


FIG. 4. Results of BRR experiments to determine maintenance levels of activity of methanogens. (A) *Methanoculleus submarinus* cell numbers in samples obtained from the BRR are plotted against time of reactor operation. Stable cell numbers after 500 h suggest that the cells in the BRR have maintenance levels of activity. (B) Methane accumulation during incubation of BRR-starved *M. submarinus* cells in serum bottles. Specific maintenance level activity of *M. submarinus* was determined from these rates and enumerations of the cells in the BRR.

were present at less than 1,000 cells per g of sediment (our limit of detection). However, we also observed that measurements made for subsamples collected from a single sample depth sometimes ranged from no cells detected to several thousand cells detected per g (data not shown). Controls with no DNA and those containing DNA extracted from nonmethanogenic archaea consistently gave negative results in our QPCR assays.

#### Methanogenic rates determined from BRR-sustained cells.

To estimate the levels at which methanogens produce methane when existing at maintenance level conditions that simulate the subsurface, a methanogen that was isolated from a hydrate formation (*Methanoculleus submarinus* [40]) was sustained in the laboratory in a BRR. Unlike most laboratory cultivation systems, a BRR functions by maintaining cells within a fixed volume and with a fixed supply of the growth-limiting substrate. During the operation of the BRR, *M. submarinus* cells increased in number until controlled by the concentration of the growth-limiting substrate (Fig. 4), resulting in a characteristic growth curve (59, 60). At this point (after 500 h in Fig. 4), the cells in a BRR were deemed to be sustaining themselves at

low specific activities; aliquots of the cell suspension were removed from the BRR, dissolved hydrogen and methane were removed from the aliquots, and then short-term accumulation of methane was measured. Methane production rates (at 24°C) during these 40-h incubations were measured in triplicate and calculated from linear regression coefficients derived from plots of headspace methane concentration against time ( $R^2$  values of 0.9525, 0.9956, and 0.9989; for pooled data, the  $R^2$  value was 0.8168). Based on the rates derived from these regression curves, compared with curves derived for sterilized controls, the mean methane production rate was estimated to be  $1.7 \times 10^{-17}$  mol methane/*M. submarinus* cell/day, or 0.017 fmol methane/*M. submarinus* cell/day.

## DISCUSSION

As for other microbes derived from environmental samples, an accounting of the numbers of methanogens present can be difficult to obtain by using cultivation-based methods. Traditional MPN analyses based on the ability of methanogens to grow and produce detectable levels of methane are labor-intensive and are often unsuccessful in cultivating most of the target cells. Similarly, fluorescence in situ hybridization approaches are labor-intensive. In competitive or MPN PCR, the measurement can be affected by biases of endpoint analysis, in which different amounts of final PCR amplicons are obtained from similar starting quantities (3). MPN PCR is also labor- and time-intensive. QPCR is sensitive, specific, and rapid (8) and has been used to enumerate archaea in water, soils, and sediments (30, 44, 58). QPCR reactions are run in replicate, with reaction and analysis times of hours; for our work, approximately three hours was required to analyze 72 samples. Since QPCR results are viewed in real time as the reaction proceeds, the bias produced by endpoint analysis is eliminated.

Our use of QPCR directed at a specific functional gene for population enumeration assumes that the DNA is derived from a living cell. DNA in dead cells or outside of cells typically has a short half-life (27), and recent investigations indicate that extracellular DNA in the upper 1 cm of marine sediments only has a ca. 9.5-year residence time (18). Because all of our samples came from HR sediments whose ages range from hundreds to millions of years, it would appear that the DNA that we extracted was most likely derived from living cells.

While many methods of detection for QPCR exist, the use of Sybr green I offers some unique advantages when working with environmental samples. The use of Sybr green I permits detection of DNA despite some variation in the internal region of the amplicon and also permits selected primers to be used for QPCR without the additional time and cost of developing and optimizing probes. Although Sybr green I will fluoresce with nonspecific PCR primer artifacts (and this can reduce the efficiency of the QPCR reaction), such data can be discriminated by using a melting curve analysis (see Fig. S3 in the supplemental material) (30), permitting this QPCR assay to attain sensitivities as low as 100 cells/g and reliably detect 1,000-cells/g levels.

The QPCR assay targeted the  $\alpha$  subunit of the MCR gene generally considered to be present in all methanogens (19, 22, 24, 39), with most methanogen isolates bearing one or two

MCR-encoding regions (52). We assumed that one *mcrA* gene copy existed per cell.

Recent studies have shown that the *mcrA* gene is also present in archaeal representatives (ANME) of consortia that perform anaerobic methane oxidation or "reverse methanogenesis" (25, 26, 44), and we expect that the *mcrA* primers that we designed may also recognize this gene in the ANME cells (see the supplemental material). In marine sediments, active anaerobic methane oxidation is restricted to a narrow band where both sulfate and methane are available, the so-called sulfate-methane interface (4). However, we expect that, as for most microbes, ANME cells may be found in sediments away from where chemical or physical conditions are optimal for their growth or metabolic activity. Previously, genes from ANME cells were not detected in clone libraries constructed of 16S rRNA genes amplified from samples acquired at sites 1244, 1245, and 1251 on HR, while methanogen clones were noted at low levels in site 1251 samples (28). While none of the samples used in our study came from the sulfate-methane interface, our enumerations of methanogens may include ANME cells. An understanding of the interstitial water chemistry in the sediments, specifically the concentrations of methane and sulfate, can help to infer whether detection of an abundance of *mcrA* genes indicates cells associated with anaerobic methane oxidation or those associated with methanogenesis.

For most of the HR samples that we examined, we could not detect methanogens. However, our estimates suggest that, in the samples in which the *mcrA* gene was detected, the numbers of methanogens were typically  $\leq 1\%$  of the total cell numbers in the sediments (Fig. 2) (28) as well as of the total cell numbers reported to occur in numerous marine sediment samples collected elsewhere (47). The findings were consistent with observations that the numbers of archaeal cells, albeit not specifically methanogens, decrease rapidly in the upper 40 m of the sediment column in ocean margin locations (28, 54).

The overall estimated numbers of methanogens in these sediments were low, but some unexpectedly high values were measured at greater depth in the sediments (Fig. 2 and 3). Our sampling densities (numbers of samples) near prominent geological features such as horizon A (site 1245) and the BSR were insufficient to permit accurate measurement of methanogen biomass at such locations, although some of the highest methanogen numbers that we detected were within several meters of these geological features. Horizon A is a three-meter-thick volcanic-ash-rich stratum that is highly transmissive, conveying fluids from deeper in the sediments, and the BSR is at the lower limit of the hydrate stability zone, a location where three phases of methane (free gas, in the dissolved state, and in a solid phase) may occur within a short vertical sequence (55a).

Despite evidence that much of the methane that occurs in sediments along coastal margins is biogenic (33), the detection of methanogens in such locations has been difficult. The few methanogens detected in the hydrate-rich sediments of HR is consistent with findings from studies examining 16S rRNA gene clone libraries from sediment samples collected in the vicinity of hydrates (32, 51). Even investigations that included 16S rRNA gene clone libraries consisting of thousands of phylotypes have noted few sequences that bear a resemblance to

known methanogens (28). Recently, the detection of methanogens in deep Nankai Trough sediments was possible by targeting *mcr* genes as opposed to 16S rRNA genes (43).

We also noted a heterogeneous distribution of methanogens in our samples, wherein subsamples from the same sediment material (i.e., the same depth and the same borehole) frequently yielded results that were different with respect to the methanogen numbers. This suggested a patchy distribution of methanogens, as has been found in other subsurface environments (7, 9).

By using the BRR to sustain *M. submarinus* cells under simulated starvation, a maintenance level rate of methane production of ca. 0.017 fmol methane/*M. submarinus* cell/day was estimated. These cells were starved of substrate, and methane was removed from the system. Therefore, the production of methane by cells from the reactor was likely thermodynamically unconstrained compared to the methane production of methanogens existing in marine sediments where they are surrounded by high levels of methane. *M. submarinus* can be thermodynamically impaired at high methane partial pressures (11), and though it was originally isolated from hydrate-bearing sediments, we do not know how representative of methanogenic communities as a whole this isolate is. Factors such as the rate of substrate introduction and quantity of substrate added likely differ between the BRR and the subsurface. As with other methods, the BRR method may overestimate or underestimate true rates depending on the degree to which it simulates in situ conditions.

The methanogenic rate that we estimated is lower than previously reported methanogenic rates, including 31.5 and 108.8 to 135 fmol methane/cell/day from environments such as lake sediments (36) and anaerobic reactors (37), respectively. Considering the Arrhenius law, which predicts a doubling of enzymatic activity for every 10°C increase in temperature, the rate determined for cells from the BRR at 24°C would correspond to about 0.004 fmol methane/*M. submarinus* cell/day if corrected for seafloor temperatures of ca. 4°C. Understanding the impacts of pressure, substrate concentration, product concentration, and the capabilities of communities of microbial cells (in comparison to the conditions when making measurements for an isolate) would further hone our understanding of microbial activities in deep sediments.

To evaluate the determined rates of methanogenesis relative to other microbial rate estimates, the rate of 0.004 fmol methane/*M. submarinus* cell/day was converted to g methane carbon produced/g cell carbon/h and compared to rates of metabolic-product formation by other microbes existing at various metabolic states as summarized by Price and Sowers (50). Using calculated or published conversion factors (i.e., 16 fg methane/fmol and 170 fg/cell [2]; 50% of the dry mass of a cell consists of carbon [1]), the activity of *M. submarinus* cells in the BRR was estimated to be  $5.9 \times 10^{-6}$  g methane carbon produced/g cell carbon/h. This rate was consistent with rates demonstrated for different cell types grown at the same temperature and exhibiting maintenance levels of activity (50). The *M. submarinus* maintenance level rate of metabolism is similar to rates estimated for sulfate-reducing and methanogenic communities in deep sediments off the Peru margin (ca.  $1 \times 10^{-5}$  g carbon/g cell carbon/h) (48).

By combining maintenance level methanogenesis rates de-



rived from the BRR with methanogen biomass values for HR samples as determined by QPCR targeting the *mcrA* gene, an estimate of the microbial methane created at in these marine sediments can be made. Most of the samples yielded methanogenesis estimates that were less than the 1,000-cells/g sediment detection limit for the QPCR assay, corresponding to  $<4.25$  fmol methane/g sediment/day (i.e.,  $<0.004$  fmol methane/cell/day  $\times$  1,000 cells/g at seafloor temperature). As noted above, this rate is based on the detection limit for the QPCR assay, and many of the samples that we analyzed were below the limit of detection for this assay. For this reason, and because some of the cells that were detected may have been ANME cells, actual in situ rates may be considerably lower than this rate. Nevertheless, with respect to previously published methanogenesis rate estimates for other seafloor locations where hydrates are important, our calculated rate for HR sediments is lower than rates reported for Cascadia margin and Blake Ridge sediments by at least three orders of magnitude and as many as nine orders of magnitude, respectively (see reference 15 for details).

Using factors that convert mass, time, and density and volume units into more practical terms (i.e.,  $10^{12}$  fmol equal 1 mmol, 365 days equal 1 year, and a sediment density of  $2.6$  g/cm<sup>3</sup> of sediment equals  $10^6$  cm<sup>3</sup>/m<sup>3</sup>), the rate that we determined for most of the samples can be expressed as  $<4.03 \times 10^{-3}$  mmol methane produced/m<sup>3</sup> sediment/year. For comparison, the modeled microbial methane production rates for deep ( $>100$ -mbsf) and shallow (a few meters below seafloor) sediments on HR are  $<0.1$  and ca. 10 mmol methane produced/m<sup>3</sup> sediment/year, respectively (12). Based on higher levels of *mcrA* gene detected in the shallower sediments, our rates of methanogenesis could also scale upwards in the shallower sediments. The detected *mcrA* genes could also belong to ANME cells, even though we did not sample at the sulfate-methane interface.

The methanogenic rates that we estimated for these sediments should take into account the amount of methane that can be biologically generated in the sediments if the sediments are the sole supply of microbial reductants and oxidants. In other words, if it is assumed that the total organic carbon (TOC) buried in the sediments is the source of the methane, then the estimated rates of methanogenesis must be consistent with both the amount of TOC available in the sediments and the time over which it can be metabolized. Generally, the TOC constitutes 1.5% of the weight of the HR sediment mass (63), equivalent to 15 mg/g. If 10% of the organic carbon in sediment TOC is available for conversion to methane (13), then as much as 1.5 mg methane/g sediment might be generated from the TOC present. For one of the deeper samples that we analyzed, coming from 375 mbsf, the sediment is estimated to be Pliocene-Pleistocene (1.6 million years of age) (55). Assuming that the methanogens in this sample were continuously active and producing on the order of 4.25 fmol methane/g sediment/day for approximately  $1.6 \times 10^6$  years, this equates to  $2.5 \times 10^{-3}$  mmol methane/g of sediment. Conversion of the methane mass term to milligrams (using 16 mg methane/mmol) yields a methanogenic productivity value of about 0.04 mg methane/g of sediment, a factor of 38 lower than the 1.5 mg methane/g sediment that might be possible according to the amount of TOC assumed to be present. In this closed-system

scenario, a methanogenic rate term sustained over geologically relevant time periods (i.e., thousands to millions of years) at a rate much higher than the one we measured would exhaust the sediment TOC that is available for conversion to methane.

Some parts of HR may act as a closed system without outside input of fluids, and in these locations, the rates we estimated are low enough to sustain methanogenic activity over long periods in the sediments, given the amount of organic carbon available. However, this view of HR as being closed is imperfect, as it assumes that the sediments would experience the gradual depletion of microbial reductants and oxidants. In fact, much of the HR subsurface is not closed and instead is a gas- and hydrate-rich environment that has the attributes of two distinctive gas-fed systems with respect to methane supply: a "focused, high-flux" system and a "distributed, low-flux" system (41, 62). To consider how the rates that we estimated compare to the active flux of methane out of HR, we can use the areal extent of the southern side of HR ( $3.7 \times 10^7$  m<sup>2</sup>) (5) and an arbitrary depth of 100 m into the sediments to derive a volume of  $3.8 \times 10^9$  m<sup>3</sup> as a zone of active methanogenesis for this location. Then, using our base rate of methanogenesis ( $<4 \times 10^{-3}$  mmol/m<sup>3</sup>/year) hypothetically occurring uniformly within that volume, we derive  $<1.5 \times 10^7$  mmol of gas/year created by methanogens. This term is 68% of the total amount of  $2.2 \times 10^7$  mmol of methane C/year estimated to be released into the overlying water from the southern side of HR (5). Thermogenic methane, a deeper biogenic zone, and dissolution of hydrates may all contribute to the difference in these approximate determinations.

None of our samples came from the fractures or horizons that represent the focused, high-flux strata. However, the low-flux system that exists elsewhere at HR would place methanogens and other microbes under conditions that are intermittently open to fluid movement. This could permit the periodic infusion of small, anaerobically oxidizable molecules (hydrogen, carbon monoxide, acetate, formate, and ammonia), along with reducible molecules (sulfate, carbon dioxide, and nitrate), to the sediments. At this time, it is difficult to determine whether other electron acceptor/donor couples (e.g., hydrogen/bicarbonate) in the sediments are present in concentrations high enough to permit methanogenesis, although such compounds are present (38). In a dynamic HR setting, the intermittent flux of appropriate molecules might allow sufficient activity to sustain chemolithotrophs, including methanogens, and the creation of more-complex organic compounds that may, in turn, support heterotrophs. Under these circumstances, there might be considerable fluctuation in the in situ rates of microbial activities.

Determining the concentrations of key electron acceptor/donor couples in the sediments will be important for completing our understanding of the biological activities in these systems. How such concentrations in the subsurface at HR change subtly over time by diffusion of fluids or dramatically through the opening and closing of fractures in the sediments will lead to better conceptual models of microbial activity. As such changes in the subsurface occur, thermodynamic conditions will determine whether the activity of methanogens and other microbes can proceed. This thermodynamic constraint has been demonstrated for methanogenic isolates from other marine environments wherein the cells require higher concentra-

tions of energy-yielding substrate (hydrogen) to remain active in the presence of high levels of methane (11).

**Conclusions.** This investigation of methanogenic activity in sediments was prompted by the importance of deriving methanogenic rate terms for computational models of hydrates. Such models are critical for understanding the distribution and dynamics of methane hydrates in locations where the methane in hydrates may contribute to climate change or may be a potential fuel source. Microbial rate terms are difficult to estimate accurately by using experimental incubation of sediments, as such measurements often yield unrealistically high rates of activity. By combining methanogenic rate data from starvation studies with a model methanogen (native to methane hydrates) and estimates of methanogen numbers in hydrate-bearing sediments by QPCR, we derived rates of methanogenesis in sea-floor sediments that were lower than many values previously derived by other methods. The rates estimated were generally consistent with methanogenic rates determined for HR by modeling of pore water chemistry, with rates that were determined for microbes sustained at maintenance levels of activity, and with the amount of methane emitted from HR sediments. The geologically dynamic nature of a system like HR would most likely result in the intermittent influx of microbial oxidants and reductants, thereby permitting fitful activities by the microbes therein, yielding methanogenic rates that may be higher or lower than those that we report. We expect that consideration of other characteristics of the sediments in methane-rich locations that were not considered in this investigation will provide improved estimates of the rate of methanogenesis for use in modeling these strata. Examples of important characteristics include in situ pressure, temperature, substrate/product concentrations, fine-scale differences in sediment properties, and how methanogen communities (rather than a single methanogen isolate) may make methane under these conditions.

#### ACKNOWLEDGMENTS

This research used samples and/or data provided by the Ocean Drilling Program (ODP). ODP is sponsored by the U.S. National Science Foundation (NSF) and participating countries under management of Joint Oceanographic Institutions (JOI), Inc. This work was supported with funding provided by the U.S. Department of Energy, Office of Fossil Energy, to the Idaho National Laboratory, now operated by Battelle Energy Alliance, under contract DE-AC07-05ID14517.

We are indebted to the scientists and crew of the RV JOIDES Resolution who collected the core samples as a part of ODP leg 204 and to the late David Boone for providing methanogen cultures from the Oregon Collection of Methanogens.

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