

ORIGINAL ARTICLE

Cultivation of methanogenic community from subseafloor sediments using a continuous-flow bioreactor

Hiroyuki Imachi¹, Ken Aoi^{1,2}, Eiji Tasumi¹, Yumi Saito¹, Yuko Yamanaka¹, Yayoi Saito^{1,2}, Takashi Yamaguchi², Hitoshi Tomaru³, Rika Takeuchi³, Yuki Morono⁴, Fumio Inagaki⁴ and Ken Takai¹

¹Subsurface Geobiology Advanced Research (SUGAR) Project, Extremobiosphere Research Program, Institute of Biogeosciences, Japan Agency for Marine-Earth Science & Technology (JAMSTEC), Yokosuka, Kanagawa, Japan; ²Department of Environmental Systems Engineering, Nagaoka University of Technology, Nagaoka, Niigata, Japan; ³Department of Earth and Planetary Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan and ⁴Geomicrobiology Group, Kochi Institute for Core Sample Research, JAMSTEC, Monobe B200, Nankoku, Kochi, Japan

Microbial methanogenesis in subseafloor sediments is a key process in the carbon cycle on the Earth. However, the cultivation-dependent evidences have been poorly demonstrated. Here we report the cultivation of a methanogenic microbial consortium from subseafloor sediments using a continuous-flow-type bioreactor with polyurethane sponges as microbial habitats, called down-flow hanging sponge (DHS) reactor. We anaerobically incubated methane-rich core sediments collected from off Shimokita Peninsula, Japan, for 826 days in the reactor at 10 °C. Synthetic seawater supplemented with glucose, yeast extract, acetate and propionate as potential energy sources was provided into the reactor. After 289 days of operation, microbiological methane production became evident. Fluorescence *in situ* hybridization analysis revealed the presence of metabolically active microbial cells with various morphologies in the reactor. DNA- and RNA-based phylogenetic analyses targeting 16S rRNA indicated the successful growth of phylogenetically diverse microbial components during cultivation in the reactor. Most of the phylotypes in the reactor, once it made methane, were more closely related to culture sequences than to the subsurface environmental sequence. Potentially methanogenic phylotypes related to the genera *Methanobacterium*, *Methanococcoides* and *Methanosarcina* were predominantly detected concomitantly with methane production, while uncultured archaeal phylotypes were also detected. Using the methanogenic community enrichment as subsequent inocula, traditional batch-type cultivations led to the successful isolation of several anaerobic microbes including those methanogens. Our results substantiate that the DHS bioreactor is a useful system for the enrichment of numerous fastidious microbes from subseafloor sediments and will enable the physiological and ecological characterization of pure cultures of previously uncultivated subseafloor microbial life.

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Introduction

Methanogenesis in subseafloor sediments is an important process in the planet's carbon cycle. A large quantity of methane is stored in the subseafloor as free gas, dissolved methane in the pore water or as hydrates and comprises approximately

500–2500 Gt of methane carbon (Milkov, 2004). Subseafloor methane has also attracted attention as an energy resource, but methane is also a powerful greenhouse gas that is critical in the history of Earth's carbon cycle and climate changes (Schoell, 1988; Kvenvolden, 1995; Overpeck and Cole, 2006). Because isotopically light methane (that is, ¹³C-depleted methane carbon) is globally distributed in subseafloor sediments, subseafloor methane may be largely mediated by microbial activities that cause enzymatic fractionation of carbon species (Milkov, 2004). Therefore, subseafloor microbial methanogenesis has been extensively investigated, largely with culture-independent molecular ecological techniques.

Correspondence: H Imachi, Subsurface Geobiology Advanced Research (SUGAR) Project, Extremobiosphere Research Program, Institute of Biogeosciences, Japan Agency for Marine-Earth Science & Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka, Kanagawa 237-0061, Japan.

E-mail: imachi@jamstec.go.jp

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Culture-independent molecular analyses have found a limited population of the known methanogenic *Archaea* (methanogens) groups in a variety of subseafloor sediments (see references in reviews by Fry *et al.* (2008); Inagaki (2010)). On average, methanogens represent only 0.1% population of the total archaeal 16S rRNA gene pool in deep subseafloor sediments (Fry *et al.*, 2008). Methane-metabolizing archaeal populations also represent <1% of the total biomass in Hydrate Ridge sediments based on the quantitative-PCR analysis of the methyl-coenzyme M reductase (*mcrA*) gene (Colwell *et al.*, 2008). Whereas, it has been hypothesized that subseafloor microbial methanogenesis might be driven by presently unidentified and/or uncharacterized life forms or via novel methanogenic pathways in subseafloor sediments (Inagaki *et al.*, 2006). Thus, to clarify the significance of microbial methanogenesis in the subseafloor environments, the *in situ* methane-producing microbial entities need to be identified with the cellular and functional evidences. Cultivation of subseafloor methanogens is a significant challenge to such identification. However, traditional batch-type cultivation efforts have always encountered the strong resistance of the numerically dominating and/or functionally important microbial components in the subseafloor sediments (for example, D'Hondt *et al.*, 2004; Toffin *et al.*, 2004). Regarding to methanogens, only three methanogens have been successfully isolated and characterized from deep-sea sedimentary environments: *Methanoculleus submarinus* (Mikucki *et al.*, 2003), *Methanococcus aeolicus* (Kendall *et al.*, 2006) and *Methanosarcina baltica* (von Klein *et al.*, 2002). In addition, Kendall and Boone (2006) reported that methanogenic enrichment cultures were obtained from anoxic marine sediments at Hydrate Ridge after a 2-year long-term incubation.

To effectively cultivate subseafloor microbes, including methanogens, we adopted a continuous-flow bioreactor technique. The bioreactor used in this study is a down-flow hanging sponge (DHS) reactor originally developed for the treatment of municipal wastewater (for example, Agrawal *et al.*, 1997; Tandukar *et al.*, 2006; Uemura and Harada, 2010). A distinctive feature of the DHS reactor is the use of a polyurethane sponge to ensure the pore space of the medium, providing an enlarged surface area for microbial habitat and a longer cell residence time. In addition, continuous-flow-type cultivation can maintain substrates at low concentrations similar to those found in the natural environment. Continuous-flow also allows the outflow of metabolic products that may inhibit microbial growth if accumulated.

In this study, a methanogenic microbial community was successfully enriched from methane-rich subseafloor sediments using a newly designed DHS reactor. We also obtained ten anaerobic microorganisms in pure culture, including four methanogenic

archaeal species, which were not cultivated directly from the natural subseafloor sediment samples but were readily cultivated from the enriched community in the reactor by conventional batch-type cultivation techniques.

Materials and methods

Sediment core sampling

In 2006, the shakedown cruise CK06-06 of the deep-sea drilling vessel *Chikyu* was conducted at approximately 80 km offshore the Shimokita Peninsula of Japan, the northwestern Pacific. During the expedition, 365 m of sediment cores comprising a continuous record of the upper sedimentary section were recovered at Site C9001 (41°10.6389'N, 142°12.081'E, 1180 m water depth; Aoike and CK06-06 Scientists, 2007; Tomaru *et al.*, 2009). For this study, we subsampled innermost sections (approximately 50 cm³) of the whole round cores from the sediment depths of 0.42, 4.66, 18.34, 48.11 and 106.7 mbsf using a sterile tip-cut plastic syringe. We then made slurries of the sediments with 200 ml autoclaved synthetic seawater (described below) without organic substances and neutralized Na₂S · 9H₂O solution under nitrogen gas. The subsampled sediments consisted of diatom-rich hemipelagic silty clay. All subsampling procedures were performed in a laminar flow clean bench onboard the *Chikyu*, and the sediment slurries were preserved at 4 °C in the dark until experiments were performed.

DHS reactor and incubation of sediment

A schematic diagram and movie of the DHS reactor are shown in Figure 1 and Supplementary Movie S1, respectively. The DHS reactor was constructed from a closed polyvinyl chloride (PVC) box (inside volume 10.7 l) with polyurethane sponge-cubes (3 cm × 3 cm × 3 cm, pore size 0.83 mm) as the carrier material for microbial cells. The sponge-cubes were encased in plastic nets to prevent crushing of the sponges. A total of 147 sponge carriers were randomly packed into the PVC box. The total pore volume of the sponges was 2.75 l, and this value was used for calculating the hydraulic retention time. The DHS reactor was operated under atmospheric pressure.

A mixture of the sediment slurry from multiple depths was used as the inoculum; that is, 20 ml of 0.42-mbsf sediment slurry, 50 ml of each other sediment slurry and 230 ml of synthetic seawater (described below) without any organic substances. The sponge-cubes were soaked with the mixed slurry manually and encased in the plastic net (Supplementary Figure S1). The encased sponges were then placed in the PVC box. This inoculation procedure was performed in a cold room maintained at 4 °C, and the slurry and PVC box were flushed by

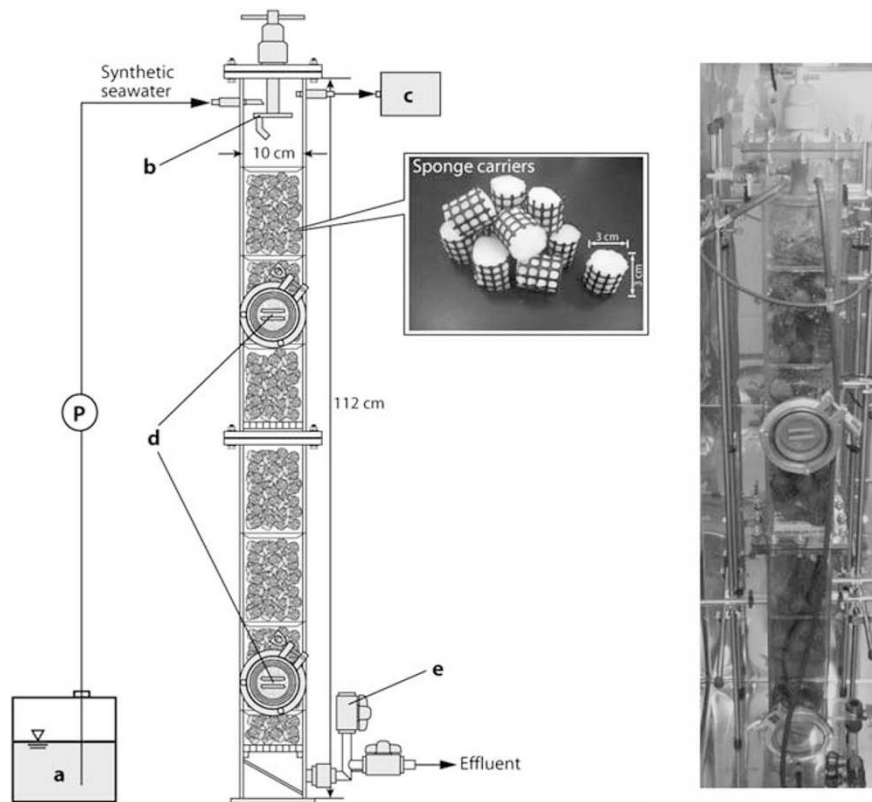


Figure 1 Schematic diagram and photographs of the DHS reactor used in this study. (a) Synthetic seawater tank, (b) distributor, (c) gas collecting bag, (d) sponge carrier sampling port and (e) port for the pH/ORP sensor.

nitrogen gas at all times. After inoculation, the PVC box was tightly closed and installed in an incubator (M-600FN, Taitech, Koshigaya, Japan) in the dark at 10 °C.

The composition of the synthetic seawater for the DHS reactor was the following (l⁻¹): glucose, 85.45 mg; sodium acetate, 58.3 mg; propionate, 29.72 mg; yeast extract, 19.73 mg; NH₄Cl, 0.53 g; KH₂PO₄, 0.1 g; MgCl₂ · 6H₂O, 4 g; CaCl₂ · 2H₂O, 1 g; NaCl, 25 g; NaHCO₃, 2 g; Na₂S · 9H₂O, 0.04 g; trace element solution (Imachi *et al.*, 2008), 1 ml; and vitamin solution (Imachi *et al.*, 2009), 1 ml. ‘Synthetic seawater’ as used in this study is defined as synthetic seawater containing these organic substrates, unless otherwise mentioned. The synthetic seawater did not contain any sulfate salt. The final concentration of organic substrates was set at 200 mg of chemical oxygen demand per liter, corresponding to 76.17 mg-C l⁻¹ as the total organic carbon (the chemical component of yeast extract was assumed to be C₅H₇NO₂). We used these organic substrates to enrich subseafloor methanogens because methanogens grow syntrophically with heterotrophic H₂-producing bacteria via interspecies H₂ transfer in many natural environments (see detailed explanation in Supplementary Text 1).

The synthetic seawater was purged by nitrogen gas, and pH was adjusted to 7.5. A total of 10 l of synthetic seawater was made at a time. The 10-l seawater bottle was stored at 10 °C. This anaerobic

synthetic seawater was supplied into the reactor from the top inlet port by a peristaltic pump (Masterflex L/S tubing pump 7550-50, Cole-Parmer, Vernon Hills, IL, USA) with viton tubings (Cole-Parmer) and distributed to the sponge carriers by a distributor unit (Supplementary Figure S2). The synthetic seawater then flowed down, passing through the sponge carriers by gravity and was finally pumped out of the PVC box. The hydraulic retention time in the reactor was 84 h, and the synthetic seawater was supplied intermittently by an automatic on/off timer (FT-011, Fine Inc., 1 min/29 min (on/off)) connected to the peristaltic pump to spread the seawater to the sponge carriers throughout the PVC box. In addition, the distributor unit was manually rotated at 3-day intervals to diffuse the seawater into all of the sponge carriers (Supplementary Figure S2). The gas phase in the reactor was sampled with an aluminum bag (AAK-5, Asone, Osaka, Japan) via viton tubing (Cole-Parmer) from the top portion of the reactor.

Chemical analysis and sampling from the DHS reactor

Detailed chemical analysis method is described in the Supplementary Text 2. For microbial community analyses and batch-type cultivation experiments, sponge carriers were sampled at 357, 560 and 761 days of operation. At each sampling, two sponge carriers were collected from each sampling port

(Figure 1), and N₂ gas was flushed during the sampling (Supplementary Figure S3). After the sampling, sponge carriers were immediately soaked in the anaerobic synthetic seawater, and sediments were removed from the sponge carriers by manual rubbing in the anaerobic synthetic seawater without organic compounds.

Nucleic acid extraction, PCR, cloning and phylogenetic analysis

Detailed methods of DNA and RNA extractions, PCR amplification and clone library construction are described in the Supplementary Texts 2 and 3. Recovered 16S rRNA and its gene clone sequences were classified into each phylotypes by a threshold of 97% sequence similarity. Representative phylotypes of *mcrA* as well as 16S rRNA and its gene sequences were subjected to similarity analysis with the BLAST program (Altschul *et al.*, 1997). The names of the phylotypes obtained from the inoculum sediment sample and enrichment samples in the DHS reactor are indicated by xxxD(or R)_A(or B or mcrA)yy (where x=sampling day, D=DNA-based library, R=RNA-based library, A=archaeal clone, B=bacterial clone, mcrA=mcrA clone and y=phylotype number). The names of the phylotypes obtained from the batch-type cultures are indicated by xxx_A(or B)y (where x=culture name, A=archaeal clone, B=bacterial clone and y=phylotype number). The phylogenetic analysis of the 16S rRNA gene sequence obtained was performed as described previously (Imachi *et al.*, 2006). A deduced McrA amino-acid sequence-based phylogenetic tree was constructed by the neighbor-joining method in the ARB program (Ludwig *et al.*, 2004) with 168 amino acid positions and the percentage of acceptance of mutations distance correction. The sequences reported in this study have been deposited in the GenBank/EMBL/DBJ database under accession numbers AB598074 to AB598076, AB598144 to AB598294 and AB598555.

Real-time PCR quantification

Quantitative real-time PCR was performed using SYBR Green I fluorescent dye as described previously (Lipp *et al.*, 2008). For archaeal and bacterial 16S rRNA gene amplifications, the primer sets ARC806F/ARC958R and Bac27F/EUB338R-I were used, respectively.

Fluorescence in situ hybridization (FISH)

Detailed sample fixation method and hybridization procedure are described in the Supplementary Text 2. The 16S rRNA-targeted oligonucleotide probes used in this study are shown in the Supplementary Table S1. To estimate the effectiveness of probes RC-III131, MBGB-380 and MBGD-318, which targeted environmental sequence

groups, the Clone-FISH method (Schramm *et al.*, 2002) was performed according to a previously described protocol (Kubota *et al.*, 2006). The plasmid-inserted sequences for the Clone-FISH were obtained from the sediment slurries used in this study and from methane seep sediments of the Nankai Trough stored in our laboratory. The clonal sequences used for the Clone-FISH were AB598074 to AB598076. Oligonucleotide probes were labeled with Cy-3, Alexa Fluor 555 or Alexa Fluor 488. To distinguish microbial cells in sediment slurry samples, the samples were counterstained with 4',6-diamidino-2-phenylindole (1 µg ml⁻¹ for 5 min at room temperature) after all of the *in situ* hybridization steps.

Batch-type cultivation

To isolate anaerobic microorganisms grown in the DHS reactor, batch-type cultivation was performed in 50-ml serum vials containing 20 ml media under an atmosphere of N₂-CO₂ (80/20 (v/v)) without shaking. The basal medium was the synthetic seawater used for the DHS reactor cultivation without organic compounds and contained one of the following energy sources: (i) each of the organic substrates in the synthetic seawater (that is, glucose, yeast extract, acetate and propionate); (ii) each of the methanogenic substrates, such as H₂, formate, trimethylamine, methanol and 2-propanol; or (iii) each of the fermentation intermediates from glucose and yeast extract, such as ethanol, lactate and butyrate. We also tested the media in the presence and absence of antibiotics (that is, ampicillin, vancomycin, kanamycin and rifampicin) to obtain archaeal cultures that might be outcompeted by the bacterial populations. Each antibiotic was added to the media at a final concentration of 50 µg ml⁻¹. For the cultivation of hydrogenotrophic methanogens using H₂ and formate, 0.01% (w/v) yeast extract and 1 mM acetate were added to the media. Initially, approximately 2 ml of the enrichment sediment slurry samples that were collected at 560 and 761 days of operation were inoculated into each medium (Supplementary Figure S3). All of the cultures were incubated at 10 °C, except for the cultivation of acetate-, propionate- and butyrate-utilizing anaerobes, for which prepared cultures were incubated at 25 °C because of their slow growth (for example, Imachi *et al.*, 2007). Isolation of microorganisms from the enrichment cultures was conducted by serial dilution in liquid culture and/or roll-tubes. The purity of the isolate was routinely checked by microscopy.

As a reference to the continuous-flow-type cultivation using the DHS reactor, conventional batch-type cultivation was also performed from the natural mixed sediment slurry using H₂ (approximately 150 kPa in the head space), formate (20 mM), methanol (10 mM), acetate (5 mM), butyrate (10 mM) or propionate (10 mM) as the energy source. The

reference cultivations were performed at 10 °C, in 100 ml serum vials containing 30 ml medium under an atmosphere of N₂-CO₂ (80/20, (v/v)) without shaking. The basal medium was the same composition as in the isolation works described above. A total of 10 ml of the sediment slurry was inoculated into each serum vial. The culture vessels were set up in duplicate for each substrate. The serum vials were sealed with butyl rubber stoppers and aluminum crimp seals. To monitor anaerobic conditions in the media, resazurin was added to the medium as a redox indicator.

Microscopy

An Olympus microscope (Olympus BX51F, Olympus, Tokyo, Japan) with a color CCD camera system (Olympus DP72) was used for the studies of cell morphology and epifluorescence.

Results

Microbial metabolism and activities during enrichment in the DHS reactor

We operated the DHS reactor at 10 °C for 826 days. The average oxidation-reduction potential (ORP) value of the effluent seawater was -287 mV (s.d. ± 56 mV, *n* = 677), indicating that reductive conditions were maintained in the reactor during operation (Supplementary Figure S4a). The average pH value was 7.4 (s.d. ± 0.1; Supplementary Figure S4b). We also analyzed the chemical characteristics of effluent seawaters and gases at an approximately 2-month interval after 179 days (Table 1). Complete consumption of glucose was observed in all of the samples examined. Acetate accumulation, probably because of microbial fermentation of glucose and/or yeast extract, was observed in all of the samples examined, whereas acetate consumption was

evident after 631 days based on the decrease in acetate concentration. No methane was detected before day 179. A small amount of dissolved methane was detected in the effluent seawater of 179- and 230-day samples. Methane was first observed in the gas phase of the reactor at 1.5 mg-Cl⁻¹ at 289 days. The methane concentration subsequently increased up to 10.1 mg-Cl⁻¹ by day 560 and fluctuated between 3.6 mg-Cl⁻¹ and 9.0 mg-Cl⁻¹ after 631 days. The stable carbon isotope compositions of methane and CO₂ in the gas phase of the reactor were between -60.1‰ and -78.1‰ and between -14.7‰ and -15.9‰, respectively. These values strongly indicate that microbial methanogenesis occurred (for example, Whiticar, 1999). We also provided propionate as a substrate, but distinct degradation of propionate was not observed until after 631 days. Any other potential fermentation intermediates such as lactate, succinate, fumarate, malate, ethanol and butyrate were not detected in the effluent seawater. The carbon mass balance was inconsistent between the influent and effluent; this inconsistency was particularly pronounced after 420 days of operation. This carbon mass imbalance may be due to the escape of dissolved carbon such as carbon dioxide produced by microbial methanogenesis and fermentation. These data indicate the existence of a methanogenic microbial community in the DHS reactor.

Abundance of Archaea and Bacteria estimated by quantitative real-time PCR

In the inoculum sediment slurry, the relative abundance of archaeal and bacterial 16S rRNA genes was found to be 2.9% and 97.1%, respectively (Supplementary Table S2). However, in the enrichment samples from the DHS reactor, the relative abundance of archaeal rRNA genes was consistently

Table 1 Changes of substance concentrations during the DHS reactor operation

	Theoretical values of influent	Sampling day of effluent seawaters and gases										
		179	230	289	357	420	491	560	631	693	761	826
TOC (mg-Cl ⁻¹)	76.17	59	69	52	68	17	42	45	17	16	21	23
Glucose (mg-Cl ⁻¹)	34.18	0	0	0	0	0	0	0	0	0	0	0
Yeast extract (mg-Cl ⁻¹) ^a	10.47	—	—	—	—	—	—	—	—	—	—	—
Acetate (mg-Cl ⁻¹)	17.06	31	36	28	40	28	31	23	16	20	18	10
Propionate (mg-Cl ⁻¹)	14.46	12	19	11	18	11	12	12	7	8	6	5
Dissolved CH ₄ in effluent (mg-Cl ⁻¹)		0.01	0.02	—	0.1	0.2	0.3	0.4	0.3	0.3	0.4	0.2
CH ₄ in gas phase (mg-Cl ⁻¹)		0	0	1.5	3.5	3.6	8.4	10.1	8.0	5.9	9.0	3.6
Carbon balance (%) ^b		77	91	69	89	22	55	59	22	21	28	30
δ ¹³ C-CH ₄ (‰) ^c		—	—	—	-74.9	-78.1	-64.5	-60.1	-60.9	-61.7	-62.8	-64.4
δ ¹³ C-CO ₂ (‰) ^c		—	—	—	-15.5	-15.0	-15.6	-15.7	-15.7	-14.7	-15.5	-15.9

Abbreviations: DHS, down-flow hanging sponge; TOC, total organic carbon.

^aYeast extract was calculated on the assumption that the chemical component is C₅H₇NO₂.

^bThe values of carbon balance were calculated by dividing total effluent-TOC including methane gases' TOC by total theoretical influent-TOC.

^cThese values were obtained gases in the gas collection bag.

—, not measured (it assumed that yeast extract was completely degraded in the DHS reactor).

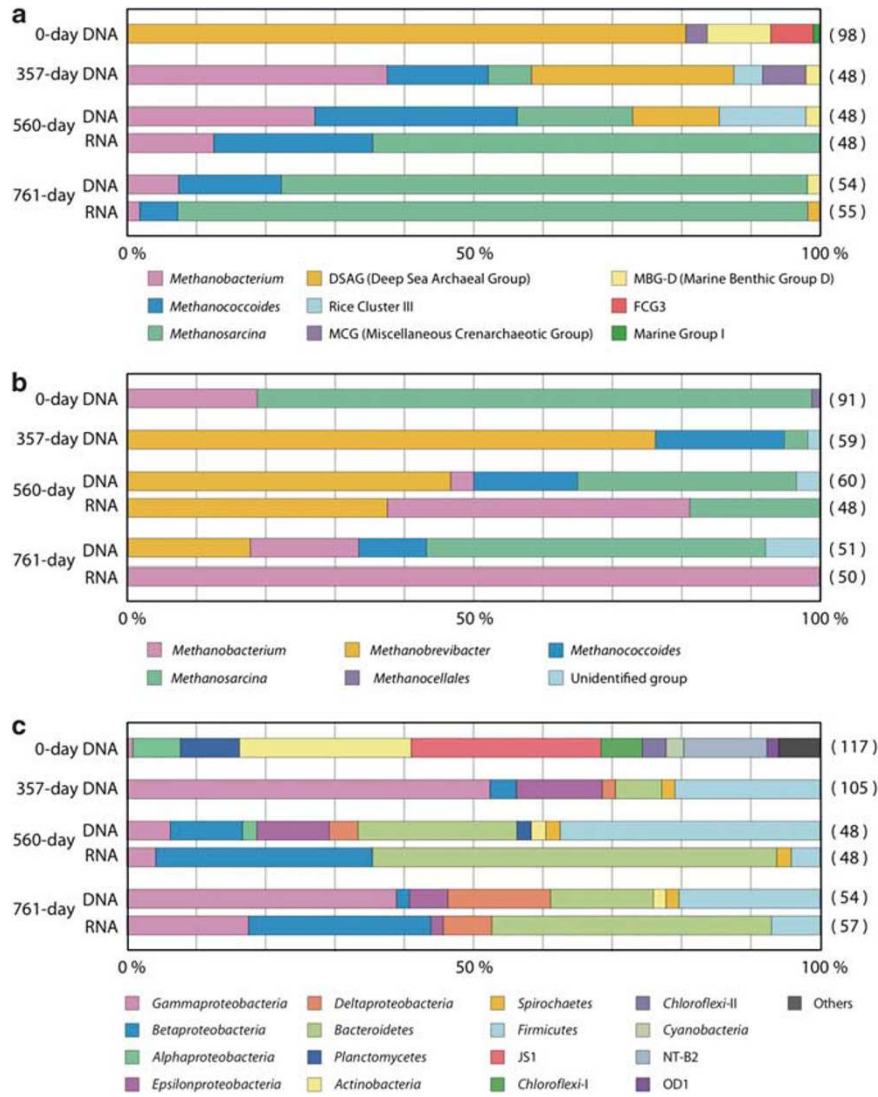


Figure 2 Archaeal, methanogens and bacterial composition shown by clone analyses. Clonal composition of (a) archaeal 16S rRNA and its gene, (b) *mcrA* mRNA and its gene and (c) bacterial 16S rRNA and its gene. Clone libraries were constructed from RNAs only made from the 561-day and 761-day samples. The numbers of sequenced clones in each clone library are shown in parentheses.

lower (0.005%, 0.009% and 0.009% at the 357, 560 and 761 days of operation, respectively).

Composition of the microbial community in the DHS reactor

To characterize the composition of the microbial community and the community shifts during the enrichment, we constructed clone libraries targeting the 16S rRNA and *mcrA* genes using samples from before and after the enrichment (Figure 2 and Supplementary Figures S5–S7, Supplementary Tables S3–S7). To identify the active microbial components, we also made clone libraries using RNA extracts obtained from the 560- and 761-day samples using reverse transcription PCR.

All of the archaeal rRNA gene phylotypes detected in the inoculum sediment sample (that is,

0-day sample in Figure 2) were closely related to the environmental phylotypes previously reported from various subsurface sediments, such as Deep-Sea Archaeal Group (alternatively designated as Marine Benthic Group-B), Marine Benthic Group-D and Miscellaneous Crenarchaeotic Group (Figure 2a and Supplementary Figure S5, Supplementary Tables S3 and S4). Among the 98 archaeal clone sequences in the inoculum sample, none of the known methanogen-related sequences were detected. During the enrichment in the DHS reactor, the archaeal composition was drastically shifted. The predominant archaeal phylotypes at 357 days (58.3% of the clones examined) were closely related to the sequences of previously isolated methanogens such as *Methanobacterium*, *Methanococcoides* and *Methanosarcina* members, with high sequence similarities (>98%) (Supplementary Table S5).

The clonal abundance of those methanogen-related phylotypes increased throughout the DHS reactor operation. In particular, the phylotype 357D_A5, related to the genus *Methanosarcina*, sharply became abundant during the DHS reactor cultivation (76.0% and 90.9% of the clones examined in the 761-day DNA and 761-day RNA-based libraries, respectively). Among the potentially methanogenic phylotypes, the phylotype 357D_A3 was closely related to the methylotrophic methanogen group *Methanococcoides*. This result implied that the microorganism might utilize methylamines eluted as secondary products from the sediments (Liu and Whitman, 2008). In fact, we detected trimethylamine in the effluent seawaters at 560 and 761 days, although the concentrations were below the quantification limit ($<0.1 \text{ mg l}^{-1}$). We also measured methanol and monomethylamine, which are substrates for known members of the genus *Methanococcoides*, but they could not be detected in the effluent seawaters. In addition to the known methanogen-related phylotypes, we also detected phylotypes belonging to uncultured archaeal lineages such as Rice Cluster III, Marine Benthic Group-D and Deep-Sea Archaeal Group after the DHS reactor operation. However, the clonal abundance of the Deep-Sea Archaeal Group group drastically decreased relative to the methanogen-related phylotypes composition during the DHS reactor operation.

mcrA gene clones were analyzed to further characterize the compositional and functional transitions of the methanogenic components (Figure 2b). The *mcrA* genes were successfully amplified from all of the samples. In the inoculum sample, the recovered phylotypes were affiliated with the genera *Methanobacterium* and *Methanosarcina* and the order *Methanocellales* (Figure 2b and Supplementary Figure S6, Supplementary Table S4). In contrast, during the enrichment, the *mcrA* gene phylotypes were potentially affiliated with not only the *Methanobacterium* and *Methanosarcina* groups but also the *Methanobrevibacter* and *Methanococcoides* groups (Supplementary Table S6). All of these *mcrA* sequences had $>90\%$ similarity to those of previously cultivated methanogens. We also detected an unidentified *mcrA* phylotype (357D_mcrA4). The H_2 -utilizing methanogen group within the genus *Methanobrevibacter* (phylotype 357D_mcrA1) dominated in the early phase of enrichment, comprising 76% of the total clones in the 357-day library, but its relative abundance decreased gradually in the course of DHS reactor operation. In contrast, the relative abundances of *Methanobacterium* and *Methanosarcina* phylotypes increased; that is, they accounted for from 0% to 15.6% and 3.4% to 49%, respectively, in the DNA-based clone libraries. In the 761-day RNA library, only the single *Methanobacterium* phylotype 560R_mcrA was detected (Figure 2b and Supplementary Table S6). Repeating the construction of the

clone library of the RNA extract from day 761 yielded the same results, which was inconsistent with the result obtained with the archaeal 16S rRNA from RNA extract from day 761. This inconsistency may be caused by possible biases associated with PCR amplification and cloning (for example, von Wintzingerode *et al.*, 1997).

The predominant bacterial 16S rRNA gene phylotypes observed in the inoculum sample were related to members of the phyla *Chloroflexi*, *Planctomycetes* and *Actinobacteria*, candidate phyla JS1 and NT-B2 and the class *Alphaproteobacteria*, which have been frequently detected as the predominant bacterial components in various subsurface sediments (for example, Fry *et al.*, 2008; Inagaki, 2010; Figure 2c and Supplementary Figure S7, Supplementary Table S4). During cultivation in the DHS reactor, the composition of bacterial rRNA gene phylotypes changed drastically. The shift in the bacterial phylotype composition before and after cultivation supports the definite but selective cultivation of bacterial populations representing indigenous subsurface bacterial components. The phylotypes within the classes *Gammaproteobacteria* and *Betaproteobacteria* and the phyla *Bacteroidetes* and *Firmicutes* dominated, and none of the sequences that were detected in the inoculum sediment was detected in the enrichment samples (Figure 2c and Supplementary Figure S7, Supplementary Table S7). The 21 phylotypes of 16S rRNA genes among the total 46 bacterial phylotypes from the enrichment samples were highly similar ($>97\%$) to the sequences of the environmental clones and cultures that were obtained from the marine or terrestrial subsurface environments (Supplementary Table S7). In addition, many phylotypes (357D_B1, 357D_B3 to B6, 357D_B9 to B12, 357D_B14, 357D_B17, 357D_B20, 357D_B22, 560D_B7, 560D_B13, 560R_B6) were closely related to anaerobic bacterial species that utilize glucose and/or yeast extract, which was consistent with the chemical data indicating the occurrence of glucose (and yeast extract) fermentation during the enrichment in the reactor (Supplementary Table S7). The bacterial phylotype composition also suggested the occurrence of aerobic and/or microaerophilic bacteria (phylotypes 357D_B7, 357D_B8, 357D_B18, 357D_B19, 357D_B21, 560D_B10, 761D_B9, 761R_8 and 761R_B14; Supplementary Table S7). Although the ORP values clearly indicated the reductive condition of the effluent seawater (Supplementary Figure S4a), the PVC box and viton tubing of the reactor system could conceivably be sources of oxygen from air because they are made of oxygen-permeable materials. When synthetic seawater containing resazurin, a redox indicator, was fed into the DHS reactor, the synthetic seawater was faintly colored pink at the top of the PVC box while the effluent seawater from the PVC box became colorless again. Thus, molecular oxygen may have contaminated the viton tubing before entering the PVC box

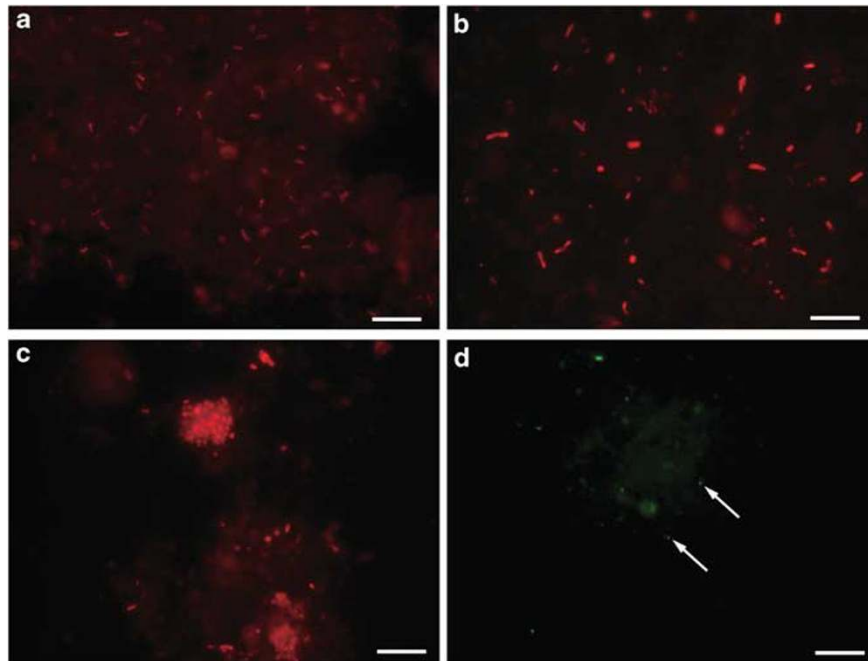


Figure 3 FISH images of the methanogenic consortium cultivated in the DHS reactor. Fluorescence photomicrographs (a–c) and (d) were obtained by the standard FISH and CARD-FISH analyses, respectively. (a) 560-day sample hybridized with the Alexa Fluor 555-labeled *Archaea* domain-specific probe ARC915, (b, c) 357-day sample hybridized with a Cy-3-labeled *Bacteria* domain-specific probe EUB338* and (d) CARD-FISH with a probe ARC915 showing fairly small coccoid cells (approximately 200–400 nm in diameter) in the 560-day sample. Cells indicated by an arrow were stained by both the ARC915 probe and 4',6-diamidino-2-phenylindole. Bars represent 10 μ m.

and could be consumed by existing aerobic microbial activities during the retention of the synthetic seawater in the sponge carriers.

Detection of microbes in the DHS reactor using FISH

The metabolically active microorganisms enriched in the DHS reactor were confirmed by FISH analysis of the sediments before and after the reactor operation. In the inoculum sample, signals were not identified using either the universal archaeal probe ARC915 or the universal bacterial probe EUB338*. In contrast, we obtained positive signals using these probes from all of the enriched sediment samples that were collected at 357, 560 and 761 days (Figure 3). All of the ARC915 probe-hybridized cells were rods (Figure 3a), while the EUB338* probe-reacted cells were morphologically variable (Figures 3b and 3c). Based on morphological features of the ARC915-positive cells and the composition of 16S rRNA and *mcrA* phylotypes in the enriched samples, we expected that the rod-shaped archaeal cells were members of the genera *Methanobacterium* and/or *Methanobrevibacter*. Thus, further FISH analysis using two different probes of ARC915 and MB1174 was conducted. Almost all of the ARC915-reactive rod-shaped cells gave positive signals with the MB1174 probe (data not shown). Positive signals using other genus- and group-specific oligonucleotide probes (targeting *Methanococcoides*, *Methanosarcina*, Rice Cluster III, Deep-Sea Archaeal Group

and Marine Benthic Group-D) were not obtained from the enrichment samples. In the CARD-FISH analysis using the ARC915 probe, we found very small coccoid-shaped cells (diameter, approximately 200–400 nm) present in the enrichment samples at 560 and 761 days of operation (Figure 3d). These small cells were presumably less active than the possible *Methanobacterium* and *Methanobrevibacter* populations but may represent different archaeal populations enriched in the DHS reactor.

Isolation of anaerobic microorganisms from the DHS reactor

To obtain pure cultures of anaerobic microorganisms, including methanogens, in the DHS reactor, conventional batch-type cultivation techniques were used. Most of the cultivation tests were conducted using the 560-day sample as an inoculum, while the cultivation of acetate- and propionate-utilizing anaerobes used the 761-day sample because the acetate and propionate degradation were clearly evident after the 631 days of operation (Table 1).

The cellular growth and substrate consumption or acid production in all of the primary cultures were confirmed after 2–8 months of incubation (Supplementary Table S8). Methane production was observed in many of the cultures, except when glucose, yeast extract or lactate were the sole energy

and carbon sources. Microscopic observation showed that these methane-producing cultures contained cells having F_{420} -like autofluorescence, indicating the presence of methanogens. In particular, F_{420} -autofluorescent cells became the predominant population in all of the hydrogen- and formate-fed cultures (more than 80% of the total microbial cells). The propionate-, ethanol- and butyrate-fed cultures contained microorganisms of various morphology types together with F_{420} -autofluorescent cells (that is, methanogens). The microscopic observations and the concomitant substrate consumption and methane production in the propionate-, ethanol- and butyrate-fed cultures suggested syntrophic growth on these organic substrates by hydrogenogenic organotrophic bacteria and hydrogenotrophic methanogens (Schink, 1997; Stams and Plugge, 2009). All of the cultures were successively transferred into fresh medium with 5% to 20% (v/v) inoculation. As the enrichment progressed, almost all of the cultures grew faster than the primary cultures. However, three cultures supplemented with acetate or propionate (cultures Ace10, AYEAV10 and Prop10) exhibited unstable growth, such as an unexpectedly long lag time (>6–8 months) for growth and stagnation of growth. Thus, molecular analyses of these cultures have not yet performed.

To identify the microorganisms in the batch-type cultures, we performed 16S rRNA gene-based clone analyses using DNA that was extracted from cultures successively transferred more than three times. Only bacterial 16S rRNA genes were obtained by PCR from the glucose-, yeast extract- and lactate-fed cultures, while both archaeal and bacterial 16S rRNA genes were obtained from the other cultures. The clone analysis revealed that phylogenetically diverse microorganisms were successfully cultivated in the batch-type cultures after the DHS reactor enrichment (Supplementary Table S8, Supplementary Figures S5–S7). A total of 38 phylotypes were obtained, and 24 phylotypes had sequences identical to those of the phylotypes that were detected in the first enrichment samples in the DHS reactor.

To isolate microorganisms found in the batch-type cultures, we performed serial dilution in both liquid and solid media. We successfully isolated four methanogens (Figure 4) and six anaerobic bacteria (Figure 5) in pure culture (see also Supplementary Table S8, Supplementary Figures S5–S7).

As a control experiment, the same natural subseafloor mixed sediments used for the DHS reactor cultivation were directly incubated in serum vials with H_2 , formate, methanol, ethanol, butyrate and propionate. However, neither growth nor chemical changes were observed after 850 days of incubation, while as a positive control, *Methanococcus aeolicus* (DSM 17508) and *Methanosarcina semesiae* (DSM 12914) could grow in the same media.

Discussion

We applied a continuous-flow bioreactor system (DHS reactor) to cultivate a methanogenic microbial community from subseafloor sediments. Using the DHS reactor system as the first step of cultivation, we successfully obtained phylogenetically diverse microorganisms including methanogens, while no growth was observed in conventional batch-type cultivation experiments using the same inoculum. Compared with previous conventional batch-type cultivation efforts that yielded only a few methanogen cultures from subseafloor sediments (von Klein *et al.*, 2002; Mikucki *et al.*, 2003; Kendall and Boone, 2006; Kendall *et al.*, 2006) and the results of our reference experiments, cultivation using the continuous-flow DHS reactor system appears very useful for cultivating the numerous fastidious microbial components in the subseafloor sedimentary biosphere.

Other reports confirm the usefulness of continuous-flow-type cultivation techniques like the DHS reactor presented in this study for the cultivation of fastidious microbial populations from natural environments. For example, several types of continuous-flow bioreactor systems have been developed and applied for the enrichment and cultivation of archaeal anaerobic methanotrophs (Girguis *et al.*, 2003, 2005; Meulepas *et al.*, 2009; Zhang *et al.*, 2010). Girguis *et al.*, (2003, 2005) developed a continuous-flow incubation system to mimic the *in situ* conditions of methane seep marine sediments and reported an increase in the archaeal anaerobic methanotroph population associated with the anaerobic oxidation of methane in the incubation system. Meulepas *et al.*, (2009) also demonstrated that the oxidation of methane rate in a continuous submerged-membrane bioreactor increased exponentially from 0.4 to 286 $\mu\text{mol g}_{\text{dry weight}}^{-1} \text{day}^{-1}$, clearly demonstrating the metabolically activated and enriched oxidation of methane consortium in the reactor.

In this study, we used polyurethane sponges as an artificial habitat for sedimentary microbes, which appear to be ideal for cultivating subseafloor microorganisms. The porous sponge mimics the porous layers (ash and sand layers) of the subseafloor sediments. Inagaki *et al.*, (2003) and Kobayashi *et al.*, (2008) reported that cultivable microbial components were generally more numerous in the porous layers than in the pelagic clay layers. In addition, the distinguishing feature of the DHS reactor is that the sponges are not submerged and freely placed in the atmosphere (Uemura and Harada, 2010). Therefore, in the DHS reactor, the influent seawater flows down into the sponges by gravity, permitting the effective exchange of the synthetic seawater fluid not only on the surface of but also inside the sponge. These advantages allow the DHS bioreactor system to yield a greater biomass in the reactor than other continuous-flow systems (Tandukar *et al.*, 2006), facilitating subsequent batch-type cultivation of the enriched microorganisms.

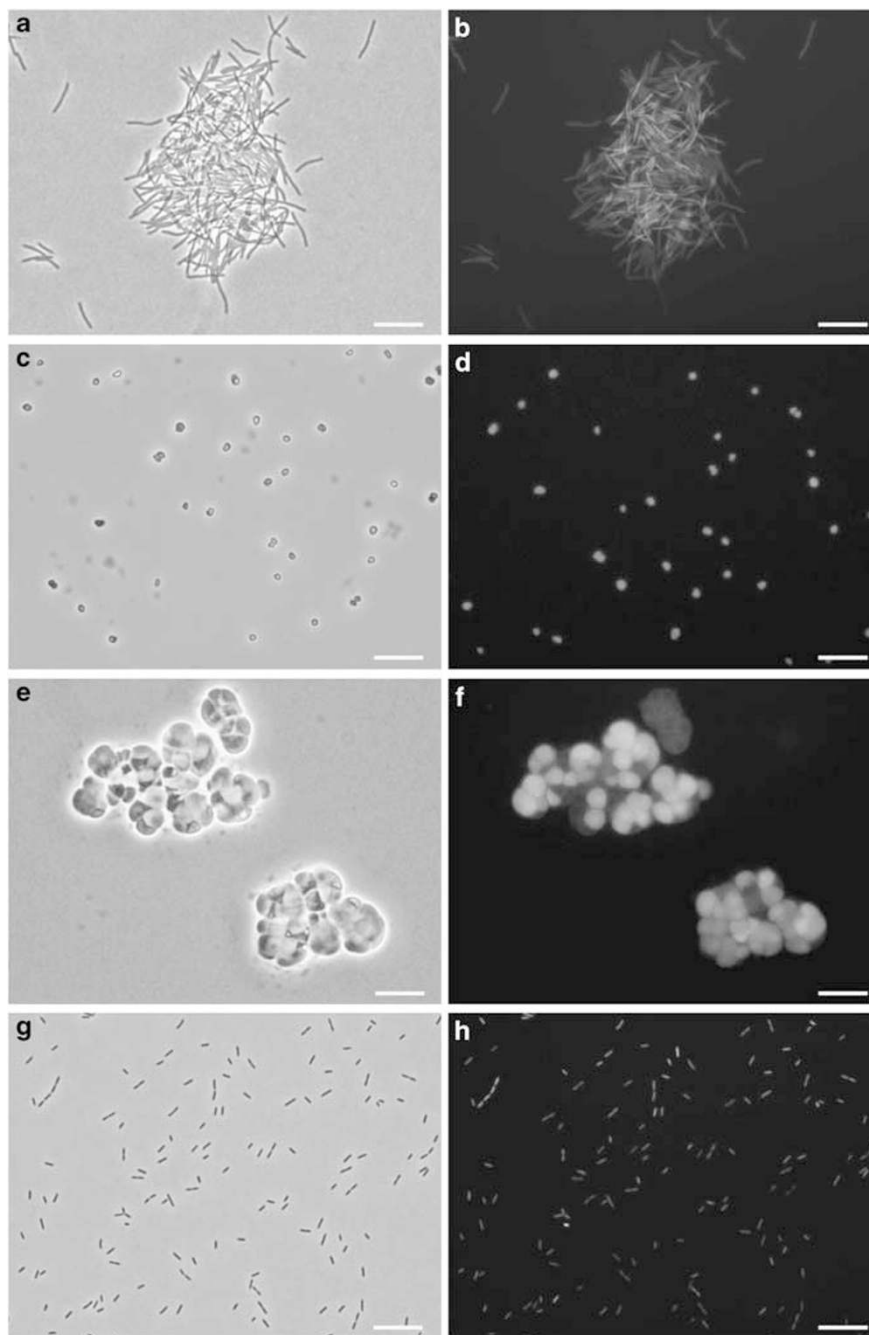


Figure 4 Photomicrographs showing cell morphologies of methanogens isolated in this study. Phase-contrast micrographs (right photos) and fluorescence micrographs (left photos) of the same fields are shown. (a, b) Strain MO-MB1, belonging to the genus *Methanobacterium*, grown on H_2/CO_2 medium at 25 °C; (c, d) strain MO-MS1, belonging to the genus *Methanosarcina*, grown on methanol medium at 25 °C; (e, f) strain MO-MCD, belonging to the genus *Methanococcoides*, grown on trimethylamine medium at 25 °C; and (g, h) strain MO-MVB, belonging to the genus *Methanobrevibacter*, grown on formate plus yeast extract medium at 20 °C. Bars represent 10 μ m.

Chemical data clearly indicated that microbial metabolism progressed in the following fashion as the DHS reactor operation progressed: (i) fermentation of glucose and yeast extract was initiated from the early stages of the reactor operation, (ii) methanogenesis occurred concomitantly with fermentation and (iii) active acetate and propionate degradations were initiated in the late stages of

reactor operation (Table 1). In addition, because the $\delta^{13}C$ values of methane gradually tended to be isotopically heavy as the reactor operation proceeded (Table 1), acetoclastic methanogenesis may contribute significantly to methane production following the hydrogenotrophic methanogenesis because of the smaller influence of stable carbon isotope fractionation on acetoclastic methanogens

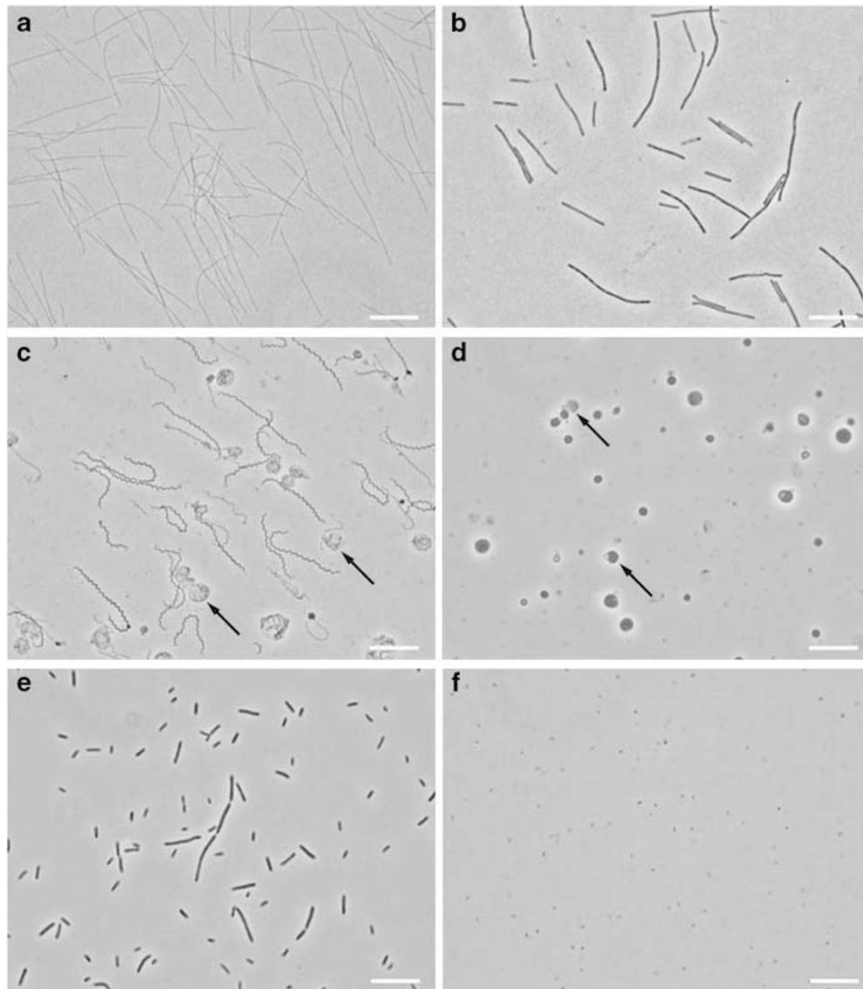


Figure 5 Phase-contrast micrographs of anaerobic bacteria isolated in this study. (a, b) Strains MO-CFX1 and MO-CFX2, belonging to the *Chloroflexi* subphylum I, grown on glucose plus yeast extract medium at 20 °C; (c, d) strains MO-SPC1 and MO-SPC2, belonging to the genus *Spirochaeta*, grown on glucose plus yeast extract medium at 10 °C. Both strains form spherical bodies that are observed in other *Spirochetes* species (indicated by arrows); (e) strain MO-SED, belonging to the genus *Sedimentibacter*, grown on yeast extract medium grown at 15 °C; and (f) strain MO-XQ, belonging to the genus *Acholeplasma*, grown on yeast extract medium at 25 °C. Bars represent 10 µm.

than on hydrogenotrophic methanogens (for example, Whiticar, 1999). This transition from hydrogenotrophic methanogenesis to acetoclastic methanogenesis is supported by several lines of evidence; (i) acetate concentrations in effluent seawater gradually decreased in the late stages of reactor operation (Table 1), (ii) the clonal frequency of the acetoclastic methanogen *Methanosarcina* increased with operational time (Figure 2) and (iii) an acetate-degrading enrichment culture containing a member of *Methanosarcina* was obtained by batch culture (AYEAV10 culture in Supplementary Table S8). On the other hand, acetate degradation can also occur via the syntrophic association of acetate-degrading bacteria and hydrogenotrophic methanogens (Hattori, 2008). In our DHS experiment, syntrophic acetate oxidation likely occurred because an acetate-degrading culture (Ace25) did not contain acetoclastic methanogen groups such as *Methanosarcina* but consisted of a hydrogenotrophic

methanogen *Methanoculleus* species and several bacterial species (Supplementary Table S8).

Contrary to our expectations, the methanogen isolates from subseafloor sediments were very closely related to previously characterized (cultivated) methanogens. Although the novelty of the isolated methanogens is unfortunately low from a taxonomic perspective, our results are consistent with previous culture-independent molecular studies, which have also detected sequences closely related to previously isolated methanogens, such as *Methanosarcina* and *Methanobrevibacter* (for example, Marchesi *et al.*, 2001; Newberry *et al.*, 2004). Among the bacterial isolates, strains MO-CFX1 and MO-CFX2 belonging to the *Chloroflexi* subphylum I, and strains MO-SPC1 and MO-SPC2 belonging to the genus *Spirochaeta* are of note (Figures 5a–d) because these bacterial components have been frequently reported as some of the predominant microbial components in marine subsurface

environments (for example, Toffin *et al.*, 2004; Inagaki *et al.*, 2006; Fry *et al.*, 2008; Webster *et al.*, 2009). To our knowledge, these strains are the first isolates of these bacterial groups from marine subsurface environments. In addition to these bacterial strains, strain MO-XQ (Figure 5f) may be one of the major anaerobic heterotrophs in organic-rich marine sediments and an ecologically significant microbial component. The most closely 16S rRNA gene sequence to strain MO-XQ was found in riser-drilling mud fluids from the same drilling site offshore the Shimokita Peninsula of Japan (GenBank accession no. AB369171; 99% sequence similarity; Masui *et al.*, 2008). The detailed isolation procedures and physiological properties of these isolates will be reported in the near future.

Given the data presented here, the reactor method is a promising cultivation technique to cultivate subsurface sedimentary microorganisms. In the future, we will further develop the new DHS reactor system for the successful cultivation of various uncultured subsurface life, targeting some key biogeochemical players in Earth's carbon and nitrogen cycling. For example, *in situ* pressure, temperature and pH are significant environmental factors controlling the habitability of life in the deep biosphere. The next generation of our bioreactor system will be further improved by integrating the controllability of these geophysical and geochemical constraints into the reactor cultivation system. These are our ongoing foci.

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