Improved methane production from brown algae under high salinity by fed-batch acclimation

Toyokazu Miura^{a,c}, Akihisa Kita^{a,c}, Yoshiko Okamura^{a,c}, Tsunehiro Aki^{a,c}, Yukihiro Matsumura^{b,c}, Takahisa Tajima^{a,c}, Junichi Kato^a, Yutaka Nakashimada^{a,c,*}

^a*Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan*

^b*Division of Energy and Environmental Engineering, Institute of Engineering, Hiroshima University, 1-4-1 Kagamiyama, Higashi-Hiroshima 739-8527, Japan*

^c*CREST, JST*

*Corresponding author at: Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan; Tel./fax: +81 82 424 4443.

E-mail address: nyutaka@hiroshima-u.ac.jp (Y. Nakashimada).

Abstract

Here, a methanogenic microbial community was developed from marine sediments to have improved methane productivity from brown algae under high salinity. Fed-batch cultivation was conducted by adding dry seaweed at 1 wt% total solid (TS) based on the liquid weight of the NaCl-containing sediment per round of cultivation. The methane production rate and level of salinity increased 8-fold and 1.6-fold, respectively, at the 10th round of cultivation. Moreover, the rate of methane production remained high, even at the 10th round of cultivation, with accumulation of salts derived from 10 wt% TS of seaweed. The salinity of the 10th-round culture was equivalent to 5% NaCl. The improved methane production was attributed to enhanced acetoclastic methanogenesis because acetate became rapidly converted to methane during cultivation. The family *Fusobacteriaceae* and the genus *Methanosaeta*, the acetoclastic methanogen, predominated in bacteria and archaea, respectively, after the cultivation.

Key words: Brown algae, fed-batch cultivation, methane, microbial community, salinity

1. Introduction

Marine macroalgae can be used as a feedstock for biomethane production because they do not compete with food crops cultivated on agricultural land (Wei et al., 2013). However, marine macroalgae contain salts (Roesijadi et al., 2010), leading to inhibition of microbial activity in macroalgae-based methane production (Oren, 1999). Indeed, methane can be produced from the feedstock by diluting salinity (Costa et al., 2012; Hinks et al., 2013; Jard et al., 2013; Vergara-Fernández et al., 2008). However, methane can also be produced without dilution of salinity by using salt-adapted microbial sources, such as marine sediments (Miura et al., 2014; Schramm and Lehnberg, 1984). Methane production under nondiluted conditions would be advantageous because of the requirement for less water and the resulting improvement in methane productivity.

To make methane production feasible, the methane production rate should be improved during methane production without dilution of salinity. Methane production consists of three steps: hydrolysis/acidogenesis, acetogenesis, and methanogenesis (McCarty and Smith, 1986). Acetogenesis and acetoclastic methanogenesis are rate-limiting steps unless the feedstocks are stable and difficult to degrade (Ito et al., 2012; Jeihanipour et al., 2011; Noike et al., 1985; Ras et al., 2011). A previous study found that acetogenesis of propionate and acetoclastic methanogenesis can become the rate-limiting steps in methane production from a brown alga at seawater salinity (Miura et al., 2014), suggesting that seaweeds are easy-to-degrade feedstocks, possibly because of the low content of lignin and cellulose (Wei et al., 2013). Thus, improvement of these rate-limiting steps is needed in order to enhance the rate of methane production.

Methane is produced in either a single-stage or two-stage digester (Nizami and Murphy, 2010). In a single-stage process, all reactions can occur in one reactor that is easy to operate compared to a two-stage digester with different microbial phases, namely acidogenic and methanogenic phases. Thus, a single-stage process is widely used. In this study, therefore, the rate-limiting steps were improved in one-stage methanization in order to increase the rate of methane production from *Saccharina japonica*, a brown alga. For this purpose, fed-batch cultivation was conducted to prepare a culture with maximal methane production for subsequent one-stage continuous operation. The microbial community was also analyzed during fed-batch cultivation.

2. Methods

2.1. Experimental design

Fed-batch cultivation was conducted to increase methane productivity by intermittently adding substrate to the marine sediment used as a microbial source. An aliquot was taken at the beginning of each cultivation to measure conductivity. The amount of methane was measured at the end of each cultivation. Volatile fatty acids (VFAs) were measured from an aliquot taken after the measurement of methane. Cultivation was conducted in triplicate. The values measured were expressed as mean $\pm$ standard deviation. The microbial community was analyzed from samples of the initial sediment and those of the culture after 10 rounds of cultivation.

2.2. Materials

Dried *Saccharina japonica* was used as a substrate (Miura et al., 2014). The marine sediment used in this study was originally sampled from the Ariake Sea in Japan. The

marine sediment was centrifuged to prepare concentrated sediment by removing the supernatant. The concentrated sediment contained 44.0 wt% total solid (TS) and 4.96 wt% volatile solid (VS).

2.3. *Fed-batch cultivation*

The concentrated marine sediment, a dilution medium, and the dry brown alga were added in amounts of 141 g, 71 mL, and 1.5 g TS, respectively, to a 700-mL vial. The dilution medium, using the brown alga as a substrate, was prepared as previously reported (Miura et al., 2014). The cultivation was conducted in triplicate at 37°C under static conditions, but was shaken following the addition of substrate. The prepared medium had 150 g liquid and 62 g TS of the sediment for a total weight of 212 g. The TS of the substrate (1.5 g TS) occupied 1 wt% of the liquid weight, corresponding to about one-tenth of the TS content of the raw substrate without drying. The TS of the sediment occupied 29 wt% of the total weight. The substrate to inoculum ratio was 1:6 on the basis of the VS. When methane production almost ceased, dry brown alga was added again.

2.4. *Chemical analysis*

The content of TS and VS, chemical oxygen demand (COD), VFAs, and gas composition were analyzed as described previously (Miura et al., 2014).

The methane production rate was calculated by the modified Gompertz equation (Gurung et al., 2012):

$$M(t) = P \cdot \exp\{-\exp[R_{\max} \cdot e/P \cdot (\lambda - 1) + 1]\} \quad (1)$$

where $M(t)$ is the cumulative methane production (mL CH₄/g VS substrate) at the time t (days), P is the maximum methane potential (mL CH₄/g VS substrate), $R_{\max}$ is the methane production rate (mL CH₄/g VS substrate/day), e is 2.71828, and λ is the lag phase (days). P , $R_{\max}$, and λ were analyzed using Solver in Excel such that the sum of the square of subtraction between $M(t)$ and experimental data was the minimum possible value.

Conductivity was measured using a conductivity meter (LAQUAtwin B-771; Horiba, Kyoto, Japan). The sample was centrifuged to measure the conductivity of the supernatant. Conductivity was converted to NaCl concentration using a standard curve prepared by plotting NaCl concentrations and conductivity.

2.5. Pyrosequencing of 16S rRNA gene amplicons

Genomic DNA was extracted from about 250 mg of sample using a NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany) and a FastPrep-24 instrument (MP Biomedicals, Solon, OH, USA) for mechanical lysis of the sample at 5 m/s for 30 s. The extraction was conducted in triplicate. The concentration of the genomic DNA solution was quantified using a spectrophotometer (Evolution 260 Bio; Thermo Fisher Scientific, Waltham, MA, USA). The DNA solution was used as a template for **polymerase chain reaction (PCR)**. PCR was conducted in a 100-μL reaction mixture containing PCR buffer for KOD FX Neo (Toyobo, Osaka, Japan), 0.4 mM dNTPs, 0.3 μM primers, 0.5 ng/μL template, and 0.02 U/μL KOD FX Neo DNA polymerase (Toyobo). The following forward (A-27F) and reverse (B-519R) primers were used for amplification of a region of the bacterial 16S rRNA gene: A-27F, 5'-adaptor A-**barcode**-AGAGTTTGATCMTGGCTCAG-3' and B-519R, 5'-adaptor

B-GWATTACCGCGGCKGCTG-3'. The following primer set was used for amplification of a region of the archaeal 16S rRNA gene (Gantner et al., 2011): A-340F, 5'-adaptor A-**barcode**-CCCTAYGGGGYGCASCAG-3' and B-1000R, 5'-adaptor B-GGCCATGCACYWCYTCTC-3'. The adaptor sequences were CCATCTCATCCCTGCGTGTCTCCGACTCAG for adaptor A and CCTATCCCCTGTGTGCCTTGGCAGTCTCAG for adaptor B. The barcode sequences in the forward primers were as follows: ACTACTATGT for bacteria and TGCTAGTCAG for archaea in the original sediment and AGACTATACT for bacteria and TGCCAGTCAG for archaea in the 10th-round culture. The bacterial 16S rRNA gene was amplified in the reaction mixture by 22 cycles of 98°C for 10 s, 57°C for 30 s, and 68°C for 15 s, following initial denaturation at 94°C for 2 min. Archaeal amplicons were prepared under the above conditions except for the following parameters: cycle number of 30, annealing at 55°C, and extension for 22 s. Amplification was confirmed by agarose gel electrophoresis on 2% gels. PCR products obtained from triplicate templates were combined and purified by using a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel).

Amplicons were sequenced using the Roche GS FLX+ system and analyzed by a commercial company (Hokkaido System Science, Hokkaido, Japan). Quality was checked by Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA). Amplicons were further purified by excision and extraction of bands from an agarose gel. The purified amplicons were quantified to make equimolar pools of bacterial amplicons and archaeal amplicons. **E**ach pool was titrated such that 1 bead contained 1 molecule of DNA, and the pools were then subjected to emulsion PCR using GS FLX Titanium SV emPCR Kit (Lib-L) (Roche, Mannheim, Germany). Pyrosequencing was

conducted on a region of the GS FLX Titanium PicoTiterPlate 70 × 75 (Roche) using a GS FLX Titanium Sequencing Kit XLR70 (Roche). Bacterial and archaeal amplicons were sequenced separately using different regions of the plate. Reads were processed by quality filters. Passed filter reads were analyzed by using the Quantitative Insights into Microbial Ecology (QIIME) software, version 1.8.0 (Caporaso et al., 2010). The passed filter reads with ambiguous bases and/or average quality scores of below 25 were trimmed. Reads having sequence lengths from 200 to 1000 bp and forward primer sequences (a 1-base difference was permissible) were used. The forward primer sequence and reverse primer sequence (a 1-base difference was permissible) were removed. The clarified reads were clustered into operational taxonomic units (OTUs) with a threshold of 97% similarity. Representative sequences in OTUs having chimeras were removed. The chimera-filtered representative OTUs were annotated using Greengenes (version 13_8), a 16 rRNA gene database, with a threshold of 90% similarity.

3. Results and discussion

3.1. Improvement in methane production by fed-batch cultivation

Fed-batch cultivation was conducted to increase the methane productivity. The dry brown alga was intermittently added to the marine sediment slurry when almost all of the added substrate was converted to methane (Fig. 1a). Methane was successfully produced, and methane production increased with each round of cultivation, although the initial sediment slurry already contained salts equivalent to a concentration of about 3% NaCl. These results indicated that methane was able to be produced under conditions in which the dry seaweed-derived salts further accumulated in the

NaCl-containing culture. Methane was produced even during the 10th round of cultivation. The 10th-round culture contained raw seaweed-derived salts because of the following reasons: (1) raw seaweed contains 10 wt% TS and 90 wt% of water, and (2) the 10th-round culture contained 10 wt% seaweed-derived TS on the basis of the liquid weight of the initial marine sediment slurry because 1 wt% TS of seaweed against the liquid was added per round of cultivation. These results indicate that methane can be produced from raw seaweed without dilution. VFAs were also analyzed at the end of each round of cultivation. Acetate barely accumulated; however, a small amount of acetate accumulated at the end of the second and third rounds of cultivation because the final step of the methanization, namely conversion of acetate to methane, was not entirely completed. Propionate accumulated until the second round of cultivation and was then consumed during the third round of cultivation. Propionate did not accumulate from the fourth round of cultivation, and no accumulation of butyrate was observed. The cultivation was continued until the 13th round, after salinity was decreased once at the 11th round to examine the effects of salinity on methane production, as described in the next section.

Methane yield per round of cultivation showed that the period needed for methane production became shorter in order to achieve the observed improvement in methane production (Fig. 1b). Methane yield was 1.2-fold higher at the end of the third round of cultivation compared with that during subsequent round because accumulated propionate was utilized, as shown in Fig. 1a. To examine the cause of the improvement in methane production, time profiles of metabolite production were investigated (Fig. 2). Interestingly, the period needed for methane production became significantly shortened, and VFAs were consumed rapidly, indicating that acetoclastic methanogenesis was

enhanced during cultivation. Therefore, enhancement of acetoclastic methanogenesis improved methane production.

3.2. Relationship between salinity and the rate of methane production

Salinity and methane production rates were plotted for each round of cultivation to examine the effects of increased salinity on methane production rates (Fig. 3). The methane production rate, as calculated from the plot in Fig. 1b, increased, and salinity measured at the beginning of each round of the cultivation also increased with each round of cultivation. The rate of methane production increased 6-fold during six rounds of cultivation. The increased rate of methane production became gradual after the sixth round of cultivation. The salinity of the sixth-round culture was equivalent to more than 4% NaCl, which was 1.4-fold higher than that of the first-round culture. These results showed that methane production was affected at a salinity equivalent to more than 4% NaCl. The methane production rate remained high at the 10th round of cultivation, despite the salinity reaching the equivalent of 5% NaCl in raw seaweed-derived salts, as described in the previous section, and the initial sediment slurry-derived salts. These results indicated that methane was able to be produced, even at a salinity equivalent to 5% NaCl, which was 1.6-fold higher than that of the first-round culture. Taking into account that the total TS addition of the dry seaweed was 10 wt% on the basis of the liquid part of the initial sediment slurry, salts derived from the dry seaweed accumulated by 2.4 wt% in the liquid because the ash content was 24 wt% on the basis of the TS of the dry seaweed. Salinity increased by the equivalent of 2% NaCl during the cultivation, suggesting that almost all ash in the seaweed was solubilized and released to the medium by the microbial community in the marine sediment. This

conclusion was also supported by the total methane yield, which reached $64.8\% \pm 0.7\%$ on the basis of the COD. The methane production rate increased 8-fold from 17.4 ± 1.5 mL CH₄/g VS of substrate/day during the first round to 141.8 ± 4.2 mL CH₄/g VS of substrate/day during the 10th round of cultivation.

An aliquot of anaerobic water was added to the 10th-round culture to examine the gradual increase in the methane production rate recovered by decreasing the salinity during the 11th round of cultivation. Interestingly, the methane production rate increased significantly during the 11th round of cultivation, and the salinity dropped to the equivalent of less than 4% NaCl. The methane production rate did not increase during further rounds of cultivation when the salinity was maintained at the equivalent of 4% NaCl. The rate at the 13th round of cultivation was 180.3 ± 11.7 mL CH₄/g VS of substrate/day, which was 27% faster than the rate at the 10th round of cultivation and 10-fold faster than the rate at the first round of cultivation. These results suggested that salinity should be retained at the equivalent of less than 4% NaCl equivalent in order to continue to increase the rate of methane production.

3.3. Microbial community

The composition of the microbial community was analyzed in order to gain insights into the improvement in methane production during fed-batch cultivation. The original sediment and the culture at the end of 10th round of cultivation were subjected to pyrosequencing of their 16S rRNA gene amplicons.

3.3.1. Bacterial community

The bacterial chimera-filtered OTUs contained a total of 12,636 and 15,908 reads for the original sediment and the 10th-round culture, respectively. The reads assigned to the same phylum were grouped to represent the number of reads per phylum. The phyla in original sediment and the 10th-round culture are listed for phyla with a relative abundance of more than 0.1% of the total reads (Tables 1 and 2). The lists covered almost all of the total reads by 99.07% and 99.81% of the total reads for the original sediment and 10th-round culture, respectively. The phylum *Proteobacteria* predominated in bacteria of the original sediment (Table 1); however, it became a minor phylum in the 10th-round culture (Table 2). The phylum *Fusobacteria* predominated in bacteria of the 10th-round culture (Table 2), but was a minor phylum in the original sediment (7 reads, 0.07% of the total reads). Reads unassigned to any phyla were present at the same extent in both samples. The phylum *Proteobacteria* tended to predominate in saline habitats, such as marine sediments because saline soil-related 16S rRNA gene sequences in the database were occupied by the phylum (Ma and Gong, 2013).

The major bacterial taxa in the original sediment and the 10th-round culture are listed for those with a relative abundance of more than 1% of the total reads (Tables 3 and 4). The 20 most predominant taxa occupied 66.93% of the total reads for the original sediment (Table 3). The most predominant taxon in the original sediment was classified into the family *Piscirickettsiaceae*. A member of the most predominant phylum *Proteobacteria* was also a major member at this next highest level of resolution. Minor taxa accounting for only 1% or less of the total reads represented about 33% of the total reads in the original sediment. On the other hand, 92.16% of the total reads were occupied by the 10 most predominant taxa in the 10th-round culture (Table 4). The

predominance of minor taxa in the original sediment (about 33% of the total reads) decreased to about 8% of the total reads in the 10th-round culture, suggesting that acclimation occurred in the bacterial community over the course of cultivation. The taxon classified into the family *Fusobacteriaceae* occupied the majority of the reads in the 10th-round culture. The reads assigned to the family *Fusobacteriaceae* consisted of one OTU. The results indicated that the major reads in the 10th-round culture were one species-equivalent classified into the family level. The predominance of the family *Fusobacteriaceae* is infrequent in methane production because the bacteria were barely detectable (Li et al., 2013; Nelson et al., 2011; St-Pierre and Wright, 2014; Ye and Zhang, 2013). The family *Fusobacteriaceae* is probably essential for producing intermediate metabolites from brown alga under increased salinity because reads classified into the family *Fusobacteriaceae* were undetectable in the original sediment. Members of the phylum *Fusobacteria* can ferment carbohydrates to produce organic acids (Nelson et al., 2013), suggesting that the family *Fusobacteriaceae* played a role in the hydrolysis/acidogenesis step of methane production in the acclimated culture, making it a member of the methanogenic microbial community.

3.3.2. Archaeal community

The archaeal chimera-filtered OTUs contained a total of 8,877 and 12,796 reads for the original sediment and 10th-round culture, respectively. The major archaeal taxa in the original sediment and 10th-round culture are listed for taxa with a relative abundance of more than 1% of the total reads (Tables 5 and 6). The 12 most predominant taxa occupied 95.73% of the total archaeal reads in the original sediment (Table 5). The unassigned taxon was the most predominant in the original sediment.

The six most common taxa occupied 98.48% of the total archaeal reads in the 10th-round culture (Table 6). The number of the major taxa decreased, and the number of reads per taxon increased during cultivation, suggesting that acclimation also occurred in the archaeal community. The genus *Methanosaeta*, the acetoclastic methanogen, was the most predominant in the 10th-round culture. The second most predominant archaea were assigned to the genus *Methanogenium*, representing the hydrogenotrophic methanogen. The third most predominant archaea were classified into the order YC-E6. The genus *Methanosarcina*, another acetoclastic methanogen, was subsequently predominant. Those taxa were minor in the original sediment as follows: *Methanosaeta* (22 reads, 0.25%), *Methanogenium* (14 reads, 0.16%), YC-E6 (4 reads, 0.05%), and *Methanosarcina* (14 reads, 0.16%). Thus, these methanogens were developed by the cultivation to comprise members of the methanogenic microbial community. The increased methanogens in the acclimated culture may have enhanced methanogenesis to improve methane production as shown in Fig. 2.

The genus *Methanosaeta* represented the more predominant acetoclastic methanogen than the genus *Methanosarcina* in the 10th-round culture, possibly due to the concentration of acetate as the substrate. The genera *Methanosaeta* and *Methanosarcina* become predominant at acetate concentrations of below 100–150 mg COD/L, corresponding to 1.7–2.5 mM, and above 250–500 mg COD/L, corresponding to 4.2–8.4 mM, respectively (De Vrieze et al., 2012). In this study, the period of exposure to the latter high concentrations of acetate changed during the cultivation as follows: above 7.4 mM between 2 and 17 days during the 20-day cultivation period in the first round of cultivation (Fig. 2a); above 4.5 mM for all of the 12-day cultivation period in the third round of cultivation (Fig. 2b), and temporary exposure to above 17

mM at 8 and 16 h in the 10th round of cultivation (Fig. 2c). These results indicate that the period of exposure to high concentrations of acetate became shortened. The shortened period of exposure to high concentrations of acetate may facilitate the cultivation of an acetoclastic methanogenic community with *Methanosaeta* as the predominant genus.

4. Conclusions

Fed-batch cultivation improved methane production from brown algae. Methane was produced from culture, even when the salinity reached the equivalent of 5% NaCl derived from the initial medium and seaweed-TS, which was comparable to the amount of salts in raw seaweed-TS. The microbial community changed to exhibit specific bacteria and predominant methanogens, indicating that microbes involved in the methane production were acclimated during fed-batch cultivation. Therefore, the fed-batch cultivation method is useful for preparing an acclimated methanogenic microbial community having improved ability to produce methane from seaweed without dilution of salinity.

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Figure captions

Fig. 1. Increase in methane productivity by fed-batch cultivation. (a) Cumulative methane production and VFA accumulation. (b) Methane yield per substrate added at each round of cultivation. Arrows, addition of substrate; values in parentheses, the number of times the addition of substrate was repeated. Water was added at the 11th addition of substrate.

Fig. 2. Time profiles of metabolite production at the first (a), third (b), and 10th (c) round of fed-batch cultivation.

Fig. 3. Relationship between salinity and methane production rate during cultivation. Water was added at the beginning of the 11th round of cultivation.

Table 1

Bacterial phyla in the original sediment.

Taxon ^a	Number of reads	Relative abundance (%)
k__Bacteria; p__ <i>Proteobacteria</i>	8025	63.51
k__Bacteria; p__ <i>Bacteroidetes</i>	816	6.46
k__Bacteria; p__ <i>Acidobacteria</i>	614	4.86
Unassigned	583	4.61
k__Bacteria; p__ <i>Planctomycetes</i>	439	3.47
k__Bacteria; p__ <i>Chloroflexi</i>	394	3.12
k__Bacteria; p__ <i>Cyanobacteria</i>	356	2.82
k__Bacteria; p__ <i>Gemmatimonadetes</i>	227	1.80
k__Bacteria; p__WS3	218	1.73
k__Bacteria; p__ <i>Actinobacteria</i>	208	1.65
k__Bacteria; p__ <i>Chlorobi</i>	139	1.10
k__Bacteria; p__ <i>Verrucomicrobia</i>	99	0.78
k__Bacteria; p__ <i>Lentisphaerae</i>	87	0.69
k__Bacteria; p__ <i>Caldithrix</i>	69	0.55
k__Bacteria; p__OP8	56	0.44
k__Bacteria; p__ <i>Nitrospirae</i>	41	0.32
k__Bacteria; p__ <i>Firmicutes</i>	37	0.29
k__Bacteria; p__GN04	33	0.26
k__Bacteria; p__ <i>Spirochaetes</i>	31	0.25
k__Bacteria; p__OP3	28	0.22
k__Bacteria; p__SAR406	18	0.14

^ak, kingdom; p, phylum.

Table 2

Bacterial phyla in the 10th round of culture.

Taxon ^a	Number of reads	Relative abundance (%)
k_Bacteria; p_ <i>Fusobacteria</i>	10762	67.65
k_Bacteria; p_ <i>Firmicutes</i>	1620	10.18
k_Bacteria; p_ <i>Bacteroidetes</i>	1207	7.59
Unassigned	675	4.24
k_Bacteria; p_WWE1	355	2.23
k_Bacteria; p_ <i>Tenericutes</i>	294	1.85
k_Bacteria; p_ <i>Chloroflexi</i>	227	1.43
k_Bacteria; p_ <i>Spirochaetes</i>	195	1.23
k_Bacteria; p_ <i>Thermotogae</i>	174	1.09
k_Bacteria; p_ <i>Fibrobacteres</i>	129	0.81
k_Bacteria; p_ <i>Proteobacteria</i>	101	0.63
k_Bacteria; p_OP9	35	0.22
k_Bacteria; p_ <i>Verrucomicrobia</i>	30	0.19
k_Bacteria; p_ <i>Synergistetes</i>	28	0.18
k_Bacteria; p_SAR406	25	0.16
k_Bacteria; p_ <i>Planctomycetes</i>	21	0.13

^ak, kingdom; p, phylum.

Table 3

The major bacteria in the original sediment.

Taxon ^a	Number of reads	Relative abundance (%)
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Thiotrichales; f__Piscirickettsiaceae; g__; s__	1888	14.94
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__[Marinicellales]; f__[Marinicellaceae]; g__; s__	712	5.63
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Chromatiales; f__; g__; s__	684	5.41
k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Desulfobacterales; f__Desulfobacteraceae; g__Desulfococcus; s__	672	5.32
Unassigned	583	4.61
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__; f__; g__; s__	568	4.50
k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Desulfobacterales; f__Desulfobulbaceae; g__; s__	512	4.05
k__Bacteria; p__Cyanobacteria; c__Chloroplast; o__Stramenopiles; f__; g__; s__	352	2.79
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; Other; Other; Other; Other	321	2.54
k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__; s__	316	2.50
k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__; s__	298	2.36
k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Desulfuromonadales; f__Desulfuromonadaceae; g__; s__	214	1.69
k__Bacteria; p__Acidobacteria; c__OS-K; o__; f__; g__; s__	206	1.63
k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Syntrophobacterales; f__Syntrophobacteraceae; g__; s__	198	1.57

k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__Myxococcales; f__; g__; s__	185	1.46
k__Bacteria; p__Acidobacteria; c__RB25; o__; f__; g__; s__	167	1.32
k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__OM60; g__; s__	159	1.26
k__Bacteria; p__Proteobacteria; c__Deltaproteobacteria; o__; f__; g__; s__	148	1.17
k__Bacteria; p__Chloroflexi; c__Anaerolineae; o__GCA004; f__; g__; s__	145	1.15
k__Bacteria; p__Proteobacteria; c__Betaproteobacteria; o__; f__; g__; s__	129	1.02

^ak, kingdom; p, phylum; c, class; o, order; f, family; g, genus; s, species.

Table 4

The major bacteria in the 10th round of culture.

Taxon ^a	Number of reads	Relative abundance (%)
k__Bacteria; p__Fusobacteria; c__Fusobacteriia; o__Fusobacteriales; f__Fusobacteriaceae; Other; Other	10672	67.09
k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__Lachnospiraceae; g__; s__	950	5.97
k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__SB-1; g__; s__	764	4.80
Unassigned	675	4.24
k__Bacteria; p__Firmicutes; c__Clostridia; o__Clostridiales; f__[Tissierellaceae]; g__ecb11; s__	441	2.77
k__Bacteria; p__WWE1; c__[Cloacamonae]; o__[Cloacamonales]; f__[Cloacamonaceae]; g__Candidatus Cloacamonas; s__	315	1.98
k__Bacteria; p__Tenericutes; c__Mollicutes; o__; f__; g__; s__	292	1.84
k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__; g__; s__	213	1.34
k__Bacteria; p__Bacteroidetes; c__Bacteroidia; o__Bacteroidales; f__Marinilabiaceae; g__; s__	176	1.11
k__Bacteria; p__Thermotogae; c__Thermotogae; o__Thermotogales; f__Thermotogaceae; g__Kosmotoga; s__A2	162	1.02

^ak, kingdom; p, phylum; c, class; o, order; f, family; g, genus; s, species.

Table 5

The major archaea in the original sediment.

Taxon ^a	Number of reads	Relative abundance (%)
Unassigned	2975	33.51
k__Archaea; p__Euryarchaeota; c__Thermoplasmata; o__E2; f__DHVEG-1; g__; s__	1197	13.48
k__Archaea; p__Crenarchaeota; c__MCG; o__; f__; g__; s__	811	9.14
k__Archaea; p__Crenarchaeota; c__Thaumarchaeota; o__Cenarchaeales; f__Cenarchaeaceae; g__Nitrosopumilus; s__	800	9.01
k__Archaea; p__Crenarchaeota; c__MCG; o__B10; f__; g__; s__	619	6.97
k__Archaea; p__Euryarchaeota; c__Methanomicrobia; o__Methanosarcinales; f__Methanosarcinaceae; g__Methanococcoides; s__	469	5.28
k__Archaea; p__[Parvarchaeota]; c__[Parvarchaea]; o__YLA114; f__; g__; s__	451	5.08
k__Archaea; p__Crenarchaeota; c__MCG; o__pGrfC26; f__; g__; s__	418	4.71
k__Archaea; p__Euryarchaeota; c__Thermoplasmata; o__E2; f__; g__; s__	209	2.35
k__Archaea; p__Euryarchaeota; c__Thermoplasmata; o__E2; f__[Methanomassiliicoccaceae]; g__; s__	196	2.21
k__Archaea; p__Euryarchaeota; c__Thermoplasmata; o__E2; f__TMEG; g__; s__	195	2.20
k__Archaea; p__Euryarchaeota; c__Thermoplasmata; o__E2; f__CCA47; g__; s__	158	1.78

^ak, kingdom; p, phylum; c, class; o, order; f, family; g, genus; s, species.

Table 6

The major archaea in the 10th round of culture.

Taxon ^a	Number of reads	Relative abundance (%)
k__Archaea; p__Euryarchaeota; c__Methanomicrobia; o__Methanosarcinales; f__Methanosaetaceae; g__Methanosaeta; s__	5316	41.54
k__Archaea; p__Euryarchaeota; c__Methanomicrobia; o__Methanomicrobiales; f__Methanomicrobiaceae; g__Methanogenium; s__	2591	20.25
k__Archaea; p__Euryarchaeota; c__Methanomicrobia; o__YC-E6; f__; g__; s__	2357	18.42
k__Archaea; p__Euryarchaeota; c__Methanomicrobia; o__Methanosarcinales; f__Methanosarcinaceae; g__Methanosarcina; s__	1403	10.96
k__Archaea; p__Euryarchaeota; c__Methanomicrobia; o__Methanomicrobiales; f__Methanomicrobiaceae; Other; Other	759	5.93
k__Archaea; p__Euryarchaeota; c__Methanobacteria; o__Methanobacteriales; f__WSA2; g__; s__	175	1.37

^ak, kingdom; p, phylum; c, class; o, order; f, family; g, genus; s, species.

Fig. 1

Fig. 2

Fig. 3