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Title

Aceticlastic and NaCl-requiring methanogen "*Methanosaeta pelagica*" sp. nov., isolated from marine tidal flat sediment.

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Running title

Marine aceticlastic methanogen, *Methanosaeta pelagica*

2 **ABSTRACT**

Acetate is a key compound for anaerobic organic matter degradation, and so far, two genera, 4 *Methanosaeta* and *Methanosarcina*, are only contributors for acetate degradation among methanogens. An aceticlastic methanogen, designated strain 03d30q^T, was isolated from a tidal flat sediment in 6 Futtsu, Japan. The phylogenetic analyses based on 16S rRNA and *mcrA* genes revealed that the isolate belonged to the genus *Methanosaeta*, but the optimal Na$^+$ concentration for growth shifted to 8 marine environments unlike the other known *Methanosaeta* species. The quantitative estimation by using a real-time PCR indicated that the 16S rRNA gene of the genus *Methanosaeta* was detected in 10 the sediments and the relative abundance ranged from 3.9% to 11.8% of the total archaeal 16S rRNA genes. Also, the amount of the genus *Methanosaeta* increased with increasing depth and was much 12 higher than that of the genus *Methanosarcina*. This is the first report of marine *Methanosaeta* species, and on the basis of phylogenetic and characteristic studies, the novel species is proposed, 14 "*Methanosaeta pelagica*" sp. nov., with types strain 03d30q^T.

2 **INTRODUCTION**

The global budget of atmospheric CH_4 is on the order of 500-600 Tg CH_4 per year (26), and

4 approximately 74% of the emitted CH_4 is derived from biological methanogenesis (5, 12, 17). Acetate is a key compound for anaerobic organic matter degradation, and it accounts for two-thirds of

6 total CH_4 generated (18). However, so far, only two genera, *Methanosarcina* and *Methanosaeta* are known as aceticlastic methanogens. *Methanosarcina* is a relative generalist that prefers methanol and

8 methylamine to acetate (2), while *Methanosaeta* is a specialist that uses only acetate (3). Analyses of the complete genome sequences of *Methanosarcina acetivorans*, *Methanosarcina mazei* and

10 *Methanosaeta thrmophila* reveled that two genera employ different enzymes to catalyze the first step of acetoclastic methanogenesis while the core steps of methanogenesis are similar in the two genera

12 (10, 14, 51). In fact, as phenotype, the distinction of affinities for acetate between two genera has been known among the scientists working in this field: *Methanosaeta* shows a much lower minimum

14 threshold for acetate utilization (7-70 μM) than *Methanosarcina* (0.2-1.2 mM) (17). Accordingly, *Methanosaeta* adapts to the low acetate environments, and it was often detected more dominantly than

16 *Methanosarcina* in the environments such as rice fields (15), landfill sites (24, 34) and anaerobic digestors (25, 49). *Methanosaeta* is widely distributed in nature and probably the predominant CH_4

18 producer on earth.

Methanogens are abundant in habitats where electron acceptors such as oxygen, nitrate and sulfate are

20 limiting. In anaerobic marine habitats, owing to the existence of sulfate in seawater, organic matter degradation is usually performed by sulfate-reducing bacteria, and anaerobic oxidation of CH_4 that is

22 produced by methanogenesis beneath the sulfate-reducing layers is also performed by ANME clusters (1, 22, 28, 42). Meanwhile, many studies in marine sediments have demonstrated that the

24 methanogenesis also occur in the high sulfate concentration layer and the anaerobic methane oxidation layer as well as the deep sulfate-depleted layer (6, 8, 45). Methylotrophic methanogens containing

26 *Methanosarcina* are regarded as major contributors to the CH_4 production here because methylated compounds are not utilized efficiently by sulfate-reducing bacteria and are termed noncompetitive

28 substrates (40). In marine sediments, particularly deep sulfate-depleted layer, CH_4 has been considered to originate from H_2/CO_2 rather than acetate (60). To date, methylotrophic, hydrogen and

30 acetate-utilizing methanogens have been isolated from the marine sediments (13, 20, 52).

As acetoclastic methanogens, *Methanosarcina* have been isolated from the marine sediments while

32 *Methanosaeta* have not yet. However, because acetate concentration of pore water in marine sediments are usually below 20 μM (19, 44), it would appear that the conditions are suitable for

34 *Methanosaeta* rather than *Methanosarcina*. In the present study, we report the isolation of an aceticlastic methanogen belonging to *Methanosaeta* from tidal flat sediment, and based on the results

36 of the phylogenetic relationship and ecological investigation, we will discuss the contribution to CH_4 production from acetate in marine sediments.

38

MATERIALS AND METHODS

2 **Microorganisms and growth conditions.** Strain 03d30q[T] (= NBRC 105920[T] = DSM 24271[T]) was isolated as follows in this study. *Escherichia coli* NBRC 3301 (K-12), *Methanosarcina barkeri*

4 NBRC 100474[T], *Methanosaeta thermophila* NBRC 101360[T], *Methanosaeta concilii* NBRC 103675[T] and *Methanosaeta harundinacea* NBRC 104789[T] were used for analyses. The cultivating media

6 were NBRC media No. 802, 837, 897, 994 and 925 (38), respectively, and the cultivating temperatures were 37°C except for *M. thermophila* (55°C).

8 **Study area, sample collection, and measurements.** Futtsu tidal flat is a foreshore sandy flat situated from the estuary of Koito River to sea in Tokyo Bay, Chiba Prefecture, Japan. Sediment

10 samples were collected on 24 June 2005 by using a peat sampler (model DIK-105A; Daiki Rika Kogyo Co., Ltd.). For the cultivation, the collected sample was transported to our laboratory in a

12 sealed nylon bag with an O_2-absorbing and a CO_2-generating agent (Anaero-Pack; Mitsubishi Gas Chemical) and inoculated to a medium as soon as possible. For the molecular analysis, the collected

14 samples in 2-ml tubes were stored at -80°C until extraction of the microbial DNA.

Temperature of the sediment was measured on site by a thermometer. Salinity and pH of pore water

16 were measured on site by a refractometer (ATC-S/Mill-E; ATAGO) and a pH meter (D-13; Horiba), respectively.

18 **Enrichment and isolation of acetoclastic methanogen.** In order to enrich and isolate aceticlastic methanogens, NBRC medium No. 1108 was used. The medium was composed of the following salts

20 and solutions (liter[-1]): 1.19 g KH_2PO_4, 0.21 g K_2HPO_4, 3.05 g $MgCl_2·6H_2O$, 0.15 g $CaCl_2·2H_2O$, 0.54 g NH_4Cl, 20 g NaCl, 6.56 g sodium acetate, 1.5 g Bacto Yeast Extract (Difco), 0.4 g Bacto Tryptone

22 (Difco), 0.14 g Coenzyme M (2-Mercaptoethanesulfonic acid sodium), 2.5 g $NaHCO_3$, 1 mg resazurin, 2 ml trace element solution (36), 10 ml vitamin solution (36), and 0.36 g $Na_2S·9H_2O$. The medium

24 was prepared in culture vessel with butyl-rubber stoppers and aluminum caps under N_2/CO_2 (80/20 [vol/vol]). For the enrichments and routine cultivations, 50-ml culture vessel containing 20 ml of the

26 liquid medium were used.

Study of phenotypic and chemotaxonomic futures. The optimum temperature, pH, and Na^+

28 concentration for growth were determined by examining the time course of CH_4 production. The pH of medium was adjusted by adding Na_2CO_3 or HCl at room temperature. Na^+ concentration range for

30 growth of each *Methanosaeta* species was examined by using each medium adding various concentration of NaCl. The gas phase of the cultures was analyzed by gas chromatography using a

32 thermal conductivity detector and a Molecular Sieve 60/80 column (both of Shimadzu).

The G + C content of extracted genomic DNA was analyzed using high performance liquid

34 chromatography (HPLC) with a reverse-phase column (31).

Observation. An Olympus AX70 microscope was used for routine observation. For the

36 observation of ultrathin section of cells, cells were prepared by using rapid freezing and the freeze-substitution method (63). After the procedures, cells were embedded, stained (63), and

38 observed using a transmission electron microscope (H-7000; Hitachi) operating at 100 kV.

4

DNA extraction, PCR, sequencing, and quantitative PCR. The extraction and purification of the genomic DNA of microorganisms were performed as previously described (37). The 16S rRNA gene of microorganisms was amplified and sequenced as previously described (35). The partial *mcrA* (alpha subunit of methyl-coenzyme M reductase) gene was amplified using primers MR1mod (5'-GAC CTS CAC TWC GTV AAC AAC-3') and ME2mod (5'-TCA TBG CRT AGT TNG GRT AGT-3') that were slightly modified primers MR1 (50) and ME2 (16) based on the genome sequence of *M. thermophila* (CP000477). The PCR amplification and sequencing of the products were performed as previously described conditions (33).

The genomic DNA of the microbial cells in the sediments was extracted using Power Soil DNA Isolation Kit (MoBio). An analysis of a quantitative PCR was performed as previously described (35) except for primer sets and standards. The following 16S rRNA gene-targeted PCR primer sets were used: Bac349F and Bac806R (55) for the domain *Bacteria*, A344F (4) and ARC915 (54) for the domain *Archaea*, MX825cm (5'- GCT AGG TGT CRG YCA CGG TGC GA-3') modified MX825c (7) and ARC915 for the genus *Methanosaeta*, and MS821c (5'- GCT CGC TAG GTG TCA GGC ATG GCG-3') (47) and ARC915 for the genus *Methanosarcina*. For the construction of the template standards for each primer set, the dilution series of the 16S rRNA gene PCR products of *E. coli* (for *Bacteria*), *M. thermophila* (for *Archaea* and *Methanosaeta*) and *M. barkeri* (for *Methanosarcina*) were used. These PCR products were used in each real-time PCR analysis to calculate the copy number of the 16S rRNA genes in the sediment samples. Two tests were performed to confirm the specificity of the real-time PCR assay. First, a melting-curve analysis was performed after the amplification. Second, the sizes of the PCR products were confirmed by gel electrophoresis. In addition to them, as for the assays of the genera *Methanosaeta* and *Methanosarcina*, the real-time PCR products were cloned and sequenced using the TOPO TA Cloning Kit (Invitrogen) and M13 primer set to confirm the specificity.

Phylogenetic analysis. Phylogenetic analyses were carried out using the16S rRNA gene sequence and the deduced amino acid sequence of the *mcrA* gene. The 16S rRNA gene sequences were aligned against an ARB data set using the ARB program (29), and the resulting alignment was manually refined based on primary and secondary structural considerations. The data set of the *mcrA* gene was aligned using program Clustal X (56). Phylogenetic trees were inferred by neighbor-joining (NJ) with the software packages of Clustal X, and 1,000 replicate data sets were used for bootstrap analysis (48, 56).

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession numbers for the sequences of 16S rRNA and *mcrA* genes are XXXXXX-XXXXXX.

RESULTS

Site description. Core samples of sandy sediments were collected from 2 points (samples 031 and 032) at low tide. Because the sample 031 was near a sea grass and the sediment was blackish in whole, the conditions were inferred to be totally anaerobic. As for the 032 sample, the sediment

gradually came to black from gray with increasing depth, and the deeper sediment probably
maintained anaerobic conditions.   Each sediment sample at 3 depths (10, 35 and 60 cm) was selected
for the molecular analysis, and the 032 samples at 35 and 60 cm in depths were used for the
enrichment of aceticlastic methanogens.   The temperature, pH and salinity of the sediments were
21°C, 7.3 and 32‰ on average, respectively.   Those of the surrounding sea water were 25°C, 7.8 and
31‰, respectively.

Enrichment and isolation.   For the primary enrichment of aceticlastic methanogens, 1 g of each
sediment sample was inoculated into the medium and incubated at 20 and 30°C.   After a 1-month
cultivation, microbial growth and CH_4 formation were observed in all cultures, and then enriched
cultures were transferred to the fresh medium.   The procedure was repeated several times.   After
that, microscopic observation indicated that *Methanosaeta*-like cells were enriched in the culture of
the sample 032 (60 cm in depth) at 30°C.   Therefore, we focused on the enrichment and tried to
obtain a pure culture.   Because growth of enriched microorganisms could not be achieved
successfully on medium solidified with 2% agar, an attempt was made to isolate microorganisms by
the serial dilution method.   We found that the *Methanosaeta*-like cells were dominant and the
transferred culture at higher dilution ($>10^7$) continuously grew in the medium removing yeast extract
and tryptone although more than 3 months were necessary for confirming its growth.   After repeating
the maximum dilution several times, an aceticlastic methanogen, designated strain 03d30q^T, was
obtained (Fig. 1A).   The purity of the isolate was verified by determination of the 16S rRNA gene
sequence amplified from the extracted DNA using the various primer sets (36) and inoculation into
following media: the medium under H_2/CO_2 (80/20 [vol/vol], 150 kPa) as substitute for N_2/CO_2; the
medium supplemented with 10 mM sodium lactate, 10 mM sodium sulfate and 10 mM sodium
thiosulfate; NBRC medium No. 325 (38) for aerobic heterotrophs.   The results showed that a trace of
contaminant was not observed and culture of strain 03d30q^T was pure.

Phylogenetic analysis.   Almost the full-length 16S rRNA gene sequences of strain 03d30q^T (1,383
bp; XXXXXXX) and *M. concilii* NBRC 104675^T (1,407 bp; XXXXXXX) were determined.
Phylogenetic analysis based on the 16S rRNA gene sequence (Fig. 2) indicated that strain 03d30q^T
belonged to the genus *Methanosaeta* and sequence similarities between the isolate and the valid
species, *M. concilii*, *M. thermophila* and *M. harundinacea* were 92.8, 92.5 and 97.0%, respectively.
In the genus *Methanosaeta*, strain 03d30q^T formed a cluster with environmental clone sequences
retrieved from saline environments such as hydrothermal sediments, cold-seep sediments, oil
reservoirs, marine methane hydrates and ancient seawater (11, 20, 32, 39, 43, 46) although it included
the sequences from terrestrial environments and *M. harundinacea*, which were isolated from a UASB
reactor treating beer-manufacture wastewater (30).   The cluster was clearly separated from the
ANME clusters that were often retrieved from marine sediments.   The sequence divergence for the
cluster was 9.4%.

In addition to the 16S rRNA gene sequence, the *mcrA* gene sequences of strain 03d30q^T (1,160 bp;
XXXXXXX), *M concilii* NBRC 103675^T (1,160 bp; XXXXXXX) and *M. harundinacea* NBRC

104789^T (1,160 bp; XXXXXXX) were also determined.   The neighbor-joining tree for McrA (Fig. 3)

demonstrated that strain 03d30q^T was also associated with the genus *Methanosaeta* and the similarities

between the isolate and *M. concilii*, *M. thermophila* and *M. harundinacea* were 82.2, 85.6 and 90.9%,

respectively.

Physiology of the isolate and Na$^+$ requirement.   Morphologically, the cells of strain 03d30q^T were

weakly auto-fluorescent rods, and many multicellular filaments were microscopically observed (Fig.

1A).   There was a tubular sheath enclosing some cells and the individual cells within the sheath had

cytoplasmic membrane (Fig. 1B).   The G+C content of the genomic DNA of strain 03d30q^T was 45.4

mol%.   Strain 03d30q^T utilized acetate as the sole energy source, and yeast extract stimulated its

growth.   Strain 03d30q^T only used acetate as energy and carbon sources.   Growth and methane

formation were not observed on H_2/O_2 (80/20; 125 kPa), formate (40 mM), methanol (20 mM),

ethanol (20 mM), methylamine (20 mM), dimethylamine (20 mM), trimethylamine (20 mM) or

dimethyl sulfide (4 mM).   Strain 03d30q^T was able to grow between 20ºC and 35ºC, and pH 6.0 and

7.8: its optimum growth conditions were 30ºC and pH 7.5.   Effect of Na$^+$ concentration in the

medium on growth as CH_4 production of strain 03d30q^T and *Methanosaeta* species were shown in Fig.

4.   The Na$^+$ concentrations for growth of strain 03d30q^T ranged from 0.20 to 0.80 M, with an

optimum of 0.28 M.   The other *Methanosaeta* species optimally grew in each medium containing the

lowest Na$^+$ concentration.   The upper Na$^+$ concentration for growth of *M. concilii*, *M. thermophila*

and *M. harundinacea* were 0.40, 0.56 and 0.34 M, respectively.   Strain 03d30q^T had a doubling time

of 12.4 day under the optimum growth conditions (30ºC, pH 7.5 and in 0.28M Na$^+$).

Environmental quantification.   For the field estimation of aceticlastic methanogens in the tidal flat

sediment, quantitative real-time PCR assays using the 16S rRNA gene copy number were performed

(Fig. 5).   The relative abundance of the genera *Methanosaeta* and *Methanosarcina* were calculated as

the percentages of the total archaeal 16S rRNA gene copy number in the DNA extracts (Table 1).

There was a trend that *Bacteria* decreased and *Archaea* increased with increasing depth.   Like the

tendency of *Archaea*, the amount of the genus *Methanosaeta* increased with increasing depth although

their proportions in the total *Archaea* ranged from 3.9 to 11.8% and did not correlate with depth.   The

amount of the genus *Methanosarcina* was less than one-tenth of that of the genus *Methanosaeta*, and

at a maximum, the proportion was 0.9% of the total *Archaea*.

DISCUSSION

Ecology   In the present study, we firstly succeeded in the isolation of aceticlastic methanogen

belonging to the genus *Methanosaeta* from marine environments.   The phylogenetic analyses based

on the 16S rRNA gene have indicated that *Methanosaeta* species inhabit saline environments such as

marine sediments, gas hydrate sediments, hydrothermal sediments, oil reservoir and natural gas filed

(11, 20, 32, 39, 43, 46), although it is difficult to distinguish clearly between saline and terrestrial

Methanosaeta species by the phylogenetic analysis (Fig. 2).   These analyses has also suggested that

Methanosaeta species detected as clonal genes in saline environments are probably the saline-adapted

species.   Our isolated strain is obviously halophilic and adapts to saline environments unlike the other

2   *Methanosaeta* species isolated so far (Fig. 4).   We firstly demonstrated that *Methanosaeta* species can grow in saline environments and saline-adapted *Methanosaeta* species are ubiquitous in saline

4   environments.   Accordingly, *Methanosaeta* species seems to contribute to anaerobic acetate degradation in saline environments as well as terrestrial environments.

6   There are much larger areas of non-seep marine sediments, but these have been much less studied. Some reports revealed that the constituent members in the sediments were similar to those in some

8   seep sediment (21, 23, 44, 57, 61, 62).   Global CH_4 production in marine sediments is very significant but the large fraction is converted to CO_2 by ANME in seep and non-seep sediments and by

10   aerobic methanotrophs in the water column (1, 41).   These CH_4 is mainly produced from methanogens living under the ANME layer and methylotrophic methanogens although hydrogen and

12   acetate are mainly consumed by sulfate-reducing bacteria in surface layer (27, 40).   However, generally sulfate is depleted at a depth of tens of centimeters to several meters, and potentially

14   methanogenesis becomes the dominant terminal oxidation process in the sulfate-depleted layer.   In addition, CH_4 production has been detected and methanogen has been retrieved from the sediments

16   with high sulfate concentration (6, 8, 19, 20, 45).   In the present study, by the real-time PCR analysis using the *Methanosaeta*-specific primer set, the 16S rRNA gene fragments of the genus were detected

18   in the tidal flat sediments and the amount increased with increasing depth (Fig. 5).   Interestingly, the amount of *Methanosaeta* was ten times higher than that of *Methanosarcina*.   The result may reflect

20   that the CH_4 production from acetate originates from *Methanosaeta* rather than *Methanosarcina* and *Methanosaeta* contributes to anaerobic organic matter degradation in tidal flat sediments.   Acetate

22   concentration of pore water in marine sediments are usually below 20 μM (20, 44).   Therefore, in marine sediments, *Methanosaeta* has an advantage in acetate utilization than *Methanosarcina* from the

24   aspect of affinity for acetate (17).

Although several strains of aceticlastic methanogens belonging to *Methanosarcina* have been isolated

26   from marine sediments (13, 20, 52, 58), isotopic evidence suggests that acetate is a minor precursor to methanogenesis and the rate of acetate oxidation to CO_2 is higher than that of CH_4 production in the

28   surface layer (44, 60).   On the other hand, there is a case that growth of *Methanosaeta* could not be observed although it was detected by the molecular approach (24).   Certainly, the enrichment and

30   isolation of *Methanosaeta* are difficult tasks among methanogens because of its slow growth.   In addition, Wellsbury *et al*. reported that it may be difficult to distinguish between abiological

32   thermogenic and biological aceticlastic methanogenesis in marine sediments (9, 59).   These facts may suggest that aceticlastic CH_4 generation in marine sediments has previously been underestimated.

34   Parkes *et al.* showed that trace of *Methanosaeta* was detected and the acetate concentration was kept at a low level in marine sediments although the rate of CH_4 production from acetate was equivalent to

36   one-quarter of that from CO_2 (44), indicating that there are sufficient acetate-utilizing microorganisms to maintain the ecology in the site.   Aceticlastic methanogens may thinly and broadly contribute

38   acetate degradation in marine sediments, particularly tidal flat sediments where a lot of organic matter

is often supplied to.

In conclusion, we firstly obtained the marine *Methanosaeta* species from the tidal flat sediment and suggested a possibility for the contributor for acetate degrading in saline environments. Further analyses for acetate degradation in tidal flat sediments such as amounts of acetate, sulfate, methanogens and sulfate-reducing bacteria are needed.

Taxonomy Characteristics of strain 03d30q^T and *Methanosaeta* species are summarized in Table 2. The phylogenetic tree based on 16S rRNA gene (Fig. 2) revealed that strain 03d30q^T belonged to the genus *Methanosaeta*, with *M. harundinaceae* as the closest relative (sequence similarity, 97.0%). Similar trend was also found for phylogenetic analysis based on McrA (Fig. 3). These results suggested that a new species should be created for the isolate (53). In addition, the G + C content of genomic DNA, the sensitivities to Na$^+$ concentration and temperature for growth differentiated strain 03d30q^T from the other *Methanosaeta* species. In particular, the difference of Na$^+$ sensitivity was a criterion for discriminating strain 03d30q^T from the other *Methanosaeta* species: strain 03d30q^T was a sole halophilic species in the genus *Methanosaeta*. For the reasons given above, strain 03d30q^T represents a novel species within the genus *Methanosaeta*, for which the name *Methanosaeta pelagica* sp. nov. is proposed.

Description of *Methanosaeta pelagica* sp. nov.

Methanosaeta pelagica (pe.la'gi.ca. L. fem. adj. *pelagica*, pertaining to the sea).

Single cells are 0.5 μm wide and 2.5-11.0 μm long, and long sheathed chains of cells form. It shows weakly blue-green autofluorescence under epifluorescence microscopy. Strictly anaerobic. Acetate is the only substrate for growth and CH$_4$ production; H$_2$/CO$_2$, formate, methanol, ethanol, methylamine, dimethylamine, trimethylamine or dimethyl sulfide do not support growth. Yeast extract is essential for growth. The optimum growth conditions are 30ºC, pH 7.5 and in 0.28M Na$^+$ concentration (1.6% NaCl). The DNA G + C content of the type strain is 45.4 mol%.

The type strain, 03d30q^T (= NBRC 105920^T = DSM 24271^T), was isolated from a tidal flat sediment in Tokyo Bay, Chiba Prefecture, Japan.

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TABLE 1.   Relative abundances of *Archaea*, and the genera *Methanosaeta* and *Methanosarcina*
16   **calculated by real-time PCR analyses.**

Sample	Depth (cm)	Abundance of the 16S rRNA gene number (%)		
		Archaea in the total Prokaryotes	*Methanosaeta* in the total *Archaea*	*Methanosarcina* in the total *Archaea*
031	10	0.5	6.6	0.2
	35	1.3	4.6	0.4
	60	2.4	4.9	0.7
032	10	0.1	11.8	0.9
	35	0.5	3.9	0.4
	60	3.0	7.8	0.2

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TABLE 2.   Comparison of properties among the species of the genus *Methanosaeta*.

Species	"*M. pelagica*"	*M. concilii*	*M. thermophila*	*M. harundinacea*
Type strain	03d30q^T	GP6^T	P$_T$^{T}	8Ac^T
Habitat	Tidal-flat sediment	Anaerobic digestor	Anaerobic digestor, mud in thermal lake	Anaerobic digestor
Optimum growth conditions:				
Temp. (°C)	30	35-40	55-60	34-37
pH	7.5	7.1-7.5	6.5-6.7	7.2-7.6
Na$^+$ conc. (M)	0.28	<0.06	<0.13	<0.02
Effect on yeast extract for growth	Require	Inhibit	Inhibit*	Require
G+C content (mol%)	45.4 (HPLC)	50.3 (Tm)	52.7 (HPLC)	55.7 (Tm)

*Yeast extract inhibits growth of some strains of *M. thermophila*.

2 **FIGURE LEGENDS**

Fig. 1. Phase-contrast micrograph of strain 03d30q^T (A; bar, 10 μm). Ultrathin section of strain
4 03d30q^T observed with a transmission electron microscope (B; bar, 2 μm).

6 **Fig. 2.** Neighbor-joining tree based on 16S rRNA gene sequence of strain 03d30qT and relatives.
The probability scores at branch points obtained are indicated by solid circles (above 95%) and open
8 circles (above 90%). The sequence obtained from isolates are indicated in bold. Environmental
clonal sequences retrieved from saline environments are indicated by grayish squares.
10 GenBank/EMBL/DDBJ accession numbers are shown in parentheses. Bar, 0.02 substitutions per
compared nucleotide site.

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Fig. 3. Neighbor-joining tree showing the relationships between strain 03d30q^T and relatives based
14 on deduced McrA sequences. The probability scores at branch points obtained are indicated by solid
circles (above 95%) and open circles (above 90%). GenBank/EMBL/DDBJ accession numbers are
16 shown in parentheses. Bar, 0.02 substitutions per compared positions.

18 **Fig. 4.** Effect of Na$^+$ concentration on CH$_4$ production of strain 03d30q^T (solid circles), *M. concilii*
(open triangles), *M. thermophila* (open squares) and *M. harundinaceae* (open circles).

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Fig. 5. Copy numbers of 16S rRNA genes of *Bacteria* (triangles), *Archaea* (squares), *Methanosaeta*
22 (circles) and *Methanosarcina* (diamonds) detected by real-time PCR analyses. Open and solid
symbols are indicated the sediment samples collected from 031 and 032 points, respectively.
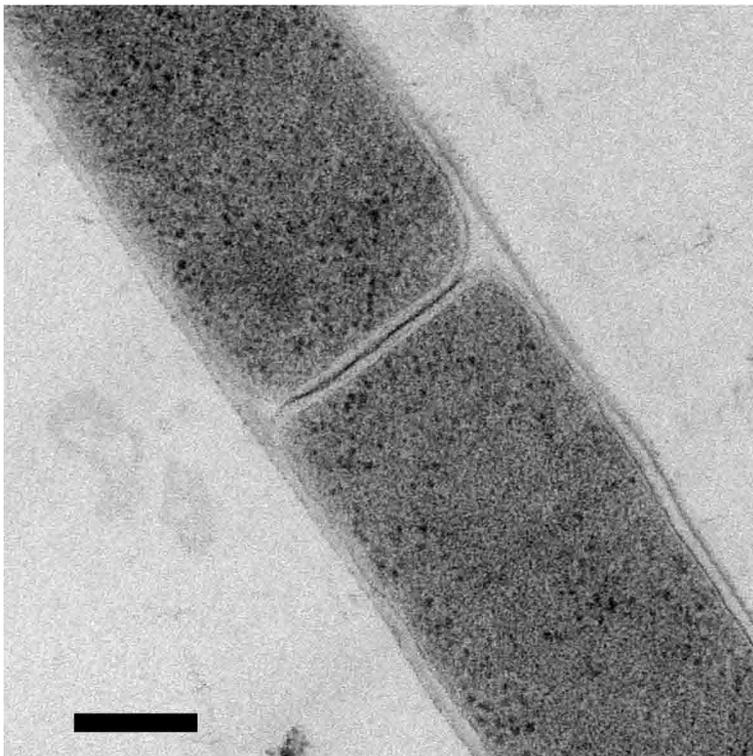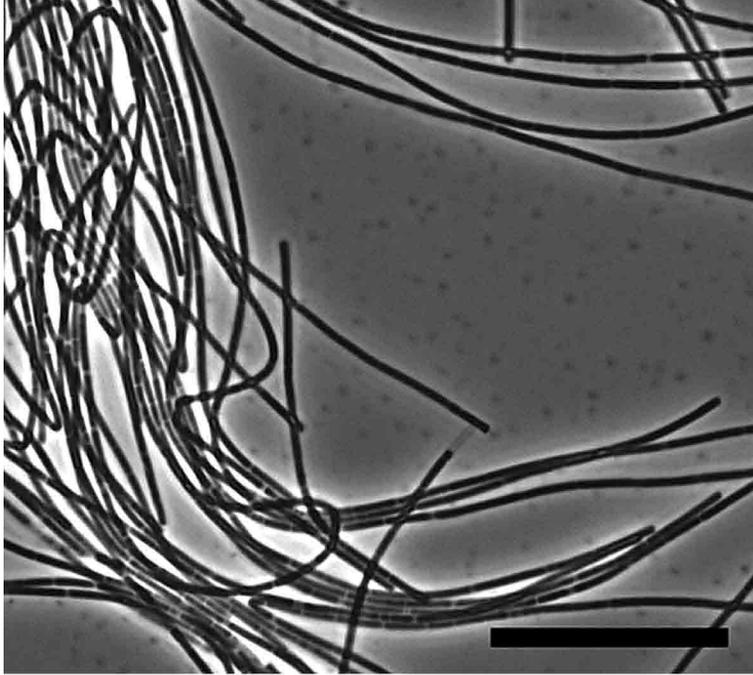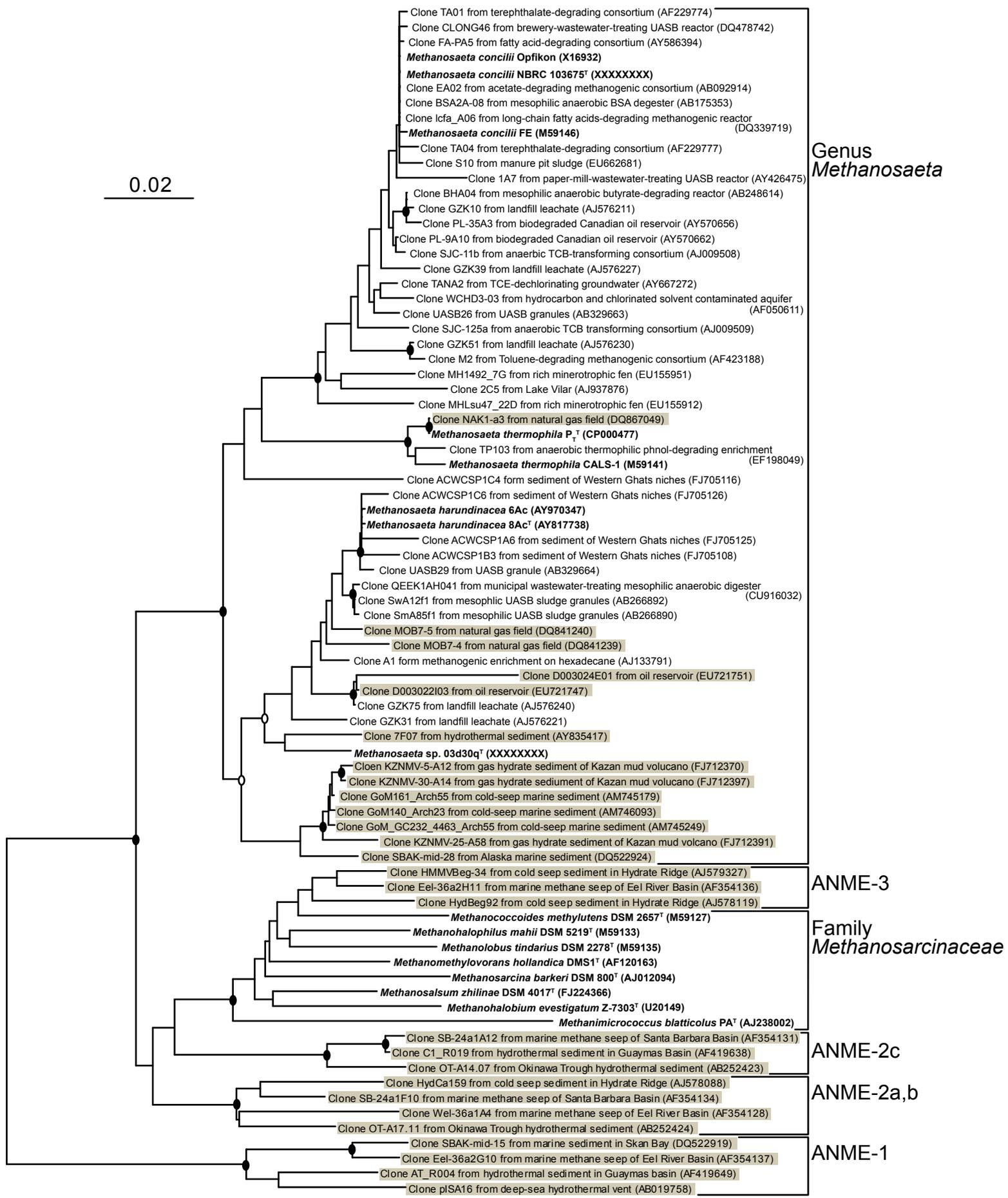
Figure 1, Mori et al.

Clone TA01 from terephthalate-degrading consortium (AF229774)
Clone CLONG46 from brewery-wastewater-treating UASB reactor (DQ478742)
Clone FA-PA5 from fatty acid-degrading consortium (AY586394)
Methanosaeta concilii Opfikon (X16932)
Methanosaeta concilii NBRC 103675^T (XXXXXXXX)
Clone EA02 from acetate-degrading methanogenic consortium (AB092914)
Clone BSA2A-08 from mesophilic anaerobic BSA degester (AB175353)
Clone lcfa_A06 from long-chain fatty acids-degrading methanogenic reactor (DQ339719)
Methanosaeta concilii FE (M59146)
Clone TA04 from terephthalate-degrading consortium (AF229777)
Clone S10 from manure pit sludge (EU662681)
Clone 1A7 from paper-mill-wastewater-treating UASB reactor (AY426475)
Clone BHA04 from mesophilic anaerobic butyrate-degrading reactor (AB248614)
Clone GZK10 from landfill leachate (AJ576211)
Clone PL-35A3 from biodegraded Canadian oil reservoir (AY570656)
Clone PL-9A10 from biodegraded Canadian oil reservoir (AY570662)
Clone SJC-11b from anaerbic TCB-transforming consortium (AJ009508)
Clone GZK39 from landfill leachate (AJ576227)
Clone TANA2 from TCE-dechlorinating groundwater (AY667272)
Clone WCHD3-03 from hydrocarbon and chlorinated solvent contaminated aquifer (AF050611)
Clone UASB26 from UASB granules (AB329663)
Clone SJC-125a from anaerobic TCB transforming consortium (AJ009509)
Clone GZK51 from landfill leachate (AJ576230)
Clone M2 from Toluene-degrading methanogenic consortium (AF423188)
Clone MH1492_7G from rich minerotrophic fen (EU155951)
Clone 2C5 from Lake Vilar (AJ937876)
Clone MHLsu47_22D from rich minerotrophic fen (EU155912)
Clone NAK1-a3 from natural gas field (DQ867049)
Methanosaeta thermophila P$_T$^{T} (CP000477)
Clone TP103 from anaerobic thermophilic phnol-degrading enrichment (EF198049)
Methanosaeta thermophila CALS-1 (M59141)

Genus *Methanosaeta*

Clone ACWCSP1C4 form sediment of Western Ghats niches (FJ705116)
Clone ACWCSP1C6 from sediment of Western Ghats niches (FJ705126)
Methanosaeta harundinacea 6Ac (AY970347)
Methanosaeta harundinacea 8Ac^T (AY817738)
Clone ACWCSP1A6 from sediment of Western Ghats niches (FJ705125)
Clone ACWCSP1B3 from sediment of Western Ghats niches (FJ705108)
Clone UASB29 from UASB granule (AB329664)
Clone QEEK1AH041 from municipal wastewater-treating mesophilic anaerobic digester (CU916032)
Clone SwA12f1 from mesophlic UASB sludge granules (AB266892)
Clone SmA85f1 from mesophilic UASB sludge granules (AB266890)
Clone MOB7-5 from natural gas field (DQ841240)
Clone MOB7-4 from natural gas field (DQ841239)
Clone A1 form methanogenic enrichment on hexadecane (AJ133791)
Clone D003024E01 from oil reservoir (EU721751)
Clone D003022I03 from oil reservoir (EU721747)
Clone GZK75 from landfill leachate (AJ576240)
Clone GZK31 from landfill leachate (AJ576221)
Clone 7F07 from hydrothermal sediment (AY835417)
Methanosaeta sp. 03d30q^T (XXXXXXXX)
Cloen KZNMV-5-A12 from gas hydrate sediment of Kazan mud volucano (FJ712370)
Clone KZNMV-30-A14 from gas hydrate sediement of Kazan mud volucano (FJ712397)
Clone GoM161_Arch55 from cold-seep marine sediment (AM745179)
Clone GoM140_Arch23 from cold-seep marine sediment (AM746093)
Clone GoM_GC232_4463_Arch55 from cold-seep marine sediment (AM745249)
Clone KZNMV-25-A58 from gas hydrate sediment of Kazan mud volcano (FJ712391)
Clone SBAK-mid-28 from Alaska marine sediment (DQ522924)

Clone HMMVBeg-34 from cold seep sediment in Hydrate Ridge (AJ579327)
Clone Eel-36a2H11 from marine methane seep of Eel River Basin (AF354136)
Clone HydBeg92 from cold seep sediment in Hydrate Ridge (AJ578119)

ANME-3

Methanococcoides methylutens DSM 2657^T (M59127)
Methanohalophilus mahii DSM 5219^T (M59133)
Methanolobus tindarius DSM 2278^T (M59135)
Methanomethylovorans hollandica DMS1^T (AF120163)
Methanosarcina barkeri DSM 800^T (AJ012094)
Methanosalsum zhilinae DSM 4017^T (FJ224366)
Methanohalobium evestigatum Z-7303^T (U20149)
Methanimicrococcus blatticolus PA^T (AJ238002)

Family *Methanosarcinaceae*

Clone SB-24a1A12 from marine methane seep of Santa Barbara Basin (AF354131)
Clone C1_R019 from hydrothermal sediment in Guaymas Basin (AF419638)
Clone OT-A14.07 from Okinawa Trough hydrothermal sediment (AB252423)

ANME-2c

Clone HydCa159 from cold seep sediment in Hydrate Ridge (AJ578088)
Clone SB-24a1F10 from marine methane seep of Santa Barbara Basin (AF354134)
Clone Wel-36a1A4 from marine methane seep of Eel River Basin (AF354128)
Clone OT-A17.11 from Okinawa Trough hydrothermal sediment (AB252424)

ANME-2a,b

Clone SBAK-mid-15 from marine sediment in Skan Bay (DQ522919)
Clone Eel-36a2G10 from marine methane seep of Eel River Basin (AF354137)
Clone AT_R004 from hydrothermal sediment in Guaymas basin (AF419649)
Clone pISA16 from deep-sea hydrothermal vent (AB019758)

ANME-1

0.02

Figure 2, Mori et al.

Methanosaeta concilii NBRC 103675^T (XXXXXXXX)

Methanosaeta thermophila P$_T$^{T} (CP000477)

Methanosaeta harundinacea NBRC 104789^T (XXXXXXXXX)

Methanosaeta sp. 03d30q^T (XXXXXXXX)

Methanohalophilus mahii DSM 5219^T (CP001994)

Methanohalobium evestigatum Z-7303^T (CP002069)
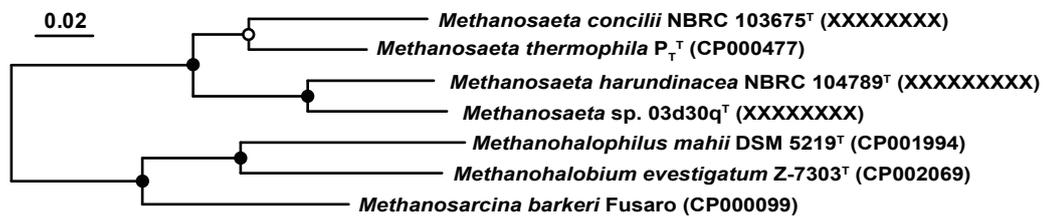
Methanosarcina barkeri Fusaro (CP000099)

0.02

Figure 3, Mori et al.

Figure 4, Mori et al.

Figure 5, Mori et al.