Running Title: Halotolerant Methanogen in Marine Sediment

**Characterization of a Halotolerant Acetoclastic Methanogen Highly Enriched
from Marine Sediment and Its Application to Removal of Acetate**

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Abstract

A marine sediment collected from Hiroshima Bay was cultured in artificial seawater, containing 0.51M NaCl and 60 mM acetate and was found to exhibit active methane production at 37°C. Following four successive serial dilutions of cultures in medium containing 0.51 M NaCl, 60 mM acetate, and antibiotics, the well-acclimated methanogen was found to exhibit growth over a range of NaCl concentration (between 0 M and 2.06 M). The specific growth rates of the highly enriched methanogen, termed strain H_A, in the absence of NaCl and in the presence of 1.54 M NaCl were estimated to be 0.037 h⁻¹ and 0.027 h⁻¹, respectively. The pH and temperature for optimum growth were determined to be 7.0–8.8 and 37°C, respectively. Although cells that had morphology similar to *Methanosaeta* sp. became dominant in the culture, methane production was still detected in the medium containing 0.51 M NaCl and other substrates such as methanol, formate, and methylamine, indicating contamination with other methanogens. The phylogenetic tree based on 16S rRNA gene sequences revealed that the strain H_A was closely related to *Methanosaeta harundinacea* 6Ac and 8Ac^T, with sequence similarity of 98% and 97%, respectively. The continuous removal of acetate with upflow anaerobic filter reactor for industrial use of strain H_A determined a methane production rate of 70 mM/d under condition of 0.51 M NaCl and successful methane production even under 1.54 M NaCl.

(Introduction)

Methane fermentation is commonly employed for anaerobic wastewater treatment and energy recovery throughout the world. Although methane fermentation is applicable for the treatment of various kinds of organic matter, including manure, food, and municipal waste, its use is restricted to organic waste with relatively low salt concentration because methanogenesis is strongly inhibited by a sodium concentration of more than 10 g/L (1). However, there are high-salt-containing organic matters that should be treated by methane fermentation (2-4). The wastewater generated by a fish-processing industry is rich in salts since fish processing industries use a large amount of salt for fish conservation (2). Although marine macroalgae are sometimes considered as hazardous organic wastes (5), ash content of the wet macroalgae can reach 3 % (w/w) (3). Although the application of methane fermentation for such high-salt-containing organic matters requires dilution with fresh water, this operation decreases the economic efficiency of the process due to requirement for increased reactor volume and aerobic treatment of the effluent subsequent to methane fermentation (4). Therefore, it is needed that the development of methane fermentation process applicable for organic matters with high salinity.

The process of methane fermentation or the production of methane from organic matter involves three steps, namely, hydrolysis/acidogenesis, acetogenesis, and methanogenesis (6). In the first step, organic matter is converted into volatile fatty acids (VFAs) by various bacteria. The next step involves the conversion of VFAs, such as propionate and butyrate, into acetate and hydrogen through the activity of propionate- and butyrate-oxidizing bacteria, respectively. Methanogenesis, the final step of methane fermentation, includes two pathways involving methanogens: the direct cleavage of

1 acetate into methane (CH₄) and carbon dioxide (CO₂), termed acetoclastic
2 methanogenesis, or the reduction of CO₂ with hydrogen, termed hydrogenotrophic
3 methanogenesis. Of these three steps, acetoclastic methanogenesis shows low salt
4 tolerance and is responsible for the high-salt intolerance of the methane fermentation
5 process (7, 8). Therefore, it is important to acquire high-salt-tolerant methanogens and
6 understand their physiology for the development of the industrial methane fermentation
7 process for high-salt containing feedstock.

8 Some of hydrogenotrophic methanogens are known to inhabit environments of
9 extremely high salt concentration, such as salt lakes and deep-sea sediments. For
10 instance, *Methanococcoides methylutens*, which was isolated from the Sumner Branch
11 of Scripps Canyon located near La Jolla, California, exhibited growth under saline
12 conditions, with optimal NaCl concentration of 0.24–0.64 M (9). *Methanohalophilus*
13 *mahii* isolated from the Great Salt Lake was another moderately halophilic methanogen,
14 which exhibited optimum growth at NaCl concentration of 2.06 M (10, 11).
15 Furthermore, the extremely halophilic methanogen *Methanohalobium evestigatum*
16 isolated from a saline lagoon in Cremaa showed growth at 4.28 M salinity (11, 12).

17 Two genera of acetoclastic methanogens are known. These include *Methanosarcina* and
18 *Methanosaeta* (13). Given that 70% of the methane generated during methane
19 fermentation is derived from the cleavage of acetate (14), an important role is played by
20 acetoclastic methanogens in this process. A few species of halophilic *Methanosarcina*
21 have been isolated to date. The optimal range of NaCl for the growth of
22 *Methanosarcina acetivorans* has been reported as 0.1–0.6 M, with growth not observed
23 in the absence of NaCl (15). *Methanosarcina mazei* Gö1 showed growth at Na⁺
24 concentration of 18,000 mg/L, corresponding to 0.79 M solution of NaCl (16, 17). On

1 the other hand, a single report available on halophilic species of *Methanosaeta* shows
2 that *Methanosaeta pelagica* 03d30q^T isolated from a tidal flat sediment exhibited
3 growth at Na⁺ concentrations of 0.20–0.80 M, with the optimum growth at 0.28 M (18).

4 In our previous study, it was found that microbial consortia acclimated from
5 coastal mud sediments produced methane from acetate at a production rate of 96.1
6 mmol/L/d at salinity of 0.51 M NaCl (19). It was also demonstrated that marine
7 sediments collected from various tidal flats in Japan exhibited high methanogenic
8 activity from brown algae under condition of 0.51 M NaCl while mesophilic granules
9 from upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater lost
10 the methanogenic activity under the saline condition (20). This observation suggested
11 that marine sediments in tidal flats were promising sources of methanogenic microbial
12 consortia that could be applied for the anaerobic treatment of organic waste with high
13 salinity. However, in the study, all tested marine sediments showed activity in
14 acetoclastic methanogenesis lower than that in hydrolytic/acetogenesis, acidogenesis
15 from butyrate and hydrogenotrophic methanogenesis. Furthermore, to determine
16 whether the methane production from the brown alga by marine sediments was
17 sustainable, when the culture was diluted to 10% (v/v) with fresh medium containing
18 the alga and salt to conduct subculture, acetoclastic methanogenesis was significantly
19 weakened, suggesting low microorganism growth rates compared to the other steps (20).

20 To overcome this constraint and practically use the marine microbial community, the
21 microbial physiology of a variety of marine methanogenic microbes needs to be
22 elucidated to optimize them further, and the appropriate bioreactor configuration for
23 high-throughput treatment needs to be determined. However, only *M. pelagica* 03d30q^T
24 has been isolated and characterized as the acetoclastic methanogen in the tidal flats so

far (18) in spite of an expectation of existence of acetoclastic methanogens with more feasible characteristics such as higher salt tolerance in different tidal flats.

In this study, therefore, we highly enriched an acetoclastic methanogen from marine sediment from a tidal flat in artificial seawater containing acetate as the sole carbon source and characterized the acetoclastic methanogens phylogenetically and physiologically. Furthermore, continuous culture with a fixed-bed reactor in which enriched methanogens were fixed on ceramic polypropylene carrier was carried out to elucidate applicability of the methanogen to industrial use.

MATERIALS AND METHODS

Source of inoculum

Marine sediment was obtained from Hiroshima Bay and preserved in the refrigerator at 4°C until use. *Methanosaeta concilii* DSM 6752 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany.

Media

DSM 334 medium of Deutsche Sammlung von Mikroorganismen und Zellkulturen (21) was employed for all experiments; the composition was as follows: 0.3 g KH₂PO₄, 1.0 g NH₄Cl, 0.1 g MgSO₄·7 H₂O, 0.08 g CaCl₂·2H₂O, 0.3 g Na₂S·9H₂O, 4 g KHCO₃, 0.3 g L-cysteine-HCl·H₂O, 1 mg resazurin, 10 ml vitamin solution, and 10 ml of trace element solution. The vitamin solution contained 2.0 mg biotin, 2.0 mg folic acid, 10.0 mg pyridoxine-HCl, 5.0 mg thiamine-HCl·2H₂O, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg Calcium-D-pantothenate, 5.0 mg *p*-aminobenzoic acid, 5.0 mg

1 lipoic acid, and 0.1 mg vitamin B₁₂. The trace element solution contained 12.8 g
2 nitrilotriacetic acid (NTA), 1.35 g FeCl₃·6H₂O, 0.10 g MnCl₂·4H₂O, 0.024 g
3 CoCl₂·6H₂O, 0.10 g CaCl₂·2H₂O, 0.10 g ZnCl₂, 0.025 g CuCl₂·2H₂O, 0.01 g H₃BO₃,
4 0.024 g Na₂MoO₄·2H₂O, 1.0 g NaCl, 0.12 g NiCl₂·6H₂O, and 0.026 g Na₂SeO₃·5H₂O.
5 For enrichment culture, 61 mM of sodium acetate and 0.51 M of sodium chloride were
6 added per liter of medium.

8 **Growth conditions**

9 A modified Hungate technique in combination with the serum bottle technique (22,
10 23) was employed for enrichment culture. Prior to the addition of cysteine, KH₂PO₄,
11 MgSO₄, CaCl₂, and Na₂S, the medium was boiled for 20 min, cooled in ice water with
12 continuous bubbling of N₂–CO₂ (80:20) gas, dispensed into serum bottles sealed with
13 black butyl rubber stoppers, and sterilized (18 min, 121°C). Concentrated aqueous
14 solutions of the remaining chemicals were separately autoclaved and injected into the
15 medium using a hypodermic syringe.

16 Enrichment was initiated at 37°C by the addition of sediment (10 g wet weight) to
17 700-ml serum vials containing 300 ml of enrichment medium; the medium contained
18 vancomycin (100 mg per liter) to inhibit the growth of non-methanogenic organisms,
19 and pH was adjusted to 7.0 by the addition of 1 M HCl. After methane production
20 subsided, 60 ml of the culture was anaerobically transferred into a new vial of sterile
21 enrichment medium. Following four such successive transfers, each enrichment culture
22 was subjected to 10-fold serial dilution from 0.1% to 10% (v/v) using medium that
23 contained vancomycin, as detailed above. The purity of the enriched cultures was
24 examined at the highest dilution at which bacterial growth was detected. Upon detection

1 of any contaminants, serial dilution was repeated. The pure cultures obtained were
2 subcultured once a month and stored at 4°C in a growth medium.

4 **Continuous culture by UAF reactor**

5 A glass column reactor (working vol., *ca.* 1000ml) packed with
6 polypropylene-ceramic mixture as a support material (a gift from Showa Herding Co.,
7 Ltd, Tokyo) developed for the fixation of acetoclastic methanogens like *Methanosaeta*
8 sp. was used for the upflow anaerobic filter (UAF) reactor for the cultivation of
9 enriched strain H_A (24). To fix the strain H_A to the carrier, the batch culture was
10 performed by inoculating 300ml of the culture broth of the strain H_A pre-cultured in
11 700-ml vials into the UAF reactor installing the cylindrical carrier (ϕ 50mm x 150mm
12 height, 4mm thickness). The gas pack filled with mixed gas (80% N₂/20%CO₂) was
13 fixed to the reservoir tank to prevent backflow from the reactor when the reservoir tank
14 has become negative pressure. To maintain the pH, 50 mM of phosphate buffer (pH6.1)
15 was supplied to the medium. Continuous cultures by UAF reactor were performed
16 under the condition of 0.51 M, 1.03 M and 1.54 M NaCl concentration, and acetate was
17 added from 15 mM/d to 130 mM/d of loading rate.

19 **Effect of pH, temperature, and salt concentration**

20 For determination of the optimum pH for growth, the pH values of the enrichment
21 medium (containing 61 mM of sodium acetate and 0.51 M of sodium chloride) were
22 adjusted to the range 5.5–8.8 using 50 mM MES [2-(N-morpholino)-ethanesulfonic
23 acid] buffer of pH 5.5–7.0 or Tris-HCl buffer of pH 7.0–8.8 and maintained at the
24 temperature of inoculation. The effect of temperature and salt concentration on methane

formation was examined by incubating cultures in acetate medium [10% (v/v) inoculum] of pH 7.0 at various temperatures (20°C to 50°C) and various concentrations of NaCl. In all experiments, triplicate cultures [10% (v/v) inoculum] in 120-ml serum vials containing 50 ml of medium were employed, and the levels of methane produced in the early stages of growth (within 7 days) were determined.

Carbon source dependence

The effect of carbon source on methane formation was examined using triplicate cultures (10% [v/v] inoculum) in 120-ml serum vials, containing 50 ml of synthetic medium of the following composition: 5 g of various carbon sources (methanol, methylamine, formate and acetate) and 0.51 M of NaCl, with pH adjusted to 7.0, at a temperature of 37°C.

Calculation of specific growth rate

Specific methane production rates (μ_{CH_4}) were calculated during the exponential phase, as described previously (25, 26):

$$\mu_{CH_4} = \frac{\ln(M_2/M_1)}{t_2 - t_1} \quad (1)$$

where μ_{CH_4} represents the specific rate of methane production per hour, and M_1 and M_2 represent methane production (in mmoles) at culture times t_1 and t_2 , respectively.

Sequence determination and phylogenetic analysis

Archaeal genomic DNA was prepared using GNOME DNA isolation kit (Qbiogene, Carlsbad, CA, USA) for use as template in PCR. 16S rRNA gene was amplified by PCR using primers targeting the conserved regions of 16S rRNA genes

1 from *M. concilii* or *Methanothermobacter thermautotrophicus* (27). The nucleotide
 2 sequences of the primers were as follows: forward primer for *M. concilii* (MC),
 3 5'-TGGTTGATCCTGCCAGAGG-3'; forward primer for *M. thermoautotrophicum*
 4 (MT), 5'-CGTTTGATCCTGGCGGAGG-3'; and the reverse primer for both strains
 5 (MR), 5'-TACGGCTACCTTGTTACGACTT-3'. The primer pairs MC and MR or MT
 6 and MR were employed for the amplification of 16S rDNA regions (corresponding to
 7 positions 9–1510 of *E. coli* rDNA) from methanogens. PCR was performed using
 8 GeneAmp™ PCR system 2400 (PE Biosystems, Foster City, CA, USA) and the
 9 following reaction conditions: initial denaturation at 95°C for 5 min, followed by 30
 10 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min, and extension at
 11 72°C for 1.5 min; the last cycle was followed by a post-run at 72°C for 2 min. The PCR
 12 mixture consisted of 25 µl of Premix Taq (Ex Taq™ version, Takara Bio Inc., Shiga,
 13 Japan), 5 pmol of each primer pair, 100 ng of purified genomic DNA, and sterile
 14 Milli-Q water to achieve a final reaction volume of 50 µl. The PCR products were
 15 cloned using pGEM-T Easy Vector System (Promega Co., Madison, WI). The
 16 sequencing reaction was performed with Thermo Sequenase fluorescent labeled primer
 17 cycle sequencing kit containing 7-deaza-dGTP (Amersham Pharmacia Biotech,
 18 Buckinghamshire, UK) and ALF-red DNA sequencer (Amersham Pharmacia Biotech).

19 Partial *mcrA* (alpha subunit of methyl-coenzyme M reductase) gene was amplified
 20 using KOD FX Neo (Toyobo, Tokyo, Japan) with the primer set MR1mod,
 21 5'-GACCTSCACTWCGTVAACAAC-3' and ME2mod
 22 5'-TCATBGCRTAGTTNGGRTAGT-3', as reported in Mori et al. (18). The PCR
 23 fragments were subsequently ligated into *Sma*I-digested pUC18, and the products were
 24 used to transform *E. coli* HST08. The partial *mcrA* gene-harboring plasmids were

1 extracted from *E. coli* transformants and sequenced by Eurofins Genomics (Tokyo,
2 Japan). Nucleotide sequence was translated to amino acid sequence, and the sequence
3 similarities were determined using a BLAST search on DDBJ
4 (<http://www.ddbj.nig.ac.jp/>) and Genetyx ver. 11 software (Genetyx, Tokyo, Japan).

5 The 16S rRNA gene and partial McrA amino acid sequences of the dominant
6 methanogen (strain HA) were aligned using Genetyx ver. 11 software. A phylogenetic
7 tree was constructed by the neighbor-joining method (28, 29) using Genetyx ver. 11
8 software. The robustness of branches in the constructed tree was evaluated using
9 bootstrap resampling method (29), for which with 1000 replicates were employed.

11 **Assessment of culture purity**

12 The purity of cultures was assessed by inoculation (10%, v/v) of samples into a
13 growth medium containing yeast extract (0.5%, w/v), Bact peptone (2%, w/v), glucose
14 (0.25%, w/v), sucrose (0.25%, w/v), cellobiose (0.25%, w/v), and xylose (0.25%, w/v),
15 instead of sodium acetate. Following inoculation, media were incubated for 30 days at
16 37°C, and cultures were examined under a light microscope.

18 **Next-generation sequencing for assessing purity of methanogen culture**

19 The 16S rRNA gene fragments were amplified by PCR using KOD FX Neo, and
20 microbial community analysis was carried out using next-generation sequencing. The
21 labeled primer set MID-149-Arc340F,
22 5'-TGATAGAGCGCCCTAYGGGGYGCASCAG-3' and Arc1000R,
23 5'-GGCCATGCACYWCYTCTC-3', as reported in Gantner *et al.* (30), were employed
24 for the amplification of archaeal 16S rRNA gene from enrichment cultures.

Next-generation sequencing of 16S rRNA gene and subsequent analysis was performed using Roche GS FLX+ (Hokkaido System Science, Sapporo, Japan).

Scanning electron microscopy

For scanning electron microscopy, cells were cultured in synthetic medium for 20 days, harvested by centrifugation at 10,000 x g for 10 min, washed three times with 0.1 M phosphate buffer (pH 7.2), and fixed with 2.5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h at 4°C. The sample was again washed three times with 0.1 M phosphate buffer (pH 7.2), fixed with 2% solution of osmium tetroxide for 2 h at 4°C, and dehydrated by successive transfer to 50%, 70%, 80%, 90%, 95%, and 99.5% solutions of ethanol and t-butyl alcohol. The sample was subsequently freeze dried, mounted on stubs of a scanning microscope with patina paint, and sputter-coated with gold. The sections were examined and photographed using JSM-5900 scanning electron microscope (JOEL Ltd., Tokyo, Japan).

Measurement of CH₄ generated

The volume of CH₄ generated during the culture of methanogens was measured by displacing the gas with a saturated solution of NaCl. The concentrations of CH₄ and acetate were determined using gas chromatography (31). The analyses were carried out using cultures in duplicate or triplicate, and average values were reported.

Chemicals

All chemicals were of reagent grade and were purchased from commercial sources. N₂-CO₂ (80:20) gas was purchased from Chugoku Teisan (Hiroshima, Japan)

1 and used without any treatment.

3 RESULTS

4 Enrichment of acetoclastic methanogens from marine sediments

5 Following the inoculation of marine sediments into medium containing acetate (60
6 mM), vancomycin (100 mg/L), and 0.51 M NaCl, the concentration of acetate increased,
7 but little methane production was observed up to 50 days; active methane production
8 along with acetate consumption began thereafter, as previously reported (20). Thus,
9 cultures were subjected to four successive serial dilutions to enrich acetoclastic
10 methanogens. Figure 1 shows the typical time course of methane production and acetate
11 consumption by the marine sediment following serial dilutions of cultures in medium
12 containing 0.51 M NaCl, 61 mM acetate, and antibiotics. The yield of methane with
13 respect to acetate consumption, as calculated by the least square method, was 0.92
14 mol/mol acetate, which nearly equaled the theoretical yield of methane from acetate by
15 acetoclastic methanogenesis ($\text{CH}_3\text{COO}^- + \text{H}_2\text{O} = \text{CH}_4 + \text{HCO}_3^-$) (32). Therefore, further
16 serial dilutions of cultures were performed.

17 Following eight successive serial dilutions of cultures in medium containing 0.51
18 M NaCl, 61 mM acetate, and antibiotics, the purity of the enriched strain (hereafter
19 termed strain H_A) was assessed by microscopic examination of cultures inoculated in a
20 complex rich medium lacking acetate; growth was not observed under such conditions.
21 The purity of strain H_A was further confirmed by community genomic analysis of
22 archaea in the enrichment culture by next-generation sequencing based on 16S rRNA
23 gene sequences. A total of 11,835 reads, with an average read length of 470 base, were
24 obtained by next-generation sequencing. Reads whose length were greater than 250 bp

1 were extracted as input reads and subjected to BLAST analysis against the 16S rRNA
2 gene database from DDBJ; taxonomy ID and the genera, family, and class of strains
3 were obtained. Community analysis was performed for 10,250 reads. The predominant
4 archaeal taxon in the culture was a strain related to *Methanosaeta harundinacea*, with
5 an abundance ratio of 99.2%. The remaining reads were of *Methanoculleus* sp. MQ-4
6 (0.5 %) and other strains (0.3%). These results indicate that a single strain was
7 predominant in the enrichment culture.

8 9 **Phylogenetic analysis**

10 As aforementioned, 16S rRNA gene sequencing analysis revealed that the strain
11 H_A was closely related to *M. harundinacea*; however, the length of DNA sequenced was
12 too short for a definite identification of the strain. Thus, a detailed analysis of 16S
13 rRNA gene sequence was performed. Amplification of 16S rRNA gene was not
14 observed using MT (the forward primer specific for 16S rRNA gene of
15 *Methanobacterium* spp.), whereas the use of MC (the forward primer specific for
16 *Methanosaeta* spp., also capable of amplifying corresponding sequences from
17 *Methanobrevibacter* spp. and *Methanogenium* spp.) (27), yielded successful
18 amplification. Restriction analysis of 24 randomly selected clones generated from the
19 PCR product using *Sma*I enzyme revealed similar restriction patterns (data not shown).
20 Therefore, a representative clone was sequenced, which yielded 1,444 bp sequence of
21 the 16S rRNA gene (accession no. LC006969). Phylogenetic analysis of 16s rRNA gene
22 demonstrated that the strain H_A was most closely related to the genus *Methanosaeta*
23 (Fig. 2), with sequence homology of 98%, 97%, 95%, and 91% observed between strain
24 H_A and *M. harundinacea* 6Ac, *M. harundinacea* 8AcT (33), *M. pelagica* (18), and *M.*

1 *concilii*, respectively (Fig. 2). Furthermore, the phylogenetic analysis based on the
2 partial McrA sequences (LC033612) demonstrated that strain H_A was associated with
3 the genus *Methanoaeta* (Fig. 3), with sequence homology of 93%, 90%, and 83%
4 observed between strain H_A and *M. pelagica*, and *M. harundinacea* 6Ac, and *M. concilii*,
5 respectively. These results support the identification of strain H_A as a species belonging
6 to the genus *Methanosaeta*.

8 **Characteristics of the enriched acetoclastic methanogen**

9 The cells of strain H_A were non-motile, straight, sheathed, gram-negative rods (Fig.
10 4A) with blunt ends (Fig. 4B). The mean dimensions of single cells were 0.5 x 2.5 µm,
11 and a few cells displayed connections with each other. Growth was observed over the
12 pH range 5.6–8.3, with optimum growth at pH 7.0 (Fig. 5A) and between the
13 temperatures 20°C and 50°C, with an optimum of 37°C (Fig. 5B). Growth was also
14 observed on acetate, methanol, formate, and methylamine in the presence of 0.51 M
15 NaCl (data not shown). This observation suggested contamination with methanogens
16 other than acetoclastic methanogens, given that methanogens capable of metabolizing
17 all the tested substrates have not yet been reported. Furthermore, the cell morphology
18 after cultivation was observed under microscope, and found that it was not a single cell.
19 This was also supported by 16S rRNA gene analysis using next-generation sequencing.
20 Further purification of acetoclastic methanogens is therefore required for a composite
21 characterization of the dominant acetoclastic methanogen. However, given that more
22 than 99% of the 16S rRNA gene reads were identical, the characteristics of the culture
23 with acetate as the sole carbon source could be considered to represent that of the major
24 acetoclastic methanogen.

Strain H_A showed growth in acetate-containing medium in the presence of 0 M–2.05 M NaCl, with optimum growth at 0 M NaCl (Fig. 6) and no growth at 2.57 M NaCl. Specific growth rate was calculated as 0.037 h⁻¹ in the absence of NaCl and as 0.027 h⁻¹ in the presence of 1.54 M NaCl. On the other hand, a culture of *M. concilii* DSM 6752, the dominant acetoclastic methanogen in UASB reactor, under identical conditions revealed growth restriction at NaCl concentration below 0.69 M (Fig. 6). These results clearly show that strain H_A has high growth rate and high salt tolerance compared with other known species of *Methanosaeta*.

Continuous culture by UAF reactor

Before the continuous culture was started, to fix the strain H_A to the carrier, the batch culture was performed by inoculating 300ml of the culture broth of the strain H_A pre-cultured in 700-ml vials into the UAF reactor filled with 700ml of fresh medium containing 0.51 M NaCl and 61 mM of acetate. Since the residual acetate concentration in the reactor reached below 10 mM at 30 days after the start of batch culture, the continuous culture was started at 35 days. Loading rate of acetate was maintained at 100 mM/d until 70 days. Thereafter, loading rate of acetate and dilution rate were increased in stepwise from 40 mM/d to 120 mM/d and from 1.5 /d to 3.5 /d, respectively, until 110 days of cultivation. During this period, the increase of methane production rate (MPR) and acetate consumption rate were observed and the maximum MPR reached 61 mM/d. Therefore, we considered that cells were certainly immobilized to carrier, and demonstrated subsequent experiments.

The methane production rate (MPR) and loading rate of acetate through the continuous culture under condition of 0.51 M NaCl and dilution rate of 2.0 /d are shown

Fig. 7A. When the loading rate of acetate was increased in stepwise from 25 mM/d to 130 mM/d, the MPR also increased. The maximum MPR of 70 mM/d was obtained at 120 mM/d of loading rate. When loading rate was increased from 120 mM /d to 130 mM/d, the MPR was slightly decreased.

Since continuous culture by UAF reactor using strain H_A under condition of 0.51 M NaCl has been successful, the concentration of NaCl was further increased from 0.51 M to 1.54 M (Fig 7B). The loading rate of acetate was maintained at 120 mM/d. When salinity was increased to 1.03 M and 1.54 M, the MPR were decreased to 42 mM/d and 14 mM/d, respectively, accompanied by increase of residual acetate concentration in the effluent.

DISCUSSION

In the present study, an acetoclastic methanogen, strain H_A, of the genus *Methanosaeta* was isolated from the marine sediment obtained from Hiroshima Bay. In general, acetate concentration is very low (below 20 µM) in the pore waters of marine sediments (18, 34, 35). The minimum threshold acetate concentration for the growth of *Methanosaeta* is of the range 7–70 µM, whereas that of *Methanosarcina* is 0.2–1.2 mM (13, 36); this provides *Methanosaeta* with a distinct advantage for growth in low-acetate environments such as marine sediments. The copy numbers of *Methanosaeta* 16S rRNA gene in tidal flat sediments have been reported to be 10 times higher than that of *Methanosarcina* (18). However, only a single strain of *Methanosaeta*, *M. pelagica* 03d30q^T, has been isolated from marine sediments (18) as opposed to several strains of *Methanosarcina* (37). This is attributable to the difficulty in enrichment of *Methanosaeta* from marine sediments, probably due to its slower growth rate compared

1 with that of *Methanosarcina* (16, 34). In addition, the concentration of acetate in the
2 culture medium for acetoclastic methanogens is typically higher than that of naturally
3 occurring marine sediments, which provides *Methanosarcina* rather than *Methanosaeta*
4 with a growth advantage. The growth rates of strain H_A under conditions of 0 M and
5 0.51 M NaCl were two-fold and seven-fold higher than that of *M. concilii* (Fig. 6).
6 Therefore, the high growth rate of strain H_A under conditions of 0.51 M NaCl is likely
7 to have contributed to its successful isolation from the marine sediment.

8 The phylogenetic tree based on 16S rRNA gene sequences revealed that strain H_A
9 was closely related to *M. harundinacea* 6Ac and 8Ac^T (33), with sequence similarity of
10 98% and 97%, respectively. The strains *M. harundinacea* 8Ac^T and 6Ac were isolated
11 from a UASB reactor in China that processed beer brewery wastewater, and their
12 optimum temperature and pH have been reported as 34–37°C and 7.1–7.5, respectively.
13 This temperature and pH is similar to that of strain H_A; however, the NaCl tolerance of
14 *M. harundinacea* was <0.34M (18, 33), which is remarkably different from that of
15 strain H_A.

16 *M. pelagica* 03d30q^T is the first strain of *Methanosaeta* isolated from marine
17 sediments (18). The strain exhibited growth under conditions of 0.20 M–0.80 M NaCl
18 but not at salinity below 0.20 M. Therefore, *M. pelagica* 03d30q^T was a halophilic
19 methanogen that required NaCl for growth. In contrast, strain H_A exhibited growth
20 under conditions of 0 M–2.05 M NaCl, with optimum growth without NaCl; this
21 observation suggests that strain H_A is halotolerant and not halophilic. Strain H_A is the
22 first halotolerant *Methanosaeta* highly enriched from marine sediments. The
23 acetoclastic methanogenesis step of methane fermentation is the rate-limiting step under
24 conditions of high salinity (20). The strain H_A exhibits high growth rates under a wide

1 range of salinity compared with the other strains of *Methanosaeta*.

2 Hence, continuous culture by UFA reactor using strain H_A was attempted under
3 condition of 0.51 M NaCl to verify whether enriched strain H_A can be employed to
4 improve acetolastic methanogenesis of industrial methane fermentation or wastewater
5 treatment under conditions of high salinity. In previous study, the 90 mM/d of MPR
6 was reported by upflow anaerobic sludge blanket reactor using the acclimated
7 methanogenic sludge from the coastal mud sediment under condition of 0.51 M NaCl,
8 when loading rate of acetate is 100 mM/d (19). In this study, the MPR of 70 mM/d
9 under condition of 0.51 M NaCl was lower compared with that of 90 mM/d reported by
10 Takeno (19). However, we successfully demonstrate continuous methane fermentation
11 under condition of 1.03 M and 1.54 M of NaCl. The MPR of 42 mM/d and 14 mM/d
12 were obtained in condition of 1.03 M and 1.54 M NaCl, respectively. As far as we know,
13 the demonstration of continuous methane fermentation from acetate under high salinity
14 condition of 1.54 M NaCl has not been reported.

15 Furthermore, filamentous cells of *Methanosaeta* can be advantages for
16 immobilization of cells in anaerobic reactors. *Methanosaeta* species are believed to be
17 the key components in anaerobic digesters not only because of their ability to use very
18 low acetate, but also their filamentous cells that serve as a scaffold for the attachment of
19 other organisms to promote the formation of UASB granules (38), an essential
20 self-immobilized organization of the functional microbes (39). Hence, the use of highly
21 enriched strain H_A in anaerobic reactors might be applicable to improve of
22 methanogenesis under condition of high salinity.

23

24

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3

Figure Legends

Figure 1. Time course of methane production and acetate consumption following serial dilutions of cultures. Symbols: open square, acetate; closed circle, methane produced. Cells were cultured and diluted in medium of pH 7.0 containing 61 mM acetate and 3% NaCl at 37°C.

Figure 2. Phylogenetic tree based on 16S rRNA gene sequence. Phylogenetic analysis and construction of the phylogenetic tree was carried out by neighbor-joining method and rooted with *Methanotermus fervidus*. Bootstrap values (>50%) are shown inside each node. A total of 1,000 bootstrap replicates were employed. [GenBank accession numbers are given in parentheses. Bar, 0.1 substitutions per compared nucleotide site.](#)

Figure 3. Phylogenetic tree of based on McrA sequences. Phylogenetic analysis and construction of the phylogenetic tree was carried out by neighbor-joining method and rooted with *Methanotermus fervidus*. Bootstrap values (>50%) are shown inside each node. A total of 1,000 bootstrap replicates were employed. [GenBank accession numbers are given in parentheses. Bar, 0.1 substitutions per compared position.](#)

Figure 4. Marine methanogens enriched from marine sludge. (A) Optical microscopic [picture](#) and (B) SEM photograph of [an](#) acetate-utilizing methanogen enriched from the marine sediment. Cells were cultured for 30 days at 37°C in medium of pH 7.0 containing 61 mM of substrates and 0.51 M NaCl.

1

2 **Figure 5.** Specific growth rate of methanogens under conditions of various pH (A) and
3 temperature (B). Cells were cultured at 37°C in media containing 61 mM sodium
4 acetate and 0.51 M NaCl. Error bars represent standard deviation of the cultures in
5 triplicate.

6

7 **Figure 6.** Specific growth rate of methanogens at various concentrations of NaCl.
8 Symbols: open square, *Methanosaeta concilii* DSM 6752; closed circle, enriched strain
9 HA. Cells were cultured at 37 °C in media of pH 7.0 containing 61 mM acetate and 0
10 M–2.57 M NaCl. Error bars represent standard deviation of the cultures in triplicate.

11

12 **Figure 7.** Methane production rate at different acetate loading rate (A) and NaCl
13 concentration (B) in the continuous culture with the reactor in which enriched
14 methanogens HA was fixed on matrix carrier. Symbols: closed circle, MPR (mM/d);
15 open triangle, pH; open square, acetate out (mM). Error bars represent standard
16 deviation of the cultures at the same loading rate or NaCl concentration.

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Figure 1, Kita et al.

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Figure 5, Kita et al.

Figure 6, Kita et al.

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