Journal of Bioscience and Bioengineering
VOL. 121 No. 2, 196–202, 2016

www.elsevier.com/locate/jbiosc

Characterization of a halotolerant acetoclastic methanogen highly enriched from marine sediment and its application in removal of acetate

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Received 22 January 2015; accepted 28 May 2015
Available online 27 June 2015

A marine sediment collected from Hiroshima Bay was cultured in artificial seawater, containing 0.51 M 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.

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[**Key words:** Halotolerant; Acetoclastic methanogen; Marine sediment; Methanosaeta; Methane fermentation; Continuous culture; Upflow anaerobic filter reactor]

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 methanogeneis 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 acetate into methane (CH_4) and carbon dioxide (CO_2), termed acetoclastic methanogenesis, or the reduction of CO_2 with hydrogen, termed hydrogenotrophic methanogenesis. Of these three steps, acetoclastic methanogenesis shows low salt tolerance and is responsible for the high-salt intolerance of the methane fermentation process (7,8). Therefore, it is important to acquire high-salt-tolerant methanogens and understand their physiology for the development of the industrial methane fermentation process for high-salt containing feedstock.

Some of hydrogenotrophic methanogens are known to inhabit environments of extremely high salt concentration, such as salt lakes and deep-sea sediments. For instance, *Methanococcoides methylutens*, which was isolated from the Sumner Branch of Scripps Canyon located near La Jolla, California, exhibited growth under saline conditions, with optimal NaCl concentration of 0.24–0.64 M

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http://dx.doi.org/10.1016/j.jbiosc.2015.05.018

(9). *Methanohalophilus mahii* isolated from the Great Salt Lake was another moderately halophilic methanogen, which exhibited optimum growth at NaCl concentration of 2.06 M (10,11). Furthermore, the extremely halophilic methanogen *Methanohalobium evestigatum* isolated from a saline lagoon in Cremea showed growth at 4.28 M salinity (11,12).

Two genera of acetoclastic methanogens are known. These include *Methanosarcina* and *Methanosaeta* (13). Given that 70% of the methane generated during methane fermentation is derived from the cleavage of acetate (14), an important role is played by acetoclastic methanogens in this process. A few species of halophilic *Methanosarcina* have been isolated to date. The optimal range of NaCl for the growth of *Methanosarcina acetivorans* has been reported as 0.1–0.6 M, with growth not observed in the absence of NaCl (15). *Methanosarcina mazei* Gö1 showed growth at Na^+ concentration of 18,000 mg/L, corresponding to 0.79 M solution of NaCl (16,17). On the other hand, a single report available on halophilic species of *Methanosaeta* shows that *Methanosaeta pelagica* 03d30q^T isolated from a tidal flat sediment exhibited growth at Na^+ concentrations of 0.20–0.80 M, with the optimum growth at 0.28 M (18).

In our previous study, it was found that microbial consortia acclimated from coastal mud sediments produced methane from acetate at a production rate of 96.1 mmol/L/d at salinity of 0.51 M NaCl (19). It was also demonstrated that marine sediments collected from various tidal flats in Japan exhibited high methanogenic activity from brown algae under condition of 0.51 M NaCl while mesophilic granules from upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater lost the methanogenic activity under the saline condition (20). This observation suggested that marine sediments in tidal flats were promising sources of methanogenic microbial consortia that could be applied for the anaerobic treatment of organic waste with high salinity. However, in the study, all tested marine sediments showed activity in acetoclastic methanogenesis lower than that in hydrolytic/acetogenesis, acidogenesis from butyrate and hydrogenotrophic methanogenesis. Furthermore, to determine whether the methane production from the brown alga by marine sediments was sustainable, when the culture was diluted to 10% (v/v) with fresh medium containing the alga and salt to conduct subculture, acetoclastic methanogenesis was significantly weakened, suggesting low microorganism growth rates compared to the other steps (20). To overcome this constraint and practically use the marine microbial community, the microbial physiology of a variety of marine methanogenic microbes needs to be elucidated to optimize them further, and the appropriate bioreactor configuration for high-throughput treatment needs to be determined. However, only *M. pelagica* 03d30q^T 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 KH_2PO_4, 1.0 g NH_4Cl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.08 g $CaCl_2 \cdot 2H_2O$, 0.3 g $Na_2S \cdot 9H_2O$, 4 g $KHCO_3$, 0.3 g L-cysteine-HCl·H_2O, 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.2H_2O, 5.0 mg riboflavin, 5.0 mg nicotinic acid, 5.0 mg calcium-D-pantothenate, 5.0 mg *p*-aminobenzoic acid, 5.0 mg lipoic acid, and 0.1 mg vitamin B_{12}. The trace element solution contained 12.8 g nitrilotriacetic acid (NTA), 1.35 g $FeCl_3 \cdot 6H_2O$, 0.10 g $MnCl_2 \cdot 4H_2O$, 0.024 g $CoCl_2 \cdot 6H_2O$, 0.10 g $CaCl_2 \cdot 2H_2O$, 0.10 g $ZnCl_2$, 0.025 g $CuCl_2 \cdot 2H_2O$, 0.01 g H_3BO_3, 0.024 g $Na_2MoO_4 \cdot 2H_2O$, 1.0 g NaCl, 0.12 g $NiCl_2 \cdot 6H_2O$, and 0.026 g $Na_2SeO_3 \cdot 5H_2O$. For enrichment culture, 61 mM of sodium acetate and 0.51 M of sodium chloride were added per liter of medium.

Growth conditions A modified Hungate technique in combination with the serum bottle technique (22,23) was employed for enrichment culture. Prior to the addition of cysteine, KH_2PO_4, $MgSO_4$, $CaCl_2$, and Na_2S, the medium was boiled for 20 min, cooled in ice water with continuous bubbling of N_2–CO_2 (80:20) gas, dispensed into serum bottles sealed with black butyl rubber stoppers, and sterilized (18 min, 121°C). Concentrated aqueous solutions of the remaining chemicals were separately autoclaved and injected into the medium using a hypodermic syringe.

Enrichment was initiated at 37°C by the addition of sediment (10 g wet weight) to 700-ml serum vials containing 300 ml of enrichment medium; the medium contained vancomycin (100 mg per liter) to inhibit the growth of non-methanogenic organisms, and pH was adjusted to 7.0 by the addition of 1 M HCl. After methane production subsided, 60 ml of the culture was anaerobically transferred into a new vial of sterile enrichment medium. Following four such successive transfers, each enrichment culture was subjected to 10-fold serial dilution from 0.1% to 10% (v/v) using medium that contained vancomycin, as detailed above. The purity of the enriched cultures was examined at the highest dilution at which bacterial growth was detected. Upon detection of any contaminants, serial dilution was repeated. The pure cultures obtained were subcultured once a month and stored at 4°C in a growth medium.

Continuous culture by upflow anaerobic filter reactor A glass column reactor (working volume, ≈1000 ml) packed with polypropylene-ceramic mixture as a support material (a gift from Showa Herding Co., Ltd., Tokyo, Japan) developed for the fixation of acetoclastic methanogens like *Methanosaeta* sp. was used for the upflow anaerobic filter (UAF) reactor for the cultivation of enriched strain H_A (24). To fix the strain H_A to the carrier, the batch culture was performed by inoculating 300 ml of the culture broth of the strain H_A pre-cultured in 700-ml vials into the UAF reactor installing the cylindrical carrier (ϕ50 mm × 150 mm height, 4 mm thickness). The gas pack filled with mixed gas (80% N_2/20% CO_2) was fixed to the reservoir tank to prevent backflow from the reactor when the reservoir tank has become negative pressure. To maintain the pH, 50 mM of phosphate buffer (pH 6.1) was supplied to the medium. Continuous cultures by UAF reactor were performed under the condition of 0.51 M, 1.03 M and 1.54 M NaCl concentration, and acetate was added from 15 mM/d to 130 mM/d of loading rate.

Effect of pH, temperature, and salt concentration For determination of the optimum pH for growth, the pH values of the enrichment medium (containing 61 mM of sodium acetate and 0.51 M of sodium chloride) were adjusted to the range 5.5–8.8 using 50 mM MES [2-(*N*-morpholino)-ethanesulfonic acid] buffer of pH 5.5–7.0 or Tris–HCl buffer of pH 7.0–8.8 and maintained at the 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–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_{CH4}) 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_{CH4} 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 from *M. concilii* or *Methanothermobacter thermautotrphicus* (27). The nucleotide sequences of the primers were as follows: forward primer for *M. concilii* (MC), 5′-TGGTTGATCCTGCCAGAGG-3′; forward primer for *M. thermoautotrophicum* (MT), 5′-CGTTTGATCCTGGCGGAGG-3′; and the reverse primer for both strains (MR), 5′-TACGGCTACCTTGTTACGACTT-3′. The primer pairs MC and MR or MT and MR were employed for the amplification of

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