Methanobrevibacter acididurans sp. nov., a novel methanogen from a sour anaerobic digester

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A novel acid-tolerant, hydrogenotrophic methanogen, isolate ATMT, was obtained from an enrichment performed at pH 5<0 using slurry from an acidogenic digester running on alcohol distillery waste. The original pH of the slurry was 5<7 and the volatile fatty acid concentration was 9000 p.p.m. Cells of isolate ATMT were Gram-positive, non-motile and 0<3–0<5 µm in size. They did not form spores. The isolate could grow in the pH range 5<0–7<5, with maximum growth at pH 6<0. The optimum temperature for growth was 35 °C. Formate, acetate, methanol, trimethylamine, 2-propanol and 2-butanol were not utilized as growth substrates. Rumen fluid and acetate were required for growth on H2/CO2. Coenzyme M and 2-methylbutyric acid were not required in the presence of rumen fluid. 16S rDNA sequence analysis confirmed the signature sequence of the genus Methanobrevibacter. Morphological and biochemical characteristics of the isolate, together with the 16S rDNA sequence analysis, clearly revealed that the isolate could not be accommodated within any of the existing species of the genus Methanobrevibacter. Therefore, it is proposed that a novel species of the genus Methanobrevibacter should be created for this isolate, Methanobrevibacter acididurans sp. nov., and the type strain is strain ATMT (= MCM B 613T = OCM 804T).

Keywords: methanogen, Methanobrevibacter, low pH, anaerobic digestion

INTRODUCTION

Anaerobic treatment of industrial wastes is becoming increasingly popular because of the generation of a valuable by-product, methane. The success of anaerobic treatment depends mainly on the optimal activity of methanogens. These bacteria are highly sensitive to changes in environmental conditions such as temperature and pH. Changes in operational parameters, e.g. an increase in the organic loading rate, lead to an increase in the volatile fatty acid concentration and a decrease in the pH of the digesting mass. These changes adversely affect the growth of methanogens (Kugelman & Chin, 1971; Pohland, 1992). This kind of effect is commonly seen with digesters running on high-strength organic wastewaters such as those arising from alcohol distilleries and food-processing units.

To overcome the problems arising from sour digestion, one of the alternatives is to utilize methanogens that tolerate low pH (~5.5–6.0) and high concentrations of volatile fatty acids. Over 65 species of methanogen belonging to 20 different genera are known today (Sowers, 1995). However, most of them grow optimally in the pH range around neutral to slightly alkaline conditions (pH 6.8–8.5). Many groups of scientists have attempted the isolation of acid-tolerant methanogens (Williams & Crawford, 1985; Ladapo & Barlaz, 1997). A methanogen identified as Methanobacterium uliginosum (König, 1984) has been reported to grow at pH 6.0–8.0. Patel et al. (1990) obtained Methanobacterium espanolae, which was able to grow at pH 5.5–6.2; this can be considered as an example of a truly acidophilic methanogen. Certain strains of Methanosarcina have been shown to grow at low pH, using methanol and H2 as the substrate.
METHODS

Source of inoculum. A two-stage anaerobic treatment process for distillery waste was developed in the laboratory in 25 l continuously stirred tank reactors. The sludge of a field-scale anaerobic digester (initiated with cattle dung as the inoculum and running on distillery waste) was used as the starter culture for establishing this process. The acidogenic digester was operated initially at a hydraulic retention time of 10 days on undiluted distillery waste neutralized to pH 7.0 with Ca(OH)₂. The loading rate was increased to reach an optimum at 18 kg COD l⁻¹ day⁻¹ with a hydraulic retention time of 4 days. Under these conditions and at ambient temperature, the biogas produced contained 15% (v/v) CH₄, the remaining gas being CO₂. The pH of the digesting slurry was 5.7±0.1. The volatile fatty acid content was 8000–10000 p.p.m. The slurry of this digester was used as the inoculum for the isolation of methanogens. Before this reason, methanogens were isolated that might have potential application in the treatment of acidic wastewaters and the recovery of sour anaerobic digesters.

Growth medium. In the light of the composition of distillery waste and the nutritional requirements of methanogens in general, DVS medium was formulated. It had the following composition (1 l of distilled water): 0.3 g KH₂PO₄, 0.3 g K₂HPO₄, 1.5 g (NH₄)₂SO₄, 0.6 g NaCl, 0.08 g CaCl₂, 2H₂O, 0.1 g MgCl₂·6H₂O, 2.0 g yeast extract, 1.0 g peptone, 1.0 g tryptone, 2.5 g sodium acetate, 2.5 g sodium formate, 1.0 ml trace-element solution (Touzel & Albagnac, 1983), 1 ml trace-vitamin solution (Wolin et al., 1963), 20 ml fatty acid mixture (Sowers & Schreier, 1995), 0.5 g cysteine hydrochloride, 0.5 g Na₂S·9H₂O and 1 mg resazurin. Coenzyme M was added at a final concentration of 50 ng ml⁻¹. The medium was prepared under a H₂/CO₂ (80:20, v/v) gas phase. The pH of the medium was adjusted using 10 M HCl or NaHCO₃. The medium was sterilized by autoclaving at 121 °C for 30 min.

Clarified rumen fluid (Ranade & Gadre, 1988b) was acidified, using 5 M HCl, to pH 5.0 under N₂ and was sterilized by autoclaving. Rumen fluid was added (10%, v/v) as a source of unknown growth factors. DVS medium supplemented with rumen fluid was termed DVS medium.

Anaerobic techniques originally developed by Hungate (1969), modified by Miller & Wolin (1974) and further described by Ranade & Gadre (1988a) were followed. All experiments were carried out in duplicate under static conditions at 35 °C unless mentioned otherwise. The vials with H₂/CO₂ (80:20, v/v) as the substrate were pressurized every other day to 1013 kPa unless mentioned otherwise.

Enrichment. Enrichments were performed in DVS medium adjusted to different pH values (4.0, 5.0 and 6.0) under the H₂/CO₂ (80:20, v/v) gas phase. Vials containing 9 ml medium were inoculated with 1 ml inoculum and incubated at 35 °C.

Isolation. Methanogens were isolated in roll tubes from the fifth transfer of the enrichment at pH 5.0 after 7 days incubation under static conditions. DVS medium at pH 5.0 in H₂/CO₂ (80:20, v/v) and solidified with 3 g% agar (Difco) was used.

Purity of the culture. Colonies obtained in the roll tubes showing methane production were transferred to liquid DVS medium in an anaerobic glove box (Forma Scientific). The gas phase in these vials was replaced with H₂/CO₂ (80:20, v/v). The organism was re-isolated on solid medium from the liquid cultures. To check whether the culture was free from aerobic contaminants, it was inoculated on nutrient agar plates. The presence of anaerobic contaminants was checked using liquid PYG medium (Holdeman & Moore, 1975) under N₂.

Maintenance. The culture was maintained by serial transfers in liquid DVS medium (pH 5.0) every 3 weeks at 10% (v/v) inoculum.

Microscopy. Cells were routinely observed under a microscope equipped with phase-contrast and a fluorescence attachment with a UV excitation filter in the range 380–420 nm (Nikon). The Gram character was determined using a standard Gram-stain kit (Hi Media). Motility was determined by the hanging-drop method, using a glass cavity slide. Spore formation was checked by using the pasteurization test and by observation of the treated cell suspension by phase-contrast microscopy. For scanning electron microscopy, cells from the exponential phase of growth were fixed using Trumps 4F:1G fixative on a glass coverslip at 4 °C overnight. Cells were washed with distilled water and dehydrated by gradual washing with an increasing gradient of ethanol. The sample was air-dried and mounted on a stub. Gold coating (20 nm thick) of the sample was done using an E 5200 Auto Sputter Coater (Bio-Rad). Observations were made on a stereoscan S120 scanning electron microscope (Cambridge Instruments).

Growth substrates. DVS medium without acetate and formate, prepared under N₂, was used for substrate-utilization studies. Anaerobic stocks of filter-sterilized substrates (acetate, methanol, formate, trimethylamine, 2-propanol and 2-butanol) were prepared and added separately at final concentrations of 50 mM. A freshly grown culture was inoculated at 10% (v/v) and vials were incubated at 35 °C for 28 days. Medium under H₂/CO₂ served as the control. The ability to use H₂/acetate as the substrate was also checked. Growth was determined on the basis of the methane produced.

Susceptibility to lysis. Cells from the exponentially growing culture were used to check susceptibility to lysis by 1% SDS and distilled water as a hypotonic solution (Boone & Whitman, 1988). Lysis was determined by visual and microscopic observation of the culture. Unexposed culture served as the control.

Bile sensitivity. The sensitivity of the culture to different bile salts was examined in DVS medium. Anaerobic stocks of sodium taurocholate, sodium deoxycholate and ox bile were prepared by dissolving the required amount of salt in a known quantity of distilled water that had been pre-boiled and cooled under a flow of N₂. The pH of the solution was adjusted and the vials were sealed under N₂. The stocks were
stereilized by autoclaving and then added separately to the medium to achieve final concentrations of 0·1, 0·1 and 2·0% (w/v), respectively. The culture was inoculated at 10% (v/v) and incubated at 35 °C for 28 days. Methane production in each vial was then checked.

**Growth experiments.** The culture was grown in DVSR medium at 35 °C with shaking at 150 r.p.m. It was observed that growth, in terms of OD at 660 nm, had a linear relationship with methanogenesis during the exponential phase and for most of the subsequent linear phase. Hence, for quantitative studies, growth was monitored by measuring the amount of methane produced. The specific growth rate was calculated from the exponential parts of the methane-formation curve. Maximum methane, 17·67 μmol (ml headspace)⁻¹, was produced in 120 h when the OD was 0·640.

**Optimum temperature.** The optimum temperature was determined in DVSR medium at pH 5·0. 72 h after incubation, the pH remained within 0·1 unit. Vials inoculated with 10% (v/v) culture were incubated at temperatures in the range 25–40 °C, using 5 °C intervals. The vials were pressurized daily with H₂/CO₂ to ensure an adequate supply of substrate. Growth was measured every other day in terms of methane produced (Boone & Whitman, 1988).

**Optimum pH.** The pH requirement was determined at the optimum temperature in DVSR medium adjusted to pH values ranging from 5·0 to 7·5 using different concentrations of NaHCO₃ or 10 M HCl. The proportion of CO₂ was kept constant at 20% (v/v) as H₂/CO₂ (80:20, v/v) in the gas phase. The pH of the medium was maintained after autoclaving. The vials were inoculated with freshly grown culture (10%, v/v). The headspace was pressurized to 101·3 kPa every day and methane production was measured. The vial headspace was then refilled for 3 min and then pressurized to maintain the pH of the broth (Patel et al., 1990). After incubation, the pH remained within 0·1 unit of the desired value. Specific growth rates were calculated on the basis of the cumulative methane production.

**Nutritional requirements.** Cells from a freshly grown culture were harvested by centrifugation at 10000 g for 10 min at 4 °C and then washed twice with anaerobic saline to prevent carry-over of nutrients. The pellet was resuspended in anaerobic saline and used as the inoculum. DVS and DVSR media were used to study the requirement for rumen fluid, acetate, coenzyme M and 2-methylbutyric acid. Growth was determined by measuring methane production.

**16S rDNA sequencing.** The DNA was extracted from 3 ml freshly grown culture (Erb & Wagner-Dobler, 1993). The 16S rRNA gene was amplified using a PCR (Perkin Elmer) as described previously (Dighie et al., 1998). The primer pair used here was M8B/M8U (Lin & Miller, 1998). The resulting 1·4 kb fragment was cloned directly in the vector pGEM-T Easy (Promega). The complete 1·4 kb sequence was obtained using universal sequencing and internal primers.

**Phylogenetic analysis.** The sequence was analysed by using BLAST and at the Ribosomal Database Project (RDP II; Maidak et al., 1999). Sequence similarities were determined at RDP II using SIMILARITY MATRIX (Maidak et al., 1999). Sequences were aligned using CLUSTAL W (Thompson et al., 1994) at the EBI site. Phylogenetic analyses were done in MEGA (Kumar et al., 1994). A phylogenetic tree was constructed using the neighbour-joining method (Saitou & Nei, 1987) by calculating the genetic distance with Kimura’s two-parameter model (Kimura, 1980) and using the Jukes–Cantor model (Jukes & Cantor, 1969). Bootstrap values were obtained from 1000 random samplings.

**Gas analysis.** Methane was analysed on a gas chromatograph (Chemito 3800; Toshiwai) equipped with a thermal conductivity detector. The column used was Porapak Q (80/100 mesh; 3·2×2·mm, SS). Hydrogen (30 ml min⁻¹) was the carrier gas. The temperature settings were as follows: oven, 50 °C; injector, 60 °C; detector, 80 °C. Pure methane (Span Gas) was used as the external standard. Data integration and analysis were done using WINCHROM EX software.

**RESULTS**

**Enrichment.**

Within 10 days of initiation of the enrichments, methane was detected in the headspace gas. When the methane concentration in the headspace gas reached 50–60% (v/v), the enrichments were transferred (10%, v/v) to fresh medium. Upon subsequent transfers, it was observed that the enrichment at pH 4·0 grew poorly and little methane was produced. The enrichment at pH 5·0 was selected for further studies as it showed better methane production than the enrichment at pH 6·0. Microscopic observations revealed coccoid cells with uniform morphology that occurred singly, in pairs and in chains. The cells consistently possessed a characteristic blue-green fluorescence when exposed to UV radiation (a colour micrograph of cells of the novel isolate is available as supplementary material in IJSEM Online; http://ijs.sgmjournals.org/). Addition of vancomycin at 100 μg ml⁻¹ during the third transfer reduced the number of non-fluorescent bacteria. Sterile rumen fluid was also added at this stage of enrichment and was found to stimulate the growth of the fluorescent bacteria. Both additions were continued in subsequent transfers. After two more transfers, isolation was undertaken.

**Isolation.**

In the roll tubes, methane was produced after 12 days incubation. Well-isolated colonies were selected and transferred to liquid medium in an anaerobic glove box. After the production of methane during growth in the broth, the purity of the isolate was checked by means of re-isolation in a roll tube. All colonies were brown and pinpoint with irregular margins. The absence of organotrophic contaminants was confirmed by failure to grow in anaerobic PYG medium and aerobic nutrient agar plates. The isolate was designated strain ATM³.

**Morphology.**

Cells were more or less round, 0·3–0·5 μm in size and occurred singly, in pairs or in chains of 8–12 cells (Fig. 1). Cells tended to form loose aggregates upon prolonged incubation. Cells were Gram-positive and non-motile. They did not form spores, as indicated by phase-contrast microscopy. Cells showed the blue-green fluorescence typical of methanogens, and this
remained consistent over a longer duration. Neither flagella nor polar fibrils were observed under scanning and transmission electron microscopy.

**Growth substrates**

Isolate ATM\(^T\) used only H\(_2/\)CO\(_2\) as the substrate for methanogenesis. No growth was observed with acetate, formate, trimethylamine, methanol, 2-propanol or 2-butanol with N\(_2\) as the gas phase. Acetate plus H\(_2\) was not utilized.

**Cell lysis**

No decrease in turbidity was observed after exposure to 1% SDS or distilled water for 10 min. Microscopic observations also revealed intact cells. Thus, the cells were found to be resistant to lysis by SDS and hypotonic solution.

**Optimum temperature**

The isolate grew at temperatures in the range 25–37 °C. The optimum temperature was 35 °C. No growth was obtained at or above 40 °C.

**Optimum pH**

The isolate grew and produced methane over a wide range of pH (5–0–7–5). On the basis of specific growth rates, the optimum pH was found to be 6–0 (Fig. 2). No growth was obtained at pH 4–5.

**Bile tolerance**

At pH 7–0, 0–1% sodium taurocholate, 0–1% sodium deoxycholate and 20% (w/v) ox bile were inhibitory. At pH 5–0, sodium taurocholate (0–1%, w/v) was inhibitory, but ox bile and sodium deoxycholate were precipitated. Thus, the isolate was found to be sensitive to bile salts.

**Nutritional requirements**

When the culture grown in DVSR medium was inoculated into DVS medium (10%, v/v), growth was greatly diminished. Furthermore, after successive transfers in DVS medium, growth ceased. A culture washed with anaerobic saline failed to grow when inoculated into DVS medium. Thus, the presence of acetate, coenzyme M and 2-methylbutyric acid in DVS medium was not sufficient for growth and maintenance of the strain. However, the addition of rumen fluid restored growth in DVS medium without acetate, coenzyme M and 2-methylbutyric acid. This showed that the addition of rumen fluid was necessary for the growth and maintenance of strain ATM\(^T\). Addition of acetate (20 mM) stimulated growth in DVSR medium.
No such effect was seen with the addition of 2-methylbutyric acid and/or coenzyme M. Organic nitrogen in the form of amino acids was required.

16S rDNA sequence analysis

Using primers for the plasmid vector and internal primers for the 16S rRNA gene, a sequence of 1359 bp was obtained. It showed the presence of the sequence 5'-TGTGAGC(A/C)ATCGCG-3', corresponding to Escherichia coli positions 375–388, which is considered as the signature sequence for the genus Methanobrevibacter.

When analysed by BLAST and at RDP II, the sequence showed a high degree of similarity to many Methanobrevibacter sequences, including many partial sequences, uncharacterized species and clones from environmental samples. For subsequent analyses, only those sequences with more than 1000 bases, excluding uncultured organisms, were compared, and a phylogenetic tree was drawn (Fig. 3). Strain ATMᵀ showed a separate lineage.

DISCUSSION

Cells of strain ATMᵀ are Gram-positive, non-motile cocci, 0.3–0.5 μm in diameter, that occur singly, in pairs or in chains. The cells do not form spores and are resistant to lysis by SDS and hypotonic solutions. The isolate is hydrogenotrophic. The optimum temperature for growth is 35 °C. All these characteristics are shared by members of the genus Methanobrevibacter (Holt et al., 1994). The presence of the signature sequence of Methanobrevibacter species in the 16S rDNA of strain ATMᵀ confirmed this assignment.

The genus Methanobrevibacter consists of seven well-characterized species, namely Methanobrevibacter ruminantium, Methanobrevibacter smithii, Methanobrevibacter arborophilicus (Holt et al., 1994), Methanobrevibacter oralis (Ferrari et al., 1994), Methanobrevibacter curvatus, Methanobrevibacter cuticularis (Leadbetter & Breznak, 1996) and Methanobrevibacter filiformis (Leadbetter et al., 1998). Isolate ATMᵀ is morphologically similar to only two species, Methanobrevibacter smithii and Methanobrevibacter ruminantium. It differs from both of these species with respect to cell size, utilization of formate as a growth substrate and temperature and pH maxima for growth. It also differs from Methanobrevibacter smithii in terms of bile tolerance and amino acid requirements.

When analysed at the RDP II, strain ATMᵀ showed a very high degree of similarity to sequences of Methanobrevibacter sp. MB9 and Methanobrevibacter sp. NT7 (which are cultured, but unidentified, methanogens from GenBank). Amongst the well-characterized species of Methanobrevibacter, it shows a maximum similarity value of 95.8% to Methanobrevibacter ruminantium, followed by a value of 94.8% for Methanobrevibacter smithii ALI. Comparison of the available 600 bp 16S rRNA gene sequence of Methanobrevibacter oralis with that of strain ATMᵀ also shows 94.8% sequence similarity. For different species of Methanobrevibacter, the similarity values are in the range 93–97%. In their analyses of faecal isolates, Lin & Miller (1998) reported similarity values of 97% and above for the isolates that they proposed as novel species. Compared to these values, the similarity values of ATMᵀ are low. Moreover, phylogenetic analysis with MEGA suggests that strain ATMᵀ is in a distinctly different lineage from the known species of Methanobrevibacter.

Thus, the results obtained in sequence analysis, along with nutritional and physiological studies, clearly suggest that strain ATMᵀ represents a novel species within the genus Methanobrevibacter.

Strain ATMᵀ stands apart from all the reported species of Methanobrevibacter with respect to pH require-
ments for growth. It grows at pH values above pH 5–0; the optimum pH for growth being 6–0. Within the genus Methanobrevibacter, only Methanobrevibacter ruminantium has been reported to grow at pH 6–0; other species growing at neutral to slightly alkaline pH values. Even Methanobrevibacter ruminantium does not grow at pH values below 6–0 (Smith & Hungate, 1958).

On the basis of the pH behaviour of the new species and the fact that it has been isolated from an environment containing high levels of volatile fatty acids, the name Methanobrevibacter acididurans sp. nov. is proposed.

Of all the acid-tolerant and/or acidophilic hydrogenotrophic methanogens growing at ambient temperature, the name Methanobrevibacter acididurans sp. nov. is proposed.

The isolation of strain ATM°F indicates the likelihood of acid-tolerant and acidophilic methanogens in other genera. It also offers an explanation for the continuous production of methane in small amounts in acidogenic digesters. Better understanding of the ecological role of this organism will help to solve the problems of sour digestion and to enhance methanogenesis under acidic conditions.

Description of Methanobrevibacter acididurans sp. nov.


Cells occur singly, in pairs or in chains. They are cocci, 0.3–0.5 μm in diameter. Cells are Gram-positive, do not form spores and are resistant to lysis by SDS and hypotonic solutions. Hydrogenotrophic. Formate, acetate, methanol, trimethylamine, 2-propanol and 2-butanol are not utilized as growth and energy substrates. Mesophilic. The optimum temperature is 35 °C. No growth occurs at or above 40 °C. The optimum pH for growth is pH 6–0. However, it can also produce methane at pH 5–0. Bile-sensitive. Rumen fluid is required for growth. Acetate and amino acids are required. Coenzyme M and 2-methylbutyric acid are not produced at pH 5–0. The optimum pH for growth being 6–0 (Smith & Hungate, 1958).

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