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Isolation and characterization of a new *Methanosarcina mazei* strain GFJ07 from a mountain forest pond

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ABSTRACT

Aims: A new methanogenic strain, named GFJ07, was isolated from a pond of mountain forests in Guangxi, China. Cells grown in liquid culture tended to form aggregates with pseudosarcina-like or irregular shape.
Methodology and Results: The optimum temperature, pH and NaCl concentration were 35 ℃, 7.0 and 0.5%, respectively. The isolate used methanol, trimethylamine, acetate and H_2-CO_2 as substrates. Analysis of the 16S rDNA sequences revealed strain GFJ07 showed the highest sequence similarity of 99.9% to *Methanosarcina mazei*.
Conclusion, significance and impact of study: The cells were Gram positive and nonmotile. Most of single cell grew as a sausage-like clinder about 0.5 μm in diameter and 1.0 μm in length.

Keywords: methanogen, archaea, *Methanosarcina*, *Methanosarcina mazei*, sausage-like

INTRODUCTION

Methanogens have attracted wide interest because they are included in archaea (Sanz and Amils, 1988; Thakker and Ranade, 2002) and they can produce methane, a greenhouse gas (Asakawa *et al*., 1995). On the other hand, they play an important role in global carbon cycle and anaerobic digestion of organic matter. Methanogens are widely distributed in strictly anaerobic environments, such as anaerobic digesters, marine sediment, lake sediment, paddy field soil and gastrointestinal tracts of animals (Ferry, 1997; Garcia *et al*., 2000; Lai *et al*., 2000). Because of the large deposition of leaves into the ponds of mountain forests, the rotting that then ensues makes these ponds the ideal location for the growth of methanogens.

The genus *Methanosarcina* were well-known methanogenic bacteria which were found for a long time. According to Bergey's manual of systematic bacteriology (second edition), the genus currently comprised ten species: *Methanosarcina barkeri*, *Methanosarcina acetivorans*, *Methanosarcina baltica*, *Methanosarcina lacustris*, *Methanosarcina mazei*, *Methanosarcina methanica*, *Methanosarcina semesiae*, *Methanosarcina siciliae*, *Methanosarcina thermophila* and *Methanosarcina vacuolata*. *M. mazei* and *M. barkeri* are the most representative mesophilic species of the genus (Clarens *et al*., 1993). The temperature tolerance of the two species were narrow and none psychrotolerant strains had been reported. Guangxi, chosen for its location in the subtropical zone has an ambient temperature ranging from below zero to 40 degree Celsius. This temperature range allows methanogens with a wide thermal tolerance to grow there. To understand the ecology of methanogens in these places, identification of methanogenic isolates here are significant.

MATERIALS AND METHODS

Source of inoculum

The strain described here was isolated from the sediment of a pond, with an altitude of 420 m, in mountain forests in Liuzhou (24°20'N, 109°19'E), Guangxi province, China. The pond has an area about 6000 m^2 and the water is 1.5 m deep at the sampling site. The sediment was collected 40 cm below the sediment-water interface and sealed in a bottle filled with N_2. The sample was stored at 4 °C and moved to laboratory immediately.

Media and culture conditions

The strict anaerobic methods of Hungate (Hungate, 1969), modified by Sowers and Noll (1995), were used throughout this study. The medium for enrichment and isolation contained (per liter of distill water) $MgCl_2 \cdot 6H_2O$ 0.3 g; KH_2PO_4 0.7 g; K_2HPO_4 0.7 g; NaCl 5.0 g; NH_4Cl 1.0 g; methanol 0.5 mL; yeast extract 0.1 g; peptone 0.1 g; L-cysteine·HCl 0.5 g; $NaHCO_3$ 0.5 g; $Na_2S \cdot 9H_2O$ 0.5 g; 0.1 % resazurin solution 0.5 mL. Trace mineral solution (Morii *et al.*, 1983) and vitamin mixture (Balch *et al*., 1979) were added to a final concentration of 1 % (v/v). The medium, without methanol, $NaHCO_3$, $Na_2S \cdot 9H_2O$ and vitamin mixture, was boiled for 10 min and cooled to room temperature under O_2-free N_2. It was then distributed to serum bottles or anaerobic tubes under N_2, and sealed

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with butyl rubber stopper and crimped by aluminium seals. Sterilization was performed at 121 °C for 20 min and the pH was 7.0. Germfree methanol, $NaHCO_3$, $Na_2S{\cdot}9H_2O$ and vitamin mixture were added before inoculation. For roll tubes, 1.7 % agar was added to liquid medium. For substrate utilization experiments, methanol (50 mM), trimethylamine (40 mM), sodium formate (25 mM), sodium acetate (25 mM) and mixture of CO_2 and H_2 (4:1) were added to medium (without organic substrates) as catabolic substrates.

Enrichment and isolation

Sample (about 5 mL) was added into a 120 mL serum bottle which contained 20 mL medium with penicillin 100 mg/L. The incubation was performed at 37 °C without shaking for 4 - 5 weeks. Then 5 mL of the culture was anaerobically inoculated into new bottles with fresh medium. After four successive transfers, rolling tube technique was performed. Colonies that formed after 2 - 3 weeks were observed under fluorescent microscope. The colonies with blue-green fluorescent were inoculated into new medium. Isolation was repeated twice or more until the pure culture was obtained. The strain was designated as GFJ07.

Effect of temperature, pH and NaCl concentration on growth

To determine the optimal pH, temperature and NaCl concentration, cultures were incubated at several pH (5 - 8), temperature (15 °C -50 °C) and NaCl concentration (0.2% - 3%, w/v) in 50 mL medium in 500 mL serum bottles. Specific growth rate was calculated from the methane production curves during exponential phase (Maestrojuan and Boone, 1991) and three parallel cultures were analyzed. Methane production were determined by gas chromatogragh (Techcomp GC7890II) equipped with a thermal conductivity detector and GDX-502 column.

Electron microscopy

Pure culture was send to Analytical & Testing Center of Sichuan University, P. R.China for transmission electron microscope photographs. Samples were negatively stained with 2 % phosphotungstic acid and examined in a FEI Tecnai G2 F20 S-TWIN transmission electron microscope.

Phylogenetic analysis

DNA of the isolate was extracted by procedure reported previously (Jarrell *et al*., 1992). The 16S rDNA was amplified by PCR from genomic DNA with primer Met86F (5'-GCTCAGTAACACGTGG-3') and Met1340R (5'-CGGTGTGTGCAAGGAG-3') (Saengkerdsub *et al*., 2007). The amplified product were purified by gel extraction, then cloned into pMD18-T Vector and sequenced. Sequencing and primer synthesis were performed at Shanghai Sangon Biological Engineering Technology & Service Co., Ltd, China. The 16S rDNA sequence was submitted to the BLAST server (http://blast.ncbi.nlm.nih.gov/) for alignment. Phylogenetic analyses were performed by ClustalX ver.1.81.

RESULTS

Morphology

On solid medium in roll tubes, colonies were irregular, white or light yellow, about 0.5 mm in diameter after incubation for 2 weeks. Cells grew in liquid culture tended to form visible aggregates (Figure 1a), but a few single cells could be observed. These aggregates exhibited pseudosarcina-like or irregular shape which was reported before (Figure 1b, c) (Asakawa *et al*., 1995; Cairó *et al*., 1992). The cells were Gram positive and nonmotile and showed the characteristic fluorescence of methanogens when irradiated at 420 nm (Figure 1b, c). Transmission electron micrographs showed that a majority of single cells grew as a sausage-like clinder about 0.5 μm in diameter and 1.0 μm in length (Figure 1d, e) which had never been reported and a few cells with irregular shape were also observed (Figure 1f).

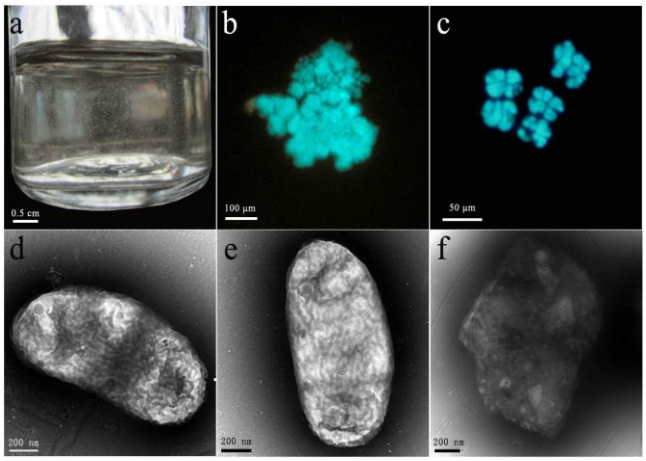

Figure 1: Photographs of strain GFJ07. (a) the visible aggregates in liquid medium, (b and c) aggregates showing blue-green fluorescence under optical microscope, (d, e and f) Transmission electron micrographs of single cell. Bars were marked on the figure respectively.

Physiology

Strain GFJ07 was able to use methanol, trimethylamine, acetate and H_2-CO_2 but not formate as substrates for growth and methane production. It grew faster on methanol than other substrates. Yeast extract and peptone could stimulate the growth of strain GFJ07 obviously. But the isolate could also grow slowly in media without yeast extract and peptone. It indicated that strain GFJ07 needed a low alimentation requirement and could synthesize all the amino acids by itself. And substance,

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such as amino acids, vitamins and nucleotide, which were abundant in yeast extract and peptone could help its growth greatly. The isolate had a wide thermal tolerance. It could grow within the temperature from 15 °C to 45 °C, with an optimum at 35 °C (Figure 2). No growth was observed at 10 °C (data not shown). Strain GFJ07 grew in a narrow pH range from 6.0 to 7.5, with an optimum at 7.0 (Figure 3). The optimum NaCl concentration for growth was 0.5 %, and the strain could grow well in the salinity up to 1.5 % (Figure 4).

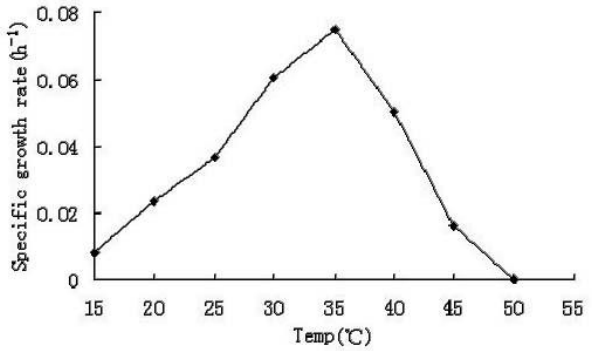

Figure 2: Specific growth rates of strain GFJ07 at different temperatures.

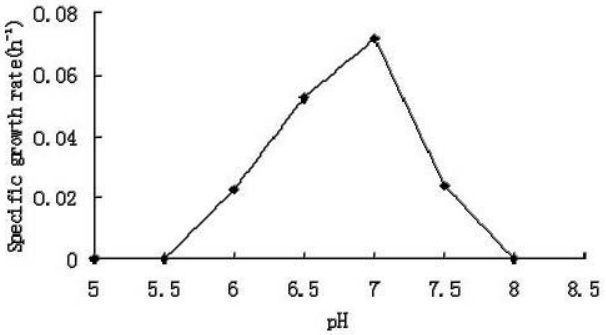

Figure 3: Specific growth rates of strain GFJ07 at different pH values.

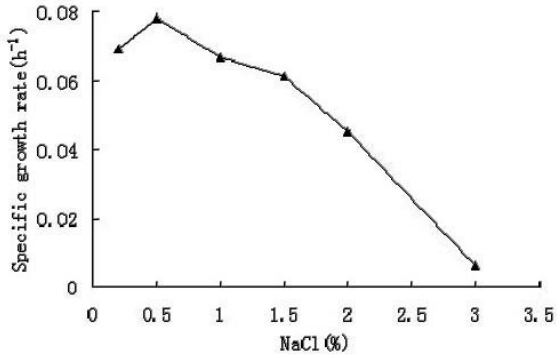

Figure 4: Specific growth rates of strain GFJ07 on different concentrations of NaCl.

Phylogeny

A 1246 bp 16S rDNA fragment of strain GFJ07 was amplified by PCR and sequenced. The sequence was aligned with partial 16S rDNA sequences of other methanogens and then a phylogenetic tree was constructed by ClustalX ver.1.81. The sequences of other methanogens were obtained from NCBI database. Analysis of the 16S rDNA sequences revealed that the sequence of strain GFJ07 showed the highest sequence homology of 99.9 % to *Methanosarcina mazei* LM5 and 01M9701. When compared with other species in the genus *Methanosarcina*, the sequence similarity ranging from 94.6 % to 98.7 %, *Methanosarcina* sp. (98.7 %), *Methanosarcina baltica* (94.6 %), *Methanosarcina lacustris* (97.7 %) and *Methnosarcina siciliae* (98.5 %). The phylogenetic tree showed the taxonomic relationship of strain GFJ07 among the selected methanogens (Fig. 5). The phylogenic analyse indicated that strain GFJ07 belonged to *M. mazei* most possibly.

DISCUSSION

Methanosarcina mazei could be distinguished from other *Methanosarcina* species by the ability to grow as single cell under appropriate condition and Xun *et al*. (1988) found MS medium could induce the disaggregation of *M. mazei* S-6^T. But the aggregation of strain GFJ07 did not disaggregate in MS medium (data not shown) which had been also reported previously (Asakawa *et al*., 1995). A possible reason was that the culture condition was not fit for the disaggregation of strain GFJ07. It was reported that the enzyme disaggregatase could release into the medium and played a role in the mechanism of disaggregation (de Macario *et al*., 1993; Liu *et al*., 1985; Xun *et al*., 1990). Therefore, the culture condition might affect the release or activity of the enzyme.

According to previous study (Cairó *et al*., 1992; Lai *et al*., 2000; Lai *et al*., 1999), the single cell of *M. mazei* strains was irregular coccus in shape. Although a few cells of strain GFJ07 showed irregular shape, most cells were sausage-like which had never been reported (Figure 1d, e). The isolate could gather in liquid medium, but the multicellular form of lamina, first discovered by Mayerhofer *et al*. (1992), was not observed here.

The favorable temperature, pH values and NaCl concentration of strain GFJ07 were basically consistent with that of other *M. mazei* strains. But strain GFJ07 had a wide thermal tolerance from 15 °C to 45 °C. It might be attributed to the large ambient temperature ranging of sampling location. Strain TMA, another eurythermal *M. mazei*, was isolated by Asakawa *et al*. (1995), which could grow from 10 °C to 50 °C.

Although it was reported that *M. barkeri, M. mazei,* and *M. vacuolata* could be distinguished from other methanogens by the characteristics that they can produce methane from trimethylamine, methanol, H_2-CO_2 and acetate (Maestrojuan and Boone, 1991), It was found later that strains of *M. mazei* could not metabolize H_2-CO_2 (Cairó *et al*., 1992; Clarens *et al*., 1993). Thus, the

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standard for using H_2-CO_2 to distinguish *M. mazei* from other species is inaccurate. From the data by others and ours, the strains of *M. mazei*, such as strain TMA, S-6^T, JC3, JC2, LYC, N2M9705, O1M9704, and GFJ07, could not use formate as substrates. This might be considered as the characteristic of *M. mazei*.

Based on the phylogenic analyse and morphological characteristics described above, the isolate was identified as a new strain of *M. mazei*, named GFJ07.

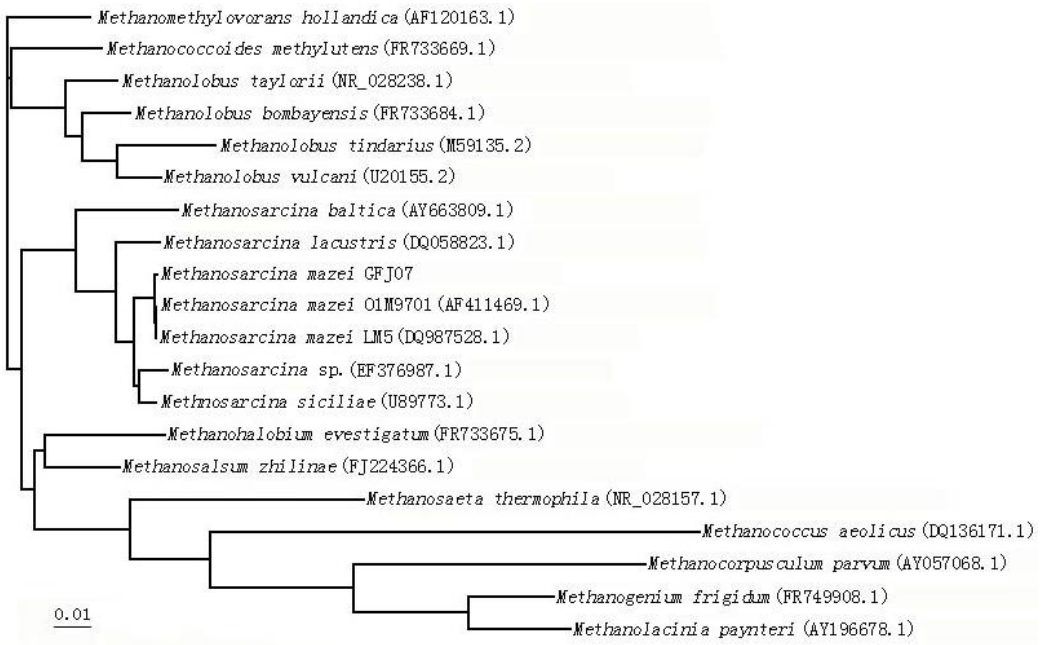

Figure 5: Phylogenetic relationship of *M.mazei* GFJ07 to other selected methanogenic bacteria based on 16s rDNA sequences. The GenBank accession numbers of the sequences are given in parentheses. The bar indicates one substitution per 100 bases.

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