Characterization of *Methanobrevibacter arboriphilicus* SA Isolated from a Paddy Field Soil and DNA-DNA Hybridization among *M. arboriphilicus* strains

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We isolated a methanogenic strain, designated strain SA (= DSM 7056), from an enrichment culture inoculated with a Japanese paddy field soil. Cells of this strain were strictly anaerobic, nonmotile, short rods and stained gram positive. The strain was able to use H₂-CO₂ or formate as a methanogenic substrate. It required vitamins, but not acetate, for growth. Growth was fastest at 35 to 40°C. Methane was produced most rapidly at pH 6.0 to 7.5. The cellular lipid composition of strain SA was similar to that of *M. arboriphilicus* A2 (= DSM 2462). The G+C content of the DNA was 26.4 mol%. Strain SA had DNA-DNA hybridization values of more than 70% with *M. arboriphilicus* DH1^T (= DSM 1125^T). On the basis of phenotypic and genotypic characteristics, we identified strain SA as *M. arboriphilicus*. In the course of our identification work, the genetic heterogeneity of *M. arboriphilicus* was revealed by the results of DNA-DNA hybridization experiments. Although strain AZ (= DSM 744) should be classified as a member of a species distinct from the species containing the other four strains studied (DH1^T, A2, DC [= DSM 1536], and SA), further phenotypic characterization will be required before a new species can be proposed.

Methane is one of the greenhouse gases, and paddy fields are reported to be an important source of atmospheric CH₄ (4). Rajagopal et al. isolated and characterized two *Methanobacterium*-like strains and two *Methanosarcina*-like strains from paddy fields in Louisiana (18). Conrad et al. isolated a *Methanobacterium*-like strain from Italian paddy fields (5). However, these strains have not been sufficiently characterized to be identified at the species level. To understand the ecology of methanogenic bacteria in paddy field soils, characterization and identification of methanogenic isolates from paddy field soils are necessary.

In this work, we isolated a methanogenic strain from a Japanese paddy field soil, characterized it, and identified it as a *Methanobrevibacter arboriphilicus* strain. Below we also discuss genomic relationships among *Methanobrevibacter arboriphilicus* strains.

MATERIALS AND METHODS

Source of inoculum and bacterial strains. The sampling site was the paddy field plot (gray lowland soil) with wheat straw incorporated at the Kyushu National Agricultural Experiment Station, Chikugo, Fukuoka, Japan. The soil sample was taken from the plow layer of flooded soil with a core sampler (volume, 100 ml; height, 5 cm; diameter, 5 cm) and was transported to a laboratory in a polyethylene bag filled with N₂. The sampling date was 12 July 1989.

Methanobrevibacter arboriphilicus DH1^T (= DSM 1125^T) (T = type strain), DC (= DSM 1536), and AZ (= DSM 744) and *Methanobacterium thermoautotrophicum* ΔH^T (= DSM 1053^T) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. *Methanobrevibacter arboriphilicus* A2 (= DSM 2462) was isolated from an anaerobic sewage digester by

Morii et al. (13). *Escherichia coli* K-12 (strain IAM 1264) was obtained from the Institute of Molecular and Cellular Bio-sciences (formerly Institute of Applied Microbiology), University of Tokyo, Tokyo, Japan.

Media and growth conditions. The basal medium used for enrichment and isolation of strain SA was a modification of LPBM (22), which contained (per liter of deionized water) 0.75 g of KH₂PO₄, 0.75 g of K₂HPO₄, 1.0 g of NH₄Cl, 0.36 g of MgCl₂ · 6H₂O, 9 ml of a trace mineral solution (13), 10 ml of a vitamin mixture solution (2), 0.5 ml of a 0.2% resazurin solution, 0.5 g of L-cysteine hydrochloride · H₂O, 0.5 g of Na₂S · 9H₂O, and 4.8 g of NaHCO₃. For cultivation of *Methanobrevibacter arboriphilicus* strains, the basal medium was supplemented with 0.1% yeast extract (Difco). For roll tubes, we added 0.1% yeast extract (Difco), 0.1% Polypeptone (Daigo Eiyo Co., Osaka, Japan), and 1.7% agar to the basal medium. The gas phase was H₂-CO₂ (4:1) pressurized to 203 kPa, and the pH was 7.0. Enrichment was performed in a 1-liter serum bottle sealed with a butyl rubber stopper and an aluminum seal containing 100 ml of medium or in a 120-ml serum bottle containing 5 ml of medium. Mass cultures were grown at 37°C in a 15-liter fermentor containing 5 liters of medium with sparging with H₂-CO₂ (4:1) (12). *Methanobacterium thermoautotrophicum* ΔH^T was cultivated as described previously (12).

To assess culture purity, we used the following media: the basal medium without NaHCO₃ but containing 0.1% yeast extract (Difco), 0.1% Polypeptone (Daigo Eiyo), and 1% glucose; VL medium (8) (10 g of Bacto Peptone [Difco], 5 g of yeast extract [Difco], 2 g of beef extract [Difco], 5 g of NaCl, 2 g of glucose, 1 mg of resazurin sodium salt, 0.5 g of L-cysteine hydrochloride · H₂O, 0.5 g of Na₂S · 9H₂O, 1 liter of distilled water; pH 7.0 to 7.2); and the medium for sulfate reducers (8) (0.5 g of K₂HPO₄, 1 g of yeast extract [Difco], 1 g of NH₄Cl, 4.5 g of Na₂SO₄, 0.06 g of CaCl₂ · 6H₂O, 0.004 g of FeSO₄ · 7H₂O, 0.06 g of MgSO₄ · 7H₂O, 5.0 g of sodium citrate · 2H₂O, 0.1 g of sodium thioglycolate, 0.1 g of

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L-ascorbic acid, 1 mg of resazurin sodium salt, 0.5 g of L-cysteine hydrochloride $\cdot$ H_2O , 0.5 g of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 1 liter of distilled water; pH 7.1 to 7.4). The media were prepared anaerobically, and the gas phase was N_2 .

Effect of temperature and pH on growth. Cultures were grown in 50 ml of the basal medium in 1-liter serum bottles. The A_{660} values of cultures were measured to determine the effect of temperature on growth. To determine the effect of pH on growth, we measured the A_{660} of cultures and CH_4 production. Media with different pH values were prepared by varying the ratio of H_2 - CO_2 mixture and the concentration of NaHCO_3 or Na_2CO_3 . The serum bottles were repeatedly repressurized to 203 kPa with the original ratios of the H_2 - CO_2 mixture, and pH values were checked during growth. The temperature was kept at 37°C.

Methane production was determined with a Shimadzu model GC-6AM gas-liquid chromatograph equipped with a flame ionization detector and a stainless steel column (3.0 mm [inside diameter] by 2.0 m) packed with Unibeads A (Gasukuro Kogyo, Inc., Tokyo, Japan). The column was kept at 70°C.

Electron microscopy. Cells grown in the basal medium for 5 days at 37°C were stained negatively with 2% phosphotungstic acid and observed with a Hitachi model H-7000 electron microscope.

Lipid analysis. Cells of strain SA were harvested at the exponential phase and washed once with deionized water. The total lipids were extracted by the method of Bligh and Dyer as modified by Nishihara and Koga (15) from frozen and thawed cells (14). The two-dimensional thin-layer chromatographic pattern of the total lipids was examined as described previously (9). The total lipids of *Methanobacterium thermoautotrophicum* ΔH^T were used as a reference. The compositions of the hydrocarbon chains of the lipids were determined by gas-liquid chromatography of alkyl chlorides (17) prepared by BCl_3 treatment (16). Gas-liquid chromatography was done with a glass column (3.0 mm [inside diameter] by 2 m) packed with OV-11 at a temperature that was increased from 120 to 340°C at a rate of 20°C/min.

G+C content and DNA-DNA hybridization. Cells of methanogenic strains were harvested at the exponential phase. The harvested cells were washed with deionized water and saline-EDTA buffer (0.15 M NaCl, 0.1 M EDTA; pH 8.0) and were stored at -20°C until they were used. Frozen cells were disrupted by freeze-pressing with an X-press (Biox Co., Nacka, Sweden). DNA was isolated and purified by the method of Marmur (10). Cells of *E. coli* were harvested from nutrient agar (Kyokuto, Tokyo, Japan) plates and washed with saline-EDTA. Frozen and thawed cells were used for isolation and purification of DNA by the method of Marmur (10).

The G+C content of the DNA was determined by high-performance liquid chromatography (HPLC) (20). DNA from *E. coli* IAM 1264 (G+C content, 51.6 mol%) was used as the reference DNA. HPLC was carried out with a Waters model 6000A apparatus and a type 5C18-AR column (Nacalai Tesque, Inc., Kyoto, Japan) eluted with a mixture containing 0.2 M $\text{NH}_4\text{H}_2\text{PO}_4$ and acetonitrile (20:1, vol/vol) at a rate of 1 ml/min and monitored with a UV detector (model UVIDEC-100-II; JASCO) at 265 nm.

DNA-DNA hybridization was performed by the membrane filter method (1). Each mixture of labeled and unlabeled DNAs was incubated at 60°C for 40 h.

Serological test. The indirect immunofluorescence reaction of strain SA with the anti-A2 S-probe prepared by Morii et

FIG. 1. Electron micrograph of negatively stained cells of strain SA. Bar = 1 μm .

al. (13) was determined by the method described by Conway de Macario et al. (6).

RESULTS AND DISCUSSION

Enrichment and isolation. A moist soil sample (about 40 g) was inoculated into 100 ml of the basal medium in a 1-liter serum bottle. The bottle was incubated with shaking at 30°C. About 80% (vol/vol) of the culture was replaced weekly by fresh medium. Bekanamycin sulfate, sodium ampicillin, and lincomycin hydrochloride (each at 20 $\mu\text{g}/\text{ml}$) were added to the seventh and eighth cultures. Serial dilutions of the eighth culture were inoculated into agar medium containing the antibiotics mentioned above, and roll tubes were prepared. The colonies that formed were inoculated into basal medium without antibiotics. After eight successive transfers, roll tubes were again prepared by using the agar medium without antibiotics. Well-isolated colonies were picked and inoculated into the basal medium. One of the cultures was used for ensuring purity. The culture was inoculated into three media without methanogenic substrates (see Materials and Methods), and the preparations were incubated statically for 55 days at 30°C. No growth was observed, and no contaminants were detected microscopically. The pure culture was designated strain SA.

Morphology. Strain SA cells were short rods that were 0.7 μm wide by 2.1 μm long (Fig. 1). The cells stained gram positive and were nonmotile. No flagella were observed. Sodium dodecyl sulfate and hypotonic conditions did not lyse cells when the method described by Boone and Whitman (3) was used.

Surface colonies on agar medium in roll tubes were 1.5 to 3.0 mm in diameter, round, entire to roughly entire, convex, and beige to ochre yellow. Cells grown in liquid culture with shaking tended to clump together.

Physiology. Strain SA grew on H_2 - CO_2 , and it was also able to utilize formate in F-complex medium (13). It could not use methanol, trimethylamine, and acetate as substrates. Vitamins were required for growth, but acetate and cysteine were not. It has been reported that the species of the genus *Methanobrevibacter* require vitamins for growth. *Methanobrevibacter ruminantium* and *Methanobrevibacter smithii* also require acetate, but *Methanobrevibacter arboriphilicus* does not (11). Yeast extract and Polypeptone stimulated cell growth of strain SA.

The generation time was 24 h (specific growth rate, 0.03 h^{-1}) in 5 liters of the basal medium in a 15-liter fermentor with sparging with H_2 - CO_2 (4:1) at 37°C.

FIG. 2. Effect of temperature on growth of strain SA. Symbols: ○, A_{660} after 65 h of incubation; ●, A_{660} after 97.5 h of incubation.

Figure 2 shows the effect of temperature on growth. Strain SA grew at 25 to 45°C but not at temperatures above 50°C or below 15°C. The growth was fastest at 35 to 40°C. Figure 3 shows the effect of pH on growth and methane production. The pH values did not change during growth. The strain grew at pH 6.0 to 9.0. Growth was fastest and methane was produced most rapidly at pH 6.0 to 7.5. Good growth was observed in basal media containing 0 to 0.1 M NaCl.

Lipids. The tetraether type of lipids was predominant (68 mol%) in the cells of strain SA. Morii et al. (14) reported that the proportion of tetraether lipids in *Methanobrevibacter arboriphilicus* A2 cells was 81 mol%. The thin-layer chromatographic pattern of the polar lipids of strain SA was very similar to that of *Methanobrevibacter arboriphilicus* A2 (14). In particular, lipids which had mobilities similar to those of gentiobiosyl caldarchaetidylinositol, archaetidylserine, and caldarchaetidylserine were found on the thin-layer chromatography plates. These lipids are characteristic of the polar lipids of species in the family *Methanobacteriaceae* (9). Aminophospholipids which had mobilities similar to those of archaetidylethanolamine and caldarchaetidylethanolamine were not detected. These lipids are found in *Methanobacterium* species but not in *Methanobrevibacter* species (9). These results strongly suggested that strain SA belongs to the genus *Methanobrevibacter*.

FIG. 3. Effect of pH on growth (Δ) and methane production (●) of strain SA after 90 h of incubation.

G+C content and DNA-DNA hybridization. The G+C contents of DNAs from strain SA and *Methanobrevibacter arboriphilicus* strains were 25.5 to 26.5 mol% (Table 1). These values are somewhat lower than the values determined by the buoyant density method for strains DH1^T (27.5 mol%) (23), A2 (29.6 mol%) (13), DC (27.7 mol%), and AZ (31.6 mol%) (2). A possible slight deviation from the linear equation in the very-low-G+C-content region between buoyant density and G+C content described by Schildkraut et al. (19) may be the cause of the difference.

Strain SA exhibited DNA-DNA hybridization values of more than 70% with *Methanobrevibacter arboriphilicus* DH1^T (Table 1). From this result and other characteristics described above, strain SA was identified as a *Methanobrevibacter arboriphilicus* strain. This is, to our knowledge, the first strain which was isolated from a paddy field soil and identified to the species level. Further investigation of the population of this species in paddy field soil will be required. Strain SA is similar to A2 in formate utilization, while type strain DH1 and two other strains, strains DC and AZ, do not utilize formate (11). The indirect immunofluorescence reaction of strain SA with the anti-A2 S-probe was 3+. However, strain SA is different from strain A2 in its optimum pH and has a tendency to clump in liquid culture.

Strains SA, DH1^T, A2, and DC exhibited hybridization values of more than 60% with each other (Table 1), which is consistent with assignment of these four strains to the

TABLE 1. G+C contents of DNAs and levels of DNA-DNA hybridization among *Methanobrevibacter arboriphilicus* strains and other strains

Strain	G+C content (mol%)	% Hybridization with labeled DNA from:				
		Strain SA	Strain DH1 ^T	Strain A2	Strain DC	Strain AZ
SA	26.4 ± 0.17 ^a	100	72.7	108	79.4	36.5
<i>Methanobrevibacter arboriphilicus</i> DH1 ^T	25.8 ± 0.58 ^a	75.6	100	76.0	66.1	39.4
<i>Methanobrevibacter arboriphilicus</i> A2	25.9 ± 0.21 ^a	87.7	59.9	100	79.6	35.3
<i>Methanobrevibacter arboriphilicus</i> DC	26.2 ± 0.13 ^a	91.8	73.7	86.1	100	42.4
<i>Methanobrevibacter arboriphilicus</i> AZ	25.5 ± 0.25 ^a	35.1	31.4	40.0	34.3	100
<i>Methanobacterium thermoautotrophicum</i> ΔH ^T	49.0 ± 0.95 ^b	1.6	1.2	2.6	1.8	2.0
<i>E. coli</i> K-12	51.6 ^c	-0.0	0.0	0.2	-0.0	-0.0

^a Mean ± standard deviation ($n = 4$).

^b Mean ± standard deviation ($n = 2$).

^c Data from reference 19.

species *Methanobrevibacter arboriphilicus*. On the other hand, strain AZ exhibited hybridization values of only 31 to 42% with these four strains of *Methanobrevibacter arboriphilicus*. The 16S rRNA oligonucleotide catalog of strain AZ has a similarity value of 0.84 with the 16S rRNA oligonucleotide catalogs of strains DH1^T and DC, although the similarity value between strains DH1^T and DC was 1.00 (2). On the basis of phylogenetic characteristics, strain AZ may be a member of a distinct species. However, we do not exclude strain AZ from *Methanobrevibacter arboriphilicus* because of the lack of differential phenotypic features. A few distinctive features of strain AZ (e.g., the presence of flagella [7] and a requirement for cysteine [21]) have been reported, but these are insufficient and inconclusive. Further information concerning the phenotypic characteristics of strain AZ and the other strains of *Methanobrevibacter arboriphilicus* will be required for taxonomic description of strain AZ.

Methanobrevibacter arboriphilicus SA has been deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany, as strain DSM 7056.

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