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One Carbon Metabolism in Methanogenic Bacteria

Cellular Characterization and Growth of *Methanosarcina barkeri*

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Abstract. Two strains of *Methanosarcina (M. Barkeri* strain MS, isolated from sewage sludge, and strain UBS, isolated from lake sediments) were found to have similar cellular properties and to have DNA base compositions of 44 mol percent guanosine plus cytosine. Strain MS was selected for further studies of its one-carbon metabolism. *M. barkeri* grew autotrophically via H_2 oxidation/CO_2 reduction. The optimum temperature for growth and methanogenesis was 37° C. H_2 oxidation proceeded via an F_{420}-dependent $NADP^+$-linked hydrogenase. A maximum specific activity of hydrodenase in cell-free extracts, using methyl viologen as electron acceptor, was 6.0 μmol min · mg protein at 37°C and the optimum pH (9.0). *M. barkeri* also fermented methanol and methylamine as sole energy sources for growth. Cell yields during growth on H_2/CO_2 and on methanol were 6.4 and 7.2 mg cell dry weight per mmol CH_4 formed, respectively. During mixotrophic growth on H_2/CO_2 plus methanol, most methane was derived from methanol rather than from CO_2. Similar activities of hydrogenase were observed in cell-free extracts from H_2/CO_2-grown and methanol-grown cells. Methanol oxidation apparently proceeded via carrierbound intermediates, as no methylotroph-type of methanol dehydrogenase activity was observed in cell-free extracts. During growth on methanol/CO_2, up to 48% of the cell carbon was derived from methanol indicating that equivalent amounts of cell carbon were derived from CO_2 and from an organic intermediate more reduced than CO_2. Cell-free extracts lacked activity for key cell carbon synthesis enzymes of the Calvin cycle, serine path, or hexulose path.

Key words: C_1 metabolism – Methanogenesis – Methylotrophy – *Methanosarcina* Chemolithotrophy – Autotrophy – Growth yields – Dehydrogenase – Archaebacteria.

The methanogenic bacteria comprise a highly specialized and unusual group of strictly anaerobic organisms. Although there is considerable morphological diversity within the group, the methanogens possess certain common characteristics. Their cell walls lack peptidoglycan (Kandler and Hippe, 1977). All taxonomically-described species contain two coenzymes not found in nonmethanogens: F_{420}, a low-molecular weight compound involved in electron transport (Cheeseman et al., 1972), and coenzyme M, a methyl carrier (McBride and Wolfe, 1971; Taylor et al., 1974). Fox et al. (1977) have recently shown that the rRNA oligonucleotide sequences of these organisms differ dramatically from those of all other life forms, and have suggested that the methanogens represent an early evolutionary divergence. Moreover, they noted fundamental differences in rRNA sequences between the *Methanobacterium* and *Methanosarcina/Methanospirillum* groups.

All described methanogens share a common energy metabolism that involves H_2 oxidation/CO_2 reduction

Abbreviations. CAPS = cycloaminopropane sulfonic acid; CH_3-S-CoM = methyl coenzyme M; DCPIP = 2,6-dichlorophenolindophenol; DEAE = diethylaminoethyl; dimethyl POPOP = 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene; DNA = deoxyribonucleic acid; dpm = disintegrations per min; DTT = dithiothreitol; EDTA = ethylenediamine tetraacetic acid; F_{420} = factor 420; G + C = guanosine plus cytosine; NAD^+ = nicotinamide adenine dinucleotide; $NADP^+$ = nicotinamide adenine dinucleotide phosphate; PBBW = phosphate buffered basal Weimer; PMS = phenazine methosulfate; PPO = 2,5-diphenyloxazole; rRNA = ribosomal ribonucleic acid; RuBP = ribulose-1,5-bisphosphate; Tris = tris-hydroxymethyl-aminomethane; μmax = maximum specific growth rate

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(Wolfe, 1971). Some species can utilize CO, formate, acetate, or methanol as methanogenic substrates (Zeikus, 1977; Daniels et al., 1977). Organisms of the genus *Methanosarcina* are regarded as the most metabolically diverse of the methanogens (Stadtman and Barker, 1951; Zeikus, 1977). Kluyver and Schnellen (1947) first isolated *Methanosarcina barkeri* and reported the fermentation of methanol, acetate, and CO to methane; however, growth data was not reported, and the original cultures were subsequently lost. Stadtman and Barker (1951) observed that impure cultures of *M. barkeri*, obtained from sediment enrichment cultures, fermented methanol according to the equation $4\,CH_3OH \rightarrow 3\,CH_4 + CO_2 + 2H_2O$. Daniels et al. (1977) reported that growing cultures of a number of methanogens, including two strains of *Methanosarcina*, were capable of oxidizing CO. Recently, several strains of *Methanosarcina* have been isolated and their general properties described (Zeikus and Bowen, 1975; Zeikus and Winfrey, 1976; Kandler and Hippe, 1977; Mah et al., 1977). We report here on detailed studies of the cellular characteristics, growth, and one-carbon metabolism of *Methanosarcina barkeri*.

Materials and Methods

Chemicals, Gases and Radioisotopes. All chemicals were reagent grade. Gases were obtained from Matheson (Joliet, Ill.) either as pure gases (N_2, H_2, Ar, He) or as mixtures of H_2/CO_2 (80/20, v/v), H_2/N_2 (80/20, v/v) or N_2/CO_2 (95/5, v/v). The following radiochemicals were obtained from Amersham Searle (Arlington Heights, Ill.): ^{14}C-methanol (58 mCi/mmol), ^{14}C-methylamine (22.4 mCi/mmol) and ^{14}C-sodium carbonate (60 mCi/mmol).

Organisms. Methanosarcina barkeri neotype strain MS was kindly provided by Dr. M. P. Bryant, Department of Microbiology, University of Illinois at Urbana. *Methanosarcina* strain UBS was isolated from sediments from Lake Mendota, Wisconsin (Zeikus and Winfrey, 1976). Both strains have been submitted to the Deutsche Sammlung von Mikroorganismen, Göttingen, FRG.

Cultivation Methods. The anaerobic culture technique of Hungate (1950) as modified by Bryant (1972) was used throughout. The organism was grown on PBBW medium, which contained the following (per 985 ml glass-distilled H_2O): KH_2PO_4, 1.5 g; $K_2HPO_4 \cdot 3\,H_2O$, 2.9 g; NH_4Cl, 1.0 g; NaCl, 0.9 g; $MgCl_2 \cdot 6H_2O$, 0.20 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; $NaSeO_3$, 0.017 mg; mineral solution (Daniels et al., 1977), 10 ml; Vitamin solution (Wolin et al., 1963), 5 ml, resazurin (0.2%) 1 ml. Medium was prepared without the phosphate salts and was thoroughly sparged with the desired O_2-free gas; if the gas phase contained CO_2, the medium was supplemented with 1.0 g $NaHCO_3$ per liter. After dispensing into 18 × 142 mm anaerobic culture tubes (Bellco, Vineland, N.J.), the medium was reduced with Na_2S and autoclaved at 103 kN/m^2. Upon cooling, phosphates and other substrates (as indicated) were added aseptically through the rubber bungs via hypodermic syringe.

Unless otherwise indicated, all cultures were incubated at 37° C. Both stock and experimental cultures were grown in tubes that contained 10 ml of medium, or in Erlenmeyer flasks with 50 – 500 ml of medium. Cultures were maintained by transfer of 10 – 20% inocula. Cultures were routinely checked for purity by both phase contrast microscopy and by inoculation of 1 ml of culture into tubes of PBBW supplemented with 0.5% glucose, 0.2% trypticase, and 0.2% yeast extract. Culture turbidity, or microscopic observation of non-sarcina cells after 7 days incubation was taken as evidence of culture contamination.

Growth studies in fermentor culture were performed in a 2 l New Brunswick Multigen fermentor that contained 1.3 l of PBBW. The culture was gassed continuously with H_2/CO_2, and $Na_2S \cdot 9H_2O$ was added manually as a concentrated solution at 4 – 8 h intervals to compensate for its loss during gassing. The exit gas rate and composition was measured after passing the effluent gas through a sulfide trap (2% zinc acetate) and a H_2O trap.

Larger quantities of cells were obtained in a 12 l New Brunswick Microferm fermentor that contained 9 l PBBW under constant gassing with H_2/CO_2, or in 20 l carboys that contained 12 l PBBW supplemented with 100 mM methanol (gas phase N_2/CO_2). Cells were harvested under N_2 at 9000 × g in a Sorvall RC-5 centrifuge equipped with a KSB continuous flow system. After centrifugation, cells were removed in an anaerobic glovebox (Coy Instruments, Ann Arbor, Mich.) and placed in a stainless steel centrifuge tube. The tube was gassed with N_2 and sealed with a neoprene stopper. Cells were either used immediately or were stored at −70° C.

Measurement of Growth and Incorporation. Growth was determined after filtration of culture samples through 0.40 µm polycarbonate membranes (BioRad, Richmond, Cal.) and washing of filters with two volumes of 50 mM K-phosphate buffer, pH 7.0. Filters were then dried to constant weight.

For incorporation experiments, dried filters were placed in glass scintillation vials that contained 0.5 ml of 0.05 N HCl. After heating the vial to dryness at 60° C, the cells were solubilized in 0.5 ml TS-2 (Research Products International, Elk Grove Village, Ill.) for 12 h at 25° C. After addition of 70 mg trichloroacetic acid and 15 ml scintillation solution, radioactivity was determined in a Packard 3375 liquid scintillation spectrometer. The scintillation solution contained 3 l toluene, 1 l Triton X-100, 16 g PPO, and 1 g dimethyl POPOP. ^{14}C-toluene (New England Nuclear, Boston, Mass.) in 0.5 ml TS-2, 70 mg trichloroacetic acid, and 15 ml scintillation solution was used as a standard.

Measurement of Gases. Methane was routinely assayed with a Varian Aerograph 600-D gas chromatograph as described by Daniels et al. (1977). In experiments with ^{14}C-substrates, analysis of CH_4, CO_2, $^{14}CH_4$, and $^{14}CO_2$ was performed by the gas chromatography-gas proportional counting method of Nelson and Zeikus (1974).

Preparation of Cell-Free Extracts. Anaerobic conditions were maintained throughout the entire procedure, and all manipulations were performed under a N_2 atmosphere at 4° C. Cells (2.5 g wet weight) were placed in a 15 ml Corex tube that contained 5 ml of 100 mM Tris HCl (pH 8.1) with 2 mM $MgCl_2$ and 2 mM DTT. After thorough mixing, the suspension was passed through a French pressure cell at 48,300 kN/m^2. The lysate was collected in a centrifuge tube, sealed with a flanged rubber bung, and centrifuged at 8,000 × g for 20 min. The supernatant was removed with a glass syringe and injected into glass vials that contained a N_2 gas phase and were sealed with soft rubber bungs. Extracts were used immediately or were stored at −70° C. The protein content of extracts was determined by the method of Lowry et al. (1951), using bovine serum albumin in 2 mM DTT as standard.

DNA Base Composition. Cells (10 g wet weight) were suspended in 100 ml of 0.15 M NaCl/0.10 M EDTA (pH 8.0). The suspension was heated to 90° C for 10 min, then quickly cooled in an ice bath. This procedure was necessary to denature an active deoxyribonuclease. The heat-treated suspension was passed through a French pressure cell at 41,400 kN/m^2 and the DNA from the resulting mixture isolated by the method of Marmur (1961). DNA base composition

was determined by the thermal denaturation procedure of DeLey (1970), using a Gilford 240 spectrophotometer equipped with a model 2527 thermoprogrammer. *Escherichia coli* type VIII DNA (Sigma) was used as a standard.

Identification of Electron Carriers. Electron carriers were identified from UV-visible absorption spectra of cell-free extracts prepared from cultures grown in the absence of resazurin. A Gilford model 240 spectrophotometer was used to obtain spectra.

F_{420} was partially purified as follows: 27.6 g (wet weight) of cells were suspended in 100 ml H_2O and heated to 90° C for 15 min. The suspension was centrifuged at 6,000 × g for 10 min. The supernatant was applied to a DEAE-cellulose column (7 × 2.5 cm bed volume) and eluted with batches of NaCl: 65 ml of 0.10 M, 100 ml of 0.20 M, 50 ml of 0.80 M, 100 ml of 1.60 M. Fractions (5 ml) corresponding to a yellow-green band (elution volume 250–320 ml) were pooled and concentrated ten-fold by vacuum evaporation at 45° C. The concentrated sample was applied to a Sephadex G-10 column (122 × 0.8 cm bed volume) and eluted with H_2O. Colored fractions (3 ml) were pooled and concentrated ten-fold by vacuum evaporation. The relulting F_{420} was analyzed by UV-visible spectrophotometry as above, and by fluorescence spectroscopy using an Aminco-Bowman spectrofluorometer.

Enzyme Assays. All assays were performed at 37° C under anaerobic conditions as described by Zeikus et al. (1977).

F_{420}-dependent hydrogenase and $NADP^+$ reductase activities were measured spectrophotometrically by the method of Zeikus et al. (1977). Hydrogenase activity was routinely measured spectrophotometrically at 578 nm by following the rate of methyl viologen reduction ($\varepsilon_{578} = 9.78\ mM^{-1}\ cm^{-1}$) in a reaction mixture that contained (in 1 ml): 100 mM CAPS pH 9.0, 10 mM methyl viologen, sufficient $Na_2S_2O_4$ to render the solution a pale blue color, and 0.05 mg of extract protein. The raction was initiated by addition of 0.40 cm^3 of H_2.

Methanol dehydrogenase was assayed spectrophotometrically under both aerobic and anaerobic conditions by a method similar to that of Anthony and Zatman (1967). PMS (with DCPIP) or F_{420} were used as electron acceptors.

Formate dehydrogenase was measured spectrophotometrically at 340 nm using NAD^+ or $NADP^+$ as electron acceptor, or at 420 nm using F_{420} as electron acceptor. The reaction mixture contained (in 1 ml): 100 mM K^+ phosphate buffer (pH 7.0), 0.25 mM $NAD(P)^+$ or 1.5 absorbance units of F_{420}, 2 mM DTT, and 0.05 mg extract protein. The reaction was initiated by the addition of 5 mM sodium formate.

RuBP carboxylase was measured by determining the rate of $^{14}CO_2$ fixation into acid-stable products. Experiments were performed in sealed 1 ml test tubes that contained 0.5 ml of the following reaction mixture: 90 mM Tris HCl (pH 8.1), 5 mM $MgCl_2$, 10 mM $NaH^{14}CO_3$ (1300 dpm/nmol), 2 mM DTT, and 0.12 mg of extract protein. The reaction mixture was preincubated for 15 min prior to addition of 5 mM sodium RuBP. At intervals over a 60 min period, the reaction was terminated by injecting 50 µl samples into 0.20 ml of 1N H_3PO_4. After gently gassing the acidified mixture with CO_2 for 5 min, 50 µl samples were counted in 10 ml of scintillation solution. RuBP carboxylase was also measured spectrophotometrically by the methods of Racker (1957) and of Leilley and Walker (1974), modified for anaerobic conditions. Spinach chloroplasts were used as positive controls in the above assays.

Hexulose-6-phosphate synthase was assayed anaerobically by the method of Ferenci et al. (1974); formaldehyde disappearance was measured by the colorimetric procedure of Nash (1953). Hydroxypyruvate reductase was determined by measuring the rate of NAD(P)H oxidation in a reaction mixture that contained 100 mM Tris HCl (pH 7.8), 0.25 mM NADH or NADPH, 2 mM DTT, and 0.10 mg extract protein; the reaction was initiated by the addition of 2 mM lithium hydroxypyruvate.

Results

Cellular Characteristics

Both *Methanosarcina* strain UBS and *M. barkeri* strain MS grew in a defined mineral medium with H_2 as sole energy source and CO_2 as sole carbon source. The strains also grew in mineral medium by fermentation of methanol or methylamine. Growth was not demonstrated in basal medium (N_2/CO_2 gas phase) supplemented with methylmercaptan (6% of gas phase) or formate (25 mM). Both strains also fermented acetate in basal medium supplemented with 0.2% trypticase and 0.2% yeast extract.

In liquid culture, both strains grew in aggregates of up to several hundred thousand roughly sperical cells, each cell approximately 2 µm in diameter. These aggregates, which were larger in cells grown on methanol than on H_2/CO_2, usually contained both healthy and lysed cells. Aggregation necessitated that cell growth be measured by dry weight determination.

The temperature optimum for growth and methanogenesis of both *M. barkeri* MS (Fig. 1) and *Methanosarcina* strain UBS (not shown) was 37–40° C. No growth was observed at temperatures below 10° C. Cells were killed by incubation at 55° C for 1 h.

DNA isolated from these strains was found to have a base composition of 43.5 and 43.9 (±1) mole percent guanosine + cytosine for *Methanosarcina* strain UBS and *M. barkeri* strain MS, respectively. Air-oxidized spectra of both strains contained an absorbance peak at 420 nm but no absorbance bands corresponding to cytochromes. Partially purified F_{420} from *M. barkeri* strain MS exhibited an absorbance peak at 389 nm (in 0.1 N HCl) or 420 nm (in 0.1 N NaOH). Analysis by fluorescence spectroscopy revealed excitation maxima at 380 nm (in 0.1 N HCl) or 420 nm (in 0.1 N NaOH). When excited at these wavelengths, single emission peaks with maxima at 500 nm (in 0.1 N HCl) or 482 nm (in 0.1 N NaOH) were observed. Both *M. barkeri* strains MS and *Methanosarcina* strain UBS displayed similar DNA base compositions and similar morphological and nutritional properties. Strain MS was selected for further study of its one-carbon metabolism.

Unitrophic Growth on H_2/CO_2 or Methanol

Figure 2 shows a typical growth curve of *M. barkeri* in fermentor culture grown under continuous gassing with H_2/CO_2. A maximum specific growth rate of 0.058 h^{-1} (corresponding to a doubling time of 11.9 h) was observed during the exponential growth phase. During this phase, methane production increased linearly, rather than exponentially, with time. The maximum efficiency of growth during the exponential growth phase was 6.4 g cells per mole methane formed. A maximum growth yield of 1.6 g (dry weight) cells per

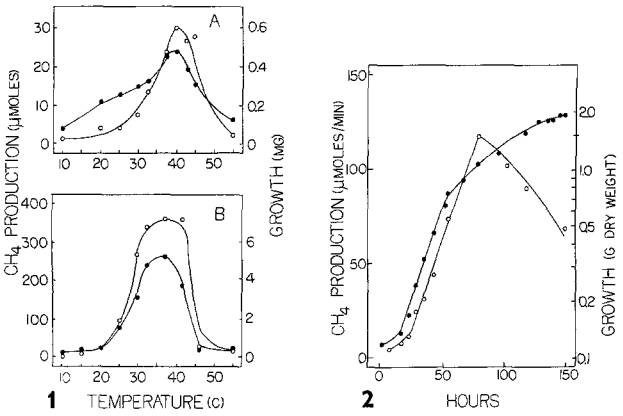

Fig. 1 A and B. Effect of incubation temperature on growth (●) and methanogenesis (○) by *M. barkeri* MS. **A** Cultivation in PBBW under H_2/CO_2. **B** Cultivation in PBBW + 50 mg CH_3OH, under N_2

Fig. 2. Growth (●) and methanogenesis (○) by *M. barkeri* MS in fermentor culture. Cells were grown in 1.3 l PBBW under constant gassing with H_2/CO_2 (20 cm^3/min)

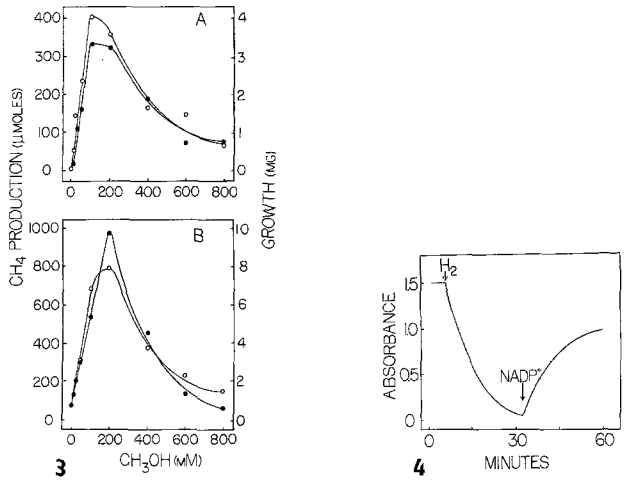

Fig. 3 A and B. Effect of methanol concentration on growth (●) and methanogenesis (○) by *M. barkeri* MS. Cells were cultivated in 10 ml PBBW plus methanol at the indicated concentration. **A** N_2 gas phase. **B** H_2/CO_2 gas phase

Fig. 4. Reduction of F_{420} by H_2 and oxidation of reduced F_{420} by $NADP^+$ in cell-free extracts of *M. barkeri*. H_2 was added at the time indicated, and F_{420} reduction was followed at 420 nm. Upon completion of the reaction, $NADP^+$ (0.25 mM) was added and its reduction measured at 340 nm

liter was obtained. This yield was not increased by further additions of Na_2S or by increasing the gassing rate of H_2/CO_2. Growth data from Fig. 2 is summarized in Table 1, and indicates that approximately four moles of CO_2 were converted to methane for each mole of CO_2 incorporated into cell material.

M. barkeri was able to grow in mineral media supplemented with methanol as sole energy and carbon source. Although it was not possible to determine the growth rate at low concentrations of methanol, the rate of methane formation was independent of methanol concentration within the range of 1 – 100 mM. The yield of methane and cell material was directly dependent upon methanol concentration within the range of 0 – 100 mM (Fig. 3A). Above 100 mM methanol, both the rate of methane formation and the yield of methane and cell material were decreased. H_2 was not produced during methanol/fermentation. Data for growth on methanol is summarized in Table 1.

Mixotrophic Utilization of Methanol, Methylamine, and H_2/CO_2

Table 2 indicates that both growth and methanogenesis in media that contained methanol under various gas phases decreased in the order $H_2/CO_2 > N_2/CO_2 \sim N_2 > H_2/N_2$. Although considerable amounts of methane were formed in cultures that contained H_2 plus methanol in the absence of CO_2, essentially no growth was observed under these conditions.

Figure 3B shows the effect of methanol concentration on *M. barkeri* during growth on methanol under a H_2/CO_2 gas phase. Yields of both methane and cell material increased as methanol concentration increased from 0 – 200 mM. At higher methanol concentrations, inhibition was observed.

The mixotrophic utilization of methanol and CO_2 as methanogenic substrates was further investigated by growing cultures under different gas phases and different concentrations of methanol, in the presence of either ^{14}C-methanol or $^{14}CO_2$. Gas production (CH_4, $^{14}CH_4$, CO_2, and $^{14}CO_2$) was measured at intervals over the 216 h growth period. Results are summarized in Table 3. The amount of methane derived from each substrate can be calculated by dividing the specific radioactivity of methane by that of the substrate. When cells were grown in the presence of $^{14}CO_2$ and unlabeled methanol, CO_2 accounted for 3 and 20% of the methane formed under N_2/CO_2 and H_2/CO_2 gas phases, respectively. Data obtained from cells grown in the presence of ^{14}C-methanol indicate that the remainder of the methane formed was derived from methanol. The fraction of methane derived from each substrate was independent of methanol concentration within the range of 10 – 200 mM. During growth on a single carbon substrate – i.e., on methanol (N_2 gas phase), or under a H_2/CO_2 gas phase in the absence of methanol – all of the methane produced was derived from the individual substrate.

Both methanol and CO_2 were incorporated into cell material during mixotrophic growth (Table 4). Methanol contributed 40 and 48% of the total cell carbon in the presence and absence of H_2, respectively.

Table 1. Growth parameters of *Methanosarcina barkeri* during cultivation on H_2/CO_2 or methanol

Parameter	Growth substrate	
	H_2/CO_2[a]	Methanol[b]
Maximum specific growth rate constant μ_{max}	0.058 h^{-1}	~0.07 h^{-1}
Yield per CH_4 (mg cell dry weight per mmole CH_4 formed)	6.37	7.2
Fraction of substrate carbon converted to CH_4[c]	0.81	0.79
Fraction of substrate carbon converted to cells[c]	0.19	0.21

[a] Data obtained from Fig. 2
[b] Cells were grown in tubes that contained 10 ml PBBW plus 100 mM methanol (gas phase N_2)
[c] Calculated from yield per CH_4. Cells were 45% carbon (determined by Galbraith Labs, Knoxville, Tenn.)

Table 2. Comparison of unitrophic and mixotrophic metabolism of methanol and H_2/CO_2 by *M. barkeri*

Growth condition[a]	Total CH_4 produced[b] (μmol)	Growth[c] (mg dry weight increase)
H_2/CO_2	105.5	0.31
$CH_3OH + N_2$	185.6	0.53
$CH_3OH + H_2/CO_2$	263.0	0.87
$CH_3OH + N_2/CO_2$	174.6	0.57
$CH_3OH + H_2/N_2$	103.7	0.09
H_2/N_2	27.3	0.09
N_2	13.4	0.06

[a] Cells were grown in PBBW for 240 h. Initial concentration of methanol, when added, was 25 mM. Initial gas phases were H_2/CO_2 (80/20, v/v, N_2/CO_2 (95/5, v/v), H_2/N_2 (80/20, v/v), or N_2. Volume of medium was 12 ml
[b] Mean value of triplicate samples
[c] Mean values of 3 pooled samples

Table 3. Mixotrophic utilization of methanol and CO_2 as methanogenic substrates[a]

A. $^{14}CO_2$ as labeled substrate

Initial $[CH_3OH]$, mM	H_2/CO_2 gas phase			N_2/CO_2 gas phase		
	SA_{CO_2}	SA_{CH_4}	SA_{CH_4}/SA_{CO_2}	SA_{CO_2}	$SA_{SA_{CH_4}}$	SA_{CH_4}/SA_{CO_2}
0	7.70	8.32	1.08			
10	8.25	1.58	0.192	13.25	0.39	0.029
40	6.73	1.36	0.202	12.29	0.37	0.030
100	6.93	1.43	0.206	9.75	0.35	0.036
200	5.88	1.22	0.207	12.68	0.35	0.028

B. $^{14}CH_3OH$ as labeled substrate

Initial $[CH_3OH]$, mM	SA_{CH_3OH}	SA_{CH_4} under different gas phases			
		H_2/CO_2	N_2/CO_2	H_2/N_2	N_2
10	17.61	12.20	22.30	14.04	15.24
40	4.40	3.35	4.32	5.14	3.91
100	1.76	1.50	1.58	1.70	1.80
200	0.88	0.72	0.98	0.86	0.86

[a] The experiment was run for 216h as described in the text
[b] Specific activities (SA) of CH_3OH, CH_4, and CO_2 expressed as dpm/nmole. SA_{CH_3OH} was calculated from the amount of CH_3OH and $^{14}CH_3OH$ originally added to the tubes. SA_{CH_4} and SA_{CO_2} are expressed as mean values of measurements at four time points — 24, 64, 110 and 216h

M. barkeri was able to ferment methylamine, although utilization required an adaptation period of several weeks. To further investigate metabolism of methylamine, cells were grown in PBBW supplemented with 50 mM ^{14}C-methanol (plus or minus 50 mM unlabeled methylamine) or 50 mM ^{14}C-methylamine (plus or minus 50 mM unlabeled methanol). Gas production was measured at intervals, and growth and incorporation of label was determined after 14 d incubation. The data are summarized in Table 5.

Table 4. Incorporation of methanol and CO_2 into cell material by growing cultures of *M. barkeri*

Media[a]	Gas phase	Label	DPM Incorporated	$SA_{substrate}$[b]	Growth (mg)	µmole incorporated per mg cells	Fraction of cell carbon derived from substrate
PBBW	H_2/CO_2	$^{14}CO_2$	965,720	27,410	1.1	32.02	1.00
PBBW +	N_2	$^{14}CH_3OH$	106,660	2,190	1.4	34.85	1.00
50mM CH_3OH	N_2/CO_2	$^{14}CO_2$	4,340,590	39,840	5.5	19.81	0.524
		$^{14}CH_3OH$	212,240	2,190	5.4	17.98	0.476
	H_2/CO_2	$^{14}CO_2$	3,510,150	27,200	6.4	20.16	0.603
		$^{14}CH_3OH$	177,150	2,190	6.1	13.28	0.397

[a] Cultures were grown for 70 h. Results expressed as pooled value of two replicate tubes
[b] Specific activity of substrate in dpm/µmol

Table 5. Metabolism of methanol and methylamine by *M. barkeri*

Growth condition[a]		CH_4 production[c]			Growth[d] mg cell dry weight increase	Incorporation[d]	
Substrate	Label[b]	µmol	dpm	dpm/µmol		dpm	dpm/mg cells
CH_3OH	$^{14}CH_3OH$	60.0	374,760	6,242	1.3	168,810	129,850
CH_3OH + CH_3NH_2	$^{14}CH_3OH$	68.8	366,150	5,322	2.2	210,970	95,900
CH_3NH^2	$^{14}{}_3NH_2$	30.0	235,040	7,835	1.3	289,240	222,490
CH_3OH + CH_3NH_2	$^{14}CH_3NH_2$	76.9	156,270	2,033	2.3	134,940	58,670

[a] Cells were pre-grown for 8 weeks in PBBW + 25 mM CH_3NH_2 (N_2 gas phase). CH_3OH (10 mM) was then added to the culture. After 2 days incubation, cells were inoculated into the experimental tubes which contained 50 mM CH_3OH and/or CH_3NH_2
[b] The specific radioactivities of the substrates were 7200 dpm/µmol for $^{14}CH_3OH$ and 9000 dpm/µmol for $^{14}CH_3NH_2$
[c] Results expressed as mean values of duplicate tubes after 9.5 d incubation
[d] Results expressed as mean values of pooled duplicate tubes after 14 d incubation

Methane production decreased in the order methanol/methylamine > methanol > methylamine. The specific activity of methane formed in cultures that contained methylamine plus ^{14}C-methanol was 15% less than that in cultures that contained ^{14}C-methanol alone; the specific acivity of methane produced in cultures that contained methanol plus ^{14}C-methylamine was 74 percent less than that in cultures that ^{14}C-methylamine alone. Similar results were observed in the incorporation of these substrates into cell material. The ratio of CH_4/CO_2 formed during methanol or methylamine fermentation was 2.7 ± 0.4.

Enzymatic Activities

Cell-free extracts of *M. barkeri* contained a hydrogenase acitivity which used F_{420} as electron acceptor. Reduced F_{420} was oxidized by extracts in the presence of $NADP^+$ but not NAD^+ (Fig. 4). Hydrogenase was further investigated using methyl viologen as electron acceptor. The maximum specific activity of hydrogen oxidation in cell-free extracts at 37°C was 6.0 µmoles/min · mg protein at the optimum pH (9.0). Similar activities were observed in cells grown in either H_2/CO_2 or methanol. Hydrogenase from H_2/CO_2-grown cells was not inhibited by addition of 20 mM methanol to the assay system. The hydrogenase activity was irreversibly inactivated by exposure to traces of molecular oxygen.

A methanol or methylamine dehydrogenase was not demonstrable in cell-free extracts, using PMS/DCPIP or F_{420} as electron acceptors. Furthermore, neither formaldehyde or formate dehydrogenase activities were observed. Extracts of *M. barkeri* did not inhibit the purified methanol dehydrogenase from *Methylobacterium organophilum*.

Cell-free extracts of *M. barkeri* lacked RuBP carboxylase activity and did not inhibit the RuBP carboxylase activity of osmotically-shocked spinach choloroplasts. Cell-free extracts of *M. barkeri* did not contain measurable quantities of 3-hexulose-6-phosphate synthase or of hydroxypyruvate reductase.

Discussion

Methanosarcina barkeri strain MS (neotype strain isolated from sewage sludge) and *Methanosarcina* strain UBS (isolated from lake sediment) had similar DNA base compositions of approximately 44 mol% G + C, and displayed close similarities in morphological and nutritional properties. These data indicate that *Methanosarcina* strain UBS is a strain of *M. barkeri*. It is likely that more one species exists within the genus. The DNA base composition of the gas-vacuolated *Methanosarcina* of Zhilina and Aleksandrushkina (1974) was reported as 51 mol% G + C.

M. barkeri was found to have several cellular characteristics in common with other methanogens. It grew as a chemolithotrophic autotroph, using H_2 as electron donor and CO_2 as electron acceptor and sole carbon source for growth. The organism contained F_{420}, an electron carrier found in all taxonomically-described methanogenic species (Zeikus, 1977) and has been reported to contain coenzyme M (Taylor et al., 1974). Cell-free extracts contained an F_{420}-dependent $NADP^+$-linked hydrogenase activity that has been demonstrated in *Methanobacterium* (Tzeng et al., 1975; Zeikus et al., 1977) species. These data indicate that *Methanosarcina* shares catabolic enzymes and coenzymes present in *Methanobacterium*. The results support the present taxonomic grouping of diverse methanogens into a single family, although rRNA sequence data (Fox et al., 1977) clearly indicates that *Methanosarcina* are in a separate group from *Methanobacterium*.

M. barkeri differs from other described methanogens in its ability to grow not only chemoautotrophically on H_2/CO_2, but also chemoorganotrophically on methanol and mixotrophically on both substrates. The organism has also been reported to grow on acetate in complex media (Mah et al., 1977). *M. barkeri* was also found to utilize methylamine as sole energy source. Growth on this substrate was relatively slow (generation time approximately 2 weeks) and required a long adaptation period. Competition experiments with ^{14}C-methanol and ^{14}C-methylamine indicated that both substrates were converted to methane, CO_2, and cell material; conversion of methanol was more rapid than that of methylamine. These data extend the range of known substrates of microbial methanogenesis.

During exponential growth in mineral medium under constant gassing with H_2/CO_2, *M. barkeri* produced 6.4 g cells per mole of methane formed. Roberton and Wolfe (1970) reported a yield of 2.4 g cells per mole methane formed by *Methanobacterium* strain M.O.H. in complex media under continuous gassing with H_2/CO_2; the relatively low yield may have been a consequence of the slow growth rate (doubling time 50 h) of the organism (Stouthamer, 1969). Taylor and Pirt (1977) obtained a yield of 0.6 – 1.6 g cells per mole methane formed by *M. thermoautotrophicum*; the relatively low yield may have been the result of the thermophilic nature of the organism. Coultate and Sundarum (1975) have reported that the molar growth yield of *Bacillus stearothermophilus* is lower at the organism's optimal temperature than at lower temperatures due to uncoupling of growth and respiration at the higher temperature.

During the fermentation of methanol by *M. barkeri*, the yields of both methane and cell material were directly dependent upon methanol concentration within the range of 0 – 100 mM; methanol was inhibitory at higher concentrations. A mean growth yield of 7.2 g cells per mol methane formed was determined. This value is slightly higher than that suggested by Stadtman (1967). The fact that similar yields were obtained for growth on methanol and H_2/CO_2 indicate that the efficiency of energy conservation from these substrates is similar.

Growth and methanogenesis in media that contained methanol varied with culture gas phase used. During growth on methanol/CO_2, methanol contributed 97 and 80%, respectively, of the methane carbon formed. These data indicate that *M. barkeri* is capable of mixotrophic growth on H_2/CO_2 and methanol. The preferential utilization of methanol as a methanogenic substrate has been obsered in mixed cultures of *M. barkeri* (Stadtman and Barker, 1951).

The observation that $^{14}CO_2$ reduction to $^{14}CH_4$ ocurred in the absence of H_2 during growth on methanol, along with the fact that free methanol, formaldehyde, or formate were not oxidized by cell-free extracts in a manner characteristic of methylotrophic bacteria, is in agreement with the unified scheme of methanogenesis involving carrier-bound one-carbon intermediates as proposed by Barker (1956). Gunsalus et al. (1976) have reported that cell-free extracts of *M. barkeri* convert methanol to $^{14}CH_3$-S-CoM. $^{14}CH_3$-S-CoM has been observed as an early product in whole cells labeled for short periods (3 – 90 s) with either $^{14}CO_2$ (in presence of H_2) or ^{14}C-methanol (unpublished observations).

Methanogenesis without measurable growth in cultures that contained H_2 plus methanol, but lacking CO_2, indicate that methanol reduction is not obligately coupled to its oxidation. Furthermore, these data indicate that H_2 regulates methanol oxidation, either directly by maintaining electron carriers in a reduced state or indirectly by altering reaction equilibria in the direction of reduced one carbon carriers (e.g. CoM derivatives) used in methanogenesis.

Although the pathway of fixation of one-carbon units into cell material by *Methanosarcina barkeri* is

not known, cell carbon synthesis in this species and in *Methanobacterium thermoautotrophicum* (Zeikus, 1977; Zeikus et al., 1977; Taylor et al., 1976) does not occur by known autotrophic or methylotrophic pathways. Methanol contributed a large amount (40 – 50 %) of the total carbon assimilated during growth on methanol/CO_2. If the fixation of ^{14}C-methanol into cell carbon occurred after its convension to CO_2, only a small amount of ^{14}C-cell material would be formed due to dilution of the label with exogenous CO_2. These data indicate that methanol is fixed into cell carbon via a compound more reduced than CO_2. During autotrophic growth on H_2/CO_2, *Methanosarcina barkeri* may fix carbon both as CO_2 and as a reduced C_1 unit derived from CO_2, possibly via the same C_1 carriers used in methanogenesis. The reduced C_1 unit can be derived directly from methanol when this substrate is added exogenously. Thus, it is possible that during growth on methanol a common one carbon carrier (e.g. a CoM derivative) is involved in both catabolism and anabolism in *Methanosarcina*. However, the data presented here do not eliminate the involvement of other one-carbon carriers.

The fixation of C_1 units at both the carboxyl and hydroxymethyl levels has been observed in *Pseudomonas* AMl and other methylotrophs utilizing the serine path (Large et al., 1961). The lack of hydroxypyruvate reductase, hexulose phosphate synthase, and RuBP carboxylase in *M. barkeri* indicate that carbon fixation does not occur via the serine or hexulose paths, or via the reductive pentose phosphate (Calvin) cycle common to most autotrophs. More detailed studies of carbon fixation in *M. barkeri* are in progress.

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