APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1986, p. 269–274
0099-2240/86/080269-06$02.00/0

Vol. 52, No. 2

Utilization of Methanol plus Hydrogen by *Methanosarcina barkeri* for Methanogenesis and Growth

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Received 27 January 1986/Accepted 29 April 1986

***Methanosarcina barkeri* grew on methanol plus H_2. Both substrates were consumed in equimolar amounts. Growth was strictly dependent on the presence of acetate, which was required for the biosynthesis of cellular constituents. Only about 0.4% of the methane produced originated from acetate. By using deuterated methanol, it was demonstrated that methanogenesis from this compound under H_2 did not occur via oxidation of methanol to CO_2 and subsequent reduction but by direct reduction with H_2. Growth yields with methanol plus H_2 and with methanol alone were not significantly different: 2.8 g of cells per mol of methanol in mineral medium and 4.6 g of cells per mol of methanol in complex medium, respectively. Growth of *M. barkeri* on methanol plus H_2 depended strictly on the presence of sodium ions in the medium. In the presence of 50 mM K^+ the K_s for Na^+ was 5 mM.**

Methanogenic bacteria are a phylogenetically diverse but nutritionally rather uniform group of strictly anaerobic bacteria. Most species grow on H_2 plus CO_2 as a source of energy and carbon. Several species, including *Methanosarcina barkeri*, are also able to grow on methyl group-containing compounds (e.g., acetate, methanol, methylamines) (1). Recently, Miller and Wolin isolated a coccus, *Methanosphaera stadtmaniae*, which is very restricted in its substrate spectrum: it only grows with H_2 plus methanol as the substrate (13, 14).

Methanol has been shown to be converted by resting cells of *M. barkeri* in two ways. First, methanol is disproportionated to CO_2 and CH_4 according to the formula (C. G. T. P. Schnellen, Ph.D. dissertation, Technical University of Delft, The Netherlands, 1947): $4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$ ($\Delta G^{0'} = -104.6$ KJ/mol of CH_4) from $CH_3OH + H_2O \rightarrow CO_2 + 6\ [H]$ and $3\ CH_3OH + 6\ [H] \rightarrow 3\ CH_4 + 3\ H_2O$. Second, in the presence of molecular hydrogen, methanol is stoichiometrically reduced to methane by the formula (3) $H_2 + CH_3OH \rightarrow CH_4 + H_2O$ ($\Delta G^{0'} = -112$ KJ/mol of CH_4).

With respect to the energy conservation and electron transfer reactions, methanogenesis from H_2 plus methanol is the simplest type of methanogenic fermentation. (i) Methanol is converted by a methyltransferase into 2-(methylthio)ethanesulfonate (23), which is a central intermediate in methanogenesis from all substrates (10, 15, 20, 23). (ii) 2-(Methylthio)ethanesulfonate is then reductively cleaved into methane and 2-mercaptoethanesulfonate (27). (iii) Since methanogenesis from methanol plus H_2 is associated with ATP synthesis by a chemiosmotic mechanism (3), the reduction of 2-(methylthio)ethanesulfonate must be the step that is coupled to energy-conserving reactions.

It has been known for a long time that *M. barkeri* utilizes methanol for growth as shown in the first equation; growth of this species on methanol-H_2, however, has never been reported. The aim of this work was to obtain evidence that *M. barkeri* can not only generate ATP from H_2-methanol, but can actually grow on this substrate combination.

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MATERIALS AND METHODS

Organism and cultivation. *M. barkeri* Fusaro (DSM 804) was obtained from the German Collection of Microorganisms (DSM), Göttingen, Federal Republic of Germany (FRG).

Media were prepared under N_2 by the anaerobic techniques described by Hungate (8) and Bryant (5). Traces of oxygen were removed by passing the gas through a heated copper column. Stock cultures were grown in 16-ml Hungate tubes with 5 ml of the medium described by Scherer and Sahm (19), except that the trace element solution SL6 (18) was used. The pH was adjusted to 6.8 with 6 N HCl, and the growth temperature was 37°C. Methanol served as the substrate in a final concentration of 200 mM. Growth was started by the addition of 2% (vol/vol) inoculum.

Growth experiments were carried out in 500-ml glass bottles filled with 100 ml of the above medium plus 10 mM sodium acetate. The atmosphere was either H_2 or N_2. Whenever CO_2 was to be added to the cultures, the medium was supplemented with 850 mg of sodium bicarbonate per liter and prepared under a stream of 80% N_2 and 20% CO_2. Growth was initiated by inoculation with a portion (10%, vol/vol) of the stock culture. The methanol concentration was 100 mM. For growth on methanol plus H_2 the culture vessels were incubated horizontally. After consumption of 10% of the added methanol, the culture vessels were incubated on a rotary shaker. During growth, the gas atmosphere was replaced twice with molecular hydrogen. When growth on methanol under N_2 and under H_2 was compared, the culture vessels under N_2 were treated in the same way but without the gas exchange.

The low-sodium-ion medium contained the following (per liter of glass-distilled water): imidazole hydrochloride, 40 mmol; KH_2PO_4, 0.5 mmol; $K_2HPO_4 \cdot 3H_2O$, 0.5 mmol; $MgCl_2 \cdot 6H_2O$, 1.7 mmol; $CaCl_2 \cdot 2H_2O$, 2.0 mmol; $(NH_4)_2 \cdot 6H_2O$, 0.1 mmol; ammonium acetate, 10 mmol; vitamin solution (28), 10 ml; trace element solution SL6 (18), 3 ml; resazurin, 1 mg; K_2S, 2.6 mmol; cysteine hydrochloride, 1.7 mmol; titanium citrate solution, 2 ml; KCl and NaCl as indicated. The pH was adjusted to 6.8 with 6 N HCl.

Cultivation conditions were as described above. The titanium citrate solution was prepared as described by Zehnder and Wuhrmann (29), except that potassium salts were used. The K_2S solution was prepared from Na_2S by adding HCl and passing the evolved H_2S into a 0.27 M KOH solution. Determination of the extracellular sodium concentration was done in a flame photometer as described previously (4).

Gas chromatographic procedures. Methane was measured by gas chromatography on a Perkin Elmer model 900 gas chromatograph (Bodenseewerk, Überlingen, FRG) by injecting 5-μl samples from the gas phase of cultures as described previously (3). The hydrogen content of the gas atmosphere was determined with a model 439 gas chromatograph (Packard Instruments, Downers Grove, Ill.) equipped with a thermal conductivity detector. The glass column (2 m by 2 mm) was packed with Porapak QS (60/80 mesh) (Riedel-de Haën, Seelze, FRG). N_2 was used as the carrier gas at a flow rate of 25 ml/min. The oven temperature was 150°C, and the temperature of the injector and detector was 220°C. Gas samples (5 μl) from the headspace of the culture vessels were injected into the gas chromatograph. The hydrogen content of the gas atmosphere was quantified by injecting standards of known hydrogen concentration.

Quantitative analysis of methanol was done by gas chromatography. A portion of the cell suspension was centrifuged at 2,000 × *g* and 4°C for 10 min, and 5 μl of the supernatant was injected into a Perkin Elmer model 3920 gas chromatograph equipped with a glass column (2 m by 2 mm) which was packed with Porapak QS (60/80 mesh; Riedel-de Haën). N_2 was used as the carrier gas at a flow rate of 25 ml/min. The temperatures of the injector, detector, and oven were 150, 200, and 130°C, respectively. Standards with various methanol concentrations were used for quantification.

Acetate was determined by gas chromatography as described previously (12).

Measurement of growth. Growth was followed by analyzing the carbon content of the cell suspension with a carbon analyzer (model Tocamaster 900; Beckman Instruments, Fullerton, Calif.). Synthetic air was used as the carrier gas at a flow rate of 300 ml/min. The oven temperature for organic and inorganic carbon was 950 and 155°C, respectively. A portion of the cell suspension was washed twice with 50 mM potassium phosphate solution, pH 4.5, and suspended in the same solution.

Depending on the carbon content of the samples, samples of 10 to 50 μl were injected into the carbon analyzer, first into the channel for total and then into the channel for inorganic carbon. The carbon content was automatically calculated and expressed in ppm. The carbon analyzer was calibrated with standards of known carbon content: $KHCO_3$ was used as the standard for inorganic carbon, and phthalic acid served as the standard for total carbon. The dry weight of the cell suspension was calculated from the carbon content by using a calibration curve in which the dry weight was plotted against the carbon content. The dry weight was determined by filtering 50 ml of cell suspension onto preweighed membrane filters (47 mm, 0.45-μm pore size; Sartorius, Göttingen, FRG) as described previously (7).

The molar growth yields (milligrams of cells per millimole of methanol or methane) were determined by plotting dry weight increase against methane increase or methanol decrease during the exponential growth phase of *M. barkeri* grown on 100 mM methanol under either H_2 or N_2.

Labeling studies. The experiments were performed in 100-ml glass bottles containing 10 ml of medium under H_2 or N_2. The final methanol concentration was 50 mM. During growth, the gas in the culture vessel was not replaced. [^{14}C]methanol (specific radioactivity, 33 nCi/μmol) and [2-^{14}C]acetate (specific radioactivity, 58.3 μCi/μmol) were added to the growth medium with a 10-μl syringe before inoculation. Determination of the radioactivity added, of $^{14}CH_4$, and of $^{14}CO_2$ was done as described by Blaut et al. (4). In the experiments with [^{14}C]methanol the unconverted or assimilated radioactivity was determined as described previously (4). For determination of the incorporated label, 1 ml of homogeneous cell suspension was passed through a membrane filter (25 mm, 0.2-μm pore size) and washed four times with 5 ml of 50 mM potassium phosphate buffer, pH 6.9. The filters were placed into a scintillation vial containing 5 ml of Quickszint 212 (Zinsser, Frankfurt, FRG). After 24 h of incubation, the vial was vigorously shaken and radioactivity was determined in a model LS 7500 liquid scintillation counter (Beckman).

Experiments with deuterated methanol. Deuterated methanol (99.5% CD_3OD) was purchased from Fluka, Buchs, Switzerland. The experiments were performed in 250-ml glass bottles filled with 20 ml of medium under H_2 or N_2.

The methanol concentration was 50 mM. Growth was started by inoculation with a preculture (10%, vol/vol) which had been grown on 200 mM CD_3OD under N_2 without acetate. After methanogenesis had ceased, isotopic analysis of the gas phase was done with a Varian MAT model CH 5 high-resolution mass spectrometer by the field ionization method. The operating conditions were: temperature, 200°C; $m/\Delta m_{10\%}$, 1,000. With this method, fragmentation of the compounds is largely reduced and relatively intense peaks of molecule ions are produced. Therefore, the spectra shown in Fig. 3 represent the original mass spectra. The headspace of the culture bottles with the frozen medium was directly connected with the mass spectrometer by means of a rubber tube.

Chemicals, gasses, and radioisotopes. All chemicals were reagent grade and purchased from Merck, Darmstadt, FRG. [2-^{14}C]acetate (sodium salt) was purchased from Amersham Buchler, Braunschweig, FRG. [^{14}C]methanol was obtained from New England Nuclear, Dreieich, FRG. Gases were obtained from Messer Griesheim, Kassel, FRG.

RESULTS

Growth of *M. barkeri* on methanol plus H_2. To prove that *M. barkeri* is able to grow on methanol plus H_2, CO_2 had to be excluded from the culture medium and from the gas atmosphere. This was necessary because the simultaneous utilization of methanol and H_2 plus CO_2 by this organism has been reported (25). However, *M. barkeri* did not grow with methanol under a hydrogen atmosphere. Since this inability was probably due to a lack of precursors for biosynthesis, 10 mmol of acetate was added per liter of medium. The growth then observed under H_2 (Fig. 1) was 37% slower than that under N_2 (Fig. 2); the doubling times were 24 and 15 h, respectively. The ratio of methane produced to methanol consumed was 0.97 in the methanol-H_2 culture and 0.78 in the methanol culture. H_2 was consumed in amounts equivalent to the amounts of methanol consumed and methane produced. The concomitant consumption of acetate was 67% under H_2 and 74% under N_2. Acetate consumption stopped when growth of these cultures ceased. The ratio of acetate consumed to methane produced was 0.1 under N_2 and 0.07 under H_2, indicating that only a very small portion of the methane produced, if any, could have originated from ace-

tate. Supplementation of the medium with 0.2% yeast extract and 0.2% Casitone (Difco Laboratories, Detroit, Mich.) led to a decrease of the doubling time to 12 h (N_2 atmosphere) or 16 h (H_2 atmosphere).

To obtain unequivocal evidence that *M. barkeri* converted CH_3OH plus H_2 directly to methane and that acetate was required only for biosynthetic purposes, control experiments were necessary.

(i) It had to be excluded that part of the methanol was oxidized to CO_2 and subsequently reduced to methane by H_2. $^{14}CO_2$ from [^{14}C]methanol could not be detected during growth (data not shown). However, this possibility could be more conclusively ruled out by fermenting CD_3OH under either H_2 or N_2. The methane species detected in a mass spectrometric analysis are shown in Fig. 3. The species CD_3H accounted for 89.2% of total methane when *M. barkeri* was grown under H_2 (Fig. 3A). Therefore, oxidation of CD_3OH to CO_2 followed by reduction with H_2 to CH_4 can be clearly excluded. Such an oxidation reaction has to take place under N_2. Reduction of partly oxidized C_1 intermediates apparently led to the formation of CD_2H_2 to a high percentage (Fig. 3B).

(ii) It had to be excluded that acetate contributed to the methane produced. This was done by adding [2-^{14}C]acetate to the culture. Acetate was primarily incorporated into the cells (Table 1). Very little radioactivity appeared in CH_4 or in CO_2. In fact, it can be calculated from the data that acetate contributed less than 0.4% to the methane produced from methanol-H_2.

Growth yield studies. Before molar growth yields could be determined, it had to be verified that growth of *M. barkeri* on methanol in a mineral medium devoid of CO_2 and supplemented with acetate was not limited with respect to cell carbon. Addition of 10 mM $NaHCO_3$, acetate, or both to a culture growing on methanol under N_2 all caused a similar decrease in doubling time and increase in molar growth yields compared with these values for the mineral medium (data not shown). The growth yields ($Y_{methanol}$ and $Y_{methane}$) of *M. barkeri* grown on methanol-H_2 or methanol alone showed no significant differences (Table 2). Supplementation of the mineral medium with 0.2% yeast extract and 0.2% Casitone, however, resulted in an increase in growth yield and a decrease in doubling time.

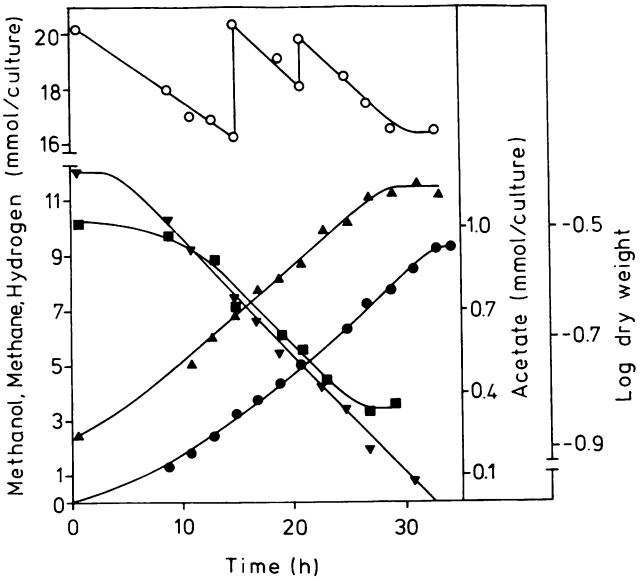

FIG. 1. Substrate consumption, product formation, and growth of *M. barkeri* on 100 mM methanol plus H_2. The organism was grown anaerobically at 37°C in a 500-ml bottle filled with 100 ml of medium. Symbols: ●, methane; ▲, dry weight; ○, hydrogen; ▼, methanol; ■, acetate.

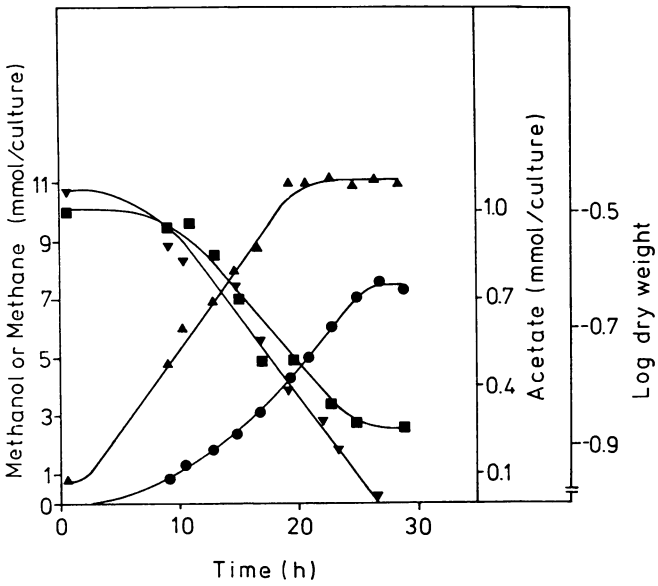

FIG. 2. Substrate consumption, product formation, and growth of *M. barkeri* on 100 mM methanol under N_2. The organism was grown anaerobically at 37°C in a 500-ml bottle filled with 100 ml of medium. Symbols: ●, methane; ▲, dry weight; ▼, methanol; ■, acetate.

Effect of sodium ions. Since methane formation and ATP synthesis from methanol-H_2 did not require Na^+ (4), the dependence of growth on this cation was tested. It is apparent (Fig. 4) that growth on methanol-H_2 as well as growth on methanol alone depended on the presence of

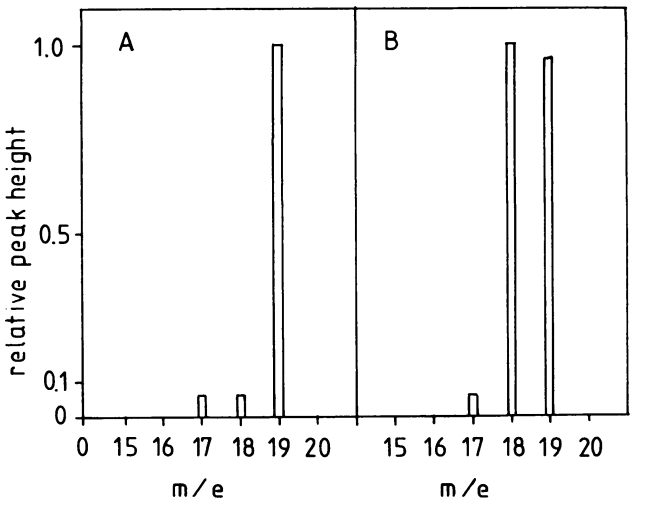

FIG. 3. Isotopic analysis of methane produced by *M. barkeri* from deuterated methanol under atmospheres of H_2 (A) or N_2 (B). The experiment was performed as described in Material and Methods. The peaks represent the following compounds: m/e = 19, CD_3H; m/e = 18, CD_2H_2; m/e = 17, CDH_3.

TABLE 1. Distribution of radioactive label in cell mass and products after growth of *M. barkeri* on methanol and [2-^{14}C]acetate under N_2 or H_2[a]

Growth conditions	CH_4 formed (mmol/liter of medium)	Conversion of label						$^{14}CH_4/^{14}CO_2$ ratio
		Incorporation		Reduction to $^{14}CH_4$		Oxidation to $^{14}CO_2$		
		Mdpm	mM	Mdpm	mM	Mdpm	mM	
50 mM methanol + H_2 + 10 mM [2-^{14}C]acetate	53.3	1.18	4.8	0.05	0.2	0.06	0.26	0.72
50 mM methanol + N_2 + 10 mM [2-^{14}C]acetate	39.3	1.14	4.7	0.05	0.2	0.09	0.39	0.53

[a] Experimental conditions were as described in Materials and Methods. The radioactivity of the [2-^{14}C]acetate added was 2.39 Mdpm under N_2 and 2.46 Mdpm under H_2. Incorporation and conversion of the label were determined after consumption of the added methanol.

sodium ions. The effect of Na^+ was largely influenced by the K^+ concentration in the medium. At 3 mM Na^+ the methane formation rate was largely suppressed by addition of potassium. This was not the case in the presence of 18 mM Na^+ (Fig. 5), indicating that inhibition of methane formation by potassium ions could be relieved by the addition of sodium.

The apparent K_s for Na^+ as determined at 50 mM K^+ was 5 mM for growth on methanol plus H_2 and 4.3 mM for growth on methanol alone (inset, Fig. 4).

DISCUSSION

M. barkeri was shown to synthesize ATP coupled to methane formation from methanol-H_2 (2). However, growth on this substrate combination was not observed (25); only when CO_2 was present as a third substrate did growth occur. Under these conditions methanol was predominantly reduced to methane and its oxidation to CO_2 was largely depressed (6, 11, 30).

We have confirmed the observations of Weimer and Zeikus (25) and deduced from our experiments that growth on methanol plus H_2 was not possible because the cells had a general shortage of precursors for cellular biosynthesis. *M. barkeri* did indeed grow on methanol-H_2 when acetate was also present as a substrate. In contrast to CO_2, acetate is not converted to methane under the conditions employed. Methane formation from acetate requires an adaptation period of the cells (9, 21, 26); the data in Table 1 demonstrate that only a negligible amount of acetate was converted to methane.

Acetate has been reported to stimulate the growth of various *Methanosarcina* strains on methanol or trimethylamine (2, 25), with 55 to 59% of the cell carbon being derived from acetate. The rest of the acetate was primarily oxidized to CO_2, providing the electrons for methane formation from methanol and trimethylamine. The results in this report show that in the presence of H_2, neither methanol nor acetate was oxidized to CO_2. Otherwise, CD_3H would not have been the predominant methane species formed from deuterated methanol. Therefore, it can be concluded that *M. barkeri* is able to grow on methanol plus H_2 if acetate is available as a source of cell carbon. *Methanosphaera stadtmaniae*, which is restricted to methanol plus H_2 as methanogenic substrates, requires acetate (in addition to CO_2) for growth as well (14). Growth of *M. barkeri* on methanol-H_2 did not require CO_2 in addition to acetate. The observed incorporation of acetate-carbon into cell material (Table 1) could be the result of incorporation of acetate as such but also of CO_2 derived from acetate. Therefore, it can be assumed that the CO_2 required for carboxylation reactions came from the oxidation of acetate.

If deuterated methanol or trimethylamine serves as the sole substrate for *M. barkeri*, 80% of the methane formed is represented by CD_3H and approximately 16% by CD_2H_2 (24). The results in this report show that in the presence of acetate, the proportion of CD_2H_2 is increased to approximately 50%. This can be explained as follows: the first oxidation product of methanol is only partly oxidized further to CO_2; a great portion of this oxidized C_1 intermediate is reduced to methane again because sufficient electrons are available. Although only a small portion of the methyl group of acetate is actually recovered as CO_2 (Table 1), it is conceivable that the partial or complete oxidation of acetate for biosynthesis generates enough electrons for the catabolic reduction of partially oxidized methanol.

Methanogenesis from methanol-H_2 and the synthesis of ATP coupled with this process have been shown not to require sodium ions (4). Growth of *M. barkeri* on methanol-H_2-acetate, however, depended on Na^+, as growth of methanogenic bacteria generally does (17). A possible explanation for this difference can be deduced from results obtained with *M. barkeri* grown on methanol alone. Here it could be demonstrated that Na^+ is involved in the oxidation of methanol to the level of formaldehyde (4). With the substrate combination methanol-H_2-acetate, Na^+ might be involved in oxidation reactions that lead from acetate to precursors of cellular constituents.

The Na^+ dependence was largely effected by the K^+ concentration in the medium. Therefore, K_s values for Na^+ are only valid for a given K^+ concentration. In accordance with the data of Perski et al. (16) and the data reported here, we propose that the determinations should be done in the presence of 50 mM K^+.

Finally, a short comment is necessary about the growth

TABLE 2. Comparison of growth yields during growth of *M. barkeri* on methanol under H_2 and N_2[a]

Growth substrate and medium[b]	$Y_{methanol}$ (g of cells/mol of CH_3OH)	$Y_{methane}$ (g of cells/mol of CH_4)
100 mM methanol + H_2		
Mineral medium	2.85 ± 0.4	2.85 ± 0.4
Complex medium	4.60 ± 0.5	4.60 ± 0.5
100 mM methanol + N_2		
Mineral medium	2.45 ± 0.2	3.26 ± 0.26
Complex medium	4.85 ± 0.4	6.46 ± 0.53

[a] Experimental conditions were as described in Materials and Methods. All experiments were done in the presence of 10 mM acetate.

[b] Complex medium was prepared by adding 0.2% yeast extract and 0.2% Casitone to the mineral medium.

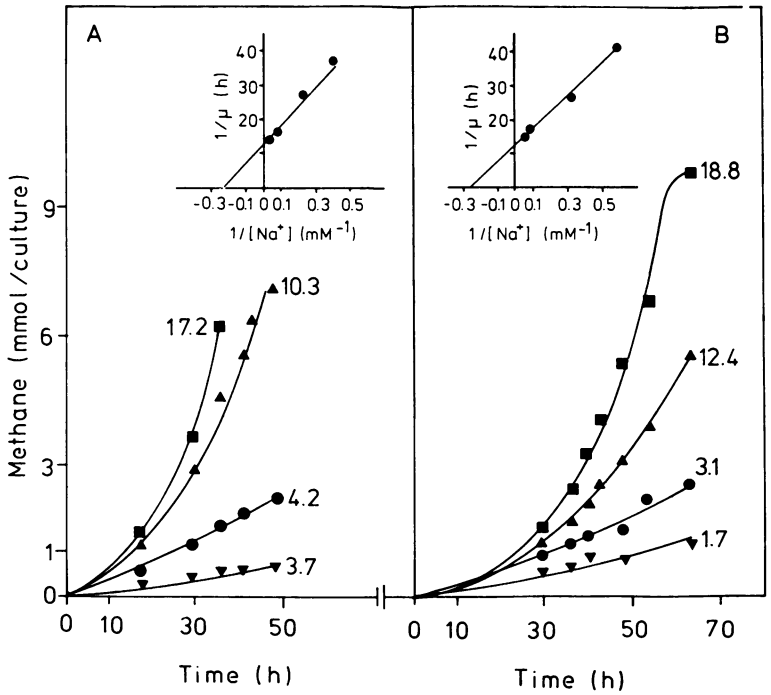

FIG. 4. Sodium dependence of methanogenesis from methanol by growing cells under either N_2 (A) or H_2 (B). The experiments were performed anaerobically at 37°C in 500-ml bottles containing 50 mM K^+ and various concentrations of Na^+ (added as NaCl) as indicated by each symbol in the figure. The insets show a double-reciprocal plot of the growth rate versus the sodium concentration.

yield data. Related to the methanol consumed, the data did not show a significant difference between the substrate combinations methanol (plus acetate) and methanol-H_2 (plus acetate). Correspondingly, the amount of cells produced per mole of methane was larger with methanol than with methanol-H_2. This could indicate that methanogenesis and growth are more effectively coupled with methanol than with methanol-H_2 or that the last step in methanol oxidation to CO_2 allows ATP synthesis by substrate-level phosphorylation, as had been proposed many years ago (22).

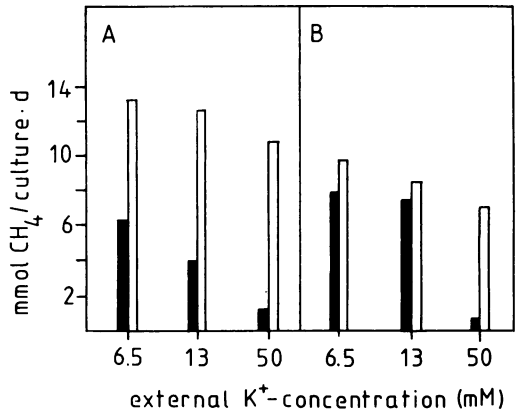

FIG. 5. Effect of potassium ions on methane formation from methanol (millimoles of CH_4 per culture per day) under N_2 (A) or H_2 (B) by growing cells of *M. barkeri* at sodium concentrations of 3 mM (solid bars) or 18 mM (open bars). *M. barkeri* was grown anaerobically at 37°C in 500-ml bottles containing 100 ml of medium. Potassium and sodium ions were added as KCl and NaCl, respectively.

ACKNOWLEDGMENTS

We are indebted to G. Schöne from the Institut für Anorganische Chemie der Universität Göttingen for the mass spectroscopic analysis and W. Schmidt from the Institut für Geobotanik der Universität Göttingen for allowing us to determine the sodium concentration with his equipment. We thank J. Czernitzky for her helpful advice on using the flame photometer.

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