JOURNAL OF BACTERIOLOGY, Jan. 1982, p. 247-254
0021-9193/82/010247-08$02.00/0

Vol. 149, No. 1

Comparison of Unitrophic and Mixotrophic Substrate Metabolism by an Acetate-Adapted Strain of *Methanosarcina barkeri*

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Received 27 May 1981/Accepted 9 September 1981

We examined the unitrophic metabolism of acetate and methanol individually and the mixotrophic utilization of these compounds by using detailed ^{14}C-labeled tracer studies in a strain of *Methanosarcina barkeri* adapted to grow on acetate as the sole carbon and energy source. The substrate consumption rate and methane production rate were significantly lower on acetate alone than during the unitrophic or mixotrophic metabolism of methanol. Cell yields (in grams per mole of substrate) were identical during exponential growth on acetate and exponential growth on methanol. During unitrophic metabolism of acetate, the methyl moiety accounted for the majority of the CH_4 produced, but 14% of the CO_2 generated originated from the methyl moiety. This correlated with the concurrent reduction of equivalent amounts of the C-1 of acetate to CH_4. $^{14}CH_4$ was also produced from added $^{14}CO_2$, although to a lesser extent than from reduction of the C-1 of acetate. During mixotrophic metabolism, methanol and acetate were catabolized simultaneously. The rates of $^{14}CH_4$ and $^{14}CO_2$ generation from [2-^{14}C]acetate were logarithmic and higher in mixotrophic than in unitrophic cultures at substrate concentrations of 50 mM. A comparison of the oxidoreductase activities in cell extracts of the acetate-adapted strain grown on acetate and of strain MS grown on methanol or on H_2 plus CO_2 indicated that the pyruvate, α-ketoglutarate, and isocitrate dehydrogenase activities remained constant, whereas the CO dehydrogenase activity was significantly higher (5,000 nmol/min per mg of protein) in the acetate-adapted strain. These results suggested that a significant intramolecular redox pathway is possible for the generation of CH_4 from acetate, that energy metabolism from acetate by *M. barkeri* is not catabolite repressed by methanol, and that the acetate-adapted strain is a metabolic mutant with derepressed CO dehydrogenase activity.

Acetate is an immediate precursor for methanogenesis in anaerobic environments, such as aquatic sediments and biogas digesters, where organic matter undergoes biodegradation to methane and carbon dioxide (11, 24, 26). The majority of methane formed in these environments originates from the methyl group of acetate. The path of acetate transformation has been determined generally by mixed-culture studies (2, 13, 18) and is explained by the following equation: $CH_3{*}COOH^+ \rightarrow CH_4{*} + CO_2{}^+$. The electrons in the methyl group of acetate are maintained intact during methanogenesis by this reaction.

Methanogens are a very diverse group of bacteria (1, 26). Nonetheless, species of only two genera have been shown clearly to grow via fermentation of acetate alone (17, 25; C. Schnellen, Ph.D. thesis, Technical University of Delft, Rotterdam, 1947). *Methanosarcina barkeri* is the most metabolically versatile methanogen in pure culture and grows via metabolism of H_2-CO_2, methanol, methylamine, or acetate (27). Documentation of acetate fermentation by *M. barkeri* was somewhat controversial (16, 30) until independent discoveries that growth of *M. barkeri* on acetate as an energy source requires a prolonged cultural adaption-strain selection process (20, 23). Winter and Wolfe (23) suggested that acetate-adapted strains retain the ability to ferment acetate even after growth on a one-carbon substrate alone.

Weimer and Zeikus (20) proposed that *M. barkeri* catabolizes both acetate and methanol or H_2-CO_2 during mixotrophic growth on combinations of substrates. Smith and Mah (16) reported that acetate catabolism by *Methanosarcina* strain 227 was catabolite repressed by H_2-CO_2 or methanol. The purpose of this investigation was to use ^{14}C-labeled tracers to examine the unitrophic and mixotrophic metabolism of acetate by a *M. barkeri* strain that was adapted to

grow on acetate as the sole carbon and energy source.

(Preliminary results were presented at the Symposium on Trends in the Biology of Fermentations for Fuels and Chemicals, 7–12 December 1980 [9].)

MATERIALS AND METHODS

Chemicals, gases, and radioisotopes. All chemicals were reagent grade. Gases were purchased from Matheson Scientific, Inc., Joliet, Ill. $Na^{14}CO_3$ (12.4 mCi/mmol), [1-^{14}C]sodium acetate (2.9 mCi/mmol), and [2-^{14}C]sodium acetate (51.0 mCi/mol) were purchased from New England Nuclear Corp., Boston, Mass. $^{14}CH_3OH$ (55 mCi/mmol) was purchased from ICN, Irvine, Calif. Both [2-^{14}C]sodium acetate and [1-^{14}C]sodium acetate were treated by the Schmidt degradation procedure (14) to confirm the label distribution between the methyl and carboxyl carbons. The [1-^{14}C]sodium acetate contained 0.1% of its total radioactivity in the methyl carbon, whereas [2-^{14}C]sodium acetate had 0.8% of its total radioactivity in the carboxyl group.

Organisms and culture conditions. *M. barkeri* strain MS was the strain originally characterized by Weimer and Zeikus (21) and then adapted to grow on acetate in complex medium (20). This strain was adapted during the course of several months to grow on acetate in defined medium by sequential transfers from complex medium to acetate-yeast extract medium containing progressively lower yeast extract concentrations, until the organism grew after transfer into acetate minimal medium. The *M. barkeri* acetate-adapted strain was grown in the phosphate-buffered basal (PBB) medium used previously (7), except that 55 mM PO_4 buffer (pH 7.0), 0.025% $Na_2S \cdot 9H_2O$, and 50 to 100 mM sodium acetate were added after autoclaving. The acetate-adapted strain of *M. barkeri* was maintained on medium containing acetate alone for more than 1 year, whereas *M barkeri* strain MS was maintained on medium containing methanol or H_2-CO_2 for several years and was not exposed to acetate. Penicillin G (200 μg/ml) was added to experimental cultures only to assure purity. Stock and experimental cultures were grown in 158-ml serum vials (Wheaton Scientific, Millville, N.J.) that contained 30 to 50 ml of PBB medium, sodium acetate, and an N_2 gas phase and were sealed with black butyl rubber stoppers and aluminum caps (Bellco Glass, Inc., Vineland, N.J.). Experiments were initiated by syringe transfer of 6 to 10% inoculum, and experimental cultures were incubated at 37°C without shaking. Substrate consumption and growth rates were determined from large-scale experiments performed in glass carboys (15 liters) that contained PBB medium supplemented with acetate or a complex medium containing (in grams per liter of distilled water): NH_4Cl, 1; K_2HPO_4, 9; KH_2PO_4, 5; $MgCl_2$, 0.1; yeast extract, 2; Trypticase, 2; resazurin, 0.001; cysteine hydrochloride, 0.05; Na_2S, 0.1. This medium was prepared without cysteine hydrochloride, Na_2S or phosphate, which were added after autoclaving. Cells used for enzyme analyses were grown in carboys containing 100 mM sodium acetate or 100 mM methanol in PBB medium or in 12-liter fermentors containing PBB medium that was gassed continuously with H_2-CO_2 (80:20). Cultures were inspected routinely for contamination by phase microscopic analysis and by examination of cell turbidity after transfer of 1 ml of culture into a complex medium that contained 0.5% glucose, 0.2% Trypticase, and 0.2% yeast extract.

Substrate and end product analysis. Each culture supernatant (0.3 ml) was acidified with 10 N H_3PO_4 (15 μl), and methanol and acetate were quantified by injection of a 2-μl sample into a Packard model 419 gas chromatograph equipped with a Chromosorb 101 column (Supelco, Bellefonte, Pa.), as described by Zeikus et al. (29). ^{12}C- or ^{14}C-labeled methane and carbon dioxide were measured by direct injection of a 0.4-ml sample into a Packard model 417 gas chromatograph linked with a Packard model 894 gas proportional counter, as described by Nelson and Zeikus (12). All values were corrected for gas solubility (except where noted) and counting efficiency and are reported as total amount formed per unit of time. Radioactive stock solutions were prepared to the appropriate specific activities by adding ^{14}C-labeled substrate to a 3.2 M solution of nonradioactive substrate and were autoclaved under N_2 before use in experimental cultures. The amount of radioactivity (in disintegrations per minute) was determined by placing a sample of solution in a vial containing Instagel (Packard Instrument Co., Downers Grove, Ill.) and measuring the sample with a Packard model PLD Prias liquid scintillation counter. Initial specific activities were calculated from the radioactivity present and the concentration of substrate measured in the culture. For endpoint analyses the total amounts of CO_2 and $^{14}CO_2$ in solutions were measured after acidification of a culture sample (3 to 5 ml) in a sealed 24.0-ml serum vial. Incorporation of radioactivity into cell material was measured by the procedure of Weimer and Zeikus (21). Cells were harvested on 0.4-μm polycarbonate filters (Bio-Rad Laboratories, Richmond, Calif.) and were washed four times with 20 ml of a solution containing 55 mM phosphate buffer (pH 7.0) and 40 mM sodium acetate. The filters were dried for 1 day at 60°C, weighed, digested in 0.6 N NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.) for 2 days at 60°C, neutralized with HCl and counted in Instagel.

Determination of yields, growth rates, and substrate utilization rates. Carboys were inoculated with cells that were pregrown on the substrate being tested for at least two successive transfers. Growth was monitored by dry weight determination. At each time point, two 100-ml samples were removed from carboys through tubing (diameter, 0.25 inch [0.635 cm]), filtered on dried and tared 0.4-μm membrane filters (Millipore Corp., Bedford, Mass.), and dried to a constant weight at 60°C. The homogeneity of the samples was ensured by vigorous mixing of the culture with a magnetic stirrer. Substrate concentrations were determined at each time point by gas chromatography. Yields were determined by the slope of the line from the plot of milligrams of cells per liter versus moles of substrate consumed per liter. The specific growth rate, yield, and rate of substrate consumption values are averages of duplicate experiments.

Analysis of enzymatic activities. Anaerobic cell extracts of *M. barkeri* and *Methanobacterium thermoautotrophicum* (used as a control) were prepared as previously described by Zeikus et al. (28). CO dehydrogenase activity was assayed by reduction of methyl

viologen in 100 mM Tris-hydrochloride (pH 8.1), as described by Daniels et al. (3). Pyruvate and α-ketoglutarate dehydrogenase were measured spectrophotometrically by reduction of methyl viologen (28), and isocitrate dehydrogenase was measured by NAD reduction, using the procedure of Weimer and Zeikus (22). The protein contents of cell extracts were determined by the method of Lowry et al. (10).

RESULTS

Unitrophic metabolism of acetate or methanol. Carboys containing 15 liters of PBB medium and either 100 mM methanol or 120 mM acetate were given a 10% inoculum of the *M. barkeri* acetate-adapted strain, and the substrate concentration and cell dry weight were monitored daily. The specific growth rate on methanol (0.037 h^{-1}) was more than twice that on acetate (0.014 h^{-1}). Likewise, the substrate consumption rate on methanol (9.4 mmol/g of cells per h) was more than twice that on acetate (3.6 mmol/g of cells per h). Notably, significant consumption of acetate continued long after the culture entered the stationary phase of growth. The concentration of acetate decreased from 105 to 70 mM, with no detectable increase in cell mass. Yields with acetate and methanol were 3.88 and 3.92 g/mol of substrate consumed, respectively. The specific growth rates, substrate consumption rates, and yields for the acetate-adapted strain of *M. barkeri* were equivalent in acetate basal medium and acetate complex medium.

Figure 1 shows a typical time course from gas production during unitrophic acetate fermentation in minimal medium and in the presence of [2-^{14}C]acetate. CH_4 and CO_2 were produced exponentially. Both $^{14}CO_2$ and $^{14}CH_4$ were produced at significant rates over the entire time studied.

The high proportion of the C-2 atom of acetate oxidized to CO_2 suggested that reducing equivalents were being generated in excess of the reducing equivalents required for cell carbon synthesis. Therefore, we examined the contributions of both acetate carbon atoms to CH_4 and CO_2 (Table 1). In these experiments, the amount of [1-^{14}C]acetate or [2-^{14}C]acetate transformed into end products or cells was determined at the end of growth. The ratio of the amount of CH_4 produced to the amount of CO_2 produced was very close to unity, and the average carbon recovery was 92 ± 3%. The average isotope recoveries were 99 ± 19% for [1-^{14}C]sodium acetate and 100 ± 9% for [2-^{14}C]sodium acetate. Both [2-^{14}C]acetate and [1-^{14}C]acetate was converted to $^{14}CH_4$ and $^{14}CO_2$. Approximately 14% of the CO_2 produced during fermentation originated from the methyl carbon of acetate, and 14% of the CH_4 produced originated from the carboxyl position. Conversely, 86% of the CO_2 was produced from the carboxyl group of acetate, and 86% of the methane was produced from the methyl moiety. More cell carbon was derived from the C-1 atom of acetate than from the C-2 atom, and this correlated with the greater amounts of CO_2 produced from the C-1 moiety.

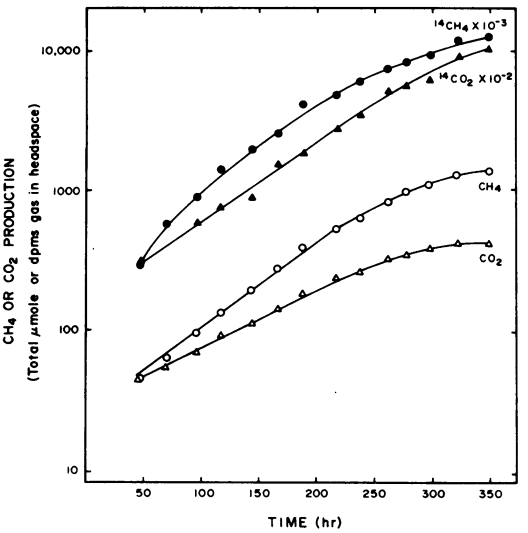

FIG. 1. $^{14}CH_4$ production and $^{14}CO_2$ production from [2-^{14}C]sodium acetate during unitrophic metabolism of the *M. barkeri* acetate-adapted strain. The experiments were performed in serum vials that contained 33 ml of PBB medium, 50 mM sodium acetate, an N_2 gas phase, 13,000 dpm of [2-^{14}C]acetate per μmol, and a 2-ml inoculum of cells pregrown on acetate.

To examine the possibility of direct CO_2 conversion to CH_4 during unitrophic acetate metabolism, we added approximately 13 μCi of $Na_2{}^{14}CO_3$ to logarithmic cultures grown in serum vials on medium that contained 50 mM sodium acetate and an N_2 gas phase. The cultures were incubated at 37°C, and at 5.5 and 20.5 h after the label was added, the gas phase was analyzed for the amount (micromoles) of CH_4, the radioactivity (disintegrations per minute) of $^{14}CH_4$, and the specific activity of $^{14}CO_2$. During this period, 140 μmol of CH_4 and 3.32×10^5 dpm of $^{14}CH_4$ were formed (specific activity, 2,370 dpm/μmol). The average specific activity of $^{14}CO_2$ was 45,500 dpm/μmol. Therefore, an estimated 5% of the CH_4 produced during this period originated from CO_2.

Mixotrophic metabolism of methanol and acetate. To determine the effect of methanol on the distribution of carbon atoms in acetate transformed to CH_4 and CO_2, an acetate-adapted strain was inoculated into a medium containing methanol and sodium acetate labeled in either the methyl position or the carboxyl position (Table 2). CH_4 and CO_2 were produced from both carbon atoms of acetate (as observed in

TABLE 1. Unitrophic metabolism of acetate by *M. barkeri* grown in minimal medium[a]

Position of label in acetate	Formation of:	Amt (μmol)	Radioactivity (dpm)	Sp act (dpm/μmol)	% Contribution of isotope	Wt of cells (mg)
$CH_3{}^{14}COOH$	Methane	920	1.60×10^{-6}	1,740	14[b]	
	CO_2	898	10.7×10^{-6}	11,900	86[b]	
	Cells		7.41×10^{-5}		50[c]	2.8
$^{14}CH_3COOH$	Methane	775	6.51×10^{-6}	8,400	86[b]	
	CO_2	768	1.17×10^{-6}	1,520	14[b]	
	Cells		3.11×10^{-5}		43[c]	1.8

[a] The experiments were performed in serum vials that contained 32 ml of PBB medium, 50 mM sodium acetate, either 13,900 dpm of [1-^{14}C]acetate per μmol or 10,900 dpm of [2-^{14}C]acetate per μmol, and the acetate-adapted strain. Cultures were incubated without shaking at 37°C for 14 days before analysis. The results represent the averages of triplicate determinations.

[b] Normalized value was calculated as follows: (specific activity of product/specific activity of substrate) × 100.

[c] Calculated as follows: (specific activity of cell carbon/specific activity of substrate) × 100, using 0.44 as the fraction of carbon in cells.

unitrophic metabolism), but the methyl moiety was oxidized to a far greater extent than in unitrophic metabolism. The ratio of radioactivity of $^{14}CO_2$ to radioactivity of $^{14}CH_4$ generated from [2-^{14}C]acetate after mixotrophic metabolism was 0.45, a threefold increase over that observed after unitrophic metabolism of acetate. The ratio of radioactivity of $^{14}CO_2$ to radioactivity of $^{14}CH_4$ generated from [1-^{14}C]acetate after mixotrophic metabolism was the same as observed after unitrophic metabolism of acetate.

We examined the simultaneous catabolism of acetate and methanol in time course experimental mixtures that contained either [2-^{14}C]acetate or $^{14}CH_3OH$. Mixotrophic metabolism of *M. barkeri* on a medium containing 50 mM sodium acetate and 50 mM methanol is shown in Fig. 2. $^{14}CH_4$ and $^{14}CO_2$ were produced exponentially from [2-^{14}C]acetate before methanol depletion from the medium. Likewise, $^{14}CH_4$ and $^{14}CO_2$ were produced exponentially from $^{14}CH_3OH$ during this same time period. However, $^{14}CH_4$ production from [2-^{14}C]acetate but not $^{14}CH_3OH$ continued to increase after 170 h, until the conclusion of the experiment. At the time of methanol depletion from the medium, the acetate concentration was 35 mM.

Rate constants for unitrophic and mixotrophic gas production. Table 3 shows the relationships between the initial medium substrate compositions and the rate constants observed for $^{14}CH_4$ and $^{14}CO_2$ production from [2-^{14}C]acetate and $^{14}CH_3OH$. The methane production rate constant for acetate (0.012 h^{-1}) was approximately the same as the growth rate constant observed in the acetate growth curves. During mixotrophic metabolism of 50 mM sodium acetate–50 mM methanol, the gas production rate constants from either $^{14}CH_3OH$ or [2-^{14}C]acetate increased significantly compared with the values observed from gas production during unitrophic metabolism of the substrates. In parallel experiments in which the methanol concentration was increased to 150 mM, the $^{14}CH_4$ production rate constants from $^{14}CH_3OH$ and [2-^{14}C]acetate were 1.5 and 0.46, respectively.

Enzymatic activities as a function of *M. barkeri* strain. Table 4 shows the specific activities of several dehydrogenases in cells of the acetate-adapted strain and in strain MS cells grown on H_2-CO_2 and on methanol. All cell extracts had high CO dehydrogenase activities, but the extracts of the acetate-adapted strain grown on acetate contained fivefold-higher specific activities than the extracts of strain MS grown on H_2-CO_2 or methanol.

TABLE 2. Mixotrophic metabolism of acetate by *M. barkeri* grown in a methanol minimal medium[a]

Position of label in acetate	Formation of:	Amt (μmol)	Radioactivity (dpm)	Sp act (dpm/μmol)	% Contribution of isotope
$CH_3{}^{14}COOH$	Methane	2,476	2.01×10^{-6}	811	5
	CO_2	1,434	12.5×10^{-6}	8,720	52
$^{14}CH_3COOH$	Methane	2,151	4.76×10^{-6}	2,210	26
	CO_2	1,361	2.12×10^{-6}	1,557	18

[a] The experiments were performed in serum vials that contained 32 ml of PBB medium, 50 mM sodium acetate, 50 mM methanol, either 16,800 dpm of [1-^{14}C]acetate per μmol or 8,530 dpm of [2-^{14}C]acetate per μmol, an N_2 gas phase, and a 2-ml inoculum of the acetate-adapted strain. The cultures were incubated without shaking at 37°C for 14 days before analysis. The results represent averages of triplicate determinations.

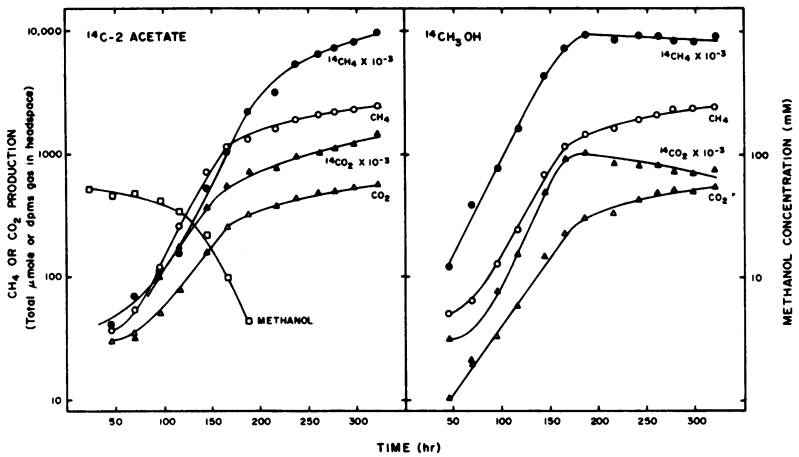

FIG. 2. $^{14}CH_4$ production and $^{14}CO_2$ production from $^{14}CH_3COOH$ and $^{14}CH_3OH$ during mixotrophic metabolism of the *M. barkeri* acetate-adapted strain on medium containing 50 mM methanol and 50 mM sodium acetate. The experiments were performed in serum vials that contained 33 ml of PBB medium, different substrate concentrations, an N_2 gas phase, either 14,000 dpm of [2-^{14}C]acetate per μmol or 12,000 dpm of $^{14}CH_3OH$ per μmol, and a 2-ml inoculum of cells pregrown on acetate.

α-Ketoglutarate dehydrogenase was not detected in extracts of the acetate-adapted strain or in strain MS, confirming the absence of a complete tricarboxylic acid cycle in *M. barkeri* (22). α-Ketoglutarate dehydrogenase activity in *M. thermoautotrophicum* extracts was not inhibited by the presence of extracts from *M. barkeri*. Pyruvate dehydrogenase and isocitrate dehydrogenase were present in *M. barkeri* grown on any of the three substrates. The levels of these enzymes were approximately the same in the acetate-adapted strain and in strain MS.

DISCUSSION

The specific growth rate, the rate of CH_4 production, and the rate of substrate utilization were substantially less for unitrophic growth of *M. barkeri* on acetate than for growth on methanol, whereas the weights of the cells produced per mole of substrate consumed were equivalent for the two substrates. The slower growth rate of *M. barkeri* with acetate as the sole carbon and energy source appears to be linked to a slower rate of substrate utilization, which may be limited either by the rate at which acetate is taken up by the cells or by the inherent reaction rate of one or more of the acetate-catabolizing enzymes. The presence of yeast extract did not affect the rate of acetate metabolism, the specific growth rate, or the yield of the acetate-adapted strain of *M. barkeri*. This indicates that these medium components do not interact with

TABLE 3. Rate constants of $^{14}CH_4$ and $^{14}CO_2$ formation by *M. barkeri* during unitrophic or mixotrophic metabolism[a]

Substrate(s)	Rate constants[b]			
	[2-^{14}C]acetate		$^{14}CH_3OH$	
	$^{14}CH_4$ (×10²)	$^{14}CO_2$ (×10²)	$^{14}CH_4$ (×10²)	$^{14}CO_2$ (×10²)
50 mM acetate	1.6 ± 0.15	0.77 ± 0.26		
50 mM methanol			2.5 ± 0.6	2.5 ± 0.3
50 mM methanol + 50 mM acetate	2.5 ± 0.07	2.4 ± 0.2	4.0 ± 0.8	4.3 ± 0.6

[a] The experiments were performed in serum vials that contained 32 ml of PBB medium, the substrates indicated, either 3,300 to 12,000 dpm of [^{14}C]methanol per μmol or 3,800 to 14,000 dpm of [2-^{14}C]acetate per μmol, an N_2 gas phase, and a 2-ml inoculum of the acetate-adapted strain. Cultures were incubated without shaking at 37°C. The values are average values ± standard deviations from triplicate experimental cultures.

[b] The rate constant (K) for $^{14}CH_4$ or $^{14}CO_2$ was derived from the following equation: $\ln P = \ln Po + KpT$, where T = time, P = radioactivity of $^{14}CH_4$ or $^{14}CO_2$ (in disintegrations per minute) in the headspace at time T, and Po is the initial amount of $^{14}CH_4$ or $^{14}CO_2$. The rate constants for $^{14}CH_4$ and $^{14}CO_2$ were obtained from the slope of a plot of ln(radioactivity of $^{14}CH_4$) or ln(radioactivity of $^{14}CO_2$) versus time. Slopes were determined by linear regression analyses of the logarithmic portions of the plots.

TABLE 4. Comparison of oxidoreductase activities in the wild-type and acetate-adapted strains of *M. barkeri*[a]

Strain	Energy source	Activity (nmol/min per mg of protein)			
		CO dehydrogenase	Pyruvate dehydrogenase	α-Ketoglutarate dehydrogenase	Isocitrate dehydrogenase
MS	Methanol	1,050 ± 400	78 ± 15	ND[b]	78 ± 15
	H_2-CO_2	1,100 ± 400	71 ± 36	ND	41 ± 2
Acetate adapted	Acetate	5,000 ± 500	78 ± 48	ND	37 ± 14

[a] Assays were conducted at 37°C as described previously (3, 2, 28) in 100 mM Tris-hydrochloride (pH 8.1). The reaction mixture for CO dehydrogenase contained 5 mM methyl viologen, 0.4 ml of 100% CO, and 5 μl of cell extract. The reaction mixture for pyruvate dehydrogenase contained 5 mM methyl viologen, 100 μM coenzyme A, 10 mM pyruvate, and 20 μl of cell extract. The reaction mixture for α-ketoglutarate dehydrogenase contained 5 mM methyl viologen, 100 μM coenzyme A, 10 mM α-ketoglutarate, and 20 μl of cell extract. The reaction mixture for isocitrate dehydrogenase contained 250 μM $NADP^+$, 5 mM $MgCl_2$, 10 mM isocitrate, and 20 μl of cell extract. Cell-free extracts contained 15 to 25 mg of protein per ml. Values are means ± standard deviations of multiple determinations.

[b] ND, Not detectable (<0.01 nmol/min per mg of protein).

acetate in the production of CH_4 or CO_2 and that the acetate-adapted strain which we used differed significantly from other *M. barkeri* strains (20, 30). Special note should be taken of the continued consumption of acetate after the cessation of growth. An accurate yield could not be obtained by sampling near the end of the logarithmic consumption of acetate since this did not coincide with the actual logarithmic phase of growth and would have resulted in an underestimation of the yield. This may explain the discrepancy between the yields which we found for growth on acetate and those reported elsewhere (17).

Previous studies of acetate fermentation by *Methanosarcina* demonstrated little if any oxidation of the methyl group or reduction of the carboxyl group to CH_4. *Methanosarcina* strain 227 transformed 2 to 3% of the methyl group of acetate to CH_4 (17). Winter and Wolfe (23) reported that *M. barkeri* strain MS oxidized 5% of the methyl group and reduced 5% of the carboxyl group of acetate. The methyl moiety of acetate was not oxidized to a significant extent except in the presence of methanol or H_2-CO_2 in previous studies with *M. barkeri* strain MS adapted to grow on acetate in complex medium (20). Our strain of *M. barkeri* that is adapted to grow on acetate as the sole carbon and energy source has been maintained on defined medium for more than 1.5 years, and the significant oxidation of the methyl group first discovered 1 year ago has occurred at similar levels until now. The range observed for the percentage of the C-2 atom of acetate transformed to CO_2 during numerous experiments was 10 to 23%. Oxidation of the methyl group of acetate is formally an eight-electron process, as is the reduction of CO_2 to CH_4 (2, 19). Thus, the acetate-adapted strain of *M. barkeri* exhibits the expected stoichiometry; namely, for every methyl converted to CO_2, one carboxyl is transformed to CH_4. It is possible that the acetate-adapted strain of *M. barkeri* is a metabolic mutant which has an accelerated flow of carbon through already existing oxidation and reduction pathways. This hypothesis needs to be tested and will be investigated in the future. In summary, it appears that two pathways of methane formation are possible during growth of *M. barkeri* on acetate. The first is consistent with the pathway described by Barker (2) and accounts for 85% of the methane produced. The second is a redox process, involving the oxidation of the C-2 atom of acetate and the reduction of either a formyl intermediate or CO_2 to methane.

Clearly, the acetate-adapted strain of *M. barkeri* was capable of simultaneous catabolism of acetate and methanol at the concentrations examined. Nonetheless, methanol significantly influenced acetate catabolism. At equal molar substrate concentrations, the rate of methane formation from acetate was greater than that observed during unitrophic metabolism. This rate was associated with an increase in the rate of oxidation of the methyl group of acetate but not with an increase in the reduction of the carboxyl group. This finding confirms previous conclusions (16, 20) concerning acetate fermentation by *Methanosarcina* and suggests that reducing equivalents generated from increased methyl group oxidation are used primarily to reduce methanol of CH_4. It is worth noting that the production of CO_2 from methanol did not decrease the ratio of $^{14}CH_4$ to $^{14}CO_2$ formed from [1-^{14}C]acetate. In medium containing 150 mM methanol and 50 mM acetate, the rate of methane formation from acetate or methanol was lower than the rate observed for unitrophic metabolism in medium containing substrate at a concentration of 50 mM. This, along with the increased cultural lag time observed, suggests

that the increased concentration of methanol was toxic to the acetate-adapted strain. However, this finding does demonstrate that the concentration of methanol can influence the rate of acetate utilization, although this influence does not appear to be the result of a phenomenon resembling catabolite repression, as reported for *Methanosarcina* strain 227 (16).

CO dehydrogenase was present in *M. barkeri* regardless of the growth substrate or strain used. The specific CO dehydrogenase activity for strain MS cells grown on methanol and H_2-CO_2 was higher than the activity previously reported for *M. barkeri* strain UBS (3). The high activity of CO dehydrogenase in methanogens suggests an important role in intermediary metabolism. It is unlikely that this enzyme functions in environmental CO consumption because cells maintained in the absence of CO for many years still synthesize the enzyme constitutively. Therefore, it is possible that the oxidation of CO by CO dehydrogenase is fortuitous and that the actual substrate of the enzyme is not CO. CO dehydrogenase has been partially purified from an obligate anaerobe and has been shown to participate in the synthesis of acetate in *Clostridium thermoaceticum* (H. L. Drake, S. I. Hu, and H. G. Wood, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, K42, p. 144). Thus, CO dehydrogenase may function in the synthesis of a two-carbon anabolic intermediate during growth of *M. barkeri* on one-carbon compounds.

The fivefold-greater CO dehydrogenase activity in the acetate-adapted strain of *M. barkeri* than in strain MS suggests a possible role for this enzyme during growth on acetate. One hypothesis for future experimentation is that CO dehydrogenase functions in the splitting of acetate to methyl and formyl intermediates. This reaction is in the reverse direction of the proposed function for CO dehydrogenase in *C. thermoaceticum*, in which CO dehydrogenase may operate as a transcarboxylase during acetate synthesis via reductive carboxylation of a methyl corrinoid (H. G. Wood, personal communication). CO dehydrogenase is present in *M. barkeri* at levels which can be considered catabolic. The activity of this enzyme in the acetate-adapted strain is comparable to the activity of a known catabolic enzyme (hydrogenase). The specific activity of hydrogenase is 6 μmol/min per mg of protein, as measured by methyl viologen reduction (21). An enzyme participating in methanogenesis from acetate would have to operate at a minimum in vivo activity of 120 nmol/min per mg of protein. CO dehydrogenase fulfills this requirement easily. In addition, corrinoid antagonists (i.e., alkyl halides) reportedly inhibit the CO dehydrogenase of *Clostridium* species (4). If this also occurs with the CO dehydrogenase of *M. barkeri*, then this inhibition may explain the inhibition of growth and methanogenesis by iodopropane when *M. barkeri* is grown on acetate as a carbon and energy source (11). Notably, *M. barkeri* contains higher levels of corrinoids than several other non-acetate-catabolizing methanogens (8).

Wild-type strains of *M. barkeri* (e.g., strains MS and UBS) isolated from nature on methanol do not grow on acetate as the sole carbon and energy source (20, 30). Winter and Wolfe (23) reported that an acetate-adapted strain of *M. barkeri* does not display a prolonged lag of many days to readapt to growth on acetate after transfer from growth on one-carbon compounds. The data presented in this paper show that our acetate-adapted strain grows on acetate as a sole carbon and energy source, that it significantly oxidizes the C-2 atom of acetate to CO_2, and that its cells contain elevated levels of CO dehydrogenase. These lines of evidence suggest that the acetate-adapted strain represents a metabolic mutant of *M. barkeri* type strain MS. Further experiments are in progress to establish this feature of the acetate-adapted strain better. Based on our understanding of the metabolic features described above for the acetate-adapted strain and the reactions proposed for CO dehydrogenase present in methanogens it is evident why *M. barkeri* strains isolated on methanol or H_2-CO_2 or *M. thermoautotrophicum*, which grows on H_2-CO_2, can transform acetate to methane at low rates (30).

Parallel to the findings reported here, several other investigations have supported the hypothesis that *Methanosarcina* is capable of simultaneous catabolism of acetate and a second energy source. Methanol, acetate, and H_2-CO_2 were simultaneously produced and transformed to methane during growth of a pectin-fermenting coculture containing *M. barkeri* and *Clostridium butyricum* (B. Schink and J. G. Zeikus, J. Gen. Microbiol., in press). Hutten et al. (6) and Scherer and Sahm (15) observed simultaneous consumption of acetate and methanol during growth of *M. barkeri*, although radioactive tracer studies were not performed.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, and by grant DE-AC02-80ER10705 from the Department of Energy, Division of Basic Biological Sciences. J.A.K. was supported by a fellowship from the National Science Foundation. R.H.W. was supported by a Public Health Service Cellular and Molecular Biology Training Grant from the National Institutes of Health.

We thank John Lobos for excellent technical assistance in adapting *M. barkeri* to grow on acetate as the sole carbon and energy source and William Kenealy for valuable discussions.

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