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Effect of H_2–CO_2 on Methanogenesis from Acetate or Methanol in *Methanosarcina* spp.

THOMAS J. FERGUSON AND ROBERT A. MAH*

Division of Environmental and Nutritional Sciences, School of Public Health, University of California, Los Angeles, California 90024

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Growth of *Methanosarcina* sp. strain 227 and *Methanosarcina mazei* on H_2–CO_2 and mixtures of H_2–CO_2 and acetate or methanol was examined. The growth yield of strain 227 on H_2–CO_2 in complex medium was 8.4 mg/mmol of methane produced. Growth in defined medium was characteristically slower, and cell yields were proportionately lower. Labeling studies confirmed that CO_2 was rapidly reduced to CH_4 in the presence of H_2, and little acetate was used for methanogenesis until H_2 was exhausted. This resulted in a biphasic pattern of growth similar to that reported for strain 227 grown on methanol-acetate mixtures. Biphasic growth was not observed in cultures on mixtures of H_2–CO_2 and methanol, and less methanol oxidation occurred in the presence of H_2. In *M. mazei* the aceticlastic reaction was also inhibited by the added H_2, but since the cultures did not immediately metabolize H_2, the duration of the inhibition was much longer.

Radioactive isotopic labeling studies indicate at least two main substrates for methane production from nonmarine fermentations in nature: (i) CO_2 reduction by H_2 to CH_4 and H_2O, and (ii) acetate splitting with reduction of the methyl group and oxidation of the carboxyl group to form CH_4 and CO_2, respectively (3, 5, 15, 18–21). In mixed methanogenic ecosystems (i.e., anaerobic digesters), both types of substrates are continuously turning over. Although H_2 is not detectable at substrate concentrations because of its rapid turnover, it accounts for ca. 30% of the methane formed in digester fermentations (12). It is also now well appreciated as an intermediate in oxidation-reduction reactions involving interspecies H_2 transfer. Its potential role in regulating other methanogenic substrates has not been reported, although it accumulates when methane formation is inhibited in anaerobic digesters or has not yet occurred in batch enrichment cultures.

Methanosarcina sp. strain 227 may utilize H_2–CO_2, acetate, methanol (10), or trimethylamine (unpublished finding) for methanogenesis; however, it preferentially metabolizes methanol before acetate in mixtures of methanol and acetate (18). Trimethylamine was metabolized before acetate by *Methanosarcina barkeri* strain Fusaro grown on mixtures of these two substrates (4). Evidence is presented that methanogenesis from acetate was limited in the presence of added H_2, but methanogenesis from methanol was not similarly affected. In *Methanosarcina mazei*, methanogenesis from acetate ceased in the presence of added H_2, and methanogenesis from H_2–CO_2 did not immediately occur. An inhibitory effect of H_2 (7, 10, 13), trimethylamine (4), or methanol (19) on the aceticlastic reaction in *Methanosarcina* spp. was previously reported. This inhibition was not entirely supported by Scherer and Sahm (17) or Hutten et al. (7). Methanol and acetate were reportedly used simultaneously (17), whereas H_2 inhibited acetate utilization only in the absence of CO_2 (7). We present new information on the effect of H_2–CO_2 on acetate and methanol utilization in *Methanosarcina* spp. and reconcile these apparent differences.

(Portions of these results were previously reported [T. J. Ferguson and R. A. Mah, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, I78, p. 108].)

MATERIALS AND METHODS

Bacterial strains. *Methanosarcina* sp. strain 227 and *M. mazei* (S-6), formerly *Methanococcus mazei* (R. A. Mah and D. A. Kuhn, submitted for publication), were maintained in stock culture as previously described (9, 10).

Culture media. Two types of mineral media were used for growth yield determinations. Medium 1 (carbonate buffered) contained the following (per liter of tap water): NH_4Cl, 1.0 g; $K_2HPO_4 \cdot 3\ H_2O$, 0.4 g; $MgCl_2 \cdot 6\ H_2O$, 0.1 g; 0.10% resazurin, 1.0 ml; $NaHCO_3$, 1.0 g. Although the media in our studies were first made up in tap water, distilled water was later substituted and adopted without any change in

our findings. The medium was prepared and dispensed in 50- to 100-ml portions into 250-ml boiling flasks or serum vials (1) under O_2-free N_2–CO_2 (70:30) and stoppered with butyl rubber stoppers that were wired in place. Media were autoclaved at 120°C and 15 lb/in^2 for 20 min. Before inoculation, 3.0 ml of a sterile, anaerobic solution of 1% Na_2S and 5% Na_2CO_3 was added to each flask. Mineral medium 2 (phosphate buffered) consisted of the same inorganic salts as medium 1, with the addition of 10.7 g of $Na_2PO_4 \cdot 7 H_2O$ and the deletion of $NaHCO_3$. The medium was prepared and dispensed under O_2-free N_2 (6). Before inoculation, 3.0 ml of 1% Na_2S per 100 ml of medium was added to each flask. The final pH of both mineral media was 6.9 to 7.1.

The complex medium for growth yield and substrate utilization studies was the 0.2% yeast extract and Trypticase (BBL Microbiology Systems, Cockeysville, Md.) medium (with sodium bicarbonate) described previously (18) except that distilled water was used in place of tap water. Acetate (0.05 to 0.10 M), methanol (0.10 M), or H_2–CO_2 (70:30; 250 KPa) were added as growth substrates where noted.

Culture conditions. Cultures were maintained by transfer at 3- to 5-day intervals for cells on complex H_2–CO_2 or methanol media, 5- to 7-day intervals for the defined H_2–CO_2 medium, and 7- to 10-day intervals for the complex acetate medium. Inocula of 1 to 2% were used. Stock cultures were grown on acetate or methanol at 37°C without shaking. Stock cultures on H_2–CO_2 and all cultures used in the substrate and growth studies were shaken at 37°C in a reciprocating-style water bath (model 2156; Research Specialties, Inc., Richmond, Calif.) operated at speed setting 6 (120 strokes per min).

Analysis of gases. Methane and carbon dioxide were analyzed as previously described (2). Hydrogen was determined by using a Loenco model 2000 gas chromatograph equipped with a thermal conductivity detector (60 mA) and a 2-ft by 0.25-in. (ca. 60.96- by 0.63-cm) aluminum column packed with 80-100 mesh Poropak Q (Applied Sciences Laboratories, State College, Pa.). The carrier gas was 100% N_2 (60 ml/min), and the injector, column, and detector were operated at 30°C for 0.10- to 0.50-ml samples.

Cell growth yields. Cell yields were determined near the peak of exponential growth for 100-ml culture volumes of strain 227 grown on H_2–CO_2. Typically the flasks were regassed twice before completion of the experiment. Cells were harvested by use of a clinical centrifuge, collected on membrane filters (Gelman 47 mm, 0.45-μm pore size), and dried to constant weight with a vacuum oven at 60 to 70°C as previously described (19).

TABLE 1. Summary of growth yield data on *Methanosarcina* sp. strain 227 grown in H_2–CO_2

Medium[a]	Yield (mg/mmol)		No. of determinations	SD
	Range	Mean		
Complex medium	6.71–10.35	8.38	20	0.88
Mineral medium 1 (carbonate buffer)	5.3–9.5	6.4	7	1.6
Mineral medium 2 (phosphate buffer)	2.79–7.90	5.40	7	1.87

[a] See the text for the composition of medium 1 and medium 2.

Labeling studies. Sterile, anaerobic, radioactive substrates were prepared in culture tubes as previously described (18). Radioactive substrates were added to yield media concentrations of 0.01 to 0.10 μCi/ml. Radioactivity in the gaseous fermentation products was determined by the method of Baresi et al. (2) by assaying the headspace. Dissolved gases were calculated from Henry's law (28).

Isotopes. Sodium [2-^{14}C]acetate (specific activity, 98 mCi/mmol); sodium [^{14}C]carbonate (specific activity, 53 mCi/mmol); and [^{14}C]methanol (specific activity, 3.9 mCi/mmol) were purchased from Schwartz/Mann, Orangeburg, N.Y.

RESULTS

Growth studies. *Methanosarcina* sp. strain 227 did not exhibit a lag in growth or methanogenesis when transferred from acetate to H_2–CO_2 in complex medium; it had a doubling time of ca. 9 h. However, growth of strain 227 on H_2–CO_2 in mineral medium required prior growth on H_2–CO_2 in complex medium containing yeast extract and Trypticase. Once growth on H_2–CO_2 was initiated, strain 227 could be transferred and maintained on mineral medium. The results of several growth yield determinations for both mineral and complex media are summarized in Table 1. Growth on H_2–CO_2 in mineral medium was characteristically slower than in complex medium, with doubling times between 12 and 24 h. The average growth yields were also considerably lower, with greater variations in yields. *M. mazei* did not grow well enough on H_2–CO_2 in complex or mineral medium to obtain any growth yield data.

Substrate utilization. Radioactive labeling experiments were performed with cells pregrown on complex acetate medium and inoculated into complex acetate medium containing either 100% N_2 or an H_2–CO_2 (70:30) atmosphere. The following results were obtained for *Methanosarcina* sp. strain 227. (i) H_2–CO_2 was used immediately as a methanogenic substrate when cells were pregrown on acetate. Once H_2 was exhausted, the reduction of CO_2 ceased. (ii) Methanogenesis from acetate lagged in the presence of H_2–CO_2, but once H_2 was exhausted, methanogenesis from acetate was rapid and complete. (iii) In the absence of H_2–CO_2, virtually no CO_2 was reduced to methane, and methanogenesis from acetate did not lag. Similar results were obtained whether the flasks contained an atmosphere of 100% N_2 or N_2–CO_2 (70:30).

These results indicated that the utilization of acetate might be regulated in response to H_2 concentration. A more refined time course study was initiated to examine methanogenesis from acetate in the presence of H_2. Strain 227 was

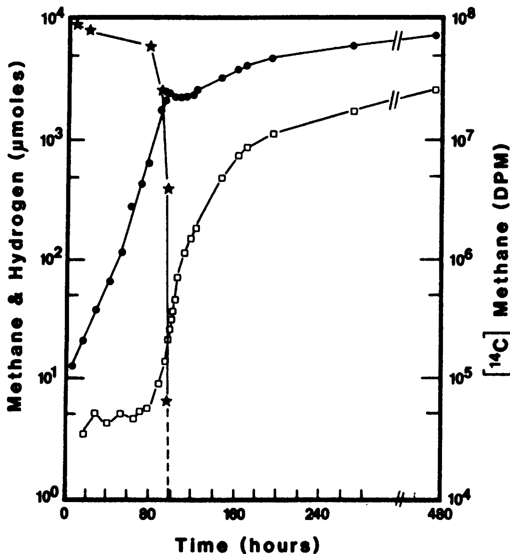

FIG. 1. *Methanosarcina* sp. strain 227 pregrown in complex medium with 0.05 M sodium acetate. Immediately after inoculation of fresh medium the flasks were gassed with H_2–CO_2 (70:30; 250 KPa). The medium contained 100 ml of complex medium, 0.05 M sodium acetate, and 10 µCi $^{14}CH_3COO^-$). Symbols: ★, H_2; ●, CH_4; □, $^{14}CH_4$.

pregrown on complex acetate medium and inoculated into fresh complex acetate medium containing [2-^{14}C]acetate and H_2–CO_2 (Fig. 1). During the first 90 h, methanogenesis, presumably from H_2–CO_2, proceeded exponentially. When H_2 was exhausted, between 98 and 99 h, less than 1% of the acetate initially present was converted to methane via the aceticlastic reaction. After 99 h the aceticlastic reaction resumed, and all methane was derived from acetate.

These results (Fig. 1) confirmed the existence of a lag period for the metabolism of acetate to methane in the presence of H_2–CO_2. To examine the effect of H_2 on a large aceticlastic population of strain 227, we grew the *Methanosarcina* sp. in acetate medium and then pressurized the flasks with H_2–CO_2 (Fig. 2). The addition of H_2–CO_2 inhibited methanogenesis from acetate. H_2 was exhausted rapidly, and methanogenesis from acetate then resumed. When H_2 was replenished (data not shown), inhibition of methanogenesis from acetate continued. In control cultures pressurized under N_2–CO_2, methanogenesis was unaffected (data not shown).

Such phenomena were not observed for all aceticlastic methanogens. In *M. mazei*, for example, acetate was not metabolized in the presence of H_2, and H_2 itself was not oxidized throughout the 12-day experimental period (Fig. 3). The addition of N_2–CO_2 had no effect on the aceticlastic reaction in *M. mazei* (Fig. 4). Zinder and Mah (28) reported a similar finding in *Methanosarcina* sp. strain TM1, a thermophilic aceticlastic methanogen that did not metabolize H_2.

Cultures of strain 227 pregrown on H_2–CO_2 for several transfers were also examined with radioactively labeled substrates. In complex acetate medium containing H_2–CO_2, very little [2-^{14}C]acetate was converted to $^{14}CH_4$ before the disappearance of H_2. However, once H_2 was depleted, methanogenesis from acetate was immediate and as rapid as in cultures pregrown on acetate. When cells pregrown on H_2–CO_2 were transferred to complex medium containing acetate as the sole methanogenic substrate, a growth lag of 4 to 8 weeks occurred. During this lag there was no visible change in culture turbidity, and little methane was produced. When growth finally occurred, it resembled that of acetate-grown cultures.

The formation of $^{14}CH_4$ and $^{14}CO_2$ by cells pregrown on acetate, methanol, or H_2–CO_2 was determined by assaying the headspace gases of cultures acidified with HCl after growth and methanogenesis had ceased (Tables 2 and 3). In Table 2, the final amount of $^{14}CH_4$ produced from [2-^{14}C]acetate by cells pregrown on acetate was similar regardless of the presence or absence of H_2. This indicated that the quantity of acetate ultimately catabolized to CH_4 was similar under these two conditions. However, the

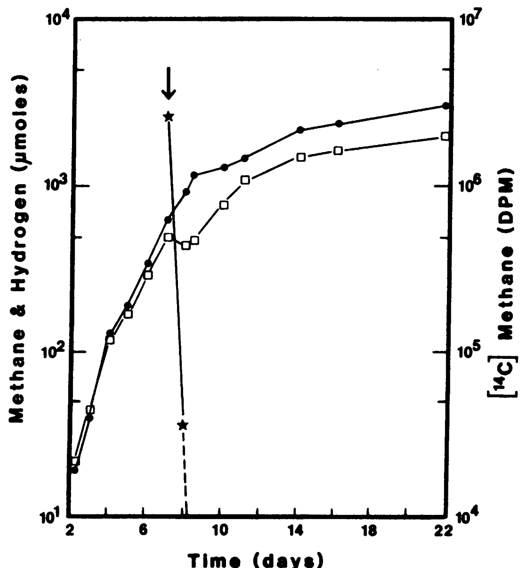

FIG. 2. *Methanosarcina* sp. strain 227 growing in complex 0.05 M sodium acetate medium with 5 µCi of $^{14}CH_3COO^-$, gassed with H_2–CO_2 (70:30; 250 KPa) as indicated by the arrow. Symbols: ★, H_2; ●, CH_4; □, $^{14}CH_4$.

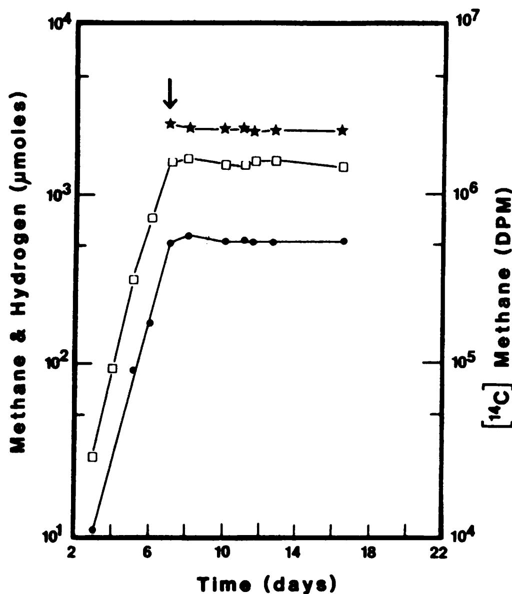

FIG. 3. *M. mazei* growing in complex 0.05 M sodium acetate medium with 5 µCi of $^{14}CH_3COO^-$, gassed with H_2–CO_2 (70:30; 250 KPa) as indicated by the arrow. Symbols: ★, H_2; ●, CH_4; □, $^{14}CH_4$.

amount of $^{14}CO_2$ produced from [2-^{14}C]acetate tended to be greater in the presence than in the absence of H_2. This showed that the methyl group of acetate may be oxidized to a greater extent when H_2 is available. The labeling of $^{14}CH_4$ in the presence of $^{14}CO_2$ and H_2 reflected the expected reduction of CO_2 by H_2. In methanol-grown cultures containing both methanol and H_2–CO_2 (Table 3), less H_2 was used to reduce CO_2 to CH_4 when methanol was present, and less methanol was oxidized when H_2 was present. By calculation based on the quantity of $^{14}CO_2$, the quantity of methane produced by oxidation and reduction of methanol (reaction 2, Table 4) accounted for approximately 53% of the radioactive methane formed in experiment 2, Table 3. The remainder of the radioactive methane (ca. 47%) was produced by reduction of $^{14}CH_3OH$ by H_2. Likewise, the oxidation of H_2 with CO_2 as the final electron acceptor was decreased by approximately 70% by the presence of methanol (Table 3). Presumably, the H_2 was diverted to methanol reduction.

DISCUSSION

The ability of *Methanosarcina* sp. strain 227 to metabolize acetate was influenced by the choice of substrate for pregrowth of cells. Strain 227 grew well on H_2–CO_2 when pregrown on acetate or methanol. Transfer of growing cells from H_2–CO_2 to acetate medium, however, resulted in an extended growth lag. The transfer from H_2–CO_2 to acetate in complex medium was facilitated when the H_2–CO_2-grown cells were first transferred to a complex medium containing both H_2–CO_2 and acetate. Thus, pregrowth of *Methanosarcina* sp. strain 227 on either H_2–CO_2 or methanol for several transfers may select for populations incapable of immediate acetate utilization. Growth lags varying between 4 and 8 weeks were observed for strain 227 pregrown on H_2–CO_2 and transferred to acetate medium. This phenomenon may be responsible for difficulties reported in culturing *M. barkeri* strain MS on acetate as the sole methanogenic substrate (25, 27). A similar lag was reported for the methanol-acetate combination (18). Conflicting reports of the simultaneous utilization of methanol-acetate (7, 8, 17) or H_2–CO_2 and acetate (7) by strains of *M. barkeri* may be explained by (i) actual metabolic differences between various aceticlastic strains or (ii) inadequate temporal observation of the initiation of methane formation without use of radioactive label. Real metabolic strain differences for utilization of these substrate combinations have not been reported. However, the simultaneous utilization of methanol and acetate by *M. barkeri* strain MS (17) may be explained by inadequate observation of the origin of methane; Scherer and Sahm (17) measured only total gas production (CH_4 and CO_2), and no radioactive substrates were employed. Similarly, the methanol-acetate studies of Hutten et al. (7) were based on gas production and not on specific measurement of radioactively labeled CH_4

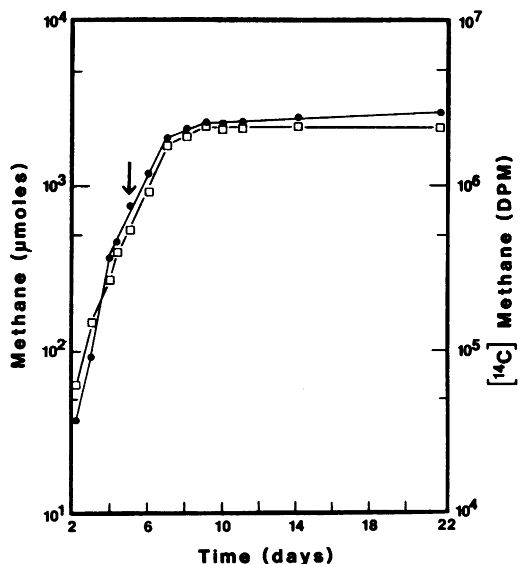

FIG. 4. *M. mazei* growing in complex 0.05 M sodium acetate medium with 5 µCi of $^{14}CH_3COO^-$, gassed with N_2–CO_2 (70:30; 250 KPa) as indicated by the arrow. Symbols: ●, CH_4; □, $^{14}CH_4$.

TABLE 2. Total radioactivity of spent cultures of *Methanosarcina* sp. strain 227 after acidification

Substrate(s)	Label	Headspace	dpm[a]		$^{14}CH_4/^{14}CO_2$ ratio	% Recovery[b]
			$^{14}CH_4$	$^{14}CO_2$		
Acetate plus H_2–CO_2	$^{14}CH_3COOH$	H_2–CO_2 plus N_2	2.1×10^7	5.4×10^5	39	97
Acetate	$^{14}CH_3COOH$	N_2	2.3×10^7	3.7×10^5	62	105
Acetate plus H_2–CO_2	$Na_2{}^{14}CO_3$	H_2–CO_2 plus N_2	2.8×10^6	1.9×10^6	1.5	85
Acetate	$Na_2{}^{14}CO_3$	N_2	6.4×10^4	4.6×10^6	0.014	84

[a] Disintegrations per minute calculations include correction for soluble gases remaining in culture fluid.
[b] Recovery was calculated as 100 [(disintegrations per minute recovered in gases)/(disintegrations per minute added)].

and CO_2. Hence, no conclusions can be made from these experiments regarding any short-term regulatory effects of H_2–CO_2 or methanol on acetate catabolism to CH_4 and CO_2. Blaut and Gottschalk (4) recently reported biphasic growth of *M. barkeri* strain Fusaro in which trimethylamine was preferentially metabolized before acetate.

Growth yields for strain 227 in complex medium containing H_2–CO_2 were similar to those reported for strain MS in defined medium (24), but higher than those reported for non-aceticlastic methanogens (16, 22). The higher H_2–CO_2 growth yields for aceticlastic versus non-aceticlastic methanogens may be due to differences in growth conditions as well as physiological characteristics. Hutten et al. (7) reported growth yields on methanol and acetate for strains MS, but Y_{CH_4} was not determined for H_2–CO_2. Growth yields previously reported for strain 227 in complex medium on methanol and acetate (18) are compared with the H_2–CO_2 yield in Table 4. The growth yield for each substrate was divided by the change in free energy for that reaction as shown in Table 4 ($Y_{CH_4}/\Delta G^{0\prime}$). This ratio showed that the energy formed per molecule of CH_4 produced was similar for each of the three substrates.

Growth of strain 227 on acetate of H_2–CO_2 in mineral medium was characteristically much slower than in complex medium. In addition, strain 227 could not be successfully transferred from mineral acetate medium to mineral H_2–CO_2 medium, probably because of some nutritional requirement as previously suggested by L. Baresi (Dr.P.H. thesis, University of California, Los Angeles, 1978). To initiate growth, it was necessary to use an inoculum of cells pregrown on H_2–CO_2 in complex medium.

The nutritional status of the cells is reflected in the differences in growth yields in mineral versus complex media. The average yield for strain 227 grown on H_2–CO_2 in mineral medium was 5.4 to 6.4 mg/mmol of methane, whereas in complex medium it was 8.4 mg/mmol of methane produced. Hutten et al. (7) reported a 1.5-fold increase in growth yield (acetate or methanol) for strain MS grown in complex versus defined media. Lower growth yields and longer generation times in mineral versus complex media corresponded to expected differences based on the availability of assimilable carbon precursors.

Growth conditions for the inoculum were important for determining the subsequent acetate-utilizing ability of *Methanosarcina* sp. strain 227. Three or four transfers of the inoculum in complex medium containing H_2–CO_2, but no acetate, resulted in diminished ability to use acetate immediately as the sole methanogenic substrate. However, three or four transfers of the inoculum in complex medium containing H_2–CO_2 alone followed by transfer to a complex H_2–CO_2 medium containing acetate showed no lag in acetate utilization upon exhaustion of H_2. Subsequent transfer of such a H_2–CO_2–acetate-grown culture to medium containing acetate as the sole methanogenic substrate also resulted in immediate growth. Winter and Wolfe also observed that pregrowth conditions affected subsequent acetate-utilizing ability for *M. barkeri* strain MS (26). It is possible that synthesis of intermediates necessary for utilization of acetate

TABLE 3. Methanol plus H_2–CO_2 mixed substrate experiment with *Methanosarcina* sp. strain 227

Substrates	Label	dpm		$^{14}CH_4/^{14}CO_2$ ratio	% Recovery[a]
		$^{14}CH_4$	$^{14}CO_2$		
Methanol plus N_2–CO_2	$^{14}CH_3OH$	1.33×10^6	5.32×10^5	2.50	89
Methanol plus H_2–CO_2	$^{14}CH_3OH$	1.55×10^6	2.76×10^5	5.63	87
Methanol plus H_2–CO_2	$Na_2{}^{14}CO_3$	1.24×10^6	9.35×10^6	0.133	95
H_2–CO_2	$Na_2{}^{14}CO_3$	4.04×10^6	1.08×10^7	0.374	134

[a] See footnote *b* of Table 2.

TABLE 4. Comparison of growth yields and changes in free energy for methanogenic reactions with *Methanosarcina* sp. strain 227

Reaction	$\Delta G^{0\prime}$ (kJ/mol of CH_4)[a]	Y_{CH_4} (mg/mmol)[b]	$Y_{CH_4}/\Delta G^{0\prime}$ ratio (g/kJ)
$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$	−31.0	2.1	−0.068
$4CH_3OH \rightarrow 3CH_4 + HCO_3^- + H^+ + H_2O$	−105.9	5.1	−0.048
$HCO_3^- + H^+ + 4H_2 \rightarrow CH_4 + 3H_2O$	−135.1	8.4	−0.062

[a] Determined from tables (25°C) compiled by Thauer et al. (23).
[b] The data for acetate and methanol yields are from Smith and Mah (18).

may take place when H_2–CO_2 and acetate are present together, perhaps by synthesis of required enzymes or cofactors by energy supplied from H_2 oxidation. Such biosynthetic requirements may not be immediately met when H_2–CO_2-grown cells are transferred to medium containing acetate as the sole methanogenic substrate. These results indicate that growth of strain 227 on H_2–CO_2 as the sole methanogenic substrate may select for a cell population with limited acetate-utilizing ability. These findings could also reflect genetic differences within the H_2–CO_2-grown population. The isolation of a *Methanosarcina* species incapable of H_2–CO_2 utilization (28) and the results reported here for *M. mazei* indicate the actual existence of genetic differences among morphologically similar methanogens. McInerney and Bryant (13) reported a similar phenomenon for strain MS grown in yeast extract medium versus rumen fluid medium.

Zeikus et al. reported that *M. barkeri* and *Methanobacterium thermoautotrophicum* were unable to "ferment" acetate in the absence of hydrogen (27). In the presence of hydrogen, very small quantities of methane were produced by the reduction of both methyl and carboxyl carbons of acetate. Consequently, they proposed the following reaction for the origin of methane from acetate: $H^+ + CH_3COO^- + 4H_2 \rightarrow 2CH_4 + 2H_2O$. This reaction is thermodynamically favorable and has a $\Delta G^{0\prime}$ of about −83.6 kJ/mol of CH_4; however, it is not as favorable as the reduction of CO_2 with H_2 (Table 4). In any case, the reduction of acetate in this manner (27) is contrary to that reported by other workers (3, 5, 18, 19) and is probably not of consequence in the net formation of methane from acetate. We did observe a slight increase in the oxidation of the methyl group of acetate in the presence of H_2–CO_2 (Table 2). An increase in methyl group oxidation was also reported by Weimer and Zeikus for strain MS grown in the presence of H_2 (24, 25). Oxidation of the methyl group of acetate increased for acetate-grown cells in the presence of H_2–CO_2.

Growth and methanogenesis from acetate by strain 227 occurred without the addition of H_2. Furthermore, very little, if any, added $^{14}CO_2$ was reduced to methane in the absence of H_2. These results are similar to those previously reported for strain 227 growing on acetate and methanol (18). Strain 227 did not produce significant quantities of methane from acetate in the presence of H_2. The transition from H_2–CO_2 to acetate utilization occurred very rapidly, perhaps in response to the changing partial pressure of H_2. This shift in methanogenesis from H_2–CO_2 to acetate was reflected in the changing specific activity of methane (Fig. 1) derived from methyl-labeled acetate. The initial radioactivity in methane may be attributed to utilization of a small amount of the acetate (less than 0.2% of the total acetate pool) by residual enzymes carried over when the cells (pregrown on acetate) were transferred to the medium. When H_2–CO_2 was added, the specific activity of CH_4 fell because unlabeled CH_4 was formed from the unlabeled H_2–CO_2, thereby diluting the $^{14}CH_4$. After the exhaustion of H_2–CO_2 (98 to 99 h), the methanogenic aceticlastic reaction resumed, and the specific activity of methane increased (Fig. 1). A similar phenomenon, i.e., a decrease in acetate utilization in the presence of H_2, was recently reported for *M. barkeri* strain MS (13). Acetate dissimilation in acetate-using cultures of strain MS did not increase until H_2 was exhausted. These results are in apparent contrast to those reported by Hutten et al. (7), who observed acetate utilization in the presence of H_2–CO_2 for strain MS, but no acetate utilization in the presence of H_2 alone. These differences may be due to the use of stationary incubation conditions which resulted in H_2 limitation to the cells because of the low solubility of H_2. This hypothesis is supported by the reported low rates of growth and methanogenesis from H_2–CO_2 (7) when strain MS was incubated without shaking (less than one-half of the rates on acetate). The reported requirement for CO_2 (7) to relieve the inhibition by H_2 may be due to H_2 removal by reduction of available CO_2 to form CH_4. Our data show that the aceticlastic reaction in *M. mazei*, which metabolized H_2 slowly if at all, remained inhibited even in the presence of CO_2. Thus, the role proposed by Hutten et al. for CO_2 as an essential carbon supply for growth and methanogenesis may be secondary to its

role as an oxidant for removal of H_2.

Although diauxic growth occurred for strain 227 on acetate and H_2–CO_2 mixtures, the current results do not support the hypothesis that catabolite repression was responsible (18), since a shift to acetate metabolism occurred before the complete exhaustion of H_2. A low H_2 concentration also accompanied the shift from H_2 oxidation to acetate utilization in *M. barkeri* strain MS (13). Methanogenesis from acetate may be initiated because of diminished availability of H_2 for CO_2 reduction or because H_2 is not an effective regulator at low concentration.

Isolation of a *Methanosarcina* species capable of methanogenesis from acetate but not H_2–CO_2 is compatible with the hypothesis that two cell populations may exist, one capable of metabolizing only acetate and the other capable of metabolizing H_2–CO_2 (28). Likewise, the present results regarding H_2–CO_2 and acetate utilization for *M. mazei* support the proposal that heritable metabolic differences do exist among the aceticlastic methanogens.

Results of the methanol–H_2–CO_2 experiments indicated that methanogenesis from these two substrates was not sequential and that methanol reduction was stimulated by the addition of H_2. Methanol reduction by H_2 was also reported for a thermophilic strain of *Methanosarcina* sp. (11, 28) as well as *M. barkeri* strain MS (24), and this pathway may be common for other strains of *Methanosarcina* spp. Methanol was apparently the preferred electron acceptor when methanol and CO_2 were both available for H_2 oxidation to generate energy for cellular metabolic needs. Under these conditions, reductants generated from methanol oxidation for anabolic reactions were also decreased (11), since methanol was mainly reduced to methane. A coccus recently isolated by Miller and Wolin (14) grew only in the presence of both H_2 and methanol, but not with either alone.

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