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Growth and Methanogenesis by *Methanosarcina* Strain 227 on Acetate and Methanol

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Methanosarcina strain 227 exhibited exponential growth on sodium acetate in the absence of added H_2. Under these conditions, rates of methanogenesis were limited by concentrations of acetate below 0.05 M. One mole of methane was formed per mole of acetate consumed. Additional evidence from radioactive labeling studies indicated that sufficient energy for growth was obtained by the decarboxylation of acetate. Diauxic growth and sequential methanogenesis from methanol followed by acetate occurred in the presence of mixtures of methanol and acetate. Detailed studies showed that methanol-grown cells did not metabolize acetate in the presence of methanol, although acetate-grown cells did metabolize methanol and acetate simultaneously before shifting to methanol. Acetate catabolism appeared to be regulated in response to the presence of better metabolizable substrates such as methanol or H_2-CO_2 by a mechanism resembling catabolite repression. Inhibition of methanogenesis from acetate by 2-bromoethanesulfonate, an analog of coenzyme M, was reversed by addition of coenzyme M. Labeling studies also showed that methanol may lie on the acetate pathway. These results suggested that methanogenesis from acetate, methanol, and H_2-CO_2 may have some steps in common, as originally proposed by Barker. Studies with various inhibitors, together with molar growth yield data, suggest a role for electron transport mechanisms in energy metabolism during methanogenesis from methanol, acetate, and H_2-CO_2.

The methanogenic bacteria are common in anaerobic environments where organic matter is undergoing decomposition. In non-gastrointestinal environments, acetate is one of the major products of fermentation and serves as a principal precursor of methane (8, 11, 13, 17, 21). In nature, acetate is converted to methane via a reaction in which the methyl group is reduced to methane while the carboxyl group is oxidized to carbon dioxide (8, 13).

We recently reported the isolation of a strain of *Methanosarcina* (strain 227) which converts acetate to methane by a decarboxylation of acetate (12). This strain converts acetate at a much greater rate than previously reported (12, 24) and does so in the absence of added H_2. Evidence is presented here that sufficient energy for growth may be obtained by the decarboxylation of sodium acetate. This is contrary to views expressed by other investigators (6, 22, 24). Additional evidence is presented to show that methanogenesis from acetate is subject to regulation by better metabolizable substrates such as methanol or H_2-CO_2 and that metabolism of methanol and acetate may have some steps in common with methanogenesis from H_2-CO_2.

MATERIALS AND METHODS

Bacterial strains. *Methanosarcina* strain 227 was isolated from a Barker-type acetate enrichment culture as described previously (12).

Culture media and growth conditions. The culture medium employed for these studies is the same as the 0.2% yeast extract (YE; low YE) medium described previously (12) except, where indicated, the acetate concentration was reduced to 0.1 M. For some experiments, sodium bicarbonate and/or sodium sulfide was omitted from the medium. When sodium bicarbonate was omitted, the medium was sometimes buffered with 0.05 M potassium phosphate, pH 6.5. On this medium, doubling times of 24 to 36 h were typical. All media were prepared under an atmosphere of oxygen-free 100% N_2. Cultures were incubated at 35 to 37°C. Culture volumes of 50 ml were employed for experiments performed in 125-ml serum bottles, whereas 100-ml volumes were employed for experiments in 200-ml round-bottomed flasks or in 300-ml nephelometer flasks (Bellco Co., Vineland, N.J.). Serum bottles and flasks were stoppered with butyl rubber stoppers. Inocula of 1 to 3% were used for all experiments.

Methane determinations. Methane was determined by gas chromatography on Loenco or Aerograph gas chromatographs as described previously (1, 12), except appropriate temperature corrections were

made.

Acetate determinations. Sodium acetate concentrations were determined by gas chromatography of 1-ml samples of culture medium acidified with 0.1 ml of 12 N HCl. A Varian Aerograph 1200 gas chromatograph with a flame ionization detector employing a column (OD, 1.82 m by 6 mm; ID, 4 mm) of 80- to 100-mesh Chromosorb 101, using helium gas saturated with formic acid at a flow rate of 94 ml/min, was used. To prevent ghosting, the column was acidified with formic acid before use. Samples of 4 μl were injected into the gas chromatograph. Known standards of various acetate concentrations were used for calibrating the gas chromatograph at the time of use.

Absorbance measurements. Absorbances of cultures in the 300-ml nephelometer flasks were measured by Klett-Summerson photocolorimetry, using a green Klett filter as described previously (12).

Growth yield determinations. Growth yields from sodium acetate were determined on 100-ml volumes of cultures as described previously (12). Growth yields on methanol were determined on 100-ml volumes of 0.2% YE medium containing 0.1 M methanol but no sodium sulfide. Reduction of the sodium sulfide concentration or its complete omission from the YE–methanol growth medium prevented lysis of the cultures at the end of growth. Inocula of 1% were used for all growth yield determinations. The cultures were sacrificed, and cells were harvested and weighed after drying to constant weight as described for growth yield determinations on acetate.

Isotopes. Sodium [1-^{14}C]acetate (specific activity, 2.5 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass. Sodium [2-^{14}C]acetate (specific activity, 98 mCi/mmol), sodium [*U*-^{14}C]acetate (specific activity, 2.0 mCi/mmol), and [^{14}C]methanol (specific activity, 3.4 mCi/mmol) were purchased from Schwarz/Mann, Orangeburg, N.Y.

Labeling studies. Sterile, anaerobic, radioactive substrates were prepared in culture tubes under 100% N_2 by adding sufficient labeled substrate to boiled water to give a final concentration of 10 μCi/ml. The tubes were stoppered with butyl rubber stoppers and autoclaved at 120°C for 15 min. Labeling studies were performed with 50-ml cultures growing exponentially on 0.1 M sodium acetate or 0.26 M methanol in 125-ml serum bottles. The medium contained 0.2% YE buffered at pH 6.5 with sodium phosphate and was reduced with 0.01% Na_2S. $NaHCO_3$ was omitted from the medium. When the methane concentration reached 250 to 500 μmol in these cultures, labeled substrates were injected into the vessels via the hypodermic needle and syringe method.

The following sets of substrates and conditions were tested on cultures growing on unlabeled sodium acetate: (i) 10 μCi of $^{14}CH_3OH$ at a final estimated specific activity of 0.002 μCi/μmol injected at zero time; (ii) 20 ml of 100% H_2 and 10 μCi of $NaH^{14}CO_3$ at an estimated final specific activity of 0.002 μCi/μmol injected at zero time; (iii) 20 ml of 100% H_2 and 10 μCi of sodium [2-^{14}C]acetate at an estimated final specific activity of 0.002 μCi/μmol injected at zero time; (iv) control cultures injected with 10 μCi of sodium [1-^{14}C]acetate or sodium [2-^{14}C]acetate to give an estimated final specific activity of 0.002 μCi/μmol at zero time.

Cultures were incubated in a water bath at 37°C for 6.5 h. A hypodermic needle was inserted through the rubber stopper to collect 1-ml gas samples for injection into an Aerograph gas chromatograph. A Packard gas proportional counter was teamed with the gas chromatograph to assay for radioactivity in methane and carbon dioxide as described previously (12, 14).

Labeling studies showing sequential utilization of methanol and acetate for methanogenesis were performed with cultures containing 0.1 M acetate and 0.025 M methanol in 50 ml of 0.2% YE medium with 0.01% Na_2S in 125-ml serum bottles. Duplicate sets of cultures contained either sodium [2-^{14}C]acetate or [^{14}C]methanol in addition to the substrate mixtures. Final specific activities were 0.002 μCi/μmol. Cultures were inoculated with a 1%, week-old culture of strain 227 on sodium acetate. The cultures were then incubated at 35°C for 12 days. $^{14}CH_4$ and $^{14}CO_2$ in the gas phase were monitored by the method of Nelson and Zeikus (14) as described previously (12).

Inhibitor studies. Anaerobic solutions of the following methanogenic inhibitors at a 0.01 M concentration were prepared under 100% N_2 in boiled distilled water containing 0.05% cysteine-hydrochloride: 2,4-dinitrophenol, viologen dyes, and 2-bromoethanesulfonic acid (2-BES). The stock chloroform solution was prepared by saturating boiled water containing 0.05% cysteine-hydrochloride with chloroform.

Inhibitor studies were performed on duplicate 100-ml cultures growing on 0.2 M sodium acetate or on 0.26 M methanol in 200-ml round-bottomed flasks according to the following procedure. A 1% inoculum of strain 227 grown on sodium acetate or on methanol was introduced into fresh medium containing the same substrate. Inhibitors were injected via the hypodermic syringe and needle technique into exponentially growing cultures to give the desired final concentrations. Daily measurements of methane were taken throughout the experiment from the time of inoculation of the culture until 10 days after addition of inhibitor.

RESULTS

Stoichiometry of the conversion of acetate to methane. Initial short-term labeling experiments indicated that acetate was split to form methane and carbon dioxide during growth under 100% N_2 on sodium acetate (12). However, quantitative data during a longer period of growth are needed to establish with certainty that significant amounts of acetate are converted to methane and carbon dioxide during growth. Hence, an experiment was performed to determine the rates of acetate disappearance and methane formation over an entire growth period of a batch culture. Duplicate 200-ml, round-bottomed flasks containing 100 ml of 0.2% YE medium (0.05 M sodium acetate) in an atmosphere of 100% N_2 were inoculated with a 1% inoculum of strain 227 grown on sodium acetate. At the time intervals shown in Fig. 1, the gas atmosphere was assayed for methane and the culture liquid was assayed for acetate as described above.

Figure 1 shows that acetate was stoichiomet-

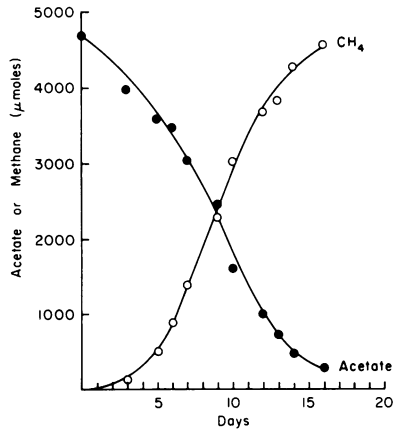

FIG. 1. *Relationship between methane production and acetate disappearance by cultures of Methanosarcina on 0.05 M sodium acetate in a 100% N_2 atmosphere.*

rically converted to methane during growth of a batch culture. The rate of acetate consumption was the same as the rate of methane formation, with a ratio of 1 mol of methane formed per mol of acetate consumed. These results show that acetate served as the energy source for growth and rule out the possibility that hydrogen from the medium (produced from yeast extract) is used to reduce acetate completely to methane as proposed by other investigators (23, 24); this latter reaction would produce 2 mol of methane per mol of acetate consumed.

Growth kinetics on sodium acetate. Because the standard-free energy change for the decarboxylation of acetate to methane and carbon dioxide is low (−7.4 kcal/mol [−31.0 kJ/mol] of acetate) compared with the standard-free energy change for the hydrolysis of ATP (−7.6 kcal/mol [−31.8 kJ/mol] [20]), the decarboxylation of acetate to methane and carbon dioxide might actually be attributed to co-metabolism, and energy for growth may instead come from the utilization of some other substrate in the culture medium (16, 24). To assess the role of acetate in the growth of strain 227, the effects of various concentrations of acetate on the growth rates and cell yields were examined.

A 1% inoculum of strain 227 grown on 0.2 M sodium acetate was introduced into 100 ml of liquid medium containing various concentrations of sodium acetate (see Fig. 2) in 0.2% YE (see above). Methane production was followed as a measure of growth (12). At the end of exponential growth, as measured by methane formation, cells were harvested, washed, dried, and weighed to determine cell yields. Figure 2 shows the kinetics of growth (methane formation) at the acetate concentrations indicated. It is apparent that methanogenesis from acetate is exponential at the higher acetate concentrations and that the doubling time for methanogenesis on acetate is decreased at acetate concentrations below 0.05 M. At the lower acetate concentrations the quantity of methane produced during the first 7 days is too low to measure accurately. However, despite this difficulty, a least-squares computation of the slopes of the lines gives specific rate constants (in reciprocal days) of 0.136, 0.241, 0.411, 0.450, and 0.442 for acetate concentrations of 0.003, 0.01, 0.03, 0.05, and 0.10 M, respectively. These values represent averages of duplicate or triplicate determinations. A plot of the specific rate constants (μ) against acetate concentrations gives roughly a hyperbolic curve (data not shown). A Hofstee plot of μ against acetate concentration (data not shown) gives a K_s for methanogenesis on acetate of approximately 5 mM. The dependence of the doubling time for methanogenesis on acetate at low concentrations of acetate indicates that energy for growth is obtained by the conversion of acetate to methane and that acetate is not simply co-metabolized in the presence of some other energy source. Because the kinetics of methane production are exponential, methane production is proportional to growth.

Since the quantity of methane formed is equal to the quantity of acetate consumed (Fig. 1), Fig. 3 shows that cell yield is a linear function of methane production and, hence, acetate utilization as determined by a regression analysis of

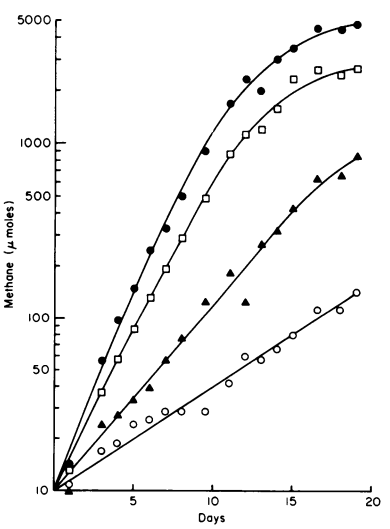

FIG. 2. *Methanogenesis by Methanosarcina strain 227 on medium containing 0.2% YE with the following concentrations of sodium acetate: 0.003 M (○); 0.01 M (▲); 0.03 M (□); 0.05 M (●). The growth curve at 0.1 M sodium acetate was similar to that at 0.05 M.*

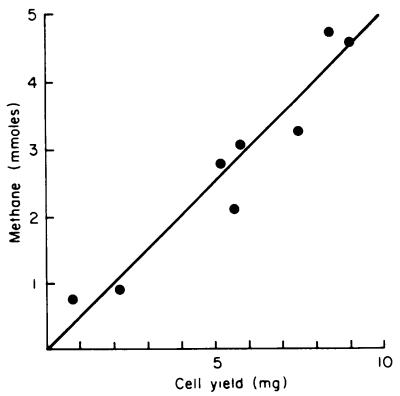

FIG. 3. *Cell yield as a function of methane production.*

the data. The cell yields at various concentrations of sodium acetate (Table 1) show that the average cell dry weight per millimole of methane formed (or acetate used) is 2.1 mg and is relatively consistent within the range of substrate concentrations tested. Each point represents results from a separate culture. For comparison, growth yields were also determined on methanol (see above). A yield constant of 5.1 mg/mmol of CH_4 was obtained (Table 2). Similar experiments on H_2–CO_2 gave molar growth yields of 8.7 ± 0.8 mg/mmol of CH_4 formed (T. Ferguson, unpublished data).

Metabolism of substrate mixtures. Initial experiments with different substrates capable of serving as energy sources for growth indicated that *Methanosarcina* strain 227 exhibited possible heterogeneity in its catabolism of the substrates H_2–CO_2, methanol, and acetate (12). Moreover, depending upon the growth substrate being metabolized, addition of new substrates resulted in differences in the metabolism of the added substrates. These phenomena were examined in greater detail to evaluate the interrelationships during the metabolism of H_2–CO_2, methanol, and/or acetate.

The behavior of strain 227 toward methanol-acetate mixtures was examined by inoculating 1 ml of an acetate-grown culture into 100 ml of 0.2% YE medium containing 0.1% methanol, 1.0% sodium acetate, and 0.03% $Na_2S \cdot 9H_2O$ in 300-ml nephelometer flasks. Absorbances and methane production were monitored as previously described (see above). The results (Fig. 4) show that the resulting growth curves (as shown by both turbidity change and methane production) on the methanol-acetate mixtures superficially resemble typical diauxic growth curves of *Escherichia coli* on glucose-lactose mixtures (10). Growth and methanogenesis in the methanol–acetate mixture during the first 3 days are characteristic of growth on methanol alone. The quantity of methane produced during this initial 3-day period accounts for all of the methanol added to the medium. Growth on methanol was followed by a lag of about 7 days, after which growth and methanogenesis at a rate characteristic of growth on acetate was observed. This

TABLE 1. *Growth yields on sodium acetate*[a]

Cell yield[b] (mg)	Methane (mmol)	Y_{CH_4}[c] (mg/mmol)
2.2	0.92	2.4
5.8	3.1	1.9
5.6	2.1	2.7
5.2	2.8	1.9
9.0	4.6	1.9
8.4	4.7	1.8
7.5	3.5	2.1

[a] From 100 ml of culture.
[b] Dry weight.
[c] Mean Y_{CH_4}, 2.1. Standard deviation, 0.33.

TABLE 2. *Growth yield studies on methanol*[a]

Cell yield[b] (mg)	Methane (mmol)	Y_{CH_4}[c] (mg/mmol)
13.4	2.75	4.9
13.7	2.94	4.7
13.9	2.48	5.6
13.1	2.95	4.4
14.0	2.35	6.0
13.7	2.70	5.1

[a] From 100 ml of culture.
[b] Dry weight.
[c] Mean Y_{CH_4}, 5.1. Standard deviation, 0.6.

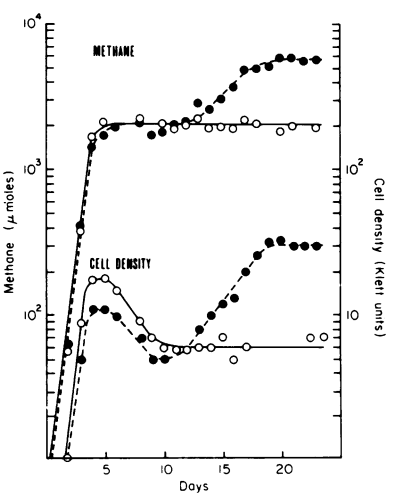

FIG. 4. *Growth characteristics of Methanosarcina strain 227 on a substrate mixture composed of 0.1% (vol/vol) methanol and 1.0% (wt/vol) sodium acetate. Symbols: ●, substrate mixture; ○, methanol only.*

graph suggests that methanol and acetate are sequentially catabolized when the inoculum is acetate grown; but, unlike the growth curves obtained with *E. coli* on glucose-lactose mixtures, strain 227 exhibited a 7-day lag period in terms of methane production and turbidity increase (which is about 2.5 generations) compared with lag periods of 0.5 to 0.8 generation due to catabolite repression by glucose on the enteric bacteria (10). However, in the presence of 0.03% $Na_2S \cdot 9H_2O$, cell lysis (as evidenced by a decrease in turbidity and a simultaneous increase in the viscosity of the culture) occurs at the end of methanol utilization (Fig. 4) and may be chiefly responsible for the long lag period during which the surviving population grows. Any effect due to catabolite repression of acetate metabolism by methanol was masked by this lysis. In any case, it appears that a shift from methanol to acetate catabolism occurred with difficulty. These findings may, in part, be responsible for the inability of previous workers to demonstrate rapid methanogenesis from acetate.

When sodium sulfide was lowered in concentration or completely omitted from the culture medium, lysis did not occur when cells metabolized methanol. The diauxic growth effect was reexamined under these conditions by introducing a 1% inoculum of acetate-grown cells into each of two replicate flasks of methanol-acetate media containing either [^{14}C]methanol or [2-^{14}C]acetate. Figure 5A and B shows that a diauxic growth effect was observed with a shorter lag time (less than 1 generation time), similar in magnitude to that reported for other organisms. $^{14}CH_4$ was produced almost exclusively from methanol during the first growth period (Fig. 5B), and no additional $^{14}CH_4$ was produced when methanol was used up after 5 days. Significant production of $^{14}CH_4$ from [2-^{14}C]acetate began in the second set of duplicate flasks (Fig. 5A) after this initial methanol-dissimilating period, although small amounts of $^{14}CO_2$ formed from the oxidation of [2-^{14}C]acetate were observed during the initial methanol-dissimilating period (data not shown).

The metabolism of radioactively labeled substrates by *Methanosarcina* strain 227 grown exponentially on sodium acetate was examined further to evaluate the metabolism of acetate and its response to regulatory phenomena. The results of duplicate or quadruplicate labeling experiments of this type are shown in Fig. 6A and B and 7A and B.

Figure 6A shows $^{14}CH_4$ formation when [2-^{14}C]sodium acetate was added to cultures growing exponentially on sodium acetate. The methyl group was immediately converted to $^{14}CH_4$ during the 11 h of incubation. Low radioactivity was

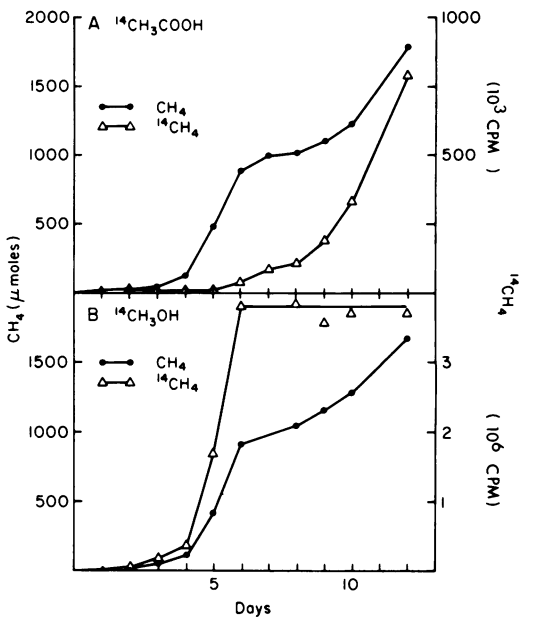

FIG. 5. *$^{14}CH_4$ and total CH_4 production during methanogenesis and growth on a mixture containing 0.025 M methanol and 0.1 M sodium acetate. Duplicate sets of cultures contained either $^{14}CH_3OH$ or sodium [2-^{14}C]acetate at equivalent specific activities in addition to the substrate mixture. (A) Substrate mixture containing sodium [2-^{14}C]acetate and unlabeled methanol; (B) substrate mixture containing unlabeled sodium acetate and [^{14}C]methanol.*

observed for $^{14}CO_2$ from [2-^{14}C]acetate (Fig. 6B), and this activity did not increase during the incubation period. The $^{14}CO_2$ produced was probably due to contamination of the added label with labeled carbonate-bicarbonate since $^{14}CO_2$ was evolved in sufficient quantities to account for this activity when mineral acid was added to the primary labeled material (sodium [2-^{14}C]acetate). However, sodium [1-^{14}C]acetate was converted exclusively to $^{14}CO_2$, and no $^{14}CH_4$ was observed (Fig. 6A and B). The rate of $^{14}CO_2$ production from sodium [1-^{14}C]acetate appears lower than the rate of $^{14}CH_4$ formation from methyl-labeled acetate probably because of the high solubility of CO_2 in the culture medium. (Since the specific activities of CH_4 and CO_2 from the two experiments were similar [424 and 454 cpm/μmol, respectively], similar rates were expected.) These data show that acetate is normally split to form CH_4 from the methyl group and CO_2 from the carboxyl group during growth on acetate.

When 0.01 M methanol was added to the 0.1 M sodium [2-^{14}C]acetate culture, a slight decrease was observed in the rate of acetate catabolism to $^{14}CH_4$ (Fig. 6A). Added $^{14}CH_3OH$ was

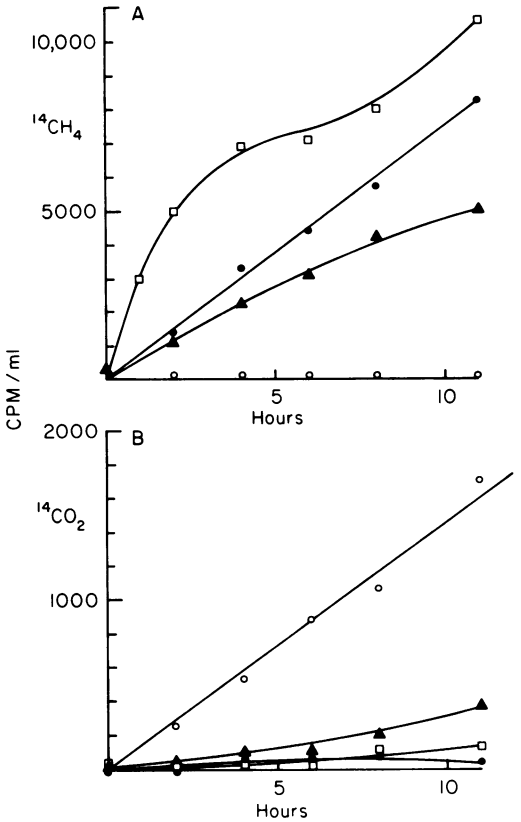

FIG. 6. *Metabolism of $^{14}CH_3OH$, $^{14}CH_3COONa$, and $CH_3{}^{14}COONa$ during growth on 0.1 M sodium acetate. Label was added at zero time; 0.01 M methanol was added to methanol-acetate cultures at zero time. (A) $^{14}CH_4$ production. Symbols: □, $^{14}CH_3OH$ plus CH_3COOH; ●, $^{14}CH_3COOH$; ▲, $^{14}CH_3COOH$ plus CH_3OH; ○, $CH_3{}^{14}COOH$. (B) $^{14}CO_2$ production. Symbols: ○, $CH_3{}^{14}COOH$; ▲, $^{14}CH_3COOH$ plus CH_3OH; □, $^{14}CH_3OH$ plus CH_3COOH; ●, $^{14}CH_3COOH$.*

also immediately metabolized to $^{14}CH_4$ by acetate cultures. However, unlike growth on methanol alone, $^{14}CO_2$ was not detected from $^{14}CH_3OH$ during growth on acetate (Fig. 6B), in agreement with our previous findings (12) and suggests that methanol is catabolized under these conditions via the acetate route or via acetate-generated intermediates. Figure 7A shows that addition of H_2 to the gas phase and $NaH^{14}CO_3$ to the medium resulted in the immediate reduction of the $NaH^{14}CO_3$ to $^{14}CH_4$. However, Fig. 7B shows that the rate of conversion of sodium [2-^{14}C]acetate to methane was depressed by addition of hydrogen over the period of incubation when compared with the rate at which sodium [2-^{14}C]acetate is metabolized to $^{14}CH_4$ in the absence of added hydrogen. These results indicate that methanol and H_2–CO_2 may be metabolized at the same time as acetate to form CH_4, provided that the acetate-metabolizing system is already operative.

The metabolism of acetate by strain 227 after growth on methanol was tested in the following experiment. A 1% inoculum of strain 227 grown and maintained on YE–methanol medium (see above) was introduced into quadruplicate flasks containing 50 ml of the same medium with 0.26 M methanol. After 48 h of incubation at 35°C, 10 μCi of $^{14}CH_3OH$ was added to two cultures, and 10 μCi of sodium [2-^{14}C]acetate was added to the two remaining cultures. At the same time, unlabeled sodium acetate was added to these latter two cultures at a final concentration of 0.1 M. All cultures were incubated for an additional 8 h in a 37°C water bath, during which time $^{14}CH_4$ and $^{14}CO_2$ production was monitored. Figure 8 shows that both $^{14}CH_4$ and $^{14}CO_2$ were produced from $^{14}CH_3OH$, but neither was produced from sodium [2-^{14}C]acetate. Hence, unlike

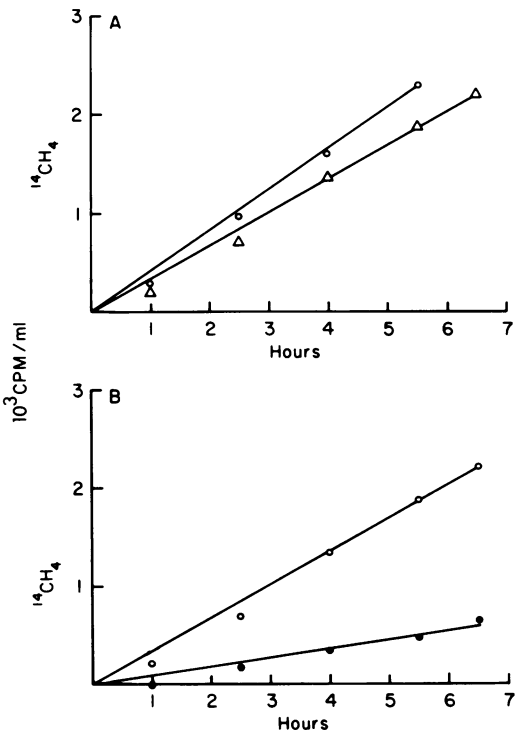

FIG. 7. *(A) Reduction of $NaH^{14}CO_3$ with molecular hydrogen during growth on sodium acetate (hydrogen was present at 22% [vol/vol] in the gas phase). Symbols: ○, H_2 plus $H^{14}CO_3^-$; △, $^{14}CH_3COOH$ (no hydrogen added). (B) Comparison of rates of metabolism of sodium [2-^{14}C]acetate in the presence and absence of added hydrogen. All specific activities were corrected for differences in the specific activities of the added label. Symbols: ○, $^{14}CH_3COOH$ (no hydrogen added); ●, H_2 plus $^{14}CH_3COOH$.*

cultures grown on acetate and presented with methanol, cultures grown on methanol and presented with acetate do not immediately metabolize acetate to CH_4 and CO_2. These data are consistent with the diauxic effect described earlier.

Inhibitor studies. Table 3 shows the results of adding known inhibitors of H_2–CO_2 conversion to methane to cultures in exponential growth on acetate. The viologen dyes were inhibitory at very low concentrations (less than 1 μM), but 2,4-dinitrophenol and 2-BES were inhibitory at a concentration of 100 μM and not at concentrations of 10 μM or less. These compounds were also inhibitory to methanogenesis from methanol at a 100 μM concentration (data not shown). KCN and $CHCl_3$ also inhibited methanogenesis from acetate at concentrations of 100 μM. These results suggest that mechanisms involved in methanogenesis from methanol and acetate may be similar to those involved in methanogenesis from H_2–CO_2. The inhibitory effect of 2-BES, an analog of coenzyme M (CoM), is particularly interesting because of the postulated role for CoM as an intermediate in methane formation from H_2–CO_2 (9, 19). CoM has been found in cell extracts from a variety of methanogenic bacteria, including *Methanosarcina barkeri* grown on H_2–CO_2 (19). The possibility that CoM may also be an intermediate in acetate dissimilation was examined after inoculating a 1% culture of strain 227 grown on YE–0.1 M sodium acetate medium into fresh medium of the same composition (Fig. 9). Ten flasks were inoculated in order to have duplicate cultures of each test condition. Addition of 70 μM 2-BES (final concentration) after 6 days of growth at 35°C resulted in 75% inhibition of methanogenesis compared with untreated controls. After an additional 3 days, CoM was added at final concentrations of 70, 500, and 1,000 μM. Two untreated cultures and two cultures containing only 70 μM 2-BES served as controls. Methane production was monitored for 6 days after ad-

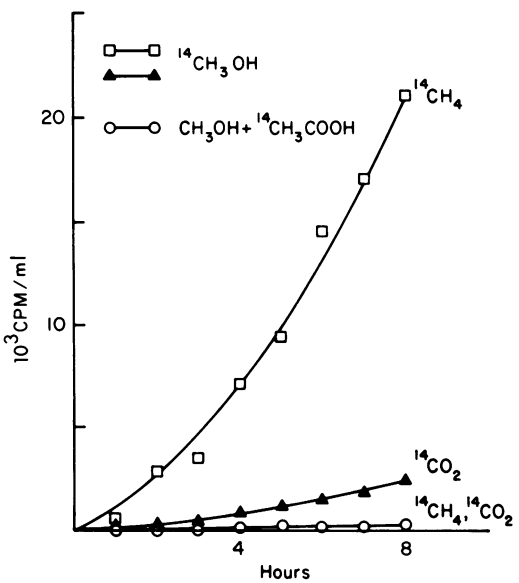

FIG. 8. *Methanogenesis from sodium [2-^{14}C]acetate during growth of a methanol culture of strain 227 on 0.26 M methanol. Counts per minute are given per milliliter of gas phase. All curves are from duplicate cultures.*

TABLE 3. *Inhibitors of methanogenesis from acetate*

Inhibitor	Acetate concn (M)	Inhibition[a]		
		100 μM	10 μM	>10 μM
Methyl viologen[b]	0.2	100	100	100
Benzyl viologen[c]	0.2	100	100	100
2,4-Dinitrophenol	0.2	100	0	0
2-BES	0.1	83	0	0

[a] Values represent percent inhibition compared with an untreated control.

[b] Methyl viologen was found to be inhibitory at the lowest concentration tested, 4.4 μM.

[c] Benzyl viologen was found to be inhibitory at the lowest concentration tested, 0.6 μM.

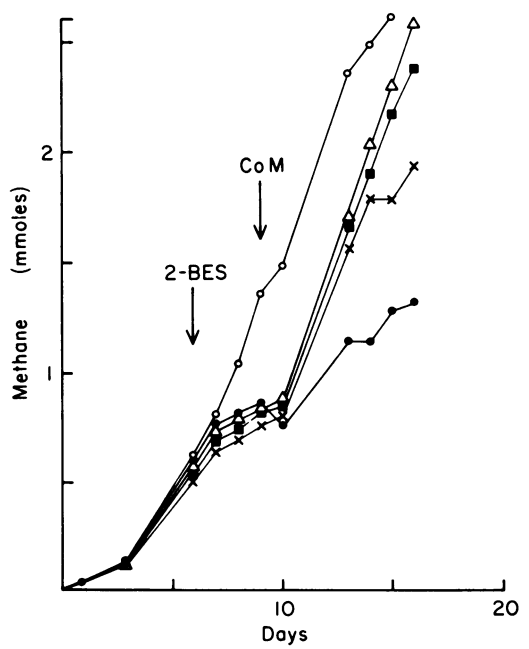

FIG. 9. *Inhibition of methanogenesis from 0.1 M sodium acetate by 2-BES and its reversal by CoM. Symbols: ○, untreated control; △, 70 μM BES plus 1,000 μM CoM; □, 70 μM BES plus 500 μM CoM; ×, 70 μM BES plus 70 μM CoM; ●, 70 μM 2-BES. The results are averages of duplicate determinations. Micromoles of methane are from 50-ml culture volumes.*

dition of CoM. Figure 9 shows that the addition of CoM to cultures containing 2-BES relieved the inhibition of methanogenesis, and methane production resumed to about its former rate. These results are anticipated if 2-BES acts specifically and competitively with CoM at some common site involved in methanogenesis from acetate and suggest that CoM is an intermediate in methanogenesis from acetate.

DISCUSSION

The capability of strains of *Methanosarcina* to utilize acetate for growth and methanogenesis is probably widespread. In addition to strain 227, we currently maintain other *Methanosarcina* strains on sodium acetate in our laboratory. They include the following: a gas-vacuolated strain (R. A. Mah, M. R. Smith, and L. Baresi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I32, p. 160); a strain obtained from M. P. Bryant; strain UBS from J. G. Zeikus; and biotype II from Zhilina. Bryant's strain was regarded by Zeikus (22) as being incapable of rapid acetate dissimilation; we find it does not differ from strain 227 in the rate of acetate dissimilation. The failure by previous workers to demonstrate rapid acetate utilization is probably attributable to conditions employed in isolating and/or maintaining the strains before inoculation into media containing acetate and perhaps to difficulties associated with cell lysis or shift down from methanol or H_2–CO_2 to acetate by the *Methanosarcina*.

Growth of broth cultures on substrates that permit faster growth (i.e., H_2–CO_2 or methanol) than does acetate seems to favor the rapid development of cultures unable to utilize acetate. However, broth cultures grown on H_2–CO_2 or methanol can be adapted to grow on acetate. The physiological evidence suggests a possible involvement of regulatory phenomena which affect the formation of methane from methanol or acetate by prior growth on methanol, acetate, or H_2–CO_2. Broth cultures grown and maintained on methanol medium containing >0.01% sodium sulfide may not grow on acetate until after a long period of time (about 2 months), perhaps, in part, because of cell lysis; only cultures grown and maintained on acetate will grow on acetate immediately and rapidly. The inability of Blaylock and Stadtman (3) to demonstrate the splitting of acetate to methane and carbon dioxide by cell-free extracts may, in part, be attributed to the use of methanol as the energy source for growing the cells examined.

The mechanisms involved in the conversion of H_2–CO_2, methanol, or acetate to methane are not well understood. Since at least one end product (methane) is the same for all three substrates, there might be some steps in common both for the conversion of these substrates to methane and for the mechanism of ATP production.

The biochemistry of CO_2 reduction by molecular hydrogen to methane has been studied the most extensively (9) and appears to involve coenzymes unique to the methanogenic bacteria (5, 19). This pathway appears to be inhibited by 2,4-dinitrophenol, viologen dyes, chloroform, and 2-BES (9). Our studies show that cells grown on methanol or acetate have some properties in common with cells grown on H_2–CO_2. Growth on acetate and methanol is inhibited by chloroform, 2-BES, 2,4-dinitrophenol, and benzyl and methyl viologens. The low inhibitory concentrations of the viologen dyes on methanogenesis from acetate or methanol (or H_2) suggest a possible interference with the terminal electron transfer reaction or a possible role for electron transport mechanisms as proposed by McBride and Wolfe for H_2–CO_2 (9, 23). If the latter is correct, then the conversion of acetate to methane may be mechanistically unusual since this implies that an intramolecular transfer of electrons from the carboxyl group to the methyl group of acetate may have occurred via an electron transport system. The inhibitory effect of the classical uncoupling agent, 2,4-dinitrophenol, on methanogenesis is not anticipated unless it acts as an alternate electron acceptor or inhibits an ATP-requiring step (9). Inhibition by 2-BES and its reversal by CoM suggest that the postulated methyl carrier for methanogenesis from H_2–CO_2, 2-mercaptoethanesulfonic acid (19), is also the methyl carrier for methanogenesis from acetate and methanol.

Our labeling studies also indicate that metabolism of methanol to methane differs according to the substrate (acetate or methanol) on which the cells are grown (12). The production of $^{14}CH_4$ and not $^{14}CO_2$ from added $^{14}CH_3OH$ when cells are grown on acetate (Fig. 6) and the depression of acetate dissimilation by methanol (Fig. 6) imply a preferential reduction of methanol by reducing equivalents generated from the metabolism of acetate, other medium components, or endogenous reserves. However, when cells are grown solely on methanol, a methanol-oxidizing system must be active to produce the necessary reducing equivalents for reduction of methanol to methane, and consequently CO_2 is generated from methanol in a stoichiometric relationship (ratio of CO_2 to CH_4, 1:3) to the methane formed. Since methanol may be reduced to methane during growth on either acetate or methanol, methanol appears to share some common intermediates (e.g., via CoM) with acetate and to lie directly or indirectly on the acetate pathway of

methane formation. These findings also indicate that separate enzymes for the initiation of acetate dissimilation must exist. The fact that acetate-grown cells sequentially utilize methanol before acetate in batch culture (Fig. 3) indicates that enzyme synthesis for acetate catabolism may eventually be switched off if both methanol and acetate are present simultaneously. When methanol is exhausted, acetate metabolism then resumes (after induction of enzymes of the acetate system).

The standard-free energy change for the splitting of acetate to CH_4 and CO_2 (−7.4 to −8.6 kcal/mol [−31.0 to −36.0 kJ/mol] [20, 22]) is insufficient for the generation of ATP (the $\Delta G'^0$ for ATP hydrolysis is 7.6 kcal/mol [−31.0 KJ/mol] [20]). This fact has led some investigators to hypothesize that growth cannot occur on acetate by methanogenic bacteria via a decarboxylation type of reaction (22, 24). Zeikus et al. (24) reported a hydrogen-dependent metabolism of acetate in which both methyl and carboxyl moieties were reduced to methane in *Methanobacterium thermoautotrophicum* and *M. barkeri*. Under these conditions, acetate-dependent growth was not reported, although it is possible that acetate may be co-metabolized under the H_2 oxidation–CO_2 reduction conditions employed by Zeikus et al. (24). The differences between our results and those of Zeikus et al. (24) may be attributed to differences in conditions of pregrowth. Our cultures were maintained on acetate before inoculation into acetate-containing media, whereas Zeikus et al. grew their cultures on methanol or H_2–CO_2. It should also be noted that in *M. thermoautotrophicum* acetate is apparently not an important substrate for methanogenesis (7).

The evidence presented here confirms our initial finding (12) that *Methanosarcina* strain 227 can metabolize acetate to CH_4 and CO_2 in the absence of added hydrogen and shows that acetate may be utilized as the sole source of energy. This is indicated by the rate at which acetate is consumed, by the quantities consumed, and by the stoichiometric conversion of acetate to methane during growth. It is also indicated by the dependence of cell yields and rates of growth on the quantity of acetate converted to methane. Methane is not formed from YE during growth on acetate since the yield of methane is equal to the quantity of acetate added and is independent of the concentration of YE (1, 12). Furthermore, strain 227 rapidly utilized acetate for growth and methanogenesis in the complete absence of cysteine or any other added organic compound (M. Smith, T. Ferguson, and R. Mah, manuscript in preparation).

In mixed culture systems such as the anaerobic sludge fermentation, acetate is split to form CH_4 and CO_2, and no H_2-dependent metabolism of acetate has been reported. The acetate-dependent growth observed in our present study demonstrates that sufficient energy must be generated from the splitting of acetate for growth and that acetate-metabolizing methanogens may use acetate as an energy source in nature.

There are a number of possible hypotheses regarding the energy available from acetate to methanogenic bacteria. (i) The amount of energy calculated for acetate dissimilation under standard conditions may not be representative of the energy available from it under physiological conditions. (ii) The efficiency of energy conversion is unusually high (an unlikely hypothesis). (iii) More than 1 mol of acetate is required for the formation of each mol of ATP. This latter hypothesis will be discussed below.

The significance of the cell yield constants for strain 227 on sodium acetate is difficult to evaluate because of the presence of other possible carbon sources in the YE and Trypticase medium. However, since acetate appears to serve as the primary, if not the only, energy source, a $Y_{acetate}$ value of 2 g of cell material per mol of acetate can be calculated as shown here and previously (M. R. Smith and R. A. Mah, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, I41, p. 87). This suggests that 2.5 mol of acetate is required to generate 1 mol of ATP if a Y_{ATP} for autotrophs of 5 g/mol (20) is assumed. However, the Y_{ATP} value is not known, is dependent upon the nutritional conditions at the time of measurement, and has a value of 10.5 g/mol in heterotrophic bacteria when all of the cell precursors for growth are provided in the medium, and the substrate serves only as an energy source; under these conditions, 5.26 mol of acetate would be required per mol of ATP generated. In either case, it appears likely that more than 1 mol of acetate is required for the generation of 1 mol of ATP, making it unlikely that substrate-level phosphorylation occurs during acetate metabolism.

The ratio of the cell yield constant on methanol (Table 2) compared with that for acetate (Table 1) is 2.5. The molar cell yield on H_2–CO_2 is 8.7 g/mol of methane formed. The ratio of cell yields on acetate, methanol, and H_2–CO_2 is thus 1:2.5:4.1 for the three substrates, respectively. Although this ratio is not in accordance with moles of electrons transferred per mole of CH_4 formed from the three substrates, it does appear to be similar to the ratios of molar ATP yields based upon the standard-free energy changes for these reactions. Given a standard-free energy change of −7.4 kcal/mol of ATP and the standard-free energy changes for the catabolism of

acetate, methanol, and H_2-CO_2 to methane (−7.4 kcal [31 kJ], −75 kcal [314 kJ], and −31 kcal [129 kJ], respectively), one might anticipate ATP yields of about 1, 10, and 4 mol of ATP per reaction (20) if the efficiency of ATP production is close to 100% for each substrate. This gives a ratio of 1:3.3:4.2 mol of ATP per mol of methane formed. Although the actual efficiency of ATP production for the three substrates is not known (e.g., 2 or more mol of acetate may be required to generate 1 mol of ATP), the results suggest that the efficiency of energy conservation in *Methanosarcina* is similar on all three substrates in our YE medium. If the mechanism(s) of ATP generation does not differ significantly when cells are grown on methanol or acetate, an electron transport or a chemiosmotic process of energy conservation seems more likely than substrate-level phosphorylation. In *M. thermoautotrophicum,* it is also unlikely that ATP formation from H_2 oxidation-CO_2 reduction comes from substrate-level phosphorylation, but rather from electron transport mechanisms (9, 20). However, it should be pointed out that intermediates resembling quinones or cytochromes that are known to be associated with chemiosmotic mechanisms in other groups of bacteria have not been detected in methanogenic bacteria (20).

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Addendum

Cultures of strain 227 maintained on methanol before inoculation into mixtures of acetate and methanol exhibited diauxic effects with radioactively labeled substrates similar to those reported here for acetate-grown cells except that traces of radioactive methane were not produced from [2-^{14}C]acetate during the initial methanol-dissimilating period. This is anticipated because a methanol-grown inoculum should be repressed for acetate catabolism but an acetate-grown inoculum should be fully induced. This rules out the possibility that two different populations of *Methanosarcina* are responsible for the observed diauxic effect. Similar diauxic effects have also been observed in our laboratory on mixtures of acetate and H_2/CO_2 (T. Ferguson, unpublished data).

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