Studies on an Acetate-Fermenting Strain of *Methanosarcina*

ROBERT A. MAH,* MICHAEL R. SMITH, AND LARRY BARESI

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

Received for publication 20 December 1977

An acetate-fermenting strain of *Methanosarcina* was isolated from an acetate enrichment culture inoculated with anaerobic sludge from a waste treatment digester. In pure culture, this organism fermented acetate in the absence of added hydrogen at rates comparable in magnitude to those found in digester systems. This rate was significantly higher than previously obtained for pure cultures of this genus. Mineral components of yeast extract were highly stimulatory for cultures growing on methanol. Comparable stimulation was not observed for cultures growing on acetate. Labeling studies indicated that acetate was converted to methane and CO₂ as predicted by previous studies on mixed cultures. Total oxidation or reduction of acetate was not the mechanism of conversion of acetate to methane by the pure culture. The ability of this strain to form colonies or to produce methane from acetate was apparently influenced by the choice of substrate and conditions used for growing the inoculum.

Under anaerobic conditions, the production of methane and carbon dioxide in natural environments may be the terminal step in the mineralization of organic compounds (6, 8, 12, 20). Numerous studies have implicated acetic acid as a principal precursor of methane (5, 6, 10, 11, 14, 17, 18). Its conversion to methane and carbon dioxide was examined in the early methanogenic enrichment cultures of N. L. Söhngen (Ph.D. thesis, Delft University of Technology, Delft, The Netherlands, 1906), C. Schnellen (Ph.D. thesis, Delft University of Technology, 1947), and Barker (1), in which acetic acid was the only source of organic carbon added to a mineral salts medium. Radioactive isotopic labeling of acetate-using “purified” cultures of *Methanosarcina barkeri* obtained from these enrichments disclosed that the methyl moiety of acetate was reduced to methane while the carboxyl moiety was oxidized to carbon dioxide (10, 14). However, this reaction has been questioned on thermodynamic grounds since the standard free energy change via decarboxylation is barely sufficient to form 1 mol of adenosine 5'-triphosphate for each mol of acetate dissolved (4, 20, 21).

*M. barkeri* is the only organism isolated in pure culture which is known to convert acetate to methane and carbon dioxide (8, 19). However, the rates of formation of methane from acetate by pure or purified cultures of *M. barkeri* are too slow (2, 14, 21) to reconcile with known rates of conversion in digester systems where the rapid turnover of acetate accounts for 70% or more of the methane produced.

Zeikus et al. (21) reported a hydrogen-dependent conversion of acetate to methane by *M. barkeri* and by *Methanobacterium thermooautotrophicum* and stated that methanogens growing on acetate required hydrogen to reduce acetate to methane.

In this paper, we describe the characteristics of a strain of *Methanosarcina* (designated strain 227) isolated from an acetate-enrichment culture which has been maintained continuously by weekly transfer in our laboratory since 1974. We show that this strain ferments acetate to methane and carbon dioxide at a rate comparable to mixed-culture systems. Moreover, methanogenic conversion of acetate by this strain is apparently via a mechanism similar to that in mixed cultures and does not appear to require external sources of H₂.

**MATERIALS AND METHODS**

**Bacterial strains.** *Methanosarcina* strain 227 was isolated from a stable Barker acetate-enrichment culture (L. Baresi, R. A. Mah, and D. Ward, submitted for publication) by serially diluting the culture into yeast extract medium (see below) containing 1% calcium acetate and a 100% N₂ gas phase. Colonies which utilized acetate with production of methane and carbon dioxide deposit crystalline calcium carbonate in and around the colonies. This results in the formation of hard, rock-like colonies distinguishable from the soft colonies, most of which do not dissipilate acetate. Colonies from the initial roll-tube cultures were picked by Pasteur pipette and transferred to fresh medium of the same composition. Samples were diluted by the syringe method of Hungate as described by Wolfe (19).
This culture procedure was continued for many transfers until the culture was judged pure by colony morphology, by numerical agreement of countable colonies following decimal dilution, by Gram staining, and by phase-contrast microscopy. Stock cultures were maintained on both liquid and solid media. Maintenance on solid media was effected by picking single colonies at the time of transfer.

**Culture media.** The defined medium was modified from the basal medium of Bryant et al. (3) and consisted of 0.1% NH₄Cl, 5% Bryant mineral solution, 0.5% vitamin solution, 0.0001% resazurin, 0.05% cysteine-HCl, and 0.15% NaHCO₃ made up in distilled water; 0.01% Na₂S·9H₂O was added aseptically just before inoculation. The medium may be supplemented with methanol (1% by volume) or with sodium acetate (0.1 to 0.2 M) as a carbon and/or energy source. Basal medium was similar to defined medium except for omission of vitamins, cobalt, and manganese.

Low-yeast-extract medium consisted of 0.02% yeast extract (Difco) and 0.2% Trypticase dissolved in a solution of 0.1% NH₄Cl, 0.04% K₂HPO₄, and 0.01% MgCl₂ in tap water. In addition, the medium contained resazurin (0.0001%), cysteine-HCl (0.05%), NaHCO₃ (0.05%), and Na₂S·9H₂O (0.01%). High-yeast-extract medium consisted of 1.0% yeast extract, 0.8% Trypticase, 0.1% NH₄Cl, 0.15% NaHCO₃, 0.05% cysteine-HCl, 0.01% Na₂S·9H₂O, and 0.0001% resazurin in distilled water. In addition, low-yeast-extract medium was supplemented with methanol (1% [vol/vol]) or sodium acetate (0.1 to 0.2 M) as an energy source. All media were prepared in an atmosphere of oxygen-free 100% N₂. The pH of the medium was adjusted to 6.4 to 6.5 prior to autoclaving. Sodium sulfide was added just prior to inoculation, and the final pH after autoclaving was 7.0. All media were dispensed into flasks or tubes closed with butyl rubber stoppers.

**Culture conditions.** Cultures were inoculated with 3- to 7-day-old liquid cultures grown on methanol in low-yeast-extract medium or 7- to 14-day liquid cultures grown on 0.12 M sodium acetate in low-yeast-extract medium. Inocula of 1 to 3% were used for all experiments. Cultures were incubated at 35°C without shaking. Stock cultures were maintained by weekly transfers to solid and liquid low-yeast-extract media containing 1% methanol or 0.12 M sodium acetate. Additional cultures were maintained on defined media supplemented with 1% methanol.

**Absorbance measurements.** Absorbances were monitored in cultures growing in 300-ml nephelometer flasks (Bellco, Vineland, N.J.) containing 100 ml of medium. Absorbances were determined with a Klett-Summerson photocolorimeter equipped with a green filter. Growth curves obtained by measuring absorbances closely resemble in shape those obtained by measuring methane production (data not shown). However, the light-scattering method was less sensitive than gas chromatographic methods, due in part to the growth of Methanoscincus in large aggregates. Even though absorbances were measured in most experiments, only the methane data will be reported here. Final absorbances were typically around 30 Klett units for cultures growing on sodium acetate and varied from 50 to 110 Klett units for cultures on methanol, depending upon the concentration of yeast extract present.

**Methane measurements.** The gas composition of the atmosphere over a culture was monitored by gas-solid chromatography as described by Baresi et al. (submitted for publication). Total gas production was determined daily by measuring the volume of gas produced (at room temperature, 23°C) by displacement of the glass-tipped plunger of an un lubricated glass syringe. Micromoles of methane were computed from the gas composition determined by gas chromatography and from the measured volumes of gas phase in the vessel and syringe. The following equation was used:

\[ \text{M}_{\text{CH}_4} = \frac{\% \text{ CH}_4 \times (V_f + V_i)}{2.24} + \text{M}_{\text{H}_2} \text{O} \]

where % CH₄ is the volume (milliliters) per 100 ml of gas phase; Vf is the volume (milliliters) of gas phase in vessel; Vi is the volume (milliliters) of gas phase removed by syringe following equilibration to atmospheric pressure; and \( M_{\text{H}_2} \text{O} \) is the sum of micromoles removed after daily equilibration—e.g., \( M_{\text{H}_2} \text{O} = \sum(\% \text{ CH}_4 \times V_i)/2.24 \). Although gas volumes were not corrected to standard temperature (273°C), computations showed that the quantity of methane was overestimated by about 8%.

**Cell yields.** Cell yields were determined at the end of growth on 100 ml of culture medium. Cells were harvested by use of a clinical centrifuge, washed twice in distilled water, and then filtered onto preweighed membrane filters. The cells and filters were dried constant weight by incubating overnight at 60°C in a vacuum oven and reweighed after drying. The difference in the dry weight of the filter before and after filtration was taken as the weight of cells.

**Labeling studies.** Labeling studies were performed by growing 100-ml liquid cultures in low-yeast-extract media supplemented with 1% methanol or 0.2 M sodium acetate. When a density of approximately 25 Klett units was reached, 10-ml aliquots were transferred to culture tubes under a 100% N₂ or 100% H₂ gas atmosphere. Radioactive substrates (0.1 μCi) were then injected into the cultures and incubated at 37°C for 24 h. Following incubation, the cultures were dried identified by injecting 2 ml of 3% HCl into the tubes. The total volume of gas produced was measured with a 20-ml syringe. Gas samples (1 ml) were then injected into an Aerograph gas chromatograph teamed with a Packard gas proportional counter. The percentage composition of the components of gas and the radioactivity appearing in each component were then measured, and the total radioactivity of each component was computed.

Alternatively, radioactivity in gases over the cultures was determined by flushing the gas phase with helium. The gases were bubbled through three successive scintillation vials, each containing 10 ml of Aquasol scintillation fluid (New England Nuclear) and 1 ml of phenethylamine to trap carbon dioxide. The gaseous effluent from the scintillation vials was passed through a 900°C combustion furnace containing copper oxide (Leco Corporation, St. Joseph, Mich.) to oxidize any methane to carbon dioxide. The resulting gas was then bubbled through a second train of three successive vials containing CO₂-trapping solution. Radioactivity in methane and carbon dioxide was then determined.
by counting the vials in a Beckman liquid scintillation counter. This second procedure was used whenever the Packard gas counter was inoperable.

Efficiencies of counting are 47% by the above procedure and 65% by the gas proportional counter. All values reported in the results are corrected to disintegrations per minute.

Isotopes. [2-14C]acetate (sodium salt) (specific activity, 56.5 mCi/mmole) and [14C]methanol (specific activity, 25.8 mCi/mmole) were purchased from Cal-Atomic (Los Angeles, Calif). [U-14C]acetate (sodium salt) (specific activity, 98 mCi/mmole) was purchased from Schwarz/Mann (Orangeburg, N.Y.).

RESULTS

* Methanosarcina* strain 227 resembles other isolates of methanosarcinae in its morphology, sensitivity to oxygen, and ability to utilize methanol, acetate, and H2/CO2 for methanogenesis. It shows a definite preference for methanol over acetate as an energy source. Like other strains, it will not ferment formate or pyruvate. It may, however, use organic sources of sulfur, since it can be maintained on yeast extract medium supplemented with methanol in the absence of added inorganic sulfur. On isolation in media containing calcium acetate, it produces hard, rock-like colonies that are distinguishable from those of organisms that do not catabolize acetate to methane and CO2 (Fig. 1).

Effect of pregrowth conditions on substrate utilization. A number of investigators have experienced difficulties in isolating strains of methanogenic bacteria that ferment acetate (20). Some of these difficulties may arise from procedures used for the enrichment and handling of these strains. If the substrates used for the enrichment or growth of these organisms influence their subsequent ability to metabolize acetate, culture problems could arise if such cells were subsequently inoculated into acetate-containing media. This was tested by pregrowing strain 227 through at least two passages on roll-tube media containing calcium acetate (i) alone or (ii) supplemented with methanol or (iii) supplemented with 70% H2-30% CO2. A single colony was picked from each of these media and decimally diluted into liquid broth. The 106, 105, and 104 broth dilutions were each inoculated into quintuplicate roll-tube media containing the above methanogenic substrates. The composition of the medium prior to substrate addition was that of Ward et al. (17a). Tubes were incubated at 35°C for 3 weeks before counts were made; they were discarded after 3 months without significant change in colony count. The percentage of colony-forming units (CFU) was calculated using the numbers of CFU obtained on H2/CO2 media as a 100% reference. The results (Table 1) represent the average of quintuplicate determinations. The highest number of CFU was always obtained on H2/CO2 medium regardless of the substrate used for growing the inoculum. The smallest percentage of CFU was observed on calcium acetate regardless of the source of the inoculum. The ability to utilize acetate, however, was greatest for cells grown exclusively on acetate and least for cells grown on H2/CO2. The population of cells capable of growing on acetate was also decreased by pregrowing on calcium acetate supplemented with methanol. These results indicate that strain 227, as initially isolated, may be heterogeneous with respect to its ability to metabolize H2/CO2, methanol, and acetate. The proportion of the population metabolizing acetate or methanol varied according to the substrates used for prior growth. However, it appears that the culture is homogeneous with respect to its ability to metabolize H2/CO2 to methane. These results suggest that enrichment techniques for isolating methanogens that employ methanol or H2/CO2 as substrates may result in a selection against acetate-utilizing methanogens and may, in part, explain some difficulties encountered by previous investigators who attempted to culture acetate-using methanogens by employing H2/CO2 or methanol for enrichment or isolation.

Nutritional studies on methanol. Initial attempts to define the optimal nutritional conditions for growth of strain 227 were carried out on media containing methanol, since methanol was preferentially used by this strain and allowed faster growth than acetate. In the routine experiments, the inoculum (strain 227) was grown on defined medium (see Materials and Methods section) containing methanol for inoculation into defined medium or on low-yeast-extract medium for inoculation into media containing yeast extract. The following 1% methanol media were inoculated: 0.2% yeast extract, 1.0% yeast extract, and defined medium. Growth was measured both by absorbance and methane production (Fig. 2). (Absorbance data, not shown, were similar to the methane data.) The control vessel, lacking methanol, showed that yeast extract was highly stimulatory to growth but could not by itself support significant growth or methane production. The doubling time of the culture on defined medium was 73 h, compared to doubling times of 11.6 and 11.1 h on low- and high-yeast-extract media, respectively. Although the final quantities of methane formed in low- and high-yeast-extract media were similar, the final amount was produced in approximately half the time on 1% yeast extract. Moreover, the cell yield at constant methanol concentration was a linear function of the concentration of yeast extract (Fig. 3) at the test concentrations indi-
cated. The results, averaged from duplicate and triplicate determinations, showed that even 0.2% yeast extract was limiting in the presence of 0.3 M methanol. The limiting factor(s) was also apparently present in Trypticase at lower concentrations.

In examining the stimulatory nature of yeast extract, the mineral content was evaluated by ashing 10 g of yeast extract in a muffle furnace at 900°C for 4 h. The ash was dissolved in sufficient distilled water to give a final concentration equivalent to 10% yeast extract, and the resulting solution was neutralized with HCl. This concentrated solution was then added to defined medium to give a final concentration equivalent to 0.2% yeast extract. Control cultures consisting of defined medium and defined medium supplemented with yeast extract were also prepared. All were inoculated at the same time with a culture of strain 227 maintained through at least three successive transfers (1% inocula) on defined medium. The results (Fig. 4) showed that yeast extract ash was approximately as stimulatory as yeast extract itself and

---

**Fig. 1.** (a) Methanosarcina strain 227 on calcium acetate, phase contrast, ×400. (b–d) Scanning electron micrographs of hard colony on calcium acetate. (b) ×50, (c) ×500, (d) ×5,000.
indicated that trace minerals, limiting in the defined medium, were supplied by yeast extract. The stimulatory effect was probably not due to the presence of cobalt, since cobalt was supplied in the defined medium and was not detectable by atomic absorption spectrometry of the ash. Effects similar to this have been observed with other methanogenic bacteria (3, 7). Experiments were in progress to identify the stimulatory component(s) of yeast extract.

Vitamins and other constituents of the defined medium were also examined to determine their effect on growth of this strain on methanol. Strain 227 (grown in defined medium) was inoculated into basal medium (Materials and Methods), basal medium supplemented with manganese, basal medium supplemented with cobalt and manganese, basal medium supplemented with vitamins, and complete defined medium. All additions to the basal medium were at concentrations equivalent to that in the defined medium. The results (Table 2) showed that strain 227 grew on the basal medium of mineral salts plus methanol, but all additions were somewhat stimulatory. Vitamins or a combination of cobalt and manganese (but not manganese alone) stimulated growth above that in the basal medium and was approximately equivalent to growth in defined medium. The results suggested that the observed stimulation of the

TABLE 1. Percentage of CFU developing on various substrates relative to those developing on H2 and CO2 as a function of pregrowth on various substrates

<table>
<thead>
<tr>
<th>Pregrowth substrate</th>
<th>% CFU (no. ( \times 10^3 )) developing ( ^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1% calcium acetate</td>
</tr>
<tr>
<td>1% calcium acetate (N2 atmosphere)</td>
<td>60 (48)</td>
</tr>
<tr>
<td>0.5% calcium acetate + 0.5% methanol (N2 atmosphere)</td>
<td>15 (20)</td>
</tr>
<tr>
<td>1% calcium acetate (H2/CO2 atmosphere)</td>
<td>18.5 (27.8)</td>
</tr>
</tbody>
</table>

\( ^* \) Figures given are averages of five replicate tubes.

- Fig. 2. Methanogenesis by strain 227 on methanol in defined and complex media containing methanol. Micromoles of methane are given for 100 ml of medium. The control consisted of low-yeast-extract medium (0.2%) to which no substrates were added. Final absorbances reached were as follows: control, 11 Klett units; 0.2% yeast extract, 51 Klett units; 1.0% yeast extract, 110 Klett units; defined medium, not examined. The results are from duplicate or triplicate determinations.
bacteria do metabolize acetate at a significant rate in a nitrogen atmosphere when cultures are isolated, maintained, and grown on acetate. Typical growth curves on yeast extract medium containing 0.12 M sodium acetate and a 100% N₂ atmosphere are shown in Fig. 5. Generation times of 60 to 72 h were usually obtained on yeast extract medium, with maximal methane production rates of 4 to 5 mmol/liter per day. Unlike growth on methanol, increasing the yeast extract concentration from 0.2 to 1.0% did not have a significant stimulatory effect when acetate served as the energy source. However, as expected, yeast extract alone did not support significant growth or methanogenesis. It was notable that growth by the pure culture of strain 227 on acetate exhibited exponential kinetics (whether observed as methane production or as an increase in cell density), in contrast to the linear kinetics of methane production observed for acetate-enrichment cultures (Baresi et al., submitted for publication). Thus, acetate metabolism to methane and carbon dioxide apparently generated sufficient energy for growth, and implies that other factors must limit methanogenesis to a linear rate in the enrichment cultures. These results indicate that strain 227 exhibited patterns of behavior toward yeast extract when growing on methanol different from when growing on acetate and suggest that the pathways for methanol metabolism and acetate metabolism may not be identical. Moreover, growth on acetate in defined medium shows that acetate can be metabolized without the participation of external sources of electron donors.

**Labeling studies.** The route of formation of methane from acetate was examined in strain 227 by use of ¹⁴C-labeled substrates (see Materials and Methods). A common source of inoculum was used in each of these studies. Following addition of ¹⁴C-labeled acetate or bicarbonate to 10 ml of cultures of strain 227 growing on sodium acetate in yeast extract medium (see Materials and Methods), cultures were incubated at 37°C for 24 h. The vessels were then acidified and assayed as described previously. The resulting

<table>
<thead>
<tr>
<th>Medium</th>
<th>Doubling time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Defined</td>
<td>73</td>
</tr>
<tr>
<td>Basal + vitamins</td>
<td>66</td>
</tr>
<tr>
<td>Basal + Co²⁺ + Mn³⁺</td>
<td>71</td>
</tr>
<tr>
<td>Basal + Mn³⁺</td>
<td>103</td>
</tr>
<tr>
<td>Basal</td>
<td>118</td>
</tr>
</tbody>
</table>

*Generation times are computed from a least-squares fit of the data and represent averages of duplicate determinations.
distribution of radioactivity in CH₄ and CO₂ is presented in Table 3. When 5 μCi of [U-¹⁴C]-acetate was added to the cultures, label was almost equally distributed between methane and carbon dioxide. These results are consistent with the hypothesis that acetate was decarboxylated to CO₂ with a reduction of the methyl carbon to methane. The observed labeling pattern is also consistent with patterns obtained in enrichment cultures, digestors, and aquatic sediments (5, 10, 14, 17, 18).

Cells were also incubated with sodium [2-¹⁴C]acetate (0.1 μCi in 10 ml of culture) or NaH¹⁴CO₃ in the presence of 100% H₂ or 100% N₂. The results (Table 3) show that virtually all of the methyl group of acetate appeared in methane with little label in CO₂ when the atmosphere over the culture consisted of nitrogen. This rules out the possibility that acetate is first completely oxidized to CO₂ and then subsequently reduced to methane, because radioactivity would appear both in methane and carbon dioxide at equivalent activities if this had occurred. The small amount of label appearing in CO₂, however, indicated that some methyl group oxidation may have occurred.

The possibility that hydrogen or its equivalent might be required for the formation of CH₄ from acetate or CO₂ was also examined in these experiments. Addition of sodium [¹⁴C]bicarbonate in the presence of 100% N₂ (Table 3) yielded little radioactivity in the methane (535 dpm);

![Graph](image)

**FIG. 5.** Methanogenesis of strain 227 on complex media with 0.2 M sodium acetate. Micromoles of methane are given for 100 ml of culture. The control consisted of high-yeast-extract (1.0%) medium to which no substrate (acetate or methanol) was added. Final absorbances (Klett units) reached were as follows: Control, 0; 0.2% yeast extract, 25; 1.0% yeast extract, 31. All experiments were performed in duplicate vessels.

**TABLE 3. Effect of hydrogen on methanogenesis from acetate**

<table>
<thead>
<tr>
<th>Labeled substrate</th>
<th>Total dpm</th>
<th>Ratio of ¹⁴CH₄ (dpm) to ¹⁴CO₂ (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition</td>
<td>Atmosphere</td>
<td>¹⁴CH₄</td>
</tr>
<tr>
<td>CH₃COOH</td>
<td>N₂</td>
<td>161,053</td>
</tr>
<tr>
<td>CH₃COOH</td>
<td>N₂</td>
<td>124,750</td>
</tr>
<tr>
<td>H⁺CO₃</td>
<td>N₂</td>
<td>535</td>
</tr>
<tr>
<td>CH₃COOH</td>
<td>H₂</td>
<td>11,108</td>
</tr>
<tr>
<td>H⁺CO₃</td>
<td>H₂</td>
<td>65,779</td>
</tr>
</tbody>
</table>

*Experiment was performed as described in the text. Asterisk indicates position of ¹⁴C label in the molecule. ¹⁴CH₄ and ¹⁴CO₂ were assayed by trapping with phenethylamine for the experiment with uniformly labeled acetate. All other labeled substrates were measured by gas proportional counter. The levels of radioactivity were adjusted for differences in counting efficiency. All values are the result of duplicate determinations using a 1% inoculum. dpm, Disintegrations per minute.
most of the radioactivity remained as CO₂ (1,400,000 dpm). This indicates that the reducing equivalents in the medium were not sufficient to produce significant quantities of CH₄ by reduction of bicarbonate. When a 100% H₂ gas atmosphere was present, the methane formed from [¹⁴C]bicarbonate was significantly labeled (66,000 dpm), indicating the presence of a functional CO₂ reduction-H₂ oxidation pathway. However, with sodium [2-¹³C]acetate in the presence of hydrogen, radioactive methane was greatly reduced, making it even less likely that acetate required external reducing equivalents to form methane, since H₂ was obviously being used for CO₂ reduction instead of acetate conversion to methane and carbon dioxide.

The pathway for methanogenesis from methanol is probably not exactly the same as that for acetate. When cells growing on substrate quantities of unlabeled acetate or methanol were presented with small quantities of radioactive acetate or methanol (Table 4), the effect of the predominating methanogenic substrate can be assessed. During fermentation of acetate, addition of [2-¹⁴C]acetate showed that it was converted almost exclusively to [¹⁴C]CH₄. If [¹⁴C]methanol (0.1 µCi) were added to a culture growing on acetate, nearly all of the methanol was reduced to methane, with little radioactivity appearing in carbon dioxide. Thus, methanol behaved in a manner similar to the methyl group of acetate. In the converse experiment, in which cells were grown on CH₃OH, addition of [¹⁴C]CH₃OH resulted in a distribution of radioactivity in CH₄ and CO₂ at an approximate ratio of 3:1. Addition of [2-¹⁴C]acetate (0.1 µCi) to a culture growing on methanol also resulted in radioactivity appearing in both methane and carbon dioxide, but the acetate fermentation was greatly depressed in this case, and the ratio of CH₄ to CO₂ was 1:2. Therefore, in the methanol-grown cultures, the methyl group of acetate behaved in a manner similar to methanol. (However, the small quantity of acetate metabolized may reflect utilization in ways other than as an energy source; e.g., a small amount may be oxidized to generate reducing equivalents for carbon assimilation.)

**DISCUSSION**

The experimental results presented in this paper show that Methanosarcina strain 227 ferments acetate by a decarboxylation reaction in which the methyl group of acetate is reduced to methane while the carboxyl group is oxidized to carbon dioxide. This reaction occurs in an atmosphere of 100% N₂ and is accompanied by cell growth. Growth and methanogenesis are consistently obtained in media containing yeast extract and acetate, but will not occur to a significant extent in yeast extract media lacking acetate or methanol. These observations indicate that acetate may serve as a source of energy for growth and that methane is not formed from it as a secondary reaction under the present culture conditions. The rate at which acetate is fermented is orders of magnitude higher than reported previously for members of this genus. (Stadtman and Barker reported that 15 weeks were required for significant quantities of methane to be formed from acetate in mineral medium [14]) Growth of strain 227 on acetate in complex medium starting with a 1% inoculum was complete within 4 weeks. Maximum rates of methanogenesis by strain 227 on acetate are typically 4 to 5 mmol of methane per liter of culture per day. Growth and methanogenesis from acetate is exponential, with doubling times of 24 to 72 h, depending upon cultural conditions.

The finding by Zeikus et al. that pure cultures of *M. barkeri* and *M. thermoautotrophicum* will not ferment acetate unless H₂ is added (20, 21) may be due to the prior growth of his cultures in an H₂/CO₂ atmosphere and/or the isolation and selection of a different strain of *Methanosarcina*. Equal quantities of methane were formed from the methyl and carboxyl groups of acetate rather than primarily from the methyl group, as observed in our pure culture experiments or in sludge digestors (6) or acetate-enrichment cultures (10, 14). In any event, under the conditions reported, even though methane was formed from

---

**Table 4. Effect of growth substrates on methanogenesis from labeled substrates**

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>Label</th>
<th>Atmosphere</th>
<th>Total dpm</th>
<th>Ratio of [¹⁴CH₄] (dpm) to [¹⁴CO₂] (dpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃COONa</td>
<td>*CH₃COONa</td>
<td>N₂</td>
<td>146,259</td>
<td>2,067</td>
</tr>
<tr>
<td>CH₃COONa</td>
<td>*CH₃OH</td>
<td>N₂</td>
<td>402,658</td>
<td>5,351</td>
</tr>
<tr>
<td>CH₃OH</td>
<td>*CH₃OH</td>
<td>N₂</td>
<td>92,169</td>
<td>29,388</td>
</tr>
<tr>
<td>CH₃OH</td>
<td>*CH₃COONa</td>
<td>N₂</td>
<td>5,203</td>
<td>10,388</td>
</tr>
</tbody>
</table>

*a* Experiment was performed as described in the text. The asterisk indicates the position of the [¹⁴C] label in the molecule. Samples were assayed by the gas proportional counter method given in the text. Results are from duplicate determinations from inocula of 1%.

*a* Primary label was not diluted by addition of unlabeled methanol or acetate.
Methanosarcina from 1182 MAH, 100% production rise complex methanogens, indicating cells equal to sources, even in the presence of yeast by nutrients derived from methanol compared to acetate. Nutrients at a linear yeast can be obtained in a defined medium.

The standard free growth of the isolate strain 227 exhibited marked differences in viable count when pregrown on one substrate and then inoculated into another.

Heterogeneity with respect to growth of initial isolates on acetate, methanol, and hydrogen/carbon dioxide is consistent with the view that the routes of metabolism for the three substrates may differ from one another. Our isolate of strain 227 exhibited marked differences in viable count when pregrown on one substrate and then inoculated into another.

Our studies have led us to hypothesize that some metabolic controlling action may be exerted by pregrowing our inoculum on one methanogenic substrate and subsequently presenting the cells with a different methanogenic substrate. There may also be a possible genetic heterogeneity in our strain with respect to the ability to use acetate. Based on these hypotheses, it is clear that the isolation and maintenance of cultures on H2/CO2, methanol, or acetate will influence the route of methanogenesis from the current substrate. Such procedures may select against organisms which can metabolize acetate in a nitrogen atmosphere.

The ability of strain 227 to use acetate as a sole energy source has been questioned on the grounds that the standard free energy for the decarboxylation of acetate to methane and carbon dioxide is insufficient to allow bacterial growth (4, 16, 20, 21). The standard free energy change for this reaction is −7.4 kcal (ca. −30.98 kJ) per mol and is barely sufficient to provide 1 mol of adenosine 5′-triphosphate per mol of acetate fermented (16). However, chemical reactions in living cells are not expected to occur under standard conditions, and the actual free energy changes are subject to variations in many factors such as ionic strength, concentrations of reactants and products, temperature, and local pH. In fact, growth and formation of adenosine 5′-triphosphate was recently reported for Desulfitobacterium acetoxidans, which oxidizes acetate to carbon dioxide with concomitant reduction of sulfur to sulfide (9). The standard free energy decrement for this reaction is only −5.7 kcal (ca. 23.86 kJ). The possibility also exists that adenosine 5′-triphosphate is generated by a chemiosmotic process, in which case 1 or more mol of acetate, growth did not apparently take place. Fortunately, rates of methanogenesis by Methanosarcina from acetate could not really be calculated from the data reported. Growth in the 100% N2 atmosphere used in our cultures and methanogenesis from acetate were not due to production of H2 from external (i.e., non-acetate) sources or organic compounds present in the complex medium, since [U-14C]acetate gave rise to 14CO2 at concentrations approximately equal to 14CH4. Furthermore, 14CH4 was not produced from H14CO3− in our complex medium when cells were incubated under N2. If hydrogen has a role in the metabolism of acetate-dissimilating methanogens, it may be to serve as an electron donor for acetate or CO2 assimilation.

The growth kinetics obtained on acetate or methanol suggest that the maximum rates of methanogenesis and growth yields are still limited by nutrients or factors other than the energy sources, even in the presence of yeast extract. Nutritional studies reported here show that growth on methanol in defined medium is slow compared to growth on methanol in media containing yeast extract. Moreover, the cell yield is a linear function of the yeast extract concentration. Nutrients in yeast extract are limiting even at a concentration of 0.2%. The limiting nutrients apparently are minerals, since an ash of yeast extract can replace it at equivalent concentrations. The limiting factor(s) is not cobalt, since defined medium containing cobalt may still be stimulated by addition of ash. Good growth on acetate in a mineral medium is more difficult to obtain than growth on methanol. Furthermore, preliminary results (M. R. Smith, unpublished data) suggest that yeast extract may be less important as a nutrient source for growth on acetate than growth on methanol.

The pathways by which acetate, methanol, and H2/CO2 are metabolized to methane appear to differ in some respects. When cells growing on methanol are exposed to methyl-labeled acetate, the methyl group of acetate is oxidized to CO2. The amount of acetate metabolized under these conditions is much less than when cells are growing on acetate alone. When cells growing on acetate are exposed to labeled methanol, the methanol is reduced without significant formation of carbon dioxide. These routes of metabolism differ from the reported routes of acetate and methanol metabolism and were observed previously in acetate enrichment cultures by Pine and Vishniac (10). The radioactively labeled methyl group of acetate is normally reduced without formation of 14CO2, and [14C]-methanol is normally oxidized and reduced with the formation of 14CH4 and 14CO2 at a ratio of 3:1. Moreover, addition of hydrogen and bicarbonate to cultures growing on labeled acetate results in a depression of the amount of acetate metabolized to methane. All of these results suggest a possible role for regulatory phenomena involving these three methanogenic substrates.

Moreover, the growth of this isolate on acetate, methanol, and hydrogen/carbon dioxide is consistent with the view that the routes of metabolism for the three substrates may differ from one another. Our isolate of strain 227 exhibited marked differences in viable count when pregrown on one substrate and then inoculated into another.

Our studies have led us to hypothesize that some metabolic controlling action may be exerted by pregrowing our inoculum on one methanogenic substrate and subsequently presenting the cells with a different methanogenic substrate. There may also be a possible genetic heterogeneity in our strain with respect to the ability to use acetate. Based on these hypotheses, it is clear that the isolation and maintenance of cultures on H2/CO2, methanol, or acetate will influence the route of methanogenesis from the current substrate. Such procedures may select against organisms which can metabolize acetate in a nitrogen atmosphere.
acetate converted to methane would be required to generate 1 mol of adenosine 5'-triphosphate. Detailed growth yield studies, to be presented in a subsequent publication, suggest that this may be the case (Smith and Mah, manuscript in preparation).

The dissimilation of acetate to methane and carbon dioxide observed in strain 227 is the same as that observed in sediments, anaerobic digestors, and acetate enrichment cultures (6, 14, 17, 18). In such ecosystems, H₂ is never present at substrate concentrations and, at best, is barely detectable. Hydrogen is therefore probably not an important substrate for generating methane from acetate in these environments, although it is an important substrate for generating methane from CO₂.

Maximum rates of methanogenesis from acetate in our pure culture of strain 227 is 4 to 5 mmol/liter per day. The rates of methane production are attributable to the relatively high cell densities (approximately 12 mg [dry weight] of cells per 100 ml of culture) obtained in these cultures. This result indicates that the methanosarcinae can be an ecologically significant source of methane from acetate in nature if they are present at sufficient densities. It further indicates that this strain is probably significant in the production of methane in the acetate-enrichment culture from which it was isolated (Baresi et al., submitted for publication). Microscopic examination of that enrichment revealed that methanosarcinae predominate.

In contrast, the rate of methane production reported for the hydrogen-dependent dissimilation of acetate by *M. thermoautotrophicum* was not sufficient to account for rates of methane production in nature (21). However, the low rates may be due to the low cell densities of the culture (0.06 optical density units at 660 nm). Direct comparison of rates of methane production as a function of cell density of strain 227 with *M. thermoautotrophicum* is not presently possible because the organisms have different light-scattering characteristics and because different methods were used for measuring cell densities. A better comparison may be made by measuring rates of methane production as a function of cell mass. It should be noted, however, that hydrogen-dependent growth was not observed on acetate for *M. thermoautotrophicum*.

A number of investigators have reported acetate enrichments in which methanosarcinae were not observed (5, 17; L. Baresi, unpublished data). Moreover, methanosarcinae are not usually observed by direct microscopic examination of sewage sludge (unpublished data), although they may be found by serial dilution of sewage sludge inoculated into roll-tube media and detected by colony formation (6; Baresi, unpublished data). The possibility therefore exists that other, as yet unisolated, species of acetate-decarboxylating methanogens exist in nature and may be even more important in methane production than the methanosarcinae. However, until actual numbers of *Methanosarcina* in digestors, muds, or other sediments are available, these organisms cannot be ruled out as important contributors to methanogenesis in these systems because of their large mass per colony-forming unit.

ACKNOWLEDGMENTS

We are indebted to Alan Miller for his technical assistance and to Jack Pangborn for the scanning electron micrographs. We also thank T. L. Glass and I. R. Kaplan for discussion and suggestions. We are grateful to the Southern California Edison Co. for their continued support. This work was supported by research grant GE-64 from the Southern California Edison Co.

LITERATURE CITED


