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# Methanogenic Cleavage of Acetate by Lysates of *Methanosarcina barkeri*

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**Cell lysates of acetate-grown *Methanosarcina barkeri* 227 were found to cleave acetate to $CH_4$ and $CO_2$. The aceticlastic reaction was identified by using radioactive methyl-labeled acetate. Cell lysates decarboxylated acetate in a nitrogen atmosphere, conserving the methyl group in methane. The rate of methanogenesis from acetate in the cell lysates was comparable to that observed with whole cells. Aceticlastic activity was found in the particulate fraction seperate from methylcoenzyme M methylreductase activity, which occurs in the soluble fraction. Pronase treatment eliminated methylcoenzyme M methylreductase activity in lysates and stimulated aceticlastic activity, indicating the aceticlastic activity was not derived from unbroken cells, which are unaffected by proteolytic treatment.**

Methane is produced from acetate (by decarboxylation), $H_2$–$CO_2$, methanol, formate, and methylamines (4, 7, 20, 27, 29). In non-gastrointestinal ecosystems acetate is the principal methanogenic precursor (1, 5, 28), and its utilization is influenced by the presence of other methanogenic substrates. For example, *Methanosarcina barkeri* 227 uses $H_2$–$CO_2$ or methanol for methanogensis before using acetate even though it is one of the few methanogens that can catabolize acetate (6, 15, 27). Not only is $H_2$–$CO_2$ preferred, but evidence now suggests that $H_2$ inhibits aceticlastic activity (2, 11, 15, 18).

Acetate-grown *M. barkeri* 227 contains enzymes and cofactors—2-mercaptoethanesulfonate (coenzyme M), methylcoenzyme M ($CH_3$-S-CoM) methylreductase, hydrogenase, and coenzyme $F_{420}$— used for the reduction of $CO_2$ by $H_2$ and at levels comparable to those in $H_2$–$CO_2$-grown cells (3). Yet information on the enzymology of the aceticlastic reaction is scant at best (31). The reason for the lack of information is probably due to the following: (i) the difficulty encountered in growing the methanogens on acetate in quantities sufficient to prepare cell lysates; (ii) the foregone conclusion that decarboxylation of acetate is not a viable energy-generating reaction (15, 24, 30); (iii) the fact that the majority of methanogens use $H_2$–$CO_2$ or formate and not acetate to produce $CH_4$ (16); and (iv) the inability of extracts from methanol or $H_2$–$CO_2$-grown cells to decarboxylate acetate. Thus, for enzyme studies in which great quantities of cell material are needed, the preferred growth substrate has been $H_2$–$CO_2$ and not acetate. In this paper I describe the first successful effort in obtaining cell lysates capable of cleaving acetate and producing $CH_4$ and $CO_2$.

(Portions of these results were previously reported [L. Baresi, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I15, p. 142]).

## MATERIALS AND METHODS

**Source of strain and culture conditions.** *M. barkeri* 227 was obtained from R. A. Mah (University of California, Los Angeles) and cultivated in the following basal medium (grams per liter): $NH_4Cl$, 0.33; $MgCl_2 \cdot 6H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.0025; $NiCl_2 \cdot 6H_2O$, 0.00047; resazurin, 0.001; L-cysteine hydrochloride · $H_2O$, 0.5; $Na_2S \cdot 9H_2O$, 0.1: yeast extract (Difco Laboratories, Detroit Mich.), 2; Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 2. For growth on acetate, the medium was supplemented with the following (grams per liter): $Na_2PO_4 \cdot H_2O$, 3.4; $Na_2HPO_4$, 2.2; sodium acetate, 8.2; $N_2$ atmosphere. When $H_2$–$CO_2$ (80:20) was the substrate, the basal medium supplement consisted of the following (grams per liter): $Na_2HPO_4$, 0.53; $NaH_2PO_4 \cdot H_2O$, 0.86; $NaHCO_3$, 5. All gases used for cell culturing, harvesting, and other anaerobic procedures were scrubbed free of oxygen by passage over copper filings heated to 350°C (3). Serum modifications of the Hungate technique were used (1, 3).

Cells were routinely cultivated at 37°C in the following: (i) 50 ml of medium in 125-ml serum bottles (Wheaton Scientific Div., Wheaton Industries, Millville, N.J.); (ii) in 400 ml of medium in 1-liter bottles (no. 219760; Wheaton Scientific) modified to accommodate syringe injection (1); (iii) in 10 liters of medium in 13-liter carboys with specially adapted metal plates that allowed continuous regulated pressure release and anaerobic syringe injection; and (iv) in 225 liters of medium in a 250-liter fermenter. A 4 to 5% inoculum was used, and cells were usually harvested within 3 weeks of inoculation (yield, 0.8 g [wet weight] per liter). The average time for incubation in the 250-liter fermenter was 4 weeks. After the second week of incubation 11 mol of acetic acid was added; the final yield was 1.5 g [wet weight] per liter.

Cells were harvested anaerobically in the late log phase (3), suspended in an equal volume of 15% glycerol–100 mM sodium phosphate buffer (pH 7)–3.3 mM 2-mercaptoethanol, and frozen in liquid $N_2$. Before use the cell suspension was centrifuged at 20,000 × *g* for 10 min.

**Preparation of cell lysates.** Lysates were prepared by suspending the cell pellets (approx 2 g [wet weight]) in 10 ml of phosphate buffer containing the following: 100 mM sodium phosphate adjusted to pH 7.0 with HCl, 0.3 M sucrose, 10 mM glutathione, 10 mM dithiothreitol (DTT), and 20 mM L-cysteine hydrochloride. The cells were then ruptured by passage through a French pressure cell (American Instrument Co., Rockville, Md.) at 108 to 121 MPa (ca. 16,000 to 18,000 lb/in$^2$). The pressure cell was loaded in an anaerobic chamber (Coy Manufacturing Co., Ann Arbor, Mich.), and the cell lysate was collected in a closed serum bottle continually flushed with $N_2$. Alternatively, cells were broken with a Braun MSK tissue disintegrator (B. Braun Melsungen

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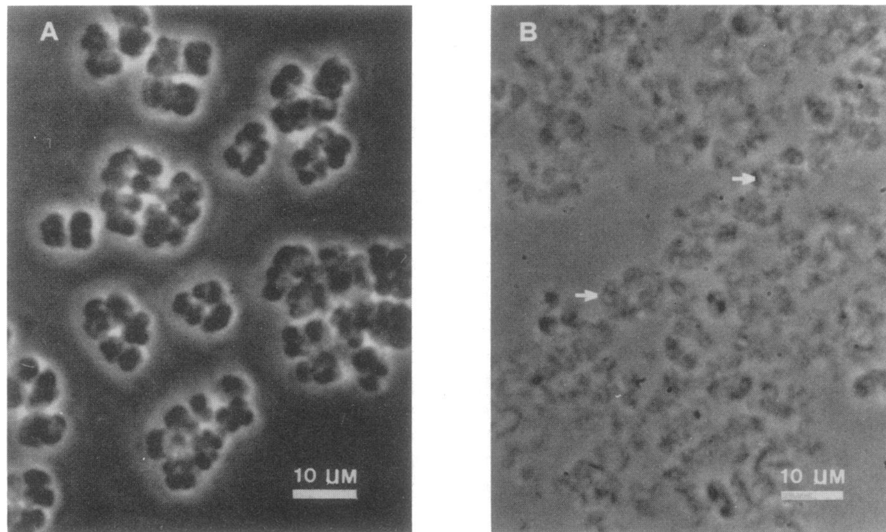


FIG. 1. Phase-contrast photomicrographs of *M. barkeri* 227 before (A) and after (B) lysis. Arrow indicates the presence of cell ghosts.

Apparatebau, Melsungen, Federal Republic of Germany). Cells were shaken for a total of 5 min in 30-s intervals with 0.45- to 0.5-mm glass beads. The homogenizer bottle was chilled and contained an oxygen-free $N_2$ atmosphere. Microscopic examination of the preparation indicated that the majority of the cell lysate consisted of broken cells.

**Assays.** The aceticlastic reaction was monitored by quantifying labeled $CH_4$ produced from methyl-labeled acetate: $^{14}CH_3COOH \rightarrow {}^{14}CH_4 + CO_2$. The contents of the reaction mixture were as follows: 400 μl of cells or cell lysate, 600 μl of 54 mM sodium phosphate buffer (pH 6.6), 15 mM DTT, 25 mM sodium acetate, and sodium [2-$^{14}$C]acetate or sodium [1-$^{14}$C]acetate as noted below. Reaction vials were placed on ice and gassed with $N_2$ 5 min before adding cells or cell lysate. The complete reaction mixture was then flushed with $N_2$ for an additional 5 min. The reaction was initiated by placing the reaction vials in a 37°C water bath or constant temperature heating block. One unit of enzyme activity is defined as the amount of enzyme that formed 1 μmol of $CH_4$ per min.

The $CH_3$-S-CoM methylreductase was assayed by the procedure of Gunsalus and Wolfe (10) as modified by Baresi and Wolfe (3). After solubilization at 90°C in 2 N NaOH for 15 min, protein was measured by the method of Lowry et al. (14) as modified by Markwell et al. (17) with bovine serium albumin as the standard.

Methane was determined by gas-solid chromatography with a Packard 428 or Varian Aerograph 1200 gas chromatograph with a flame ionization detector and a 180- by 4-cm glass column packed with Super Q (Alltech Associates Inc., Arlington Heights, Ill.) (3). Labeling studies were accomplished by gas chromatography with a thermal conductivity detector followed by a Packard gas proportional counter or by combusting the gas chromatograph effluent and traping $CO_2$ in 10 ml of Aquasol scintillation fluid containing 1 ml of phenethylamine. Gas samples for radioactive gas analysis were obtained by acidifying the reaction mix with phosphoric acid before gas sampling.

**Proteolytic hydrolysis.** Proteolytic hydrolysis of cell lysate was performed by mixing equal volumes of *M. barkeri* lysate with either 0.5% trypsin, 0.5% papain, or 0.5% pronase in their respective buffers (see below). The lysate was incubated at 37°C for 20 min while flushing with $N_2$ and then adjusted to pH 6.6 to 6.8 with anaerobic HCl or NaOH. The pronase buffer solution consisted of 20 mM phosphate buffer (pH 7.5), 26 mM NaCl, 5.4 mM KCl, 10 mM $CaCl_2$, 2 mM $MgCl_2 \cdot 6H_2O$, and 10 mM DTT. The papain buffer solution contained 40 mM phosphate buffer (pH 6.6), 5 mM cysteine, 0.1 mM EDTA, 0.06 mM 2-mercaptoethanol, and 10 mM DTT. The trypsin buffer solution consisted of 46 mM Tris, (pH 8.1), 11.5 mM $CaCl_2$, and 10 mM DTT.

**Chemicals.** $CH_3$-S-CoM was synthesized by W. E. Ellefson or B. Whitman by the procedure of Romesser and Balch (21). Glutathione, *N*-tris(hydroxymethl)methyl-2-aminoethanesulfonic acid, 2-(*N*-morpholine)ethanesulfonic acid, 2-mercaptoethanol, DTT, papain, trypsin, and ATP were purchased from Sigma Co., St. Louis, Mo. Pronase was purchased from Calibiochem-Behring Corp., San Diego, Calif. Sodium [2-$^{14}$C]acetate (1 to 3 Ci/mol) and sodium [1-$^{14}$C]acetate (1 to 3 Ci/mol) were purchased from New England Nuclear Corp., Boston, Mass.

TABLE 1. Methane production in cell lysates of *M. barkeri* 227 grown on acetate[a]

| Atmosphere in reaction vial | Total dpm in: | | Methane production (nmol/min) |
|---|---|---|---|
| | $^{14}CH_4$ | $^{14}CO_2$ | |
| $N_2$ | 150,000 | 4,000 | 2.6 |
| $H_2$ | <4,000 | 67,000 | 0.5 |

[a] Sodium [2-$^{14}$C]acetate (2.5 μCi) was used as the radiotracer. For the determination of label in $CH_4$ or $CO_2$, reaction mixtures were acidified and sampled after 3 h. Identical volumes of the same cell lysate were used for each test.

## RESULTS

**Cell lysis.** Cell breakage was confirmed either by microscopic observation, by quantitation of soluble protein released after disruption, or by quantitation of released $CH_3$-S-CoM methylreductase (a soluble enzyme).

Figure 1 shows the appearence of *M. barkeri* 227 before (Fig. 1A) and after (Fig. 1B) lysis. After disruption the lysate consisted of large membrane particles, cell ghosts, and adherent cell wall material. The extent of lysis was usually determined by quantifying the amount of protein released

TABLE 2. Effect of culture conditions on methanogenesis from cell lysates of *M. barkeri* 227

| Species | Growth substrate | Methane production from acetate (nmol of $CH_4$/mg of protein)[a] | $CH_3$-S-CoM methylreductase (mU of $CH_4$/mg of protein)[b] |
|---|---|---|---|
| *M. barkeri* 227 | Acetate | 828 | 7.7–18.4 |
| *M. barkeri* 227 | $H_2$–$CO_2$ (2 generations) | 360 | 9.6 |
| *M. barkeri* 227 | $H_2$–$CO_2$ (>8 generations) | ND[c] | 12.1 |
| *M. thermoautotrophicum* | $H_2$–$CO_2$ | ND | 50.4 |

[a] Methane determinations reported here were after 3 h. Methanogenesis from acetate was linear over the entire incubation period.
[b] $CH_3$-S-CoM methylreductase activity of supernatant from cell lysate (10,000 × *g* for 15 min).
[c] ND, Nondetectable (detectable limits, 8 nmol of $CH_4$).

after lysis. Between 85 and 90% of the total cell protein was released into the supernatant (20,000 × *g* for 30 min) after cellular disruption with either the French pressure cell or the Braun cell homogenizer. These determinations indicate that the lysate consisted mainly of broken cells.

**Effect of atmosphere on methanogenesis from acetate by cell lysates.** Aceticlastic activity in lysates was dependent on the composition of the atmosphere. With $N_2$ (Table 1), substantial quantities of $^{14}CH_4$ and very little $^{14}CO_2$ were produced from [2-$^{14}$C]acetate (less than 3% of the consumed label ended up in $CO_2$). $^{14}CH_4$ represented less than 5% of the total radioactivity in gases when [1-$^{14}$C]acetate was the substrate. Most of the label from [1-$^{14}$C]acetate was found in $^{14}CO_2$. Acetate was decarboxylated in cell lysates without the addition of ATP. An increase in oxidation of the methyl group of acetate to $CO_2$ was observed in the presence of $H_2$ (more than 94% of the [2-$^{14}$C]acetate ended up in $CO_2$, whereas 6% was converted to $CH_4$). Increased oxidation of acetate to $CO_2$ has also been reported for cultures exposed to $H_2$ (9). Hydrogen inhibited by 80% the rate of methanogenesis in lysates from acetate-grown cultures.

The growth substrate used for culturing *M. barkeri* also markedly influenced the aceticlastic activity of the *M. barkeri* lysate. Cells grown on acetate then transfered to medium in which $H_2$–$CO_2$ was the sole energy source exhibited decreased aceticlastic activity after two generations (Table 2). Cultures transfered more than eight times on $H_2$–$CO_2$ in the absence of added acetate did not show detectable aceticlastic activity, even though low levels of acetate (<2 mM) contributed by yeast extract and Trypticase were present in the medium. *Methanobacterium thermoautotrophicum* grown in acetate-supplemented medium with $H_2$–$CO_2$ contained no detectable aceticlastic activity. On the other hand, methyl reductase activity was present regardless of the growth substrate (Table 2) in both *M. barkeri* and *M. thermoautotrophicum*.

**In vitro properties of the aceticlastic reaction.** $CH_4$ was produced from acetate by cell lysates in an $N_2$ atmosphere at a constant rate for at least 300 min (Fig. 2). Without added acetate, little or no $CH_4$ was produced. (Trace amounts of $CH_4$ were sometimes formed from endogenous precursor pools; this could be eliminated by the preincubation of the extract in an $N_2$ atmosphere for 15 min at 37°C before use.) Methane formation from acetate increased linearly with increasing amounts of cell lysate (0.11, 0.35, 0.9, 1.3, 1.8, and 2.9 μmol of $CH_4$ was formed from 0.4, 0.8, 1.6, 2.4, 3.2, and 4.8 mg of protein, respectively, in 2.5 h). The apparent $K_m$ for acetate was 5.2 mM. Methane was produced from acetate in a pH range of 5.5 to 8.0 (optimum pH, 6.5). The optimum pH for the reaction was not influenced by the choice of buffer [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, 2-(*N*-morpholine)ethanesulfonic acid, or phosphate], but phosphate-buffered reactions produced greater methanogenic activity and less oxidation of the acetate methyl group to $CO_2$ than did the other buffers tested. The optimum concentration of phosphate buffer was about 36 mM.

**Inhibitor studies.** Several compounds previously shown to inhibit in vivo methanogenesis from acetate or $H_2$ were tested for their ability to inhibit the aceticlastic reaction in vitro. Complete inhibition of methanogenesis from acetate by methyl or benzyl viologen occurred at concentrations as low as 1 μM. The $CH_3$-S-CoM analog 2-bromoethanesulfonate, a potent inhibitor of the $CH_3$-S-CoM methylreductase reaction, also proved effective in inhibiting the production of $CH_4$ from acetate (50% inhibition at 10 μM). The one-carbon analog of $CH_4$, chloroform, the electron transport inhibitors

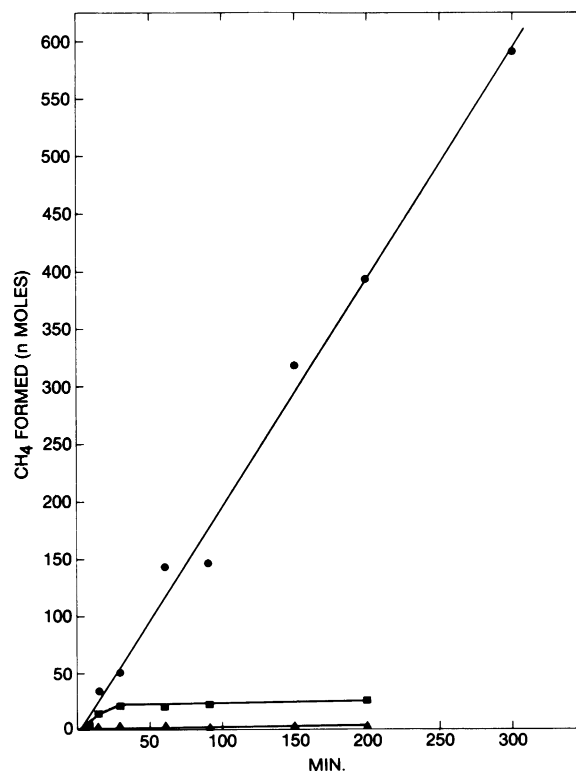


FIG. 2. Effect of acetate on methanogenesis in cell lysates incubated with an $N_2$ atmosphere. The reaction mixture contained 400 μl of cell lysate (3.2 mg/ml), 0.25 μCi of $^{14}CH_3COONa$, and 600 μl of 15 mM DTT, 54 mM sodium phosphate buffer (pH 6.6), with 25 mM sodium acetate (●), without sodium acetate (■), and without sodium acetate and preincubated (▲).

TABLE 3. Localization of methanogenesis

| Prep[a] | dpm/ml of lysate | | Methane synthesis from acetate (μmol/ml of lysate) | Sp act (mU/mg of protein) | Protein (mg/ml) | Total vol (ml) | Total activity (mU) | Yield (%) |
|---|---|---|---|---|---|---|---|---|
| | $^{14}CH_4$ | $^{14}CO_2$ | | | | | | |
| Cells | 155,000 | 3,200 | 4.1 | 12.7 | 1.8 | 4 | 92 | 100 |
| Cell lysate | 125,000 | 13,000 | 3.9 | 12.0 | 1.8 | 4 | 87 | 95 |
| Centrifuged cell lysate[b] | | | | | | | | |
| Pellet | 297,000 | 39,500 | 14.0 | 130.0 | 0.6 | 1 | 78 | 85 |
| Supernatant | 6,000 | 1,200 | 0.1 | 0.3 | 1.9 | 3 | 2 | 2 |

[a] Sodium [2-$^{14}$C]acetate (0.25 μCi) was used as the radiotracer. Methane was determined after 3 h. Methanogenesis was linear for the incubation period. Each incubation mixture contained 1 ml of cellular preparation.
[b] Centrifugation at 20,000 × g for 15 min.

2,4-dinitrophenol and sodium azide (does not inhibit whole cells [M. Smith, personal communication]) caused 50% inhibition of methanogenesis in crude extracts at 4, 5, and 10 μM, respectively. No aceticlastic activity was found when cells or cell lysate reagents were prepared aerobically, but the inhibitory concentrations of oxygen were not determined.

**Stability of cells or cell lysates.** The most active aceticlastic reactions were obtained with freshly lysed cells. Cells frozen at −10°C for less than a month lost 96 to 98% of their original activity. On the other hand, cells stored in liquid $N_2$ maintained 80 to 88% of their original activity even when stored for over 2 years. Freshly prepared lysates produced higher levels of aceticlastic activity than those stored on ice or frozen. Freezing the lysate or prolonged storage at 4°C eliminated the aceticlastic activity and stimulated the rate of acetate methyl group oxidation to $CO_2$.

**Location of aceticlastic activity.** Under the proper conditions, aceticlastic activity of lysates was similar to that of whole cells (Table 3). After centrifugation of the lysate (10,000 × g, 10 min), methanogenic activity from acetate was associated with the pellet (85%). Only 2% of the methanogenic activity from acetate was present in the soluble fraction. Unlike the intact cells, the cell lysate and pellet possessed elevated activity for methyl group oxidation (<3, 9, and 12%, respectively).

The lysate was fractionated further by discontinuous gradient centrifugation (Table 4). Methanogenic activity from acetate was distributed throughout fractions 2 (1 M sucrose) and 3 (a distinct band between the 1 and 2.2 M sucrose bands), whereas the majority of $CH_3$-S-CoM methylreductase activity was in the first fraction (0.3 M sucrose). As much as 87% of the total aceticlastic activity resides in fractions 2 and 3, yet fractions 2 and 3 contain less than 17% of the original cell lysate protein. Microscopic examination of the fractions revealed cell ghosts in fractions 2 and 3 and unbroken cells in the pellet. The pellet contained only a small fraction of the total methanogenic activity of the lysate and, along with fraction 2, exhibited substantial activity for methyl group oxidation. Thus $CH_3$-S-CoM methylreductase activity was separated from aceticlastic activity by sucrose gradient centrifugation.

**Effects of proteolytic enzymes on methanogenesis from acetate.** Lysates from acetate-grown cells were subjected to proteolytic hydrolysis in an attempt to distinguish particulate enzyme activity from soluble enzyme activity. $CH_3$-S-CoM methylreductase is a soluble enzyme in strain 227 (3) and was thus used as a marker for the presence of soluble enzyme activity. As expected, when cell lysates where treated with trypsin or papain, methyl reductase activity was destroyed (Table 5). As much as 88% of the $CH_3$-S-CoM methylreductase activity was lost when lysates were subjected to hydrolysis by papain. Pronase destroyed $CH_3$-S-CoM methylreductase, but stimulated methanogenic activity from acetate two- to threefold. Trypsin was ineffective in destroying aceticlastic activity, whereas papain reduced the aceticlastic activity by 30%. The inhibitory effect of papain and the stimulatory effect of pronase provide further evidence that the aceticlastic activity found in the lysate is not due to intact cells, which are unaffected by these treatments, but due to the presence of accessible enzyme.

Proteolytic enzymes also exhibited an inhibitory effect on oxidation of the methyl carbon of acetate to $CO_2$ (indicating that the oxidation of acetate methyl group to $CO_2$ was mediated by a soluble enzyme system). This was first noticed with a crude preparation showing low methanogenic activity and increased levels of methyl group oxidation. This particular preparation produced only 0.1 nmol of $CH_4$ per min with as much as 21% of the methyl group of acetate oxidized to $CO_2$ (normally around 5% of the methyl group of

TABLE 4. Distribution of $CH_3$-S-CoM methylreductase and aceticlastic activity in a discontinuous sucrose gradient[a]

| Fraction | Vol (ml) | Total protein (mg) | $CH_3$-S-CoM methylreductase activity | | | Methanogenic activity from acetate | | | Distribution (%) of label[b] in: | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Sp act (mU/mg of protein) | Total activity (mU) | Yield (%) | Sp act (mU/mg of protein) | Total activity (mU) | Yield (%) | $^{14}CH_4$ | $^{14}CO_2$ |
| 1 | 3.5 | 14.7 | 17.0 | 254 | 88 | 0.1 | 0.7 | 2 | ND | ND |
| 2 | 3.0 | 1.8 | 1.7 | 3 | 1 | 8.5 | 15.3 | 45 | 90 | 11 |
| 3 | 3.3 | 2.0 | 2.2 | 4 | 1 | 7.3 | 14.5 | 42 | 84 | 8 |
| 4 | 1.5 | 0.6 | 2.5 | 2 | 1 | 5.1 | 3.0 | 9 | | |
| Pellet | 0.7 | 1.6 | 0.3 | 1 | 1 | 0.9 | 1.4 | 4 | 86 | 15 |
| Lysate | 4.0 | 22.4 | 12.9 | 288 | 100 | 1.5 | 34.4 | 100 | 94 | 5 |

[a] Sodium [2-$^{14}$C]acetate (0.25 μCi) was used for labeling. Centrifugation was at 1,000 × g for 15 min. The initial sucrose concentrations for fraction 1, 2, and 4 were 0.3, 1.0, and 2.2 M anaerobic sucrose, respectively. Fraction 3 was collected as a distinct band at the interface between fractions 2 and 4. Unbroken cells were not present by microscopic examination of fractions 1 through 4. Methane production was linear throughout the incubation period.
[b] Averages of two experiments. ND, None detected.

acetate was oxidized to $CO_2$). When the lysate was treated with pronase the rate of $CH_4$, production rose to 1.1 nmol of $CH_4$ per min (an 11-fold increase), whereas the quantity of labeled $CO_2$ decreased from 21% to less than 0.5%.

Sucrose gradient fractions of the lysate were also affected by pronase (Table 5). $CH_3$-S-CoM methylreductase activity in fraction 2 (1 M sucrose) showed a marked decrease (3.7 to 0.3 mU of $CH_4$ per mg, 93%) when treated with pronase, but caused a fivefold increase in methanogenic activity from acetate (2.1 to 10.7 mU of $CH_4$ per mg).

## DISCUSSION

This is the first report of cell lysates from *M. barkeri* producing $CH_4$ from acetate. Thus the aceticlastic reaction becomes accessable to enzymological investigations directed toward a better understanding of this very important reaction in methanogenesis. The components necessary for the cleavage of acetate to $CH_4$ were found associated with the particulate fraction of the cell lysates. This fraction was separated from most of the soluble $CH_3$-S-CoM methylreductase activity by discontinuous sucrose gradient centrifugation and proteolytic digestion. Protein digestion of the crude extract reduced both the $CH_3$-S-CoM methylreductase and acetate oxidation activities while enhancing the aceticlastic activity. The effect of proteolytic enzymes on aceticlastic activity in sucrose gradients together with microscopic examination of the fractions ruled out unbroken cells as the source of methane from acetate. However, the sedimentation of aceticlastic activity in 1 M sucrose by centrifugation at 1,000 × $g$ indicates that aceticlastic activity is associated with a large complex.

Acetate was decarboxylated in cell lysates by a reaction in which the methyl group of acetate was reduced to $CH_4$ and the carboxyl group was oxidized to $CO_2$: $^{14}CH_3COOH \rightarrow {}^{14}CH_4 + CO_2$. Similar results have been obtained for cells growing on acetate (7, 15, 20, 29). The $K_m$ for acetate in the enzyme preparation was 5.12 mM, which compares favorably with that found for cells (27). Hydrogen inhibited methanogenesis from acetate in cell lysates; this is contrary to earlier reports of stimulation by $H_2$ (of methanogenesis from acetate) in intact cells (11, 32).

The inhibitory effects of $H_2$ on in vitro methanogenesis from acetate agrees with similar effects reported for intact cells (2, 15, 18, 30, 33). The presence of $H_2$ during growth regulated both the catalytic efficiency and the amount of aceticlastic activity present. In the presence of $H_2$ the rate of methanogenesis from acetate decreased in both cells and cell lysates. Aceticlastic activity in lysates also decreased with the number of generations cells were grown on $H_2$–$CO_2$ for energy, even if they were initially grown on acetate. The fact that cultures grown on $H_2$–$CO_2$ must be readapted to grow on acetate suggests that the aceticlastic enzyme may be an inducible enzyme system in *M. barkeri* 227. Hydrogen also stimulated oxidation of the acetate methyl carbon to $CO_2$ in cell lysates. These effects are in agreement with in vitro findings (9, 15, 33) and substantiate these effects at the enzyme level.

Treatment of lysates with pronase showed that oxidation of acetate to $CO_2$ and $CH_4$ formation were separable activities and that oxidation of acetate methyl groups to $CO_2$ was probably mediated by a soluble enzyme system (13). Pronase destroyed the activity of soluble enzymes such as $CH_3$-S-CoM methylreductase, but stimulated methanogenesis from acetate. The effect of pronase treatment on soluble enzymes other than $CH_3$-S-CoM methylreductase or acetate oxidation was not measured.

TABLE 5. Effect of proteolytic enzymes on methanogenic activity of lysates from acetate-grown *M. barkeri*[a]

| Prepn | $CH_3$-S-CoM methylreductase (mU of $CH_4$/mg of protein) | Methane from acetate (mU of $CH_4$/mg of protein) |
|---|---|---|
| Papain buffer | 4.8 | 2.6 |
| Papain plus papain buffer | 0.6 | 1.8 |
| Trypsin buffer | 3.9 | 2.4 |
| Trypsin plus trypsin buffer | 0.8 | 2.4 |
| Pronase buffer | 3.8 | 2.4 |
| Pronase plus pronase buffer | 1.0 | 4.5 |
| Fraction 1 | | |
| Pronase buffer | 2.0 | 0.1 |
| Pronase plus buffer | 0.3 | 0.1 |
| Fraction 2 | | |
| Pronase buffer | 3.7 | 2.1 |
| Pronase plus buffer | 0.3 | 10.7 |
| Fraction 3 | | |
| Pronase buffer | 1.3 | 8.8 |
| Pronase plus buffer | 0.6 | 11.4 |

[a] Fractions 1, 2, and 3 were collected after discontinuous sucrose gradient centrifugation at 2,500 × $g$ for 20 min and contained initial sucrose concentrations of 0.3, 1, and 2.3 M anaerobic sucrose, respectively.

Pronase treatment of cell lysates enhanced methanogenic activity from acetate. It is significant that neither trypsin nor papain increased methanogenic activity from acetate, although all of the proteolytic enzymes would be expected to destroy soluble enzymes or to affect methanogenesis by unbroken cells in the same manner. The reason for the specific enhancement of methanogenesis from acetate by pronase is not clear, but selective effects of proteases have been reported for membrane-bound proteins (5, 12, 25). In *Methanobacterium ruminantium* and *M. thermoautotrophicum* $CH_3$-S-CoM methylreductase (22, 23), hydrogenase (19), and the hydrogenase-ATP synthetase complex (8) are closely associated with the particulate fraction. In this study the aceticlastic components were also associated with the particulate fraction.

In vitro methanogenesis from acetate was found to be inhibited by the same inhibitors and at similar concentrations reported for in vivo systems (2, 27). Like $CH_3$-S-CoM methylreductase, the in vivo aceticlastic reaction was inhibited by 2-bromoethanesulfonate (27). $CH_3$-S-CoM is the methyl carrier in the terminal step of methanogenesis from $H_2$–$CO_2$ and methanol for *M. barkeri* 227 (26). Whether $CH_3$-S-CoM is envolved in the aceticlastic reaction cannot be ascertained from these studies.

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