NOTES

Kinetics of Acetate Utilization by Two Thermophilic Acetotrophic Methanogens: Methanosarcina sp. Strain CALS-1 and Methanothrix sp. Strain CALS-1

HANG MIN† AND STEPHEN H. ZINDER*

Department of Microbiology, Stocking Hall, Cornell University, Ithaca, New York 14853-7201

Received 6 June 1988/Accepted 26 October 1988

The kinetics of acetate utilization were examined for washed concentrated cell suspensions of two thermophilic acetotrophic methanogens isolated from a 58°C anaerobic digester. Progress curves for acetate utilization by cells of Methanosarcina sp. strain CALS-1 showed that the utilization rate was concentration independent (zero order) above concentrations near 3 mM and that acetate utilization ceased when a threshold concentration near 1 mM was reached. Acetate utilization by cells of Methanothrix sp. strain CALS-1 was concentration independent down to 0.1 to 0.2 mM, and threshold values of 12 to 21 μM were observed. Typical utilization rates in the concentration-independent stage were 210 and 130 nmol min⁻¹ mg of protein⁻¹ for the methanosarcina and the methanothrix, respectively. These results are in agreement with a general model in which high acetate concentrations favor Methanosarcina spp., while low concentrations favor Methanothrix spp. However, acetate utilization by these two strains did not follow simple Michaelis-Menten kinetics.

Acetate has been shown to be the precursor of two-thirds or more of the methane produced in mesophilic (4, 10, 13) and thermophilic (9, 21) anaerobic digesters. Two methanogenic genera, Methanosarcina and Methanothrix, are known to decarboxylate acetate to CH₄ and CO₂ (5). Thermophilic isolates of Methanosarcina spp. (15, 22) and Methanothrix spp. (20), as well as a rod-shaped thermophile called the TAM organism (1), which may represent a novel genus, have been described previously.

Evidence supports a model in which Methanosarcina spp. can use acetate more rapidly than corresponding Methanothrix spp. when the concentration is high (i.e., a higher Vₘₐₓ for acetate), while Methanothrix spp. are favored by low acetate concentrations (i.e., a lower apparent Kₘ for acetate). For example, Schönheit et al. (11) found an apparent Kₘ near 3 mM for acetate utilization by Methanosarcina Barkeri Fusaro, while Huser et al. (3) estimated an apparent Kₘ for growth of Methanothrix soehngenii Opikon near 0.7 mM. Zinder et al. (21) found that Methanosarcina spp. were numerous in a 58°C anaerobic digestor soon after startup, when acetate concentrations were in the range of 2 to 5 mM, while Methanothrix spp. were numerous several months later, when acetate concentrations were 0.3 to 1.5 mM. In their studies on a thermophilic sludge blanket anaerobic bioreactor using acetate as a substrate, Wiegent and de Man (16) showed that a population of mainly Methanosarcina spp. could be maintained if the feeding rate was adjusted to keep acetate concentrations high, while Methanothrix spp. were favored by low acetate concentrations. In this study, we compare progress curves for acetate consumption by two thermophilic acetotrophic methanogens isolated from a 58°C anaerobic digestor (22).

Methanosarcina sp. strain CALS-1 (21) was grown at 58°C and Methanothrix sp. strain CALS-1 (20) was grown at 60°C in 1-liter bottles containing 500 ml of defined growth medium with 40 mM sodium acetate as the growth substrate (20). Mid-to late-logarithmic-growth-phase cultures were brought into an anaerobic growth chamber (Coy Laboratory Products, Ann Arbor, Mich.), and cells were washed with a clinical centrifuge in growth medium lacking acetate, sulfide, and bicarbonate but containing 20 mM BES [N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid] buffer (final pH, 6.8) at 60°C and 20 mM NaCl. After the second centrifugation, the cell pellet was suspended in an appropriate amount of BES-buffered medium and was dispensed in 10-ml amounts into 37-ml serum vials. The vials were sealed with butyl rubber stoppers (Belco Biotechnology, Vineland, N.J.) and were brought out of the anaerobic chamber.

The vial headspaces were flushed with 70% N₂-30% CO₂ (Matheson Gas Products, Secaucus, N.J.) and scrubbed over hot copper coils, and Na₂S·9H₂O and NaHCO₃ were added to 0.1 and 1.0 g/liter, respectively. Sodium acetate was added to the desired concentration, and then all suspensions were incubated in a static water bath at 58°C, the temperature of the digestor from which they were isolated.

For experiments involving acetate utilization by Methanosarcina sp. strain CALS-1, acetate concentrations were measured using a high-pressure liquid chromatograph (HPLC) equipped with a fast acid analysis column (Bio-Rad Laboratories, Richmond, Calif.). Since experiments on acetate utilization by Methanothrix sp. strain CALS-1 involved concentrations near the detection limit of the HPLC (10 to 20 μM), acetate disappearance was measured radiometrically with [14C]CH₃COO⁻ (56 μCi/mmole; Amersham Corp., Arlington Heights, Ill.) as substrate. In a typical experiment, washed cell suspensions were first incubated with ca. 1 mM

* Corresponding author.
† Present address: Department of Environmental Science, Zhejiang Agricultural University, Hangzhou, People’s Republic of China.
Methanosarcina acetate was added to an unlabeled acetate. When HPLC analysis had shown that nearly all of the acetate had been consumed, a second addition of acetate was made, along with ca. 4 μCl of 14CH₃COO⁻, and the initial acetate concentration was measured by HPLC. This procedure ensured that the 10- to 20-min initial lag in acetate utilization by the cell suspensions did not interfere with determining utilization kinetics. Remaining 14CH₃COO⁻ was monitored by taking 50-μl samples and placing them, along with 100 μl of 1 M HCl to drive off bicarbonate as CO₂, into 10 ml of ACS scintillation cocktail (Amersham) in scintillation vials and counting samples with a LS-230 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). 14C-label in selected samples was fractionated by using a Bio-Rad HPX-87H HPLC column, as previously described (18).

Protein was released from cell suspensions by disruption for several minutes with 0.5-mm glass beads (VWR Scientific, Rochester, N.Y.) with a vortex Genie mixer (Fisher Scientific Co., Rochester, N.Y.) until no intact cells were seen in samples viewed by phase-contrast microscopy. Cell protein was assayed by the Coomassie brilliant blue method with reagents purchased from Bio-Rad. The protein standard was bovine serum albumin, and all protein values were corrected for the anomalously high absorbances given by bovine serum albumin, as described in the instruction manual accompanying the Bio-Rad reagents.

Figure 1 shows a representative progress curve for acetate utilization by a concentrated cell suspension of Methanosar-cina sp. strain CALS-1. After an initial lag, acetate was consumed until it reached a concentration slightly greater than 1 mM, at which point acetate consumption ceased. Since the cessation could have been due to nutrient depletion or adverse conditions, a second addition of sodium acetate was made. After a brief lag, this acetate was consumed at a similar initial rate, followed by cessation at a similar concentration. Until acetate concentrations of 3 to 4 mM were reached, acetate disappearance was zero order (linear). Attempts to fit these results to the Michaelis-Menton equation in the integrated and nonintegrated forms (12) failed. Even if the threshold value was arbitrarily defined as zero, a fit to Michaelis-Menton kinetics was not obtained unless a high degree of cooperativity was invoked (12). This was because the rate of decrease of utilization with decreasing concentration was much more rapid than that predicted by the Michaelis-Menton equation. This experiment was repeated more than 10 times with different cell suspensions, and similar results were obtained. Acetate thresholds were seen in every experiment, and threshold concentrations generally varied from 1.0 to 1.5 mM, but values as high as 2.5 mM and as low as 0.8 mM were obtained with different cell suspensions of Methanosarcina sp. strain CALS-1. A representative rate of acetate utilization during the zero-order utilization phase was 210 nmol min⁻¹.

To determine whether these short-term results were relevant to growing cultures of Methanosarcina sp. strain CALS-1, cultures were inoculated into growth medium containing various initial concentrations of acetate, and acetate consumption and methanogenesis were monitored for 40 days. Final acetate concentrations varied from 0.2 to 0.7 mM, but in each culture, acetate reached a final concentration which did not decrease further upon prolonged incubation (Table 1).

It has previously been demonstrated that acetate utilization by cells of Methanothrix sp. strain CALS-1 was linear below concentrations of 1 mM (20). Therefore, consumption of acetate was examined radiometrically with 14CH₃COO⁻ as a substrate. Figure 2 shows a representative progress curve for acetate utilization by Methanothrix sp. strain CALS-1. Acetate utilization was linear until concentrations near 100 μM were reached, and then there was a rapid decrease.

![FIG. 1. Acetate utilization by washed and concentrated cells of Methanosarcina sp. strain CALS-1. At the arrow, a second addition of acetate was made.](image-url)

![FIG. 2. Acetate utilization by washed and concentrated cells of Methanothrix sp. strain CALS-1.](image-url)

### TABLE 1. Final concentration of acetate in cultures of Methanosarcina sp. strain CALS-1 grown at 58°C in medium containing various initial concentrations of acetate

<table>
<thead>
<tr>
<th>Acetate concn (mM)</th>
<th>Initial</th>
<th>Final*</th>
<th>Days to reach final concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.3</td>
<td>0.60 ± 0.27</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>0.23 ± 0.15</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>0.39 ± 0.05</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>9.2</td>
<td>0.19 ± 0.01</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>17.8</td>
<td>0.38 ± 0.12</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>41.2</td>
<td>0.72 ± 0.08</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

*Concentration after which no further decrease was detected (± standard deviation for duplicate samples). Incubations were carried out for 40 days.
reduction in rate until a threshold value of radioactivity remained, representing an acetate concentration near 18 μM. This experiment was repeated more than 10 times with essentially identical results with threshold concentrations ranging from 15 to 25 μM. Incubations as long as 200 min did not result in significant further decreases in the remaining radioactivity.

Since it was quite possible that this residual label represented impurities in the label preparation not utilized by *Methanothrix* sp. strain CAL-1 rather than acetate, samples taken during this latter phase were fractionated on a HPLC column for organic acid analysis, and the various fractions were analyzed for radioactivity (18). It was found that over 80% of the residual counts (1,050 cpm) were associated with acetate, while nearly all of the remaining counts (210 cpm) were associated with a fraction comigrating with succinate and lactate. This analysis was performed on four separate samples, and the counts comigrating with lactate-succinate were also found in samples taken prior to metabolism, indicating that they represented an impurity in the label preparation. Therefore, the majority of the residual radioactivity was apparently acetate, indicating corrected threshold values of 12 to 21 μM acetate.

As was the case for *Methanosarcina* sp. strain CALS-1, the presence of a threshold concentration for acetate utilization and the rapid decrease in rate as the threshold concentration was approached were inconsistent with Michaelis-Menten kinetics for acetate utilization by *Methanothrix* sp. strain CALS-1. A representative rate for acetate utilization by *Methanothrix* sp. strain CALS-1 was 130 nmol min⁻¹ mg of protein⁻¹.

These results are consistent with the general model in which high acetate concentrations favor *Methanosarcina* sp. strain CALS-1 while low concentrations favor *Methanothrix* sp. strain CALS-1. The rates of acetate utilization, i.e., 210 and 130 nmol min⁻¹ mg of protein⁻¹ for *Methanosarcina* sp. strain CALS-1 and *Methanothrix* sp. strain CALS-1, respectively, are consistent with their doubling times at 58°C, which were 14 h (19) and 26 h (20), respectively. These results can be obtained from batch growth experiments. Lower concentrations of acetate, may not be relevant to natural situations in which lower acetate concentrations are present. For example, low acetate concentrations could induce a high-affinity uptake system. Indeed, thresholds for acetate utilization by *Methanosarcina* sp. strain CALS-1 were found to be lower in growth experiments than in short-term experiments.

Schönheit et al. (11) found that progress curves for acetate uptake by the mesophile *Methanosarcina Barkeri* Fusaro did follow Michaelis-Menten kinetics and no threshold was described, although this may be because the progress curve presented was terminated when values near 1 mM were reached. The differences between their results and ours may represent differences in growth and assay conditions or strain differences. As an example of the latter, Yang and Okos (17) found that cultures of *Methanosarcina Barkeri* MS converted only a small percentage of 7.4 mM acetate to methane after 30 days of incubation, while *Methanosarcina mazei* S6 cultures nearly completely catabolized the same concentration of acetate within 8 days. Our results resemble those of Ahring and Westermann (2), who examined progress curves for acetate uptake by the TAM organism when it was part of a butyrate-catabolizing consortium. They found that acetate uptake was linear down to concentrations near 0.6 mM and that there was a threshold of 25 to 75 μM.

The apparent *Kₚ* reported for growth on acetate by *Methanothrix soehngenii* Opfikon (3) of 0.7 mM is considerably greater than the concentration at which the rate of acetate utilization decreased for *Methanothrix* sp. strain CALS-1, 0.1 to 0.2 mM. It is of interest that Kohler and Zehnder (7) found high levels of acetate thiokinase in crude extracts of *Methanothrix soehngenii*, with an apparent *Kₚ* for acetate near 0.7 mM. They suggested that acetate activation by this enzyme determined the overall affinity of the organism for acetate. Perhaps the acetate thiokinase in *Methanothrix* sp. strain CALS-1 has a lower *Kₚ* value or is not the rate-limiting enzyme for acetate utilization. Kenealy and Zeikus (6) found high levels of acetate kinase and phosphotransacytase in *Methanosarcina Barkeri*, and the acetate kinase had an apparent *Kₚ* for acetate near 3 mM. This difference in the mechanism of acetate activation may explain the higher apparent *Kₚ* for acetate utilization of this organism.

Thresholds have been previously described for hydrogen utilization by methanogens. Lovley (8) found that several mesophilic methanogenic cultures were unable to consume hydrogen at partial pressures below 6 to 12 Pa, depending on the culture. Such thresholds may represent points at which the substrate no longer provides enough energy to support its utilization. This energy may be required for transport, activation, and especially ATP synthesis from the substrate. The approximately 50-fold higher threshold concentration for acetate utilization by *Methanosarcina* sp. strain CALS-1 versus *Methanothrix* sp. strain CALS-1 may indicate that *Methanosarcina* spp. conserve more energy from methanogenesis from acetate than do *Methanothrix* spp. By using standard calculations for ΔG° values (14), it can be estimated that when the acetate concentration is 1 mM, approximately 11 kJ more free energy is available per mol for conservation by an acetotrophic methanogen at 58°C than when the acetate concentration is 20 μM. Congruent with this idea is the higher growth yield on acetate reported for *Methanosarchina thermophila* TM-1 (1.8 g [dry wt] mol of CH₄⁻¹) (22) versus that for *Methanothrix* sp. strain CALS-1 (1.1 g [dry wt] mol of CH₄⁻¹) (20). That the acetate threshold concentration values vary from experiment to experiment for each organism suggests that physiological factors, as well as thermodynamic considerations, can play a role in determining the actual threshold concentrations for acetate utilization. Furthermore, the threshold for acetate utilization by *Methanosarcina* sp. strain CALS-1 did not change after a second addition of acetate (Fig. 1), even though the reaction products had accumulated to nearly double their concentrations after the first addition. If the threshold was a purely thermodynamic phenomenon, the threshold would have been considerably higher after the second addition.

This research was supported by grant DE-FG02-85ER13370 from the U.S. Department of Energy. Hang Min was, in part, supported by the National Science Foundation of the People’s Republic of China.

The excellent typing of Shirley Cramer is acknowledged.

LITERATURE CITED

