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## Carbon and hydrogen isotope fractionation by moderately thermophilic methanogens

DAVID L. VALENTINE,<sup>1,\*</sup> AMNAT CHIDTHAISONG,<sup>2,†</sup> ANDREW RICE,<sup>2,3,‡</sup> WILLIAM S. REEBURGH,<sup>2</sup> and STANLEY C. TYLER<sup>2</sup><sup>1</sup>Department of Geological Sciences, University of California, Santa Barbara, CA 93106, USA<sup>2</sup>Department of Earth System Science, University of California, Irvine, CA 92697, USA<sup>3</sup>Department of Chemistry, University of California, Irvine, CA 92697, USA

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**Abstract**—A series of laboratory studies were conducted to increase understanding of stable carbon ( $^{13}\text{C}/^{12}\text{C}$ ) and hydrogen (D/H) isotope fractionation arising from methanogenesis by moderately thermophilic acetate- and hydrogen-consuming methanogens. Studies of the aceticlastic reaction were conducted with two closely related strains of *Methanosaeta thermophila*. Results demonstrate a carbon isotope fractionation of only 7‰ ( $\alpha = 1.007$ ) between the methyl position of acetate and the resulting methane. Methane formed by this process is enriched in  $^{13}\text{C}$  when compared with other natural sources of methane; the magnitude of this isotope effect raises the possibility that methane produced at elevated temperature by the aceticlastic reaction could be mistaken for thermogenic methane based on carbon isotopic content. Studies of  $\text{H}_2/\text{CO}_2$  methanogenesis were conducted with *Methanothermobacter marburgensis*. The fractionation of carbon isotopes between  $\text{CO}_2$  and  $\text{CH}_4$  was found to range from 22 to 58‰ ( $1.023 \leq \alpha \leq 1.064$ ). Greater fractionation was associated with low levels of molecular hydrogen and steady-state metabolism. The fractionation of hydrogen isotopes between source  $\text{H}_2\text{O}$  and  $\text{CH}_4$  was found to range from 127 to 275‰ ( $1.16 \leq \alpha \leq 1.43$ ). Fractionation was dependent on growth phase with greater fractionation associated with later growth stages. The maximum observed fractionation factor was 1.43, independent of the  $\delta\text{D}-\text{H}_2$  supplied to the culture. Fractionation was positively correlated with temperature and/or metabolic rate. Results demonstrate significant variability in both hydrogen and carbon isotope fractionation during methanogenesis from  $\text{H}_2/\text{CO}_2$ . The relatively small fractionation associated with deuterium during  $\text{H}_2/\text{CO}_2$  methanogenesis provides an explanation for the relatively enriched deuterium content of biogenic natural gas originating from a variety of thermal environments. Results from these experiments are used to develop a hypothesis that differential reversibility in the enzymatic steps of the  $\text{H}_2/\text{CO}_2$  pathway gives rise to variability in the observed carbon isotope fractionation. Results are further used to constrain the overall efficiency of electron consumption by way of the hydrogenase system in *M. marburgensis*, which is calculated to be less than 55%. Copyright © 2004 Elsevier Ltd

### 1. INTRODUCTION

Methane,  $\text{CH}_4$ , is an environmentally important greenhouse gas and is an economically important fuel. Methane is produced in nature by four principle processes, biogenesis (as the end product of microbial metabolism; Ferry, 1993), thermogenesis (chemical degradation of organic material at elevated temperature and pressure; Schoell, 1988), geogenesis (as the result of interaction between geologic fluids with chemically reduced rocks; Horita and Berndt, 1999; Lollar et al., 2002), and ignigenesis (as a byproduct of combustion). The rate at which  $\text{CH}_4$  is released to the atmosphere is well-constrained and is currently ca.  $5.0 \times 10^{14}$  g per year (e.g., Cicerone and Oremland, 1988). The overall rate of  $\text{CH}_4$  production in nature is certainly greater than the release rate to the atmosphere, but is poorly constrained due to difficulties in quantifying the impact of microbially mediated  $\text{CH}_4$  oxidation (methanotrophy). The global rate of methanogenesis can be estimated as the sum of the global oxidation rate ( $6.9\text{--}9.2 \times 10^{14}$  g  $\text{y}^{-1}$ ; Reeburgh,

1996; Valentine, 2002) and the global atmospheric flux ( $5.0 \times 10^{14}$  g  $\text{y}^{-1}$ ). Of the total  $1.2\text{--}1.4 \times 10^{15}$  g of  $\text{CH}_4$  produced annually, the majority of this  $\text{CH}_4$  is produced biogenically, likely greater than 85%.

Biogenic  $\text{CH}_4$  production (hereafter referred to as methanogenesis) occurs at all temperatures between freezing and boiling (Valentine and Boone, 2000). The majority of  $\text{CH}_4$  currently released to the atmosphere is produced near the surface, at temperatures between 0 and 50°C. Abundant  $\text{CH}_4$  is also produced in environments with elevated temperatures (moderately thermal environments, defined here as having temperatures from  $\sim 50\text{--}110^\circ\text{C}$ ), including geothermal springs, hydrothermal vents, and waste digestors. The most important of these moderately thermal methanogenic environments are deeply buried sediments, which are heated from below by the geothermal gradient (comprising much of the “deep biosphere”). The primary energy source for heterotrophic microbes in such environments is the organic carbon initially deposited with the sediment. Little is known about biogenic  $\text{CH}_4$  production in moderately thermal subsurface environments, much of our knowledge comes from hydrocarbon exploration and from studies of other moderately thermal environments including waste digestors. Biogenic  $\text{CH}_4$  produced in moderately thermal settings is generally distinguished from thermogenic  $\text{CH}_4$  by the carbon and hydrogen isotopic content of the  $\text{CH}_4$  as well as by comparing the abundance of  $\text{CH}_4$  to ethane and propane (Schoell, 1980).

\* Author to whom correspondence should be addressed (valentine@geol.ucsb.edu).

† Present address: The Joint Graduate School of Energy & Environment, King Mongkut's University of Technology Thonburi, Bangkok, Thailand.

‡ Present address: Joint Institute for the Study of the Atmosphere and Ocean, Department of Oceanography, University of Washington, Seattle, WA, USA.

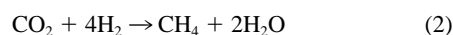
Table 1. Experiments presented in this study.

Experiment	Organism	Purpose	Substrate	Variables <sup>a</sup>	Presented in:	System <sup>b</sup>
A1-4	<i>M. thermophila</i>	<sup>13</sup> C fractionation	Acetate	g	Table 2, 4, E-1, Figs. 1–4	o, c
B1-4	<i>M. thermophila</i>	D/H fractionation	Acetate		Table 4, E-2, Fig. 9	o
C1-4	<i>M. marburgensis</i>	<sup>13</sup> C fractionation	H <sub>2</sub> /CO <sub>2</sub>	t, h, g, l, m	Table 3, 6	o
D1-3	<i>M. marburgensis</i>	D/H fractionation	H <sub>2</sub> /CO <sub>2</sub>	t, g, e, m	Table 3, Figs. 10–13, E-3	o

<sup>a</sup> Variables tested: temperature (t); H<sub>2</sub> concentration (h); growth phase (g); light level (l); hydrogenase efficiency (e); metabolic activity (m).

<sup>b</sup> System approximated as: open (o); closed (c). Tables E-1, E-2 and E-3 refer to tables presented in the electronic annex (Elsevier website, Science Direct).

Biogenic CH<sub>4</sub> is produced by two primary pathways, the aceticlastic reaction (Eqn. 1), and CO<sub>2</sub> reduction (Eqn. 2). The reduction of CO<sub>2</sub> can be accomplished with either hydrogen (H<sub>2</sub>) or formate (HCOO<sup>-</sup>) acting as reductant.



Several other methanogenic pathways exist, but are thought to be less important quantitatively (Cicerone and Oremland, 1988). The relative importance of the two primary methanogenic pathways varies depending on the environment. In terrestrial environments with moderate temperatures, the aceticlastic reaction accounts for up to 70% of all CH<sub>4</sub> produced with CO<sub>2</sub> reduction accounting for ~30%. In moderately thermal environments this ratio changes, and CO<sub>2</sub> reduction is often quantitatively more important than the aceticlastic reaction (Fey et al., 2003). In permanently cold marine sediments, CH<sub>4</sub> is thought to be derived primarily from CO<sub>2</sub> reduction (Whiticar et al., 1986). The relative importance of the aceticlastic reaction versus CO<sub>2</sub> reduction is not known for deep, moderately thermal environments. Interestingly, many thermophilic methanogens have been isolated capable of CO<sub>2</sub> reduction, while only a handful of such organisms are known to carry out the aceticlastic reaction. Furthermore, no extreme thermophiles capable of performing the aceticlastic reaction have been isolated.

Methane produced in deep subsurface environments generally migrates along the concentration gradient toward the ocean and atmosphere, often being physically or chemically trapped in the subsurface. Trapping of CH<sub>4</sub>, such as in the case of CH<sub>4</sub> hydrates, allows for the buildup of a large subsurface reservoir which may act as a capacitor (Dickens, 2003). Massive CH<sub>4</sub> releases from the subsurface reservoir may have impacted Earth's climate at several times in the past, including the Proterozoic (Kennedy et al., 2001), the early Cretaceous (Jahren et al., 2001), the terminal Paleocene thermal maximum (Dickens et al., 1997), the Permian/Triassic boundary (Krull and Retallack, 2000), the late Jurassic (Padden et al., 2001) as well as during the Quaternary (Kennett et al., 2002). Deep subsurface CH<sub>4</sub> is likely a major source of such CH<sub>4</sub>.

Given the importance of methanogenesis in moderately thermal environments, the importance of isotopic distributions in characterizing CH<sub>4</sub> sources, uncertainties regarding carbon and hydrogen isotopic fractionation in thermophilic methanogens, uncertainties regarding the role of acetate in moderately thermal environments, and the importance of CH<sub>4</sub> in rapid climate change, we performed a series of laboratory studies to increase

understanding of the physical, chemical and biologic factors controlling stable isotope fractionation in moderately thermophilic methanogens. A series of four studies were performed to quantify carbon isotope fractionation during methanogenesis from acetate (Experiment A), hydrogen isotope fractionation during methanogenesis from acetate (Experiment B), carbon isotope fractionation during CO<sub>2</sub>/H<sub>2</sub> methanogenesis (Experiment C), and hydrogen isotope fractionation during CO<sub>2</sub>/H<sub>2</sub> methanogenesis (Experiment D). Table 1 provides a general guide to these experiments.

## 2. MATERIALS AND METHODS

### 2.1. Organisms and Culture Conditions

Pure cultures of *Methanosaeta thermophila* strain CALS-1 (DSMZ 3870; Zinder et al., 1987) and strain P<sub>T</sub> (OCM 778; Kamagata and Mikami, 1991) were used for experiments to study carbon and hydrogen isotope fractionation during methanogenesis from acetate. Cultures were grown at 61°C in crimp-top bottles using a modified Hungate technique (Hungate, 1969) in a defined mineral salts medium initially containing 30 mM acetate as the sole energy source. The medium contained (per liter): 0.4 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g NH<sub>4</sub>Cl, 0.1 g MgCl<sub>2</sub> · 6H<sub>2</sub>O, 0.05 g CaCl<sub>2</sub> · 2H<sub>2</sub>O, 1 mg resazurin, 1.0 g NaHCO<sub>3</sub>, 0.36 g Na<sub>2</sub>S · 9H<sub>2</sub>O, 0.15 g CoM, 0.04 mg biotin, 5.0 mg sodium EDTA dihydrate, 1.5 mg CoCl<sub>2</sub> · 6H<sub>2</sub>O, 1.0 mg MnCl<sub>2</sub> · 4H<sub>2</sub>O, 1.0 mg FeSO<sub>4</sub> · 7H<sub>2</sub>O, 1.0 mg ZnCl<sub>2</sub>, 0.4 mg AlCl<sub>3</sub> · 6H<sub>2</sub>O, 0.3 mg Na<sub>2</sub>WO<sub>4</sub> · 2H<sub>2</sub>O, 0.2 mg CuCl<sub>2</sub> · 2H<sub>2</sub>O, 0.2 mg NiSO<sub>4</sub> · 6H<sub>2</sub>O, 0.1 mg H<sub>2</sub>SeO<sub>3</sub>, 0.1 mg H<sub>3</sub>BO<sub>3</sub>, and 0.1 mg Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O.

*Methanothermobacter marburgensis* (formerly *Methanobacterium thermautotrophicum* strain Marburg-OCM 82; Wasserfallen et al., 2000) was used in studies of CO<sub>2</sub>/H<sub>2</sub> methanogenesis. The organism was originally isolated from a thermophilic waste digester in Marburg, Germany (Fuchs et al., 1978); closely related species have been observed in a variety of moderately thermal environments including petroleum reservoirs (Orphan et al., 2000) and thermal springs. *M. marburgensis* is chemoautotrophic and is capable of reducing CO<sub>2</sub> as its sole carbon and energy source. The organism was grown in MS medium (Boone et al., 1989), or with modified MS mineral medium as noted. MS medium (complex) contains exogenous organic material while the only organic component in modified MS mineral medium (defined) was mercaptoethane sulfonate (100 mg per liter).

### 2.2. Carbon Isotope Fractionation Studies

#### 2.2.1. Carbon isotope fractionation from acetate

Four experiments (A-1 through A-4) were performed in sealed serum bottles to quantify the isotopic fractionation factors associated with aceticlastic methanogenesis by *M. thermophila*; experiments were designed to assess the discrimination against both the methyl position of acetate (which gives rise to CH<sub>4</sub>) and the whole acetate molecule. Inoculum for experiments with strain CALS-1 was obtained from late stationary phase cultures that had been thoroughly purged of CH<sub>4</sub> (so as not to interfere with the isotopic measurements). Three crimp-top serum bottles designated experiments A-1, A-2, and A-3 (1.05 L, 1.05 L, and 1.2 L, respectively), each containing basal medium (382 mL,

Table 2. Initial experimental conditions for cultures of *Methanosaeta thermophila* strain CALS-1 (Experiment A).

Condition	A-1	A-2	A-3
pH	6.49	6.56	6.51
Acetate Concentration (mM)	33.9	31.0	32.2
$\delta^{13}\text{C}$ -Acetate (whole molecule-‰)	N/A	-25	-25
$\delta^{13}\text{C}$ -Acetate (methyl position-‰)	-31.0	-29.1	-28.3
$\text{PCH}_4$ (Pa)	2	1	1

387 mL, and 447 mL-respectively), were inoculated with 20 mL of a stationary phase culture of *M. thermophila* strain CALS-1. Cultures were sampled immediately after inoculation for  $\text{CH}_4$  concentration, acetate concentration,  $\delta^{13}\text{C}$ - $\text{CH}_4$ ,  $\delta^{13}\text{C}$ - $\text{Ac}_{\text{bulk}}$ , and  $\delta^{13}\text{C}$ - $\text{Ac}_{\text{methyl}}$ . Initial experimental conditions are given in Table 2.

After the initial liquid and gas samples had been removed, all three bottles were transferred to an incubator and maintained at 61 °C. Periodic sampling for  $\text{CH}_4$  concentration, acetate concentration,  $\delta^{13}\text{C}$ - $\text{CH}_4$ ,  $\delta^{13}\text{C}$ - $\text{Ac}_{\text{bulk}}$ , and  $\delta^{13}\text{C}$ - $\text{Ac}_{\text{methyl}}$  was performed daily, or every other day, from each of the three bottles for a period of 2 weeks. The temperature during sampling was maintained between 51 and 61 °C so as to not interrupt growth. The sampling procedure involved first removing a small (0.2–5 mL) sample of headspace gas, and measuring the  $\text{CH}_4$  mixing ratio. Then, a known overpressure of  $\text{N}_2$  was added (to avoid drawing a vacuum) and a large volume gas sample (20–240 mL) was removed for analysis of  $\delta^{13}\text{C}$ - $\text{CH}_4$ . After gas samples were removed, liquid samples were taken for analysis of acetate concentration and isotopic composition.

To analyze possible changes in fractionation during different growth phases, the bottle representing experiment A-1 was completely purged of  $\text{CH}_4$  after each sampling. The bottle was purged for 4 min at a temperature of 51–61 °C (5–10  $\text{L}\cdot\text{min}^{-1}$ ), then incubated for 15 min (61 °C) with occasional vigorous shaking. Finally, the bottle was purged for an additional 4 min (at 51–55 °C), vented to atmospheric pressure, and then a known volume of  $\text{CO}_2$  was added as an overpressure. The partial pressure of residual  $\text{CH}_4$  was generally near 2 Pa. Samples for lipid isotope analysis were taken from bottle A-1 after the substrate concentration fell below 10 mM, so as to ensure sufficient biomass for isotope analysis. Growth phase was inferred from the rate of acetate depletion and  $\text{CH}_4$  production.

A second strain of *M. thermophila*, strain  $\text{P}_T$ , was also used to study isotopic fractionation from the aceticlastic reaction (Experiment A-4) to determine if fractionation differs between two strains of the same species. Culture conditions were identical to those described for strain CALS-1 except replicate cultures were grown in 125 mL serum bottles. Methane samples were collected after the  $\text{CH}_4$  reached 6% so as to avoid significant isotopic enrichment of the residual acetate.

### 2.2.2. Carbon isotope fractionation from $\text{H}_2/\text{CO}_2$

Four experiments (C-1 through C-4) were performed using an  $\text{H}_2$ -controlling bioreactor (Valentine et al., 2000a) to assess the impact of  $\text{H}_2$  partial pressure, temperature, media organic content, and light level on carbon isotope fractionation. Studies were designed to test the hypothesis that  $\text{H}_2$  partial pressure impacts carbon isotope fractionation,

while controlling for variability in temperature and media organic content. Experiments were further designed to explore observations presented by Botz et al. (1996) of greater fractionation in titanium (dark) versus glass (illuminated) bioreactors. Inoculum for each experiment was obtained from 300 mL cultures grown to completion (substrate depletion,  $\text{OD}_{598} \approx 0.3$ ) in sealed 1 L bottles under a mixture of  $10^5$  Pa  $\text{H}_2$ ,  $3 \times 10^4$  Pa  $\text{CO}_2$ , and  $7 \times 10^4$  Pa  $\text{N}_2$ . After inoculation into the bioreactor, the total gas flow rate, the partial pressure of  $\text{CO}_2$  and  $\delta^{13}\text{C}$ - $\text{CO}_2$  were held constant for the duration of each experiment. The mixing ratio of  $\text{H}_2$  was set by mass flow controllers and confirmed by both upstream and downstream measurements (before entering the reactor vessel and in the exhaust stream). Methane concentrations in the exhaust gas were measured before each experiment; background  $\text{CH}_4$  was always found to be below 0.03 Pa. The downstream  $\text{CH}_4$  concentration was also below 0.03 Pa during an uninoculated control experiment. Experimental conditions are shown in Table 3.

A sterilized glass reactor vessel with a total working volume of 230 mL was filled to a final volume of 200 mL for each experiment. The apparatus is similar to that described by Valentine et al. (2000a,b) though alterations were necessary because  $\text{H}_2$  was being fed into the vessel, not sparged from the vessel. Gases were fed to the culture using mass flow controllers. Flow rate calibrations were confirmed using a primary standard airflow calibrator (The Gilibrator, Gilian Instruments Corp., W. Caldwell, NJ). Gas flowed through a copper column maintained at 200 °C to remove residual oxygen before entering the reactor vessel.

## 2.3. Hydrogen Isotope Fractionation Studies

### 2.3.1. Hydrogen isotope fractionation from acetate

Four experiments (B-1 through B-4) were performed in sealed serum bottles to quantify the hydrogen isotope fractionation factors associated with aceticlastic methanogenesis by *M. thermophila*; experiments were designed to assess the fractionation between both the methyl-bound hydrogens of acetate and the hydrogen in ambient water with the resulting  $\text{CH}_4$ . Inoculum for experiments with *M. thermophila* was obtained from late stationary phase cultures that had been thoroughly purged of  $\text{CH}_4$  (so as not to interfere with the isotopic measurements). Three 250-mL crimp-top serum bottles designated Experiments B-1, B-2, and B-3, each containing 125 mL basal medium, were inoculated with 5 mL of a stationary phase culture of *M. thermophila* strain CALS-1. Cultures were grown at 61 °C. The  $\delta\text{D}$ - $\text{H}_2\text{O}$  of the bulk media in Experiments B-1, B-2 and B-3 was -92.1, +1227.2 and +239.7‰, respectively. Liquid and gas samples were taken immediately after inoculation for analysis of  $\text{CH}_4$  concentration (<0.1 Pa),  $\delta\text{D}$ - $\text{H}_2\text{O}$ , and  $\delta\text{D}$ -Ac. Liquid and gas samples were then taken every other day for 4 d for analysis of  $\text{CH}_4$  concentration,  $\delta\text{D}$ - $\text{CH}_4$ ,  $\delta\text{D}$ -Ac, and  $\delta^{13}\text{C}$ - $\text{CH}_4$ . The experiments with strain CALS-1 were stopped after 4 d, while still at an early stage of growth, due to poor culture growth.

A second strain of *M. thermophila*, strain  $\text{P}_T$ , was also used to study hydrogen isotopic fractionation from  $\text{H}_2\text{O}$ /acetate (Experiment B-4, same culture as Experiment A-4). Culture conditions were identical to those described for strain CALS-1 except that replicate cultures were grown in 125 mL serum bottles. Methane samples were removed after the  $\text{CH}_4$  reached 6% so as to avoid significant isotopic enrichment of the residual acetate. The initial  $\delta\text{D}$ - $\text{H}_2\text{O}$  of the culture media was  $-71 \pm 16.6\%$ .

Table 3. Conditions for experiments involving *M. marburgensis* in a flow-through bioreactor (Experiments C and D).

Experiment	Gas flow rate ( $\text{mL}\cdot\text{min}^{-1}$ )	$\text{CO}_2$ upstream (%)	$\text{H}_2$ upstream (Pa)	$\text{CH}_4$ upstream (Pa)	Temperature range (°C)	Light/dark	pH	Volume of inoculum (mL)	Culture volume (mL)	Medium type
C-1	29.7	18.4	310	0.03	40–75	L	7.7	20	200	Complex
C-2	28.7	19.1	290	0.03	40–75	L	7.7	200	200	Complex
C-3	29.7	18.4	350	0.03	65	L/D	8.3	200	200	Defined
C-4	30.1	20.1	$8 \times 10^4$	0.03	65	L	8.3	N/A	200	Defined
D-1	50/15	30	$7 \times 10^4$	0	65	L	7.8	6	260	Defined
D-2	15	30	$7 \times 10^4$	0	65	L	7.6	6	250	Defined
D-3	15	30	$7 \times 10^4$	0	45–75	L	8.1	N/A	250	Defined

### 2.3.2. Hydrogen isotope fractionation from $H_2/CO_2$

Three experiments (D-1 through D-3) were performed using an  $H_2$ -controlling bioreactor to determine the influence of growth phase on D/H fractionation in  $H_2$  and  $CH_4$ , the impact of varying  $\delta D-H_2$  on  $\delta D-CH_4$ , and the impact of temperature and catabolic rate on D/H fractionation in  $H_2$  and  $CH_4$ . A sterilized glass reactor vessel with a total working volume of 300 mL was filled to a final volume of 250–260 mL for each experiment. The apparatus is similar to that described previously (Valentine et al., 2000a).

Inoculum for each experiment was obtained from 10 mL cultures grown to completion in sealed 20 mL tubes under a mixture of  $10^5$  Pa  $H_2$ ,  $3 \times 10^4$  Pa  $CO_2$ , and  $7 \times 10^4$  Pa  $N_2$ . The total gas flow rate was set to  $55 \text{ mL min}^{-1}$  (normalized to STP) at the beginning of Experiment D-1, and was changed to  $15 \text{ mL min}^{-1}$  after the 31st hour of experimentation due to analytical difficulties in quantifying  $\delta D-CH_4$  at relatively low mixing ratios. Experiments D-2 and D-3 were performed with a flow rate of  $15 \text{ mL min}^{-1}$ . The mixing ratio of  $H_2$  was set by mass flow controllers and confirmed by both upstream and downstream measurements (before entering the reactor vessel and in the exhaust stream). Methane concentrations in the exhaust gas were measured before each experiment; background  $CH_4$  was always found to be below 0.03 Pa. Experimental conditions are given in Table 3. Experiment D-3 was a continuation of Experiment D-2, performed with the same culture immediately after Experiment D-2 had ended.

### 2.4. Isotope Systematics and Calculation of Fractionation Factors

All carbon isotope values are given in the per mil notation (‰) relative to the V-PDB standard (Pee Dee belemnite carbonate, as established by the International Atomic Energy Agency [IAEA] in Vienna, Austria; Coplen, 1995; Gonfiantini et al., 1995). All hydrogen isotope values are given in the per mil notation (‰) relative to the V-SMOW standard (Vienna Standard Mean Ocean Water as established by the IAEA).

Fractionation factors reported in this study are expressed using either the  $\alpha$  or  $\epsilon$  notation. In the case where isotopic equilibrium is achieved,  $\alpha$  is defined as:

$$\alpha = R_a/R_b \quad (3)$$

where  $R_a$  and  $R_b$  are the isotope ratios of reactants and products, respectively. In the case where isotope fractionation arises from kinetic processes,  $\alpha$  is defined as:

$$\alpha = r_l/r_h \quad (4)$$

where  $r_l$  and  $r_h$  are the reaction rates for the species containing the light and heavy isotope, respectively. In cases where fractionations are small (less than 10‰; O'Neill, 1986) the fractionation factor,  $\epsilon$ , can be used:

$$\epsilon = (\alpha - 1) \times 1000 \quad (5)$$

In all cases here, fractionation factors represent net fractionation associated with a series of physical and chemical steps; however, fractionation factors are presented as if they were associated with a single step. Fractionation factors associated with specific and individual reactions remain constant; such fractionation factors reflect isotope discrimination due to kinetic and/or thermodynamic isotope effects. Fractionation factors associated with multistep reactions are variable and may be expressed at different levels. See Hayes (1983, 2001) for a detailed consideration of these issues.

#### 2.4.1. Carbon isotope fractionation in *M. thermophila*

To calculate the carbon isotope fractionation factors associated with acetate methanogenesis by *M. thermophila*, we apply both closed- and open-system models. Models of closed-system behavior are considered first, open-system behavior is considered later. For further details on open and closed isotopic systems see Hayes (1983, 2001).

An isotopic system is considered to be closed if no material crosses its boundaries. As a reaction proceeds, the fractional yield of the product increases and the isotopic composition of the products and reactants vary in a systematic fashion, controlled by the fractionation

factor,  $\alpha$ . Because of mass balance constraints reactants become isotopically enriched as the reaction proceeds, and the isotopic composition of the pooled product tends toward the initial isotopic composition of the reactant. Fractionation factors associated with acetate methanogenesis (Experiment A) can be determined as a closed system using the approach of Gelwicks et al. (1989, 1994):

$$\delta_{af} = \delta_{ai} - \epsilon_m [\ln(1 - f)] \quad (6)$$

where  $f$  is the fractional yield based on the consumption of acetate,  $\delta_{af}$  is the  $\delta^{13}C$  of the methyl position of acetate at any  $f$ ,  $\delta_{ai}$  is the initial  $\delta^{13}C$  of the methyl position of acetate, and  $\epsilon_m$  is the fractionation factor between the methyl position of acetate and  $CH_4$ . An equation of identical form can be constructed for the conversion of the whole acetate molecule:

$$\delta_{wf} = \delta_{wi} - \epsilon_w [\ln(1 - f)] \quad (7)$$

where  $\delta_{wf}$  is the  $\delta^{13}C$  of the whole acetate molecule at any  $f$ ,  $\delta_{wi}$  is the initial  $\delta^{13}C$  of the whole acetate molecule, and  $\epsilon_w$  is the fractionation factor between the whole acetate molecule and product. This equation assumes no isotope exchange between the carboxyl position of acetate and  $CO_2$ . An independent equation can be constructed based on the isotopic composition of the pooled product,  $CH_4$ :

$$\delta_{mf} = \delta_{ai} + \epsilon_m (1 - f) [\ln(1 - f)]/f \quad (8)$$

where  $\delta_{mf}$  is the  $\delta^{13}C$  of pooled  $CH_4$  at any  $f$ . The isotopic fractionation factor,  $\epsilon_m$ , can be determined by regressing  $\delta_{af}$  against  $\ln(1 - f)$ , or  $\delta_{mf}$  against  $(1 - f) [\ln(1 - f)]/f$ . Similarly,  $\epsilon_w$  can be determined by regressing  $\delta_{wf}$  against  $\ln(1 - f)$ . In each case  $\epsilon$  is determined by the slope of the linear regression.

For Experiment A the fractional yield,  $f$ , is determined by following changes in acetate concentration. In the case of Eqn. 6 and 7,  $f$  at any given time is determined from the ratio of the acetate concentration at that time to initial acetate concentration and is independent of the quantity of liquid or gas removed during sampling. In the case of Eqn. 8, determination of  $f$  requires that all acetate removed during sampling be accounted for. Similarly, in the case of Eqn. 6 and 7,  $\delta_{af}$  and  $\delta_{wf}$  at any given time are equal to the  $\delta_{af}$  and  $\delta_{wf}$  values measured at that time. However, in the case of Eqn. 8,  $\delta_{mf}$  represents the  $\delta^{13}C$  of all  $CH_4$  produced, and is thus dependent on gas removed from the system. The value of  $\delta_{mf}$  is determined using an isotopic mass balance accounting for all  $CH_4$  removed from the system.

An isotopically open system is one which reactants are constantly added and products constantly withdrawn. Stated another way, the supply of substrate for a reaction is unlimited relative to the appearance of product. During the early stages of growth Experiments A-1, A-2, A-3, and A-4 can be treated as open systems. Because  $CH_4$  produced by the acetate reaction is generated exclusively from the methyl position of acetate (Pine and Barker, 1956), and because the fractionations involved are relatively small, the isotopic enrichment factor,  $\epsilon_m$ , is used here for acetate methanogenesis. Methane production by *M. thermophila* during the early stages of growth reasonably approximates conditions of unlimited acetate, as does the regular purging of headspace  $CH_4$ , as in Experiment A-1.

#### 2.4.2. Carbon isotope fractionation in *M. marburgensis*

Carbon isotopic fractionation factors for *M. marburgensis*, measured in Experiment C, were calculated using an open-system approach, assuming  $CO_2$  as an infinite reservoir and the constant removal of  $CH_4$ . This assumption is valid based upon the low conversion of  $CO_2$  to  $CH_4$  ( $\leq 3\%$ ) and the rapid gas flow through the culture system. In addition, the upstream  $\delta^{13}C-CO_2$  was assumed to be equal to the downstream  $\delta^{13}C-CO_2$ , an assumption supported by the distribution of  $\delta^{13}C-CO_2$  in the various experiments, and by the results of Chidthaisong et al. (2002). The measured  $\delta^{13}C-CO_2$  was assumed to be the same as that available for methanogenesis. Although fractionation occurs during  $CO_2$  dissolution (thermodynamic) and during gas transfer (kinetic), the fractionations are small ( $\approx 1-2\%$ ; Zhang et al., 1995) and were not quantified for the experimental conditions employed. Stable isotope fractionation factors ( $\alpha$ -factors) for coexisting  $CO_2-CH_4$  pairs were

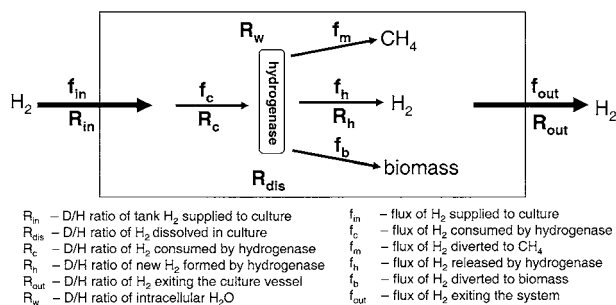


Fig. 1. Schematic diagram for the calculation of hydrogenase efficiency in *M. marburgensis*.

then calculated directly according to Eqn. 3, where  $\alpha$  is a numerical representation of fractionation for the entire methanogenic pathway.

#### 2.4.3. Hydrogen isotope fractionation in *M. marburgensis*

During H<sub>2</sub>/CO<sub>2</sub> methanogenesis the ultimate source of carbon-bound hydrogen in CH<sub>4</sub> (e.g., CH<sub>4</sub>-bound hydrogen) is thought to be H<sub>2</sub>O (Daniels et al., 1980; Spencer et al., 1980), though the immediate precursors are not completely understood. There are several potential sources of CH<sub>4</sub> hydrogen, including H<sub>2</sub>O, H<sub>2</sub>, and methyl-bound hydrogen (e.g., from acetate, methyl amines, methyl thiols, and methanol). Furthermore, CH<sub>4</sub> hydrogen may cycle through the proton pool, through biologic reductants such as coenzyme F<sub>420</sub> and coenzyme M, and through amino acid residues. A combination of kinetic and equilibrium isotope effects give rise to the ultimate hydrogen isotope distributions in CH<sub>4</sub>. Because  $\delta$ D varies by several hundred per mil in these studies, and because the  $\delta$  notation is nonlinear in this range (O'Neil, 1986), D/H ratios ( $r = D/H$ ) are needed to calculate fractionation factors between H<sub>2</sub>O and CH<sub>4</sub>:

$$\alpha_{(H_2O-CH_4)} = R_{H_2O}/R_{CH_4} \quad (9)$$

and between H<sub>2</sub>O and H<sub>2</sub>:

$$\alpha_{(H_2O-H_2)} = R_{H_2O}/R_{H_2} \quad (10)$$

The  $\alpha$  notation, as applied here, quantifies the difference in isotopic composition between two hydrogen-containing molecules linked biochemically. For example, in Eqn. 10,  $\alpha$  only applies to the H<sub>2</sub> produced by the hydrogenase system (see section 2.4.4), not to the H<sub>2</sub> entering the system. The use of this notation does not presume a predominance of kinetic or equilibrium isotope effects, it is intended only to quantify empirical observations of net isotopic fractionation resulting from methanogenesis.

#### 2.4.4. Determination of hydrogenase efficiency in *M. marburgensis*

To determine the efficiency of the hydrogenase system in *M. marburgensis*, a mass balance was constructed based on the experimental design (Eqn. 11). A schematic of the system as well as definitions of the system variables are given in Figure 1.

$$f_{out}R_{out} = f_{in}R_{in} + f_hR_h - f_cR_c \quad (11)$$

Eqn. 11 assumes there are no other significant sources or sinks of H<sub>2</sub> besides hydrogenase in the system. The gross H<sub>2</sub> consumption term in Eqn. 11 ( $f_c \times R_c$ ) can be expressed as the sum of individual production and consumption terms, yielding Eqn. 12:

$$f_{out}R_{out} = f_{in}R_{in} + f_hR_h - (f_m + f_b)R_c \quad (12)$$

The D/H ratio of H<sub>2</sub> consumed (activated) by hydrogenase ( $R_c$ ) can be expressed in terms of the D/H ratio of dissolved H<sub>2</sub> ( $R_{dis}$ ), given the fractionation factor ( $\alpha_c$ ) associated with H<sub>2</sub> uptake by hydrogenase (e.g.,  $R_{dis} = \alpha_c \times R_c$ ).

$$f_{out}R_{out} = f_{in}R_{in} + f_hR_h - (f_m + f_b)(R_{dis}/\alpha_c) \quad (13)$$

Because of the low energy yields associated with methanogenesis, biomass yields tend to be low (Schonheit et al., 1980). Therefore we assume  $f_b$  is negligible. Eqn. 13 can then be rearranged to solve for  $f_h$  (Eqn. 14):

$$f_h = (f_{out}R_{dis} - f_{in}R_{in} + f_mR_{dis}/\alpha_c)/(R_h - (R_{dis}/\alpha_c)) \quad (14)$$

The efficiency of the hydrogenase system can be defined in terms of the fluxes (Eqn. 15), where  $E$  approaches one at high efficiency, and  $E$  approaches zero at low efficiencies:

$$E = 1 - f_h/(f_h + f_m) \quad (15)$$

To calculate  $E$  (as a function of  $R_h$  and  $\alpha_c$ ), the flux of reducing equivalents diverted to CH<sub>4</sub> is assumed to be equal to four times the rate of CH<sub>4</sub> production (see Eqn. 1). We further assume  $R_{dis} = R_{out}$ ,  $R_w = D/H$  of the culture media,  $R_h$  and  $\alpha_c$  remain constant over time, and each H<sub>2</sub> is taken up only once.

### 2.5. Analytical Methods

Methane concentration analyses were performed using a gas chromatograph equipped with a flame ionization detector (Mini-2, Shimadzu Corp., Kyoto, Japan). For Experiments A and B, discrete gas samples were removed by syringe from experimental bottles, and measured immediately. For Experiments C and D, the gas chromatograph was integrated into the gas exhaust line. Acetate was quantified in Experiments A and B by HPLC as previously described (Valentine et al., 2000b). Hydrogen was quantified in Experiment C by performing known volume dilutions of the exhaust gas and quantifying using a reducing gas analyzer, as previously described (Valentine et al., 2000b).

For Experiments A and B, discrete samples for quantification of  $\delta^{13}C$ -CH<sub>4</sub> and  $\delta$ D-CH<sub>4</sub> were collected by syringe, transferred into evacuated serum bottles, and stored for later isotopic analyses. Sample bulbs (Experiment C) or stoppered-serum bottles (Experiment D) were incorporated into the exhaust line in the case of the bioreactor studies. Bulbs and serum bottles were flushed for at least 40 min with the exhaust gas before removal for isotopic analysis.

Analyses for  $\delta^{13}C$ -CH<sub>4</sub> and  $\delta^{13}C$ -CO<sub>2</sub> for Experiment C were performed in the Isotope Biogeochemistry Laboratory at the University of Hawaii using the technique of Sansone et al. (1997). For all other experiments, isotopic analyses of CH<sub>4</sub> ( $\delta^{13}C$ ,  $\delta$ D), CO<sub>2</sub> ( $\delta^{13}C$ ), H<sub>2</sub> ( $\delta$ D), and H<sub>2</sub>O ( $\delta$ D) were performed at the University of California Irvine using techniques described previously (Tyler et al., 1997; Rice et al., 2001; Chidthaisong et al., 2002). Typical uncertainties, quantified by replicate measurements of standards, for quantification with these techniques are:  $\delta^{13}C$ -CO<sub>2</sub> ( $\pm 0.4\%$ ),  $\delta^{13}C$ -CH<sub>4</sub> ( $\pm 0.3\%$ ),  $\delta$ D-H<sub>2</sub> ( $\pm 7.3\%$ ),  $\delta$ D-CH<sub>4</sub> ( $\pm 4.6\%$ ), and  $\delta$ D-H<sub>2</sub>O ( $\pm 1\%$ ).

Acetate samples were purified using HPLC by collecting the acetate fraction from each run. After purification the samples were split into two aliquots. The first aliquot was added to a strong NaOH solution and dried in a quartz tube under vacuum. The tubes were flame sealed under vacuum and the acetate was pyrolyzed at 400 °C, converting the carboxyl carbon to CO<sub>2</sub> and the methyl carbon to CH<sub>4</sub> (Blair et al., 1985). The CH<sub>4</sub> was separated from the CO<sub>2</sub> in a vacuum line, combusted to CO<sub>2</sub>, and analyzed by IRMS as above. The second aliquot was used to measure total acetate (methyl plus carboxyl carbons). After being dried the sample was combusted at 900°C in a sealed quartz tube filled with 0.2 g CuO wire and silver foil. Tubes were cleaned of organic residue by heating at 900°C for 2 h before sample addition. The isotopic composition of the resulting CO<sub>2</sub> was determined in the same manner as described above. The isotopic composition of the carbon in the carboxyl position was calculated from the isotopic mass balance of the methyl carbon and total carbon. Typical uncertainty for quantification of acetate  $\delta^{13}C$ , quantified by replicate measurements of standards, was  $\pm 2\%$ .

Samples for lipid analysis were collected from Experiment A-1, and centrifuged aerobically at 6500  $\times$  g for 1 h to form cell pellets. The supernatant was decanted and the pellets transferred to small glass vials. The vials were warmed under a slow stream of N<sub>2</sub> until dry, and were then stored in an airtight jar containing anhydrous CaSO<sub>4</sub>. Isoto-

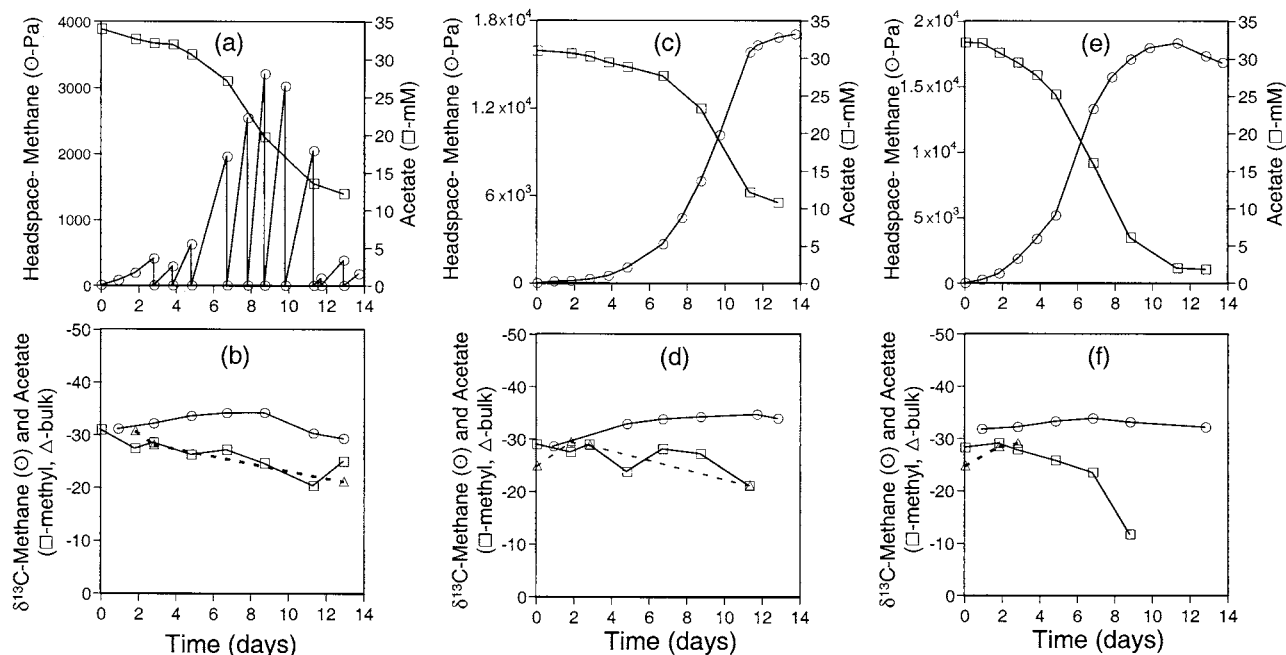


Fig. 2. Variations in the partial pressure (Pa) and  $\delta^{13}\text{C}$  of headspace methane ( $\circ$ ) as well as the concentration (mM) and  $\delta^{13}\text{C}$  of aqueous acetate (methyl position,  $\square$ ; whole molecule,  $\triangle$ ) during growth of *M. thermophila* strain CALS-1 in sealed-bottle Experiments. (a, b) Experiment A-1; (c, d) Experiment A-2; (e, f) Experiment A-3. For Experiment A-1, the headspace was purged after each sampling, giving rise to the sawtooth pattern in (a). For Experiments A-2 and A-3  $\text{CH}_4$  was allowed to build up over the course of the experiment, except that some samples were removed for concentration and isotope analysis, and inert gas was added to maintain a positive headspace pressure.

pic ( $^{13}\text{C}/^{12}\text{C}$ ) analyses of lipid biomarkers for Experiment A were performed at Woods Hole Oceanographic Institution using the technique of Hinrichs et al. (1999).

### 3. RESULTS AND DISCUSSION

#### 3.1. Carbon Isotope Fractionation

##### 3.1.1. Methanogenesis from acetate

Results from Experiments A-1, A-2 and A-3 are given in Figure 2. For Experiments A-2 and A-3,  $\text{CH}_4$  concentrations built up in the headspace of the experimental vessel over the course of the experiment (Figs. 2c,e), though samples were removed frequently for the various analyses. The headspace  $\text{CH}_4$  in Experiment A-1 (Fig. 2a) displays an unusual sawtooth pattern due to frequent purging. A simple mass balance assumes the sum of  $\text{CH}_4$  quantified in these bottles and the  $\text{CH}_4$  removed from these bottles is equal to the acetate consumed. Calculations for Experiments A-2 and A-3 are within 10%, and are consistent with the expected result. Results of a mass balance for Experiment A-1 do not fall within experimental error ( $\text{CH}_4$  is underestimated by 30–40% relative to acetate consumption), presumably because of calculation errors associated with frequent purging of  $\text{CH}_4$  from the headspace.

In each experiment the cultures grew at different rates. Based on the rate of  $\text{CH}_4$  production and acetate consumption, all cultures appear to have gone through the typical stages of microbial growth. These stages include a lag phase before growth occurs, an exponential phase in which cell growth is unhindered and metabolic products appear in an exponential

fashion, a stationary phase when cell metabolism continues but the population comes to steady state (growth rate = death rate), and a death phase when cells die and metabolism ceases. The culture in Experiment A-3 utilized almost all of the acetate supplied, while the cultures in Experiments A-1 and A-2 only consumed acetate to  $\sim 10$  mM. *M. thermophila* consumes acetate to a threshold value near  $20 \mu\text{M}$  (Min and Zinder, 1989), therefore another factor must have inhibited growth at the end of the experiment.

The  $\delta^{13}\text{C}$  of the ether lipid archaeol, which constituted  $\sim 80\%$  of the total extractable lipids, was found to be  $-30\%$  in a sample from Experiment A-1 taken on day 13 (K. Hinrichs, personal communication).

**3.1.1.1. Determination of  $\epsilon_m$ .** Isotopic fractionation factors ( $\epsilon_m$ ) for the conversion of the acetate methyl to  $\text{CH}_4$  were determined using a closed-system approach (Eqn. 6) by comparing the  $\delta^{13}\text{C}$  of the residual acetate to the fractional yield (Fig. 3). The fractionation factors calculated for conversion of methyl acetate to  $\text{CH}_4$  range from 6.1 to 10‰ ( $\text{CH}_4$  depleted relative to acetate).

Isotopic fractionation factors ( $\epsilon_m$ ) for the conversion of the acetate methyl to  $\text{CH}_4$  were determined independently using a closed-system approach by comparing the  $\delta^{13}\text{C}$  of the pooled  $\text{CH}_4$  (calculated by mass balance) to the fractional yield of  $\text{CH}_4$  (also determined by mass balance). As given in Eqn. 8 and shown in Figure 4, the linear regression of  $(1 - f)\ln(1 - f)/f$  versus  $\delta^{13}\text{C}$  of pooled acetate yields a slope of  $\epsilon_m$ . Results indicate two distinctive trends, one corresponding to the early

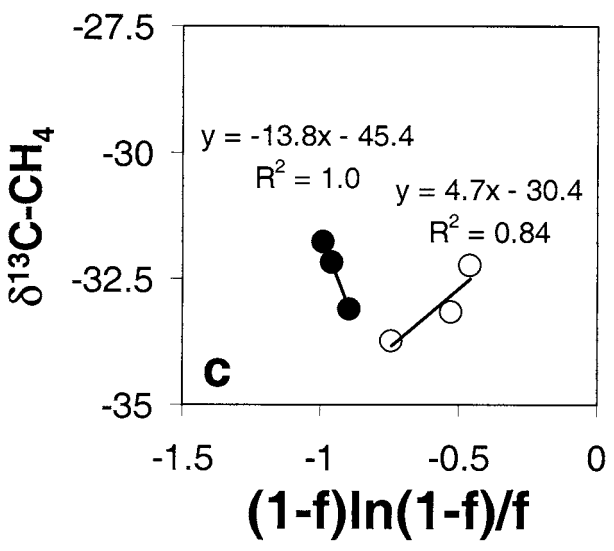
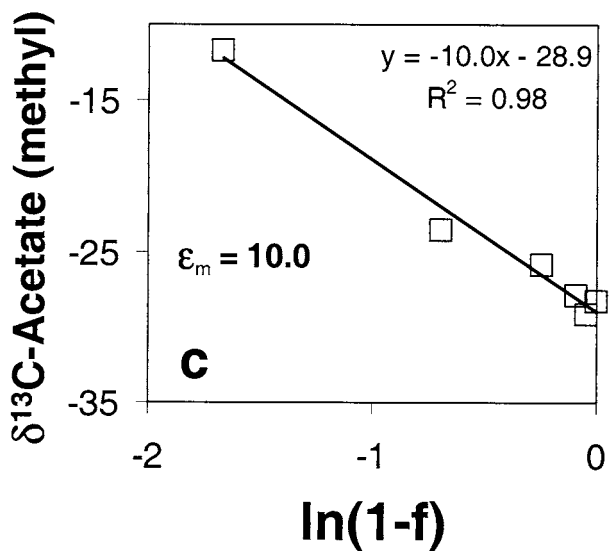
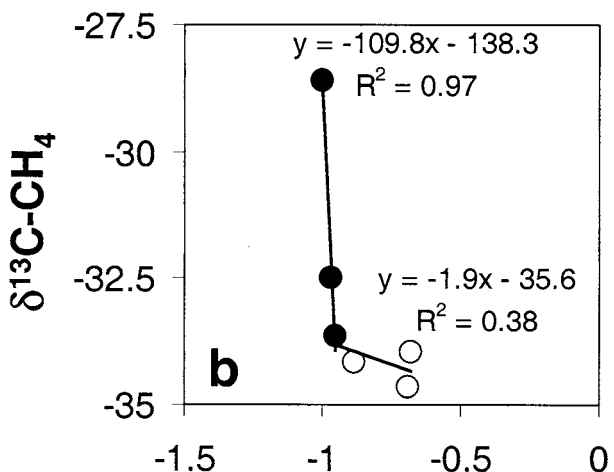
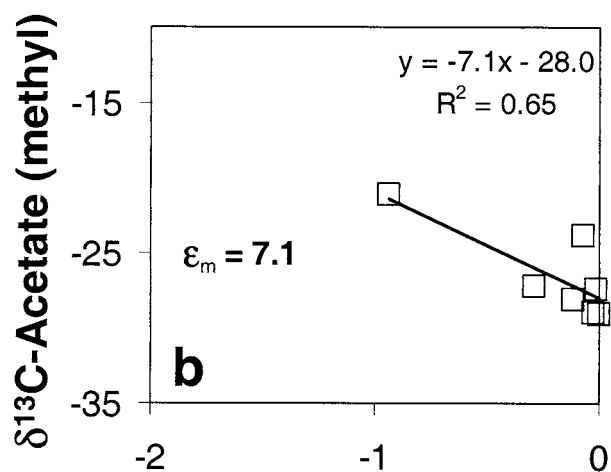
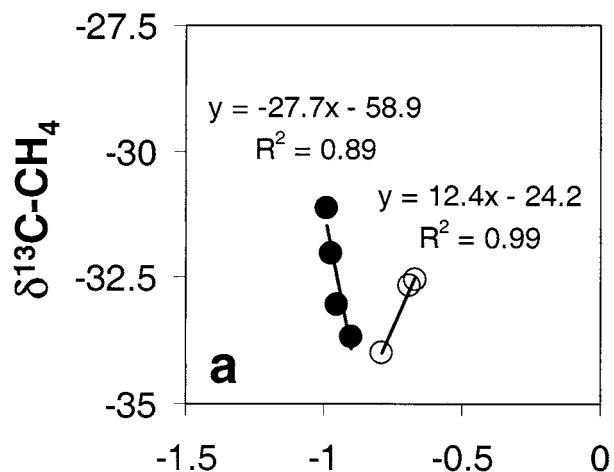
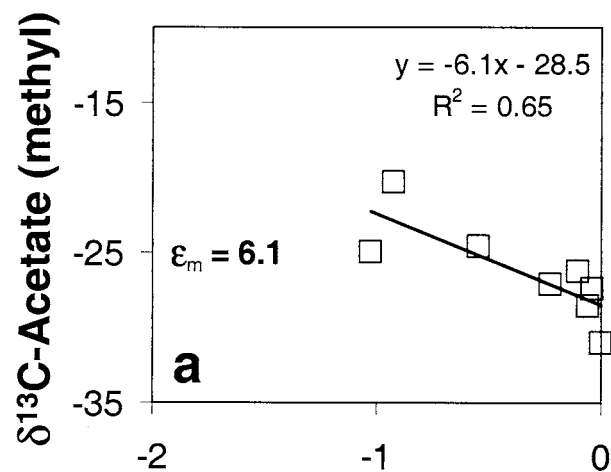


Fig. 3. Determination of  $\epsilon_m$  for Experiments A-1 (a), A-2 (b) and A-3 (c) using a closed-system approach. Both  $\delta^{13}\text{C-acetate}$  (methyl) and  $f$  were measured directly.

Fig. 4. Independent determination of  $\epsilon_m$  for Experiments A-1 (a), A-2 (b) and A-3 (c) using a closed-system approach. Both  $\delta^{13}\text{C-CH}_4$  and  $f$  were back-calculated from experimental data using mass balance. Closed circles represent samples taken between days 1 and 7 while open circles represent samples taken between days 7 and 14.

Table 4. Results of isotope fractionation experiments with *M. thermophila* strain P<sub>T</sub> (Experiments A-4 and B-2).

Culture	PCH <sub>4</sub> (Pa)		δ <sup>13</sup> -CH <sub>4</sub> (‰)	δD-CH <sub>4</sub> (‰)	Acetate (mM)		δ <sup>13</sup> C-Ac (methyl)	δ <sup>13</sup> C-Ac (bulk)	δD-H <sub>2</sub> O (‰)
	Initial	Final	Final	Final	Initial	Final	Initial	Initial	Initial
P <sub>T</sub> (1)	1	7000	-29.28	-295.06	50	44	-22.18	-24.71	-73 ± 17
P <sub>T</sub> (2)	1	6000	-27.16	-297.45	50	44	-25.54	-25.87	-73 ± 17

stages of growth, and one corresponding to the later stages of growth. The breakpoint between the trends occurs midway through the experiments, and further seems to correspond to the transition from exponential product accumulation to linear accumulation. Results indicate that expressed fractionation increases from early growth phases to later growth phases. The negative slope observed during early growth results from the gradually increasing  $\epsilon_m$ . Fractionation factors calculated for only the latter stages of growth from Experiments A-1 and A-3 yield  $\epsilon_m$  values of 12.4 and 4.7‰, respectively.

In addition to a closed-system approach,  $\epsilon_m$  was also calculated by approximating an open system (Eqn. 5). For Experiment A-1 this is a reasonable approach because the headspace was purged regularly. The difference between  $\delta^{13}\text{C-CH}_4$  and  $\delta^{13}\text{C-Ac}_{\text{methyl}}$  is indicative of the instantaneous isotopic fractionation. The average  $\epsilon_m$ , calculated from Experiment A-1 (Fig. 2b), is 5.8‰. Unlike Experiment A-1, Experiments A-2 and A-3 cannot be treated as open systems for the entire course of the experiment; each experiment shows significant <sup>13</sup>C enrichment of the residual acetate toward the end of the experiment. Excluding the latter time points, analysis of Experiments A-2 and A-3 (Figs. 2d,f) are in agreement with Experiment A-1, with  $\epsilon_m$  of 6.5 and 5.9‰ respectively. The average  $\epsilon_m$  from these experiments is 6.1‰, and compares well with the average  $\epsilon_m$  of 7.7‰ calculated using a closed-system approximation (from Fig. 3).

Carbon isotope fractionation was also measured during the early growth stages in two replicate experiments with *M. thermophila*, strain P<sub>T</sub>. Results from this experiment are summarized in Table 4. Samples taken during the early phase of growth approximate an open system, as only 12% of the initial acetate had been consumed at the time of sampling. Assuming an open system,  $\epsilon_m$  averaged 4.4‰ in these experiments, and is similar to strain CALS-1.

By equally considering each approach and each experiment, an average  $\epsilon_m$  of  $7.0 \pm 2.6$ ‰ is calculated, corresponding to an  $\alpha$  of 1.007.

**3.1.1.2. Determination of  $\epsilon_w$ .** Isotopic fractionation factors ( $\epsilon_w$ ) for the conversion of the whole acetate molecule to CH<sub>4</sub> and CO<sub>2</sub> were determined using a closed-system approach (Eqn. 7) by comparing the  $\delta^{13}\text{C}$  of the residual acetate to the fractional yield (Fig. 5). The fractionation factors calculated for conversion of acetate to products are 8.9 to 9.5‰ (CH<sub>4</sub> and CO<sub>2</sub> depleted relative to acetate).  $\epsilon_w$  represents the average fractionation against the methyl and carboxyl positions of acetate. The similar values determined for  $\epsilon_w$  and  $\epsilon_m$  seemingly indicate there is no intramolecular ordering of acetate during acetoclastic methanogenesis by *M. thermophila*. Similarities in the isotope enrichment patterns of acetate and methyl acetate (Figs. 2b,d) provide a visual indication that fractionation may

be similar in magnitude at both the methyl and carboxyl positions. This observation is consistent with the closed-system approach above (Fig. 5). However, there is also an indication of isotopic depletion in total acetate during the early stages of growth (Figs. 2b,d), possibly due to enzymatically mediated isotope exchange of the carboxyl position with CO<sub>2</sub> (O'Leary and Yapp, 1978). Results here are not sufficient to definitively determine the importance of isotopic exchange at the carboxyl position of acetate. In fact, it is possible that acetate experiences intramolecular fractionation during consumption, and that isotopic equilibration between CO<sub>2</sub> and the carboxyl po-

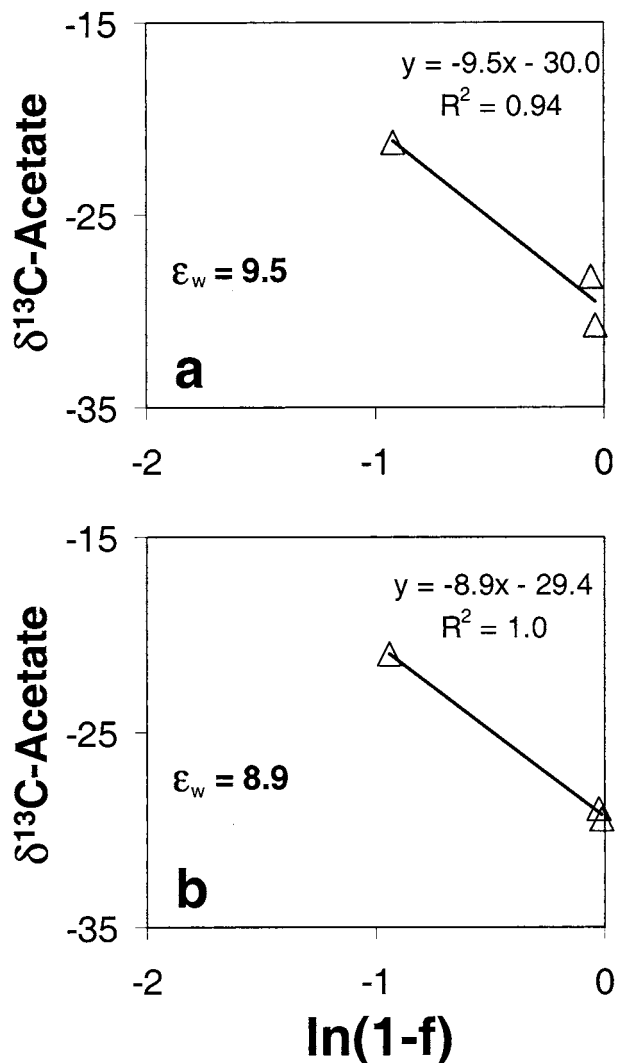


Fig. 5. Determination of  $\epsilon_w$  for Experiments A-1 (a) and A-2 (b) using a closed-system approach.



Table 5. Compilation of carbon isotope fractionation ( $\alpha$ ) factors for methanogenesis by pure cultures.

Organism	Substrate <sup>a</sup>	Strain number DSMZ/OCM <sup>b</sup>	Temp (°C)	H <sub>2</sub> (Pa)	Growth phase	System type	Reference	Max $\alpha$ Substrate -CH <sub>4</sub>
<i>Methanothermobacter marburgensis</i>	H <sub>2</sub> /CO <sub>2</sub>	2133/82	65	8 × 10 <sup>4</sup>	log	open	Fuchs et al. (1979)	1.034
<i>Methanosarcina barkeri</i> (MS)	H <sub>2</sub> /CO <sub>2</sub>	800/38	36	8 × 10 <sup>4</sup>	log	open	Krzycki et al. (1987)	1.049
<i>Methanobacterium ivanovii</i> (Ivanov)	H <sub>2</sub> /CO <sub>2</sub>	2611/140	37	8 × 10 <sup>4</sup>	log	open	Belyaev et al. (1983)	1.037
<i>Methanobacterium thermotrophicum</i> ( $\Delta$ H)	H <sub>2</sub> /CO <sub>2</sub>	1053/ <sup>c</sup>	65	2.4 × 10 <sup>5</sup>	early log	closed	Games et al. (1978)	1.025
<i>Methanosarcina barkeri</i>	H <sub>2</sub> /CO <sub>2</sub>	<sup>d</sup>	40	8 × 10 <sup>4</sup>	mid-log	open	Games et al. (1978)	1.045
<i>Methanobacterium bryantii</i> (M.o.H.)	H <sub>2</sub> /CO <sub>2</sub>	863/110	40	8 × 10 <sup>4</sup>	mid-log	open	Games et al. (1978)	1.061
<i>Methanococcus thermolithotrophicus</i> (SN-1)	H <sub>2</sub> CO <sub>2</sub>	2095/138	55 <sup>e</sup>	8 × 10 <sup>4</sup>	stationary	open	Botz et al. (1996)	1.069
<i>Methanococcus vannielii</i>	H <sub>2</sub> /CO <sub>2</sub>	1224/148	35 <sup>e</sup>	8 × 10 <sup>4</sup>	stationary	open	Botz et al. (1996)	1.079
<i>Methanococcus igneus</i>	H <sub>2</sub> /CO <sub>2</sub>	5666/ <sup>c</sup>	85 <sup>e</sup>	8 × 10 <sup>4</sup>	late log	open	Botz et al. (1996)	1.068
<i>Methanobacterium formicicum</i> (MF)	H <sub>2</sub> /CO <sub>2</sub>	1535/55	34	2.4 × 10 <sup>5</sup>	<sup>f</sup>	closed	Balabane et al. (1987)	<1.05 <sup>g</sup>
<i>Methanothermobacter marburgensis</i>	H <sub>2</sub> /CO <sub>2</sub>	2133/82	65	310	SL <sup>h</sup>	open	Present study	1.064
<i>Methanothermobacter marburgensis</i>	H <sub>2</sub> /CO <sub>2</sub>	2133/82	65	8 × 10 <sup>4</sup>	log	open	Present study	1.031
<b>Aceticlastic and Methylotrophic Methanogenesis</b>								
<i>Methanosarcina barkeri</i> strain MS	Ac	800/38	37	N/A		closed	Krzycki et al. (1987)	1.022
<i>Methanosarcina barkeri</i> strain 227	Ac	1538/35	37	N/A		closed	Gelwicks et al. (1994)	1.022
<i>Methanosaeta concilii</i>	Ac	N/A	25	N/A		closed	Chidthaisong, unpublished	1.017
<i>Methanosaeta thermophila</i> strain CALS-1	Ac	3870/N/A	61	N/A	all	both <sup>i</sup>	Present study	1.007 <sup>i</sup>
<i>Methanosaeta thermophila</i> strain P <sub>T</sub>	Ac	6194/778	60	N/A	early log	closed	Present study	1.007
<i>Methanosarcina barkeri</i> strain MS	Me	800/38	37	N/A		closed	Krzycki et al. (1987)	1.079
<i>Methanococcoides burtonii</i>	TMA	6242/468	20	N/A		closed	Summons et al. (1998)	1.076
<i>Methanosarcina barkeri</i>	TMA	800/38	37	N/A		closed	Summons et al. (1998)	1.053

<sup>a</sup> Ac (acetate), Me (methanol), TMA (trimethylamine).

<sup>b</sup> DSMZ = German Collection of Microorganisms and Cell Cultures, OCM = Oregon Collection for Methanogens.

<sup>c</sup> Strain not currently available through OCM.

<sup>d</sup> Authors unable to determine which strain was used (likely strain MS).

<sup>e</sup> Cultures grown in titanium fermentor.

<sup>f</sup> Samples taken from sealed vials over 3 days of growth.

<sup>g</sup> Maximum value estimated using  $\Delta\delta^{13}\text{C}(\text{CO}_2\text{-CH}_4)$  assuming influence of biomass to be negligible.

<sup>h</sup> SL = substrate limited.

<sup>i</sup> Both open- and closed-system approaches used to calculate an average value for  $\alpha$ .

sition of acetate occurs simultaneously. The dual effect of these processes can not be determined from the limited data presented here.

**3.1.1.3. Potential mechanisms of fractionation.** Several previous studies have considered fractionation during aceticlastic methanogenesis at moderate temperatures, both in pure culture and in environmental samples (Table 5 and references therein). This work represents the first analysis of carbon isotope fractionation associated with the aceticlastic reaction at elevated temperature. The  $\epsilon_m$  of 7‰ associated with aceticlastic methanogenesis in *M. thermophila* is much lower than has been previously observed for methanogenesis from any substrate, and is not an artifact of the culturing system, as both open- and closed-system approaches yield similar fractionation factors.

Only two genera of methanogens, *Methanosarcina* and *Methanosaeta* (sometimes referred to as *Methanothrix*), are known to perform the aceticlastic reaction (Eqn. 1; Fig. 6).

Both genera contain mesophilic as well as moderately thermophilic species. Previous experiments considering carbon isotopic fractionation associated with the aceticlastic reaction have focused on the genus *Methanosarcina*, and have been performed at moderate temperatures. Results from such experiments indicate an  $\epsilon_m$  of ~22‰ (Table 5).

The primary ecological difference between the *Methanosarcina* and the *Methanosaeta* seems to be that *Methanosaeta* specialize in the aceticlastic reaction, to the extent they are unable to use any other substrates for catabolism. The *Methanosarcina* are capable of using a variety of other substrates, and are considered to be metabolic generalists among the methanogens (Galagan et al., 2002). These two genera employ different catabolic pathways for the aceticlastic reaction (Fig. 6); as a result the *Methanosaeta* are capable of utilizing acetate to a threshold value near 20  $\mu\text{M}$ , whereas the acetate threshold for the *Methanosarcina* is near 500  $\mu\text{M}$  (Min and Zinder, 1989). The difference in catabolic pathways is consistent with

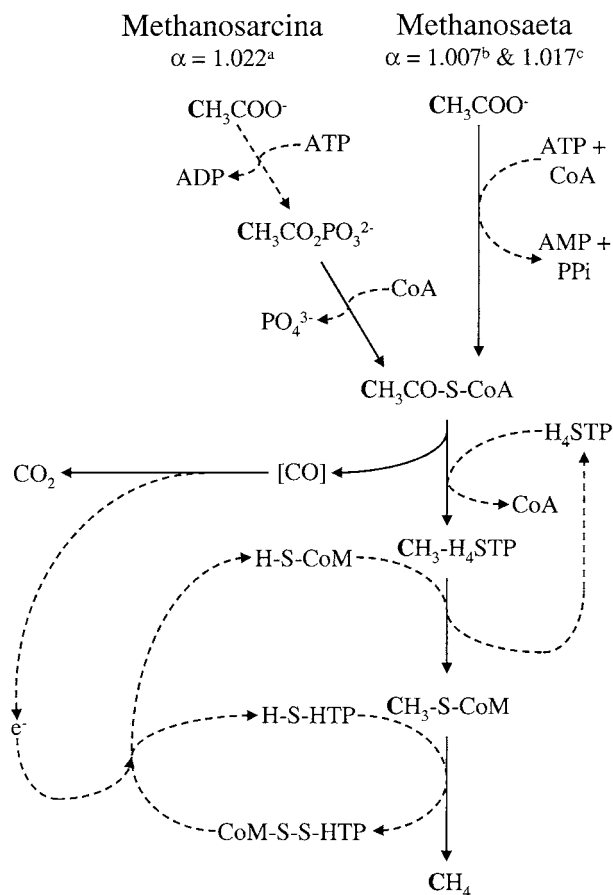


Fig. 6. Catabolic pathways of aceticlastic methanogens. The *Methanosarcina* utilize a two step pathway to fix acetate to acetyl CoA, while the *Methanosaeta* utilize only one step. The pathways converge at acetyl CoA. The solid lines represent steps where bonds to acetate carbon (carbon originating at either position) are formed or broken, while the dashed lines indicate steps which do not directly involve these carbon atoms. Both carbon atoms of acetate are generally conserved; the carboxyl position goes to  $\text{CO}_2$  while the methyl position goes to  $\text{CH}_4$ . Key to abbreviations: ATP (adenosine triphosphate), PPI (inorganic pyrophosphate), CoA (coenzyme A),  $\text{H}_4\text{STP}$  (tetrahydrosarcinapterin), CoM (coenzyme M), and H-S-HTP (*N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine). <sup>a</sup>Krzycki et al. (1987), Gelwicks et al. (1994). <sup>b</sup>This study. <sup>c</sup>Chidthaisong, unpublished results. Based on information in Ferry (1993).

the smaller expressed fractionation in thermophilic *Methanosaeta* than in mesophilic *Methanosarcina*. However, experiments with mesophilic *Methanosaeta* indicate an  $\epsilon_m$  of  $\sim 17\%$  (Chidthaisong et al., unpublished results), thus differences in the enzymatic pathway are unable to completely account for these observations. A comparison to thermophilic *Methanosarcina* would be useful in determining the impact of temperature on fractionation associated with the aceticlastic reaction.

One possible explanation for the low  $\epsilon_m$  values expressed by *M. thermophila* is that the uptake or fixation of acetate is an irreversible process. Assuming reaction by Eqn. 1 is the only fate for intracellular acetate, no fractionation is possible arising from the catabolic pathway once a biochemical steady-state is achieved. It is also possible that these initial steps of acetate transport and fixation are somewhat reversible, a situation

quantified by Rees (1973) for sulfate reduction, and discussed in greater detail in section 3.1.2. This idea is further consistent with the growth-phase effect apparent in Figures 2 and 4. For example, it is possible the extent of metabolic reversibility is lower during exponential growth due to elevated rates of metabolism causing a limitation on intracellular acetate delivery. These sorts of rate effects have been considered for phytoplankton (Bidigare et al., 1997), sulfate reducing bacteria (Rees, 1973), and  $\text{CO}_2$ -utilizing methanogens (Valentine, 2000).

**3.1.1.4. Environmental implications.** Geologic  $\text{CH}_4$  is typically categorized as thermogenic if  $\delta^{13}\text{C}-\text{CH}_4$  is heavier than  $-50\%$  or biogenic if  $\delta^{13}\text{C}-\text{CH}_4$  is lighter than  $-60\%$  (Cicerone and Oremland, 1988). The environmental implication of the minimal carbon isotope fractionation associated with aceticlastic methanogenesis by *M. thermophila* is that  $\text{CH}_4$  produced by *Methanosaeta* at elevated temperature is likely to appear isotopically enriched relative to other sources of  $\text{CH}_4$ . The actual isotopic content will depend on the isotopic content at the methyl position of acetate, as well as alternative fates for acetate in the environment (e.g., syntrophic oxidation, assimilation into biomass, mineral sorption). Based on carbon isotopes alone,  $\text{CH}_4$  produced at elevated temperatures by the aceticlastic reaction is likely to appear thermogenic in origin. The true environmental importance of acetate-derived  $\text{CH}_4$  in geologic settings remains unclear due to a lack of knowledge about aceticlastic methanogenesis at elevated temperature and pressure. The importance of the aceticlastic reaction in geologic settings cannot be ruled out based on the carbon isotopic content of the  $\text{CH}_4$ .

The apparent isotopic equilibration between the carboxyl position of acetate and  $\text{CO}_2$  observed at early points in Experiment A may help to explain the isotopic composition of organic acids in petroleum source waters and other geologic settings. Organic acid samples collected from oil field formation waters in the San Joaquin Basin, for example, display an enrichment in the carboxyl position relative to the other carbon positions (Franks et al., 2001), similar to observations here. A mechanism of microbially catalyzed isotope exchange (O'Leary and Yapp, 1978) may account for such observations. Recent evidence also indicates that acetate produced from oil-prone source rocks at elevated temperature ( $200\text{--}360^\circ\text{C}$ ) leads directly to  $^{13}\text{C}$ -enrichment of the carboxyl position (Dias et al., 2002). Microbially catalyzed exchange could further act on organic acids after cooling, altering the initial  $\delta^{13}\text{C}-\text{Ac}_{\text{carboxyl}}$  by way of exchange with ambient  $\text{CO}_2$ . Isotope exchange of  $^{13}\text{C}$ (carboxyl)-labeled acetate has also been observed by de Graaf et al. (1996) in methanogenic, but not sulfidic sediments. Carbon isotope exchange at the carboxyl position of acetate is known to be catalyzed by acetyl CoA synthase (Spormann and Thauer, 1989; Raybuck et al., 1991), and the equilibrium isotope effect for decarboxylation reactions is  $\sim 1.0027$  (carboxyl position is enriched in  $^{13}\text{C}$  relative to  $\text{CO}_2$ ) at  $25^\circ\text{C}$  and pH 7.5 (O'Leary and Yapp, 1978).

### 3.1.2. Methanogenesis from $\text{H}_2/\text{CO}_2$

Carbon isotope fractionation experiments with *M. marburgensis* were designed to determine how environmental factors

Table 6. Results of carbon isotope fractionation experiments involving *M. marburgensis* in a flowthrough bioreactor (Experiment C).

Time (h)	Temp (°C)	H <sub>2</sub> up <sup>a</sup> (Pa)	H <sub>2</sub> down <sup>b</sup> (Pa)	CH <sub>4</sub> down <sup>b</sup> (Pa)	Light/dark	δ <sup>13</sup> C–CO <sub>2</sub> down <sup>b</sup> (‰)	δ <sup>13</sup> C–CH <sub>4</sub> down <sup>b</sup> (‰)	α <sub>CO<sub>2</sub>–CH<sub>4</sub></sub>
Results from Experiment C-1								
4	65	310	226 <sup>c</sup>	21	L	–43.50	–94.10	1.056
7	55	310	218 <sup>c</sup>	23	L	–44.09	–91.47	1.052
13	45	310	242 <sup>c</sup>	17	L	–44.04	–90.32	1.051
16	75	310	286 <sup>c</sup>	6	L	–43.43	–96.75	1.059
19	40	310	282 <sup>c</sup>	7	L	–44.39	–92.33	1.053
Results from Experiment C-2								
7.5	65	290	193 <sup>c</sup>	25	L	–43.54	–94.21	1.056
13	55	290	178 <sup>c</sup>	29	L	–43.95	–93.47	1.055
24.5	45	290	182 <sup>c</sup>	26	L	–43.81	–93.45	1.055
32	75	290	276 <sup>c</sup>	3	L	–44.32	–97.73	1.059
Results from Experiments C-3 and C-4								
13	65	350	345 <sup>c</sup>	1.3	L	–43.99	–96.23	1.058
24	65	350	342 <sup>c</sup>	2.1	D	–43.90	–97.70	1.060
35	65	350	344 <sup>c</sup>	4.1	L	–44.23	–73.15	1.031
48	65	350	316 <sup>c</sup>	8.5	D	–44.18	–99.97	1.062
78	65	350	270 <sup>c</sup>	20	L	–44.52	–102.17	1.064
80	65	8.0 × 10 <sup>4</sup>	7.9 × 10 <sup>4d</sup>	210	L	<sup>e</sup>	–68.06	1.026
83	65	8.0 × 10 <sup>4</sup>	7.9 × 10 <sup>4d</sup>	300	L	–43.98	–70.59	1.029
86	65	8.0 × 10 <sup>4</sup>	7.8 × 10 <sup>4d</sup>	400	L	–43.76	–71.99	1.030
97	65	8.0 × 10 <sup>4</sup>	7.8 × 10 <sup>4d</sup>	510	L	<sup>e</sup>	–65.87	1.023

<sup>a</sup> Upstream of the culture vessel.

<sup>b</sup> Downstream of the culture vessel.

<sup>c</sup> Measured directly.

<sup>d</sup> Calculated from CH<sub>4</sub> assuming 4:1 stoichiometry.

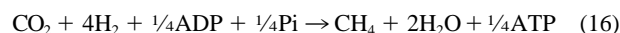
<sup>e</sup> Sample lost: value assumed to be average of δ<sup>13</sup>C–CO<sub>2</sub> for Experiments C-3 and C-4.

such as temperature (Experiments C-1 and C-2), H<sub>2</sub> partial pressure (Experiments C-3 and C-4), media organic content (Experiment C-2 and C-3), and light level (Experiment C-3) impact isotopic fractionation between CO<sub>2</sub> and CH<sub>4</sub>. Results from these experiments are summarized in Table 6. These results fail to demonstrate any significant impact of light level, temperature or media organic content on isotopic fractionation. The factor controlling isotope fractionation in these experiments was H<sub>2</sub> partial pressure. Low H<sub>2</sub> partial pressures (Experiments C-1 through C-3) yield large fractionation factors between CO<sub>2</sub> and CH<sub>4</sub>. Increasing the H<sub>2</sub> partial pressure (Experiment C-4) results in a decrease in the enrichment factor by roughly a factor of two. The average α-factor was 1.055 for experiments with low levels of H<sub>2</sub> (290–350 Pa H<sub>2</sub>); the average α-factor was 1.027 for experiments with elevated levels of H<sub>2</sub> (8.0 × 10<sup>4</sup> Pa H<sub>2</sub>). Previous studies of H<sub>2</sub>/CO<sub>2</sub> methanogenesis with other strains demonstrated a growth-phase dependence in carbon isotope fractionation. The “H<sub>2</sub> effect” observed here is likely related to the “growth-phase effect” observed by Botz et al. (1996) and the “cell specific rate effect” reported by Zyakun (1996).

**3.1.2.1. Differential reversibility.** Here we propose that variations in the carbon isotopic fractionation factor are controlled by the extent of enzymatic reversibility, and that the extent of enzymatic reversibility is controlled by the Gibbs Free Energy of catabolism. In essence, we hypothesize that the observed fractionation factors are indicative of the extent of reversibility of H<sub>2</sub>/CO<sub>2</sub> methanogenesis under different environmental and physiologic conditions. The extent of reversibility in multistep (nonbranching) enzymatic processes, as has been shown for dissimilatory sulfate reduction, impacts the extent to which

fractionations are expressed from each enzymatic step (Kaplan and Rittenberg, 1964; Rees, 1973; Detmers et al., 2001). While the fractionation for any given catabolic step remains constant, the extent to which fractionation is expressed in the final product depends on the reversibility of the pathway (Hayes, 1983, 2001; Rees, 1973). We propose the partial pressure of H<sub>2</sub> gas is the primary controlling factor on the free energy of catabolism, and thus on the magnitude of rate constants for reverse reactions and on catabolic reversibility. This hypothesis is outlined schematically in Figure 7.

The Gibbs Free Energy change for H<sub>2</sub>/CO<sub>2</sub> methanogenesis (Eqn. 2) is approximately –131 kJ mol<sup>–1</sup> under standard conditions, including 10<sup>5</sup> Pa H<sub>2</sub>. Such conditions are unusual in nature, and methanogenesis generally occurs close to the theoretical minimum free energy yield (Lovley, 1985; Cordruwisch et al., 1988; Hoehler et al., 2001; Chong et al., 2002; Valentine, 2001) of –10 to –20 kJ mol<sup>–1</sup>. Furthermore, catabolism is coupled to energy conservation, by way of a chemiosmotic potential (equivalent to ¼ – ½ of an ATP; Eqn. 16), meaning the overall free energy change for Eqn. 16 occurs close to zero (indicating that thermodynamic efficiency approaches 100%) under environmental (H<sub>2</sub>-replete) conditions (Hoehler et al., 1998; Hoehler et al., 2001).



Under conditions of excess H<sub>2</sub> methanogens are capable of conserving some of the excess energy (de Poorter et al., 2003), but a larger portion is likely lost in the catabolic pathway and in concentration gradients (Sorensen et al., 2001). At the experimental conditions of this study, the Gibbs Free Energy yield (ΔG') for catabolism is calculated to be –97 kJ mol<sup>–1</sup>

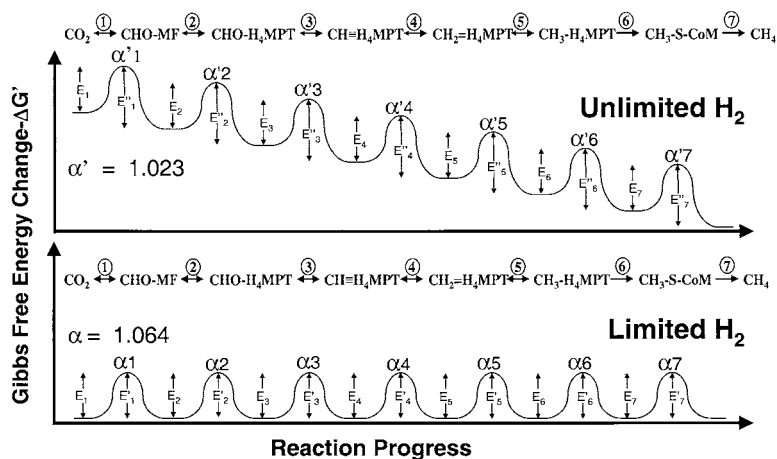


Fig. 7. Schematic representation of the Gibbs Free Energy change associated with catabolism during  $\text{H}_2/\text{CO}_2$  methanogenesis (Eqn. 16), focusing on the activation energies for the forward and reverse reactions. Each of the seven catabolic reactions (shown at bottom and in Fig. 8) are outlined for conditions of excess  $\text{H}_2$  (top) and limited  $\text{H}_2$  (lower). Under conditions of excess  $\text{H}_2$ , the activation energies for the reverse reactions (given as  $E''_{\#}$ ) are greater than the activation energies for the forward reactions (given as  $E'_{\#}$ ). Under conditions of limited  $\text{H}_2$ , the activation energies for the forward ( $E'_{\#}$ ) and reverse ( $E''_{\#}$ ) reactions are similar. The reaction rate constants will therefore change in response to the change in  $\Delta G'$  (according to the Arrhenius equation). Reversibility is more likely to be achieved under limited  $\text{H}_2$  conditions. This model assumes reversibility of the enzymatic pathway until step 6, and each step includes energetic couplings (see Thauer, 1998, for energetic details of each step). This figure represents all the activation energies as being equal, which is unlikely; each step likely consists of multiple peaks and valleys. Greater differentials between forward and reverse activation energies are expected for reductive/oxidative steps (1, 4, 5 and 7). Late stages of growth resemble low  $\text{H}_2$  conditions because rapid  $\text{H}_2$  consumption by dense cultures draws the dissolved and intracellular  $\text{H}_2$  lower, and the  $\Delta G'$  experienced by the organism is decreased (e.g., much of the  $\Delta G'$  is lost to the  $\text{H}_2$  concentration gradient; Sorenson et al., 2001). MF = methanofuran,  $\text{H}_4\text{MPT}$  = tetrahydromethanopterin, CoM = coenzyme M. Further details on the biochemistry and enzymology of methanogenesis are given in Figure 8.

(assuming reaction by Eqn. 16 and conditions from Experiment C-4,  $t = 97$  h;  $\alpha = 1.023$ ) under excess  $\text{H}_2$  and  $-42 \text{ kJ mol}^{-1}$  (assuming reaction by Eqn. 16 and conditions from Experiment C-3,  $t = 78$  h;  $\alpha = 1.064$ ) for conditions of limiting  $\text{H}_2$ . Both calculations assume the formation of  $1/4$  ATP requires  $20 \text{ kJ mol}^{-1}$ .

This “differential reversibility” hypothesis requires that the first five steps of the methanogenic pathway be fundamentally reversible under low  $\text{H}_2$  conditions. This assumption is well supported by a variety of biochemical and environmental data (Donnelly et al., 1985; DiMarco et al., 1986; Donnelly and Wolfe, 1986; Schworer and Thauer, 1991; Vorholt and Thauer, 1997; Thauer, 1998; Gartner et al., 1994; Shima et al., 2002). Perhaps the strongest support for this assumption comes from the observation that methylotrophic methanogens and sulfate-reducing archaea utilize steps 1–5 in the reverse direction during catabolism. The sixth step in methanogenesis involves methyl transfer from  $\text{H}_4\text{MPT}$  to an intermediate, followed by transfer of that methyl to CoM. The methyl transfer from the intermediate to CoM is thought to be irreversible (Gartner et al., 1993; Gartner et al., 1994) and we hypothesize that this step serves as the ultimate isotopic bottleneck during  $\text{H}_2/\text{CO}_2$  methanogenesis.

In the case of *M. marburgensis* the differential reversibility of catabolism may be further related to the expression of different enzyme systems under different  $\text{H}_2$  levels (Fig. 8). The reduction of  $\text{CH} \equiv \text{H}_4\text{MPT}$  to  $\text{CH}_2 = \text{H}_4\text{MPT}$  (Fig. 8, step 4) is linked to the oxidation of  $\text{H}_2$  under condition of excess  $\text{H}_2$ , and is linked to the oxidation of coenzyme  $\text{F}_{420}$  under limited  $\text{H}_2$  (Nolling et al., 1995; Nolling and Reeve, 1997; Reeve et al.,

1997). The  $\text{H}_2$ -linked reduction of  $\text{CH} \equiv \text{H}_4\text{MPT}$  to  $\text{CH}_2 = \text{H}_4\text{MPT}$  proceeds with a free energy yield  $11 \text{ kJ mol}^{-1}$  more exergonic (Thauer, 1998) than the equivalent  $\text{F}_{420}$ -linked reduction. Although both reactions are reversible, the  $\text{F}_{420}$ -linked reaction may be “more” reversible than the  $\text{H}_2$ -linked reaction due to the difference in activation energies of the reverse reactions. The less favorable  $\Delta G'$  associated with the reversal of the  $\text{H}_2$ -linked reaction (e.g., the oxidation of  $\text{CH}_2 = \text{H}_4\text{MPT}$  to  $\text{CH} \equiv \text{H}_4\text{MPT}$ ) could create an isotopic bottleneck, allowing only steps 1–4 to express their full fractionation. In contrast, greater reversibility of the  $\text{F}_{420}$ -linked reaction would allow greater expression of the fractionation associated with steps 1–6.

The idea of differential reversibility in the methanogenic pathway controlling fractionation can be further applied to physical transport processes responsible for supplying substrate to the cell. We have previously considered the possibility of high metabolic rates causing a closed-system effect with respect to intracellular  $\text{CO}_2$  (e.g., a steady-state isotopic enrichment in the intracellular  $\text{CO}_2$  pool; Valentine, 2000). This mechanism is effectively a physical analog of the chemical reaction discussed above and has been considered quantitatively for other metabolic systems (Rees, 1973; Hayes, 1983, 2001). Basically, high cell-specific metabolic rates may draw down the intracellular concentration of  $\text{CO}_2$ , effectively creating a situation of irreversible flux into the cell (once  $\text{CO}_2$  enters the cell there is no fate but conversion to  $\text{CH}_4$ —thus no net fractionation is possible). This idea is consistent with results presented here, and could well act in concert with differential reversibility of the catabolic pathway.

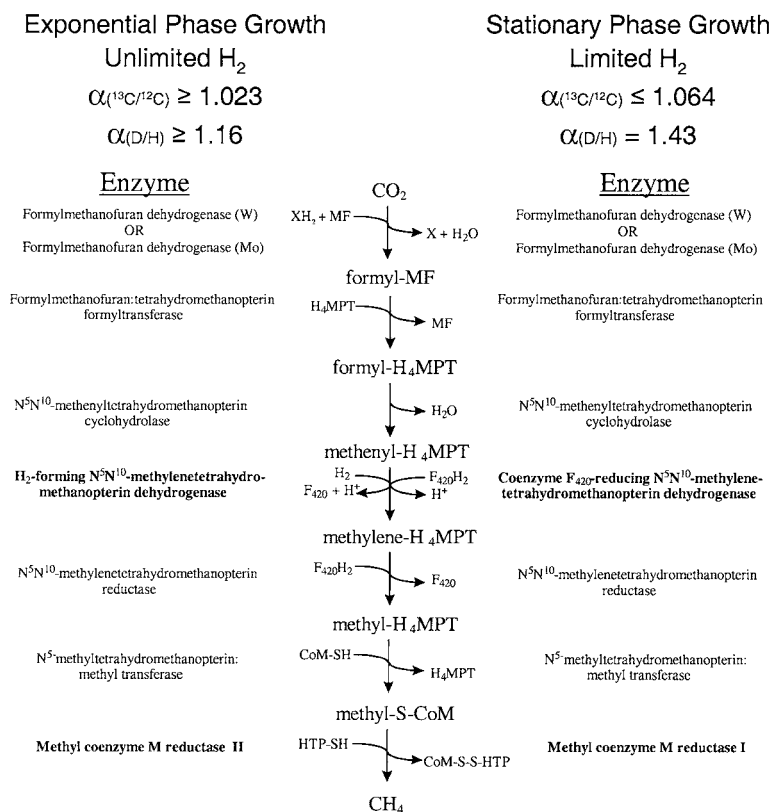


Fig. 8. The central catabolic pathway of methanogenesis in *M. marburgensis* and the enzymes which catalyze each step. Factors which differ between  $\text{H}_2$  limited and  $\text{H}_2$  unlimited conditions are given in bold. The catabolic pathway is based on Thauer (1998) while the enzymatic information is taken from Reeve et al. (1997). X signifies an unknown electron donor.

The “differential reversibility” hypothesis predicts that a variety of factors impact the fractionation of carbon isotopes during  $\text{H}_2/\text{CO}_2$  methanogenesis, including enzyme expression patterns, cell-specific catabolic rate, cell density, kinetics of  $\text{H}_2$  transport, energy conservation mechanisms, and the  $\Delta G'$  of catabolism. Furthermore, the fractionation associated with  $\text{H}_2/\text{CO}_2$  methanogenesis is likely to vary between species. Despite these many complications, the “differential reversibility” effect may hold true across a variety of environmental conditions, as it correctly predicts that methanogens in energy stressed environments (such as permanently cold marine sediments) tend to display larger fractionation factors than do methanogens in other environments, such as the rumen. This effect is prevalent during dissimilatory sulfate reduction and during  $\text{H}_2/\text{CO}_2$  methanogenesis, both anaerobic processes proceeding with minimal Free Energy yields. This effect may also be prevalent in other forms of “low energy” anaerobic metabolism such as acetoclastic methanogenesis, homoacetogenesis, anaerobic  $\text{CH}_4$  oxidation, iron (III) reduction and fermentation.

**3.1.2.2. Relation to previous studies.** Observations of  $\text{H}_2$ -dependent fractionation of carbon isotopes during  $\text{H}_2/\text{CO}_2$  methanogenesis (Experiment C) may be used to critically analyze previously published fractionation factors. Several early studies of isotopic fractionation (Games et al., 1978; Fuchs et al., 1979; Belyaev et al., 1983; Balabane et al., 1987; Krzycki et al., 1987; Botz et al., 1996) considered methanogenesis under high  $\text{H}_2$

levels, and in early stages of growth (Table 5). Such results may be biased by the choice of experimental conditions, and may not represent the range of fractionations achieved by these organisms in nature. In particular, Fuchs et al. (1978) reported a fractionation factor of 1.034 for the same strain as was used in this study.

## 3.2. Hydrogen Isotope Fractionation

### 3.2.1. Methanogenesis from acetate

Studies of hydrogen isotope fractionation arising from the acetoclastic reaction were designed to quantify the impact of  $\delta\text{D}$ -Acetate,  $\delta\text{D}$ - $\text{H}_2\text{O}$  and the isotope effect on the  $\delta\text{D}$  of the resulting  $\text{CH}_4$ . However, technical problems with the quantification of  $\delta\text{D}$  in acetate only allow for comparison of  $\delta\text{D}$ - $\text{H}_2\text{O}$  with  $\delta\text{D}$ - $\text{CH}_4$ . Results comparing the  $\delta\text{D}$ - $\text{CH}_4$  produced by *M. thermophila* (Experiments B-1 through B-4) with the  $\delta\text{D}$ - $\text{H}_2\text{O}$  in the culture media are given in Figure 9. The regression analysis ( $R^2 = 0.996$ ) is given for Experiments B-1, B-2, and B-3, which were amended with the same stock solution of acetate. Results from Experiment B-4 are also given, though data is not included in the regression analysis because the bottles were amended with acetate from a different source than in Experiments B-1 through B-3. The slope of the regression line indicates that 19% of the  $\text{CH}_4$ -bound hydrogen originates from a source in isotopic equi-

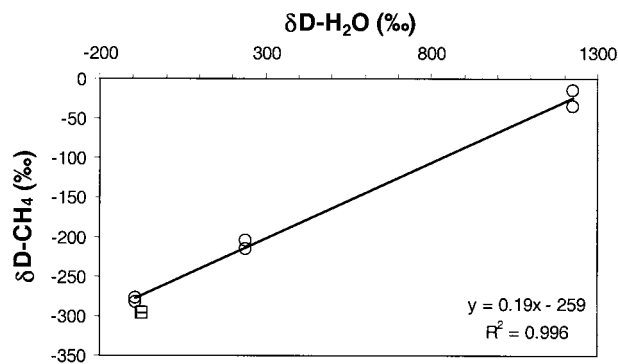


Fig. 9. The correlation between  $\delta\text{D-CH}_4$  produced by *M. thermophila* strains CALS-1 (circles) and  $P_T$  (squares) and the  $\delta\text{D-H}_2\text{O}$  of corresponding culture media (Experiments B-1 through B-4).

librium with  $\text{H}_2\text{O}$ , which is slightly lower than the expected value of 25%. This calculation assumes all acetate has the same initial  $\delta\text{D}$ , and that D/H fractionation is constant during addition of the fourth hydrogen. The methyl position of acetate can only supply up to 75% of the  $\text{CH}_4$ -bound hydrogen (Fig. 6; 3 hydrogen atoms), and other sources of hydrogen (not in isotopic equilibrium with  $\text{H}_2\text{O}$ ) are not certain. The Y intercept of the curve ( $-259\text{‰}$ ) is representative of both the acetate substrate ( $\delta\text{D-Ac}$ ) and the kinetic isotope effect associated with the aceticlastic reaction. Given the uncertainties in quantification of  $\delta\text{D-Ac}$ , we are unable to further constrain these two primary factors. Despite the use of different stock solution, the results from strain  $P_T$  are similar to those from strain CALS-1.

One possible cause of the difficulty in accurately quantifying  $\delta\text{D}$  in acetate is that of isotope exchange. Hydrogen atoms bound to a carbon adjacent to a carboxyl group are able to exchange with  $\text{H}_2\text{O}$  (protons) through a keto-enol tautomerization reaction (Amyes and Richard, 1996; Richard et al., 2002). Exchange occurs more rapidly at low pH, and rates are likely to also be dependent on temperature. Because acetate purification was performed in aqueous solution at pH 3, we suspect some D/H exchange between water and acetate may have occurred at this step. The linear correlation (0.19) between  $\delta\text{D-CH}_4$  and  $\delta\text{D-H}_2\text{O}$  (Fig. 9) indicates little exchange occurred during heat sterilization and early growth. Rapid exchange has been observed in anoxic methanogenic (but not sulfidic) sediments, and has been attributed to enzymatically mediated exchange (de Graaf et al., 1996).

### 3.2.2. Methanogenesis from $\text{H}_2/\text{CO}_2$

Hydrogen isotope experiments with *M. marburgensis* (D-1, D-2) were performed to determine whether varying  $\delta\text{D-H}_2$  would impact  $\delta\text{D-CH}_4$  at conditions with a constant  $\delta\text{D-H}_2\text{O}$ . In Experiments D-1 and D-2,  $\text{H}_2$  gas was supplied with two distinctive values of  $\delta\text{D-H}_2$ ,  $-692$  and  $-190\text{‰}$ , respectively. Results of these two experiments are presented in Figures 10 and 11, respectively. The isotopic fractionation between  $\text{H}_2\text{O}$  and  $\text{CH}_4$  was constant at  $270$ – $275\text{‰}$  ( $\alpha = 1.43$ ) in the latter half of Experiment D-1. The  $\delta\text{D-H}_2$  remained constant throughout Experiment D-1 and did not deviate measurably from the value of the source  $\text{H}_2$  ( $-692\text{‰}$ ). The difference between  $\text{H}_2\text{O}$

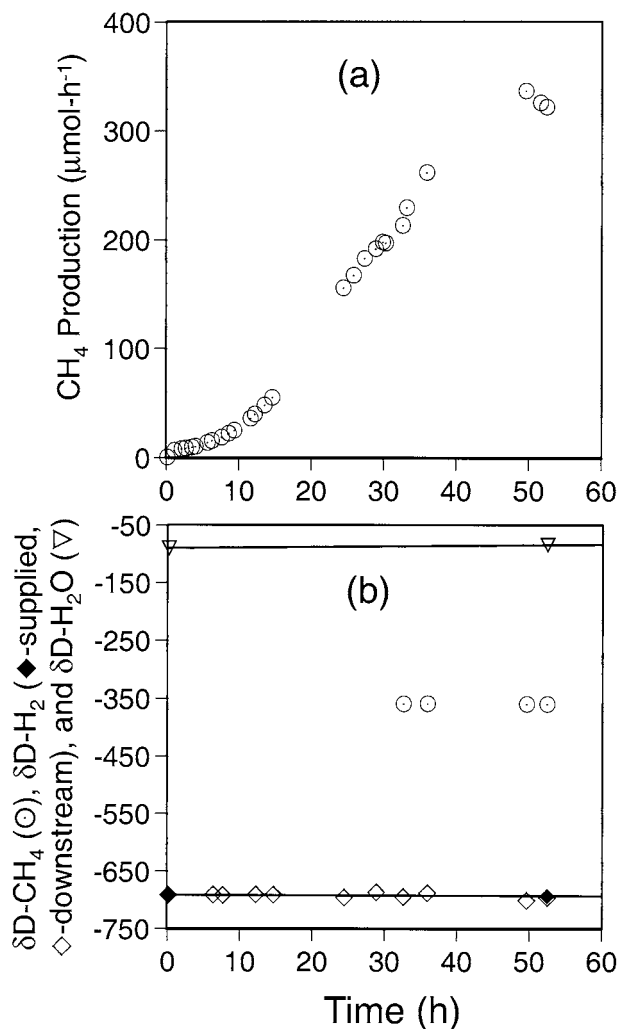


Fig. 10. Methane production and hydrogen isotope variations during growth of *M. marburgensis* in a flowthrough bioreactor (Experiment D-1). The rapid and continuous supply of substrate, and the constant removal of product, approximates an open system. Variations in the rate of methane production ( $\odot$ ) are given in (a). Variations in  $\delta\text{D-CH}_4$  ( $\odot$ ),  $\delta\text{D-H}_2$  ( $\blacklozenge$ , supplied;  $\diamond$ , downstream) and  $\delta\text{D-H}_2\text{O}$  ( $\nabla$ ) are given in (b). The initial flow rate of gas entering the vessel was  $55 \text{ mL min}^{-1}$ , and was decreased to  $15 \text{ mL min}^{-1}$  after 31 h due to analytical considerations. The  $\alpha(\text{H}_2\text{O to CH}_4)$  was identical for each of the (4) sampling times, 1.43.

and  $\text{CH}_4$  was more variable in Experiment D-2, ranging from  $-127$  to  $-275\text{‰}$  ( $\alpha = 1.16$ – $1.43$ ). The magnitude of the fractionation was dependent on the growth phase of the culture with fractionation increasing throughout the exponential growth phase, and finally stabilizing at a difference of  $275\text{‰}$  during the stationary phase. The  $\delta\text{D-H}_2$  of the residual  $\text{H}_2$  exiting the vessel varied throughout Experiment D-2, following the same pattern as the  $\delta\text{D-CH}_4$  produced during the experiment. The  $\delta\text{D-H}_2$  of the residual  $\text{H}_2$  was initially similar to the source  $\text{H}_2$  ( $-190\text{‰}$ ), and became more depleted over the course of the experiment to a final  $\delta\text{D-H}_2$  of  $-263\text{‰}$ . At the maximum rate of metabolism, during stationary phase, less than 8% of the total  $\text{H}_2$  supplied to the vessel was consumed. The time scale for isotopic exchange for these experiments can

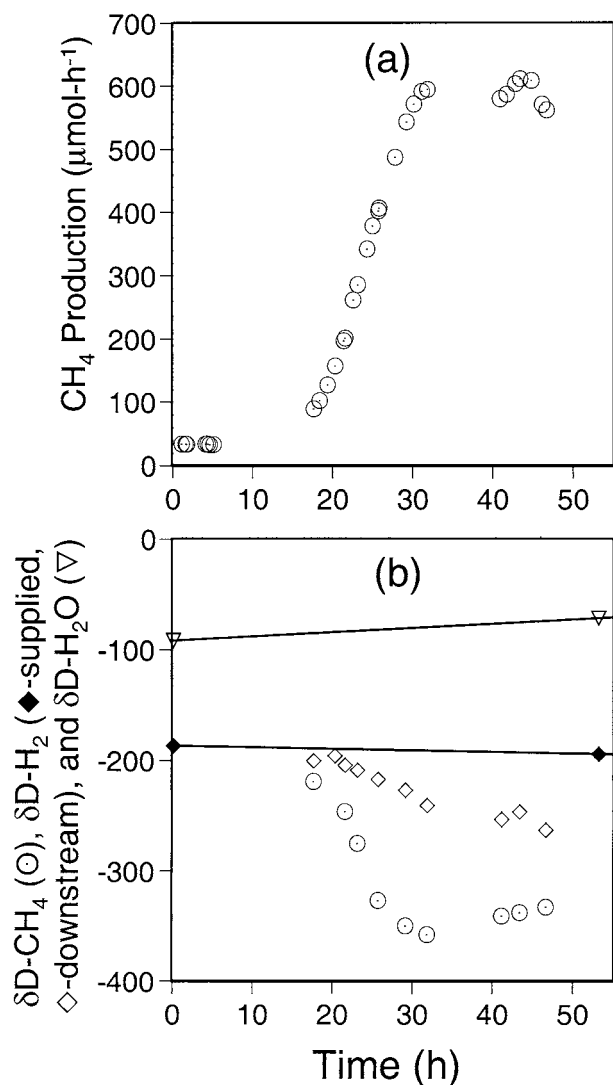


Fig. 11. Methane production and hydrogen isotope variations during growth of *M. marburgensis* in a flowthrough bioreactor (Experiment D-2). Conditions approach those of an open system. Variations in the rate of methane production (○) are given in (a). Variations in  $\delta\text{D-CH}_4$  (○),  $\delta\text{D-H}_2$  (◆, supplied; ◇, downstream) and  $\delta\text{D-H}_2\text{O}$  (▽) are given in (b). The flow rate of gas entering the vessel was maintained at  $15\text{ mL min}^{-1}$  over the course of the experiment. The  $\alpha(\text{H}_2\text{O to CH}_4)$  ranged from 1.16 to 1.43 over the course of the experiment. The  $\text{H}_2$  exiting the vessel is depleted by as much as 71% relative to the  $\text{H}_2$  entering the vessel, and displays a growth-phase dependence.

be constrained based on the residence time of gas in the system, and is on the order of seconds.

Hydrogen isotope experiments were also performed with *M. marburgensis* (D-3) to determine the impact of temperature on  $\delta\text{D-CH}_4$  at a constant  $\delta\text{D-H}_2$  (supplied) and  $\delta\text{D-H}_2\text{O}$ . Results are presented in Figure 12. The  $\delta\text{D}$  of  $\text{CH}_4$  produced by the culture is strongly correlated with both the  $\text{CH}_4$  production rate and the temperature ( $R^2$  of 0.92 and 0.90, respectively). The residual  $\delta\text{D-H}_2$  exiting the vessel also correlates strongly with both the  $\text{CH}_4$  production rate and temperature ( $R^2$  of 0.88 and 0.89, respectively). Assuming no significant changes in cell density over the course of this experiment, the isotopic frac-

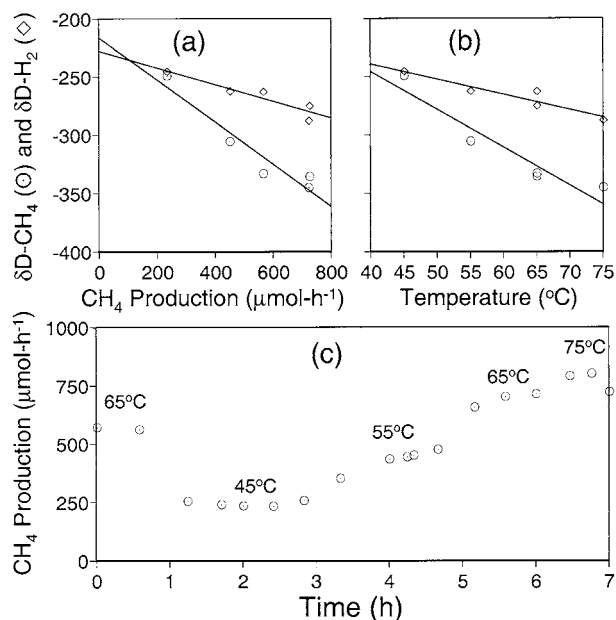


Fig. 12. The impact of temperature and metabolic rate on hydrogen isotope fractionation during growth of *M. marburgensis* in a flowthrough bioreactor. Variations in  $\delta\text{D-CH}_4$  (○) and  $\delta\text{D-H}_2$  (◇) measured downstream from the bioreactor are shown as a function of  $\text{CH}_4$  production rate (a) and temperature (b). The methane production rate is shown as a function of time in (c), with temperature included. The flow rate was maintained at  $15\text{ mL min}^{-1}$  for the duration of the experiment. This experiment was performed over the course of 7 h so as to minimize potential changes in cell density; a 25% increase in methane production rate between 30 min and 6 h (both at  $65^\circ\text{C}$ ) indicates slight growth may have occurred. (a)  $\delta\text{D-CH}_4$  (○) vs.  $\text{CH}_4$  production rate ( $R^2 = 0.92$ );  $\delta\text{D-H}_2$  (◇) vs.  $\text{CH}_4$  production rate ( $R^2 = 0.88$ ). (b)  $\delta\text{D-CH}_4$  (○) vs. temperature ( $R^2 = 0.90$ );  $\delta\text{D-H}_2$  (◇) vs. temperature ( $R^2 = 0.89$ ). The  $\alpha(\text{H}_2\text{O to CH}_4)$  ranged from 1.24 to 1.42 over the course of the experiment.

tions observed in this experiment also correlate to the cell-specific metabolic rate. This assumption is supported by the relatively short (7 h) duration of this experiment, and by the similarity in  $\text{CH}_4$  production rates at the beginning of the experiment and after 6 h (both at  $65^\circ\text{C}$ ).

**3.2.2.1. The source of  $\text{CH}_4$ -bound hydrogen.** Experiments on  $\text{H}_2/\text{CO}_2$  methanogenesis (Experiment D) were designed to test whether the  $\delta\text{D-H}_2$  supplied to methanogens impacts the  $\delta\text{D-CH}_4$  produced, at constant  $\delta\text{D-H}_2\text{O}$ . The maximal observed fractionation factor ( $\alpha$ ) between  $\text{CH}_4$  and  $\text{H}_2\text{O}$  was 1.43, and was independent of the  $\delta\text{D-H}_2$  provided. These results suggest, but do not prove,  $\delta\text{D-H}_2\text{O}$  is the primary factor controlling  $\delta\text{D-CH}_4$  produced by *M. marburgensis* during the stationary phase of growth. Our results are consistent with those of Daniels et al. (1980) who used 81 atom% deuterated  $\text{H}_2\text{O}$  to determine the ultimate source of  $\text{CH}_4$ -bound hydrogen during methanogenesis; results indicated the hydrogen source was  $\text{H}_2\text{O}$ , with a corresponding  $\alpha(\text{H}_2\text{O-CH}_4)$  of  $1.5 \pm 0.2$ . However, results from several biochemical studies indicate  $\text{H}_2$  hydrogen is added to the carbon atom directly during at-least one step of metabolism (step 4, Fig. 8; Schworer et al., 1993; Schleucher et al., 1994; Klein et al., 1995a,b; Hartmann et al., 1996). Results from this study and from Daniels et al. (1980) do

not exclude H<sub>2</sub> as a source of CH<sub>4</sub>-bound hydrogen, due to the rapid isotopic equilibration between H<sub>2</sub>O and H<sub>2</sub>. The possibility remains hydrogen from H<sub>2</sub> is incorporated into CH<sub>4</sub>— if the intracellular H<sub>2</sub> isotopically equilibrates with H<sub>2</sub>O before being incorporated into CH<sub>4</sub>.

**3.2.2.2.  $\delta D$ -CH<sub>4</sub> growth phase effect.** A significant “growth phase” effect was observed in Experiment D-2 (Fig. 11). Fractionation factors ranged from 1.16 early in the experiment and showed a gradual increase to a maximum of 1.43 late in the experiment. Experiment D-3 further linked variations in fractionation ( $1.24 \leq \alpha \leq 1.42$ ) to temperature and/or catabolic rate (Fig. 12). We suggest the observed variations in  $\delta D$ -CH<sub>4</sub> are caused primarily by changes in the enzymatic addition of hydrogen to the carbon substrate. As seen in Figure 8 and discussed in section 3.1.2.1, *M. marburgensis* is capable of producing multiple enzymes which perform the same catabolic function. One such function is the reduction of methenyl-H<sub>4</sub>MPT to methylene-H<sub>4</sub>MPT, which involves the addition of a hydrogen atom to the carbon substrate (Fig. 8, step 4). Two enzymes are capable of performing this reaction, and their regulation is controlled by H<sub>2</sub> availability and/or growth phase (Nolling et al., 1995; Nolling and Reeve, 1997; Reeve et al., 1997).

The first enzyme (H<sub>2</sub>-forming N<sup>5</sup>,N<sup>10</sup>-methylene-tetrahydro-methanopterin dehydrogenase) receives reducing power directly from H<sub>2</sub> and is preferentially expressed at early stages of growth and under conditions of excess H<sub>2</sub>. This enzyme catalyzes the heterolytic cleavage of H<sub>2</sub> and the subsequent hydride shift to a conjugated carbocation reaction center (Schleucher et al., 1994; Klein et al., 1995b; Hartmann et al., 1996; Thauer et al., 1996). Hydrogen from H<sub>2</sub> is affixed directly to the growing methane moiety, though isotope exchange occurs (Schworer et al., 1993; Klein et al., 1995a,b; Hartmann et al., 1996). There is no deuterium isotope effect associated with this reaction (Klein et al., 1995b).

The second enzyme (coenzyme F<sub>420</sub>-dependent N<sup>5</sup>,N<sup>10</sup>-methylene-tetrahydro-methanopterin dehydrogenase) receives reducing power from the reduced form of coenzyme F<sub>420</sub>, and is expressed under conditions of H<sub>2</sub> limitation. This enzyme catalyzes a reversible face-specific hydride transfer. The expression pattern of these enzymes (Nolling et al., 1995; Nolling and Reeve, 1997; Reeve et al., 1997) matches the observed variability in  $\alpha$ (H<sub>2</sub>O/CH<sub>4</sub>).

One possible mechanism by which these enzymes could impact the  $\delta D$ -CH<sub>4</sub> involves distinctive fractionations associated with the H<sub>2</sub>- and F<sub>420</sub>-linked enzymes. Greater fractionation associated with hydrogen addition by way of F<sub>420</sub> is consistent with experimental observations, and could be related to the two-step reduction, first from H<sub>2</sub> to F<sub>420</sub>H<sub>2</sub> (Nambiar et al., 1983; Yamazake et al., 1985; Schauer et al., 1986; Klein and Thauer, 1995) and then to methylene-H<sub>4</sub>MPT. During the early stages of Experiment D-2 the observed fractionation during CH<sub>4</sub> production was rather small ( $\alpha = 1.16$ ), as might be expected if the H<sub>2</sub>-linked enzyme is dominant and expresses no fractionation. The experimental results are consistent with the F<sub>420</sub>-linked enzyme expressing an  $\alpha$ (H<sub>2</sub>O/CH<sub>4</sub>) of  $\sim 2.1$ , assuming all other fractionations remain constant and no further isotope exchange.

**3.2.2.3. Hydrogenase efficiency.** Results from Experiment D can be further used to constrain H<sub>2</sub> activation and release by the action of hydrogenase. The isotopic evidence clearly indicates the hydrogenase system is not completely efficient, as the H<sub>2</sub> leaving the culture vessel is depleted in deuterium relative to the H<sub>2</sub> entering the vessel; hydrogenase enzymes are well known for rapid reversibility and for catalysis of D-H exchange between H<sub>2</sub> and water (Rose, 1970). The efficiency of the hydrogenase system, E, can be estimated using the experimental data, by assuming a fractionation for the formation of H<sub>2</sub> from H<sub>2</sub>O,  $\alpha$ (H<sub>2</sub>O-H<sub>2</sub>), and a fractionation for the uptake of H<sub>2</sub> by hydrogenase ( $\alpha_c$ ). The mass balance is detailed in section 2.4.4. Although one uptake hydrogenase (H<sub>2</sub>-forming N<sup>5</sup>,N<sup>10</sup>-methylene-tetrahydro-methanopterin dehydrogenase, as in section 3.2.2.2) displays no fractionation during H<sub>2</sub> uptake (e.g.,  $\alpha = 1$ ; Klein et al., 1995b), it is likely other uptake hydrogenases in *M. marburgensis* exhibit greater fractionation factors.

Figure 13a displays the relationship between the isotopic fractionation for H<sub>2</sub> production,  $\alpha$ (H<sub>2</sub>O-H<sub>2</sub>), and the efficiency of the hydrogenase system (E), at three different values of  $\alpha_c$ . Results indicate the hydrogenase system in *M. marburgensis* is highly inefficient, even as  $\alpha$ (H<sub>2</sub>O-H<sub>2</sub>) approaches 10. Likely values of  $\alpha$ (H<sub>2</sub>O-H<sub>2</sub>) are at least 3 and possibly higher. Assuming an  $\alpha$ (H<sub>2</sub>O-H<sub>2</sub>) of 3.1 (which also corresponds to the equilibrium isotope fractionation factor for H<sub>2</sub>/H<sub>2</sub>O at 65°C; Horibe and Craig, 1995), *M. marburgensis* (as observed in this study) would activate 8 H<sub>2</sub> molecules for each CH<sub>4</sub> produced; one H<sub>2</sub> molecule would be released for every one catabolized. Results also indicate significant variations in uptake efficiency at different temperatures (Fig. 13b). The hydrogenase system appears most effective at 65°C, the optimal growth temperature for this strain, and is least efficient at 45°C. There is also a possibility that the efficiency of the hydrogenase system is dependent on growth phase, though the data is not sufficient to prove this.

**3.2.2.4. Environmental relevance.** Sugimoto and Wada (1995) performed a series of incubation experiments with paddy soil to discern the relationship between  $\delta D$ -H<sub>2</sub>O and  $\delta D$ -CH<sub>4</sub> in an environment where both H<sub>2</sub>/CO<sub>2</sub> and acetate are important CH<sub>4</sub> precursors. These authors attributed temporal variations in  $\delta D$ -CH<sub>4</sub> to changes in the relative contributions of acetate versus H<sub>2</sub>/CO<sub>2</sub> methanogenesis, and used such variations to estimate the fraction of CH<sub>4</sub> derived from H<sub>2</sub>/CO<sub>2</sub> versus acetate. Sugimoto and Wada (1995) observed increasing fractionation between  $\delta^{13}C$ -CH<sub>4</sub>/ $\delta^{13}C$ -CO<sub>2</sub> and between  $\delta D$ -CH<sub>4</sub>/ $\delta D$ -H<sub>2</sub>O over the course of their incubations. Results presented here raise a further possibility that such variations were caused by changes in substrate availability for the methanogenic community over the course of the incubation, as results from Experiments C and D are consistent with their observations. Changes in the structure of the methanogenic community might also impact temporal variation in  $\delta D$ -CH<sub>4</sub> (Chidthaisong et al., 2002).

Few culture studies have considered hydrogen isotope fractionation during H<sub>2</sub>/CO<sub>2</sub> methanogenesis. Calculations using data presented by Balabane et al. (1987) indicate a hydrogen isotope fractionation of  $1.67 \pm 0.05$  between source H<sub>2</sub>O and the resulting CH<sub>4</sub> for pure cultures of *Methanobacterium for-*



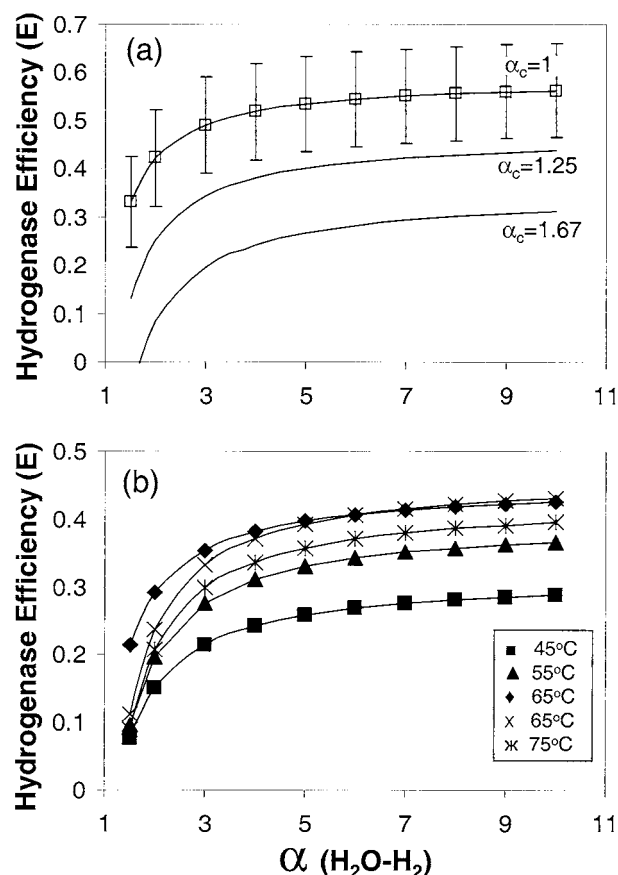


Fig. 13. The calculated efficiency (E) of the hydrogenase uptake system expressed as a function of the fractionation between intracellular  $\text{H}_2\text{O}$  and  $\text{H}_2$  produced,  $\alpha(\text{H}_2\text{O}/\text{H}_2)$ . (a) All data from Experiments D-2 and D-3 were used to calculate an average efficiency (E), which is plotted as a function of  $\alpha(\text{H}_2\text{O}/\text{H}_2)$ . Error bars represent  $\pm$  one standard deviation from the mean. Efficiency is plotted for three different values of  $\alpha_c$  (fractionation associated with  $\text{H}_2$  uptake by hydrogenase) (b) Individual data points from Experiment D-3, corresponding to different temperatures, are plotted as a function of  $\alpha(\text{H}_2\text{O}/\text{H}_2)$ . Maximum efficiency is apparent at the optimum growth temperature for this strain, 65°C. Calculations assume  $\alpha_c = 1$  (i.e., no fractionation during uptake of  $\text{H}_2$ ).

*micicum*. Results from Experiment D indicate this fractionation may serve as an upper limit for this strain, but that lesser fractionations may be observed at other environmental/growth conditions.

Results from  $\text{H}_2/\text{CO}_2$  experiments (Experiment D) may help to explain a long-standing discrepancy between the  $\delta\text{D}-\text{CH}_4$  in biogenic natural gas reservoirs and the  $\delta\text{D}-\text{CH}_4$  from terrestrial environments and ruminants. Biogenic natural gas ( $\text{CH}_4$ ) tends to be depleted by only  $\sim 160\%$  relative to source  $\text{H}_2\text{O}$  (Schoell, 1980), whereas  $\text{CH}_4$  produced in wetlands and ruminants tends to be depleted by 300–400% relative to source  $\text{H}_2\text{O}$  (Sugimoto and Wada, 1995; Waldron et al., 1999; Bilek et al., 2001; Chidthaisong et al., 2002). A depletion of 160% falls within the range observed in this study (Figs. 11 and 12). A large proportion of biogenic natural gas is formed in moderately thermal environments, where organisms such as *M. marburgensis* are active (Orphan et al., 2000). Thus, results from this study are consistent with moderately thermophilic  $\text{H}_2/\text{CO}_2$  methanogens

as producers of biogenic natural gas with a relatively enriched deuterium content.

Results from Experiment D can be used to consider the hypothesis proposed by Burke (1993) that hydrogen isotopic fractionation during  $\text{H}_2/\text{CO}_2$  methanogenesis in nature is controlled by ambient  $\text{H}_2$  levels, and that high  $\text{H}_2$  levels are correlated to deuterium-depleted  $\text{CH}_4$ . The proposed mechanism behind this hypothesis is the depletion of the intracellular  $\text{H}_2\text{O}$  pool by  $\text{H}_2$  uptake under high  $\text{H}_2$  conditions. Results from Experiment D-2 indicate that natural variability associated with growth phase and variations in the catabolic pathway are likely to play a dominant role in controlling the  $\delta\text{D}$  of  $\text{CH}_4$  produced. These results further demonstrate large variations in  $\delta\text{D}-\text{CH}_4$ , at constant extra-cellular  $\text{H}_2$  concentration.

#### 4. CONCLUDING REMARKS

Methane produced from acetate by *M. thermophila* at elevated temperatures is fractionated only slightly in  $^{13}\text{C}$  ( $\sim 7\%$ ) from the methyl position of acetate. Given the potential importance of thermophilic *Methanosaeta* in nature, biogenic  $\text{CH}_4$  produced from acetate at elevated temperature may be easily mistaken for thermogenically derived  $\text{CH}_4$  based on carbon isotopes alone.

Methane produced by  $\text{H}_2/\text{CO}_2$  methanogenesis at elevated temperature displays a broad range of carbon isotope fractionation ( $\alpha$ ) factors, ranging from 1.023 to 1.064. Low levels of  $\text{H}_2$  and steady-state metabolism yield greater isotopic fractionations. We propose that differential reversibility in the catabolic pathway, driven by changes in the activation energies for individual catabolic steps, provides the most consistent explanation of these results. This “differential reversibility” hypothesis is further consistent with a variety of pure culture and environmental observations.

Fractionation during  $\text{H}_2/\text{CO}_2$  methanogenesis is commonly referred to as a kinetic isotope effect. If the differential reversibility hypothesis holds true, the fractionation arising from  $\text{H}_2/\text{CO}_2$  methanogenesis is better described as a mixed kinetic and equilibrium isotope effect. It is possible the equilibrium component of the isotope effect is greater than the kinetic.

The  $\delta\text{D}$  of  $\text{CH}_4$  derived from  $\text{H}_2/\text{CO}_2$  at elevated temperature is highly variable, even within one species, with fractionation ( $\alpha$ ) factors ranging from 1.16 to 1.43. The extent of fractionation appears to be controlled by the catabolic rate, and is thus dependent on environmental conditions. The biochemical basis for this “growth phase effect” may be linked to variations in expression of hydrogenase isoenzymes or cell-specific metabolic rate. The relatively small fractionation between  $\text{H}_2\text{O}$  and  $\text{CH}_4$  at high temperature is consistent with environmental data and can be used to explain the relative deuterium enrichment of many biogenic gas reservoirs compared to  $\text{CH}_4$  produced at moderate temperatures.

Hydrogen isotopic fractionation between  $\text{H}_2\text{O}$  and  $\text{H}_2$  occurs rapidly, on the order of seconds, in the presence of  $\text{H}_2$ -metabolising microbes. This evidence indicates that uptake of hydrogen by *M. marburgensis* is a readily reversible process. Calculations based on experimental results indicate that the hydrogenase system in *M. marburgensis* is less than 50% efficient, meaning that less than half the  $\text{H}_2$  molecules (reducing equivalents) taken up by *M. marburgensis* are converted to

CH<sub>4</sub>, the majority are released later. D/H fractionation and exchange processes remain largely uncharacterized and may serve as important controls on hydrogen cycling in anoxic environments.

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