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Carbon and hydrogen isotope fractionation by moderately thermophilic methanogens

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Abstract—A series of laboratory studies were conducted to increase understanding of stable carbon ($^{13}C/^{12}C$) 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}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 H_2/CO_2 methanogenesis were conducted with *Methanothermobacter marburgensis*. The fractionation of carbon isotopes between CO_2 and 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 H_2O and 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 $\delta D\text{-}H_2$ 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 H_2/CO_2. The relatively small fractionation associated with deuterium during H_2/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 H_2/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%.

1. INTRODUCTION

Methane, 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 CH_4 is released to the atmosphere is well-constrained and is currently ca. 5.0×10^{14} g per year (e.g., Cicerone and Oremland, 1988). The overall rate of 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 CH_4 oxidation (methanotrophy). The global rate of methanogenesis can be estimated as the sum of the global oxidation rate ($6.9–9.2 \times 10^{14}$ g y^{-1}; Reeburgh, 1996; Valentine, 2002) and the global atmospheric flux (5.0×10^{14} g y^{-1}). Of the total $1.2–1.4 \times 10^{15}$ g of CH_4 produced annually, the majority of this CH_4 is produced biogenically, likely greater than 85%.

Biogenic CH_4 production (hereafter referred to as methanogenesis) occurs at all temperatures between freezing and boiling (Valentine and Boone, 2000). The majority of CH_4 currently released to the atmosphere is produced near the surface, at temperatures between 0 and 50°C. Abundant CH_4 is also produced in environments with elevated temperatures (moderately thermal environments, defined here as having temperatures from ~ 50–110°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 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 CH_4 produced in moderately thermal settings is generally distinguished from thermogenic CH_4 by the carbon and hydrogen isotopic content of the CH_4 as well as by comparing the abundance of CH_4 to ethane and propane (Schoell, 1980).

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Table 1. Experiments presented in this study.

Experiment	Organism	Purpose	Substrate	Variables[a]	Presented in:	System[b]
A1-4	*M. thermophila*	^{13}C fractionation	Acetate	g	Table 2, 4, E-1, Figs. 1–4	o, c
B1-4	*M. thermophila*	D/H fractionation	Acetate		Table 4, E-2, Fig. 9	o
C1-4	*M. marburgensis*	^{13}C fractionation	H_2/CO_2	t, h, g, l, m	Table 3, 6	o
D1-3	*M. marburgensis*	D/H fractionation	H_2/CO_2	t, g, e, m	Table 3, Figs. 10–13, E-3	o

[a] Variables tested: temperature (t); H_2 concentration (h); growth phase (g); light level (l); hydrogenase efficiency (e); metabolic activity (m).
[b] 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_4 is produced by two primary pathways, the aceticlastic reaction (Eqn. 1), and CO_2 reduction (Eqn. 2). The reduction of CO_2 can be accomplished with either hydrogen (H_2) or formate ($HCOO^-$) acting as reductant.

$$CH_3COO^- + H^+ \rightarrow CH_4 + CO_2 \quad (1)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (2)$$

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_4 produced with CO_2 reduction accounting for ~30%. In moderately thermal environments this ratio changes, and CO_2 reduction is often quantitatively more important than the aceticlastic reaction (Fey et al., 2003). In permanently cold marine sediments, CH_4 is thought to be derived primarily from CO_2 reduction (Whiticar et al., 1986). The relative importance of the aceticlastic reaction versus CO_2 reduction is not known for deep, moderately thermal environments. Interestingly, many thermophilic methanogens have been isolated capable of CO_2 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_4, such as in the case of CH_4 hydrates, allows for the buildup of a large subsurface reservoir which may act as a capacitor (Dickens, 2003). Massive CH_4 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 Jurrasic (Padden et al., 2001) as well as during the Quaternary (Kennett et al., 2002). Deep subsurface CH_4 is likely a major source of such CH_4.

Given the importance of methanogenesis in moderately thermal environments, the importance of isotopic distributions in characterizing CH_4 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_4 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_2/H_2 methanogenesis (Experiment C), and hydrogen isotope fraction during CO_2/H_2 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_T (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_2PO_4, 0.5 g NH_4Cl, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 1 mg resazurin, 1.0 g $NaHCO_3$, 0.36 g $Na_2S \cdot 9H_2O$, 0.15 g CoM, 0.04 mg biotin, 5.0 mg sodium EDTA dihydrate, 1.5 mg $CoCl \cdot 6H_2O$, 1.0 mg $MnCl_2 \cdot 4H_2O$, 1.0 mg $FeSO_2 \cdot 7H_2O$, 1.0 mg $ZnCl_2$, 0.4 mg $AlCl_3 \cdot 6H_2O$, 0.3 mg $Na_2WO_4 \cdot 2H_2O$, 0.2 mg $CuCl_2 \cdot 2H_2O$, 0.2 mg $NiSO_4 \cdot 6H_2O$, 0.1 mg H_2SeO_3, 0.1 mg H_3BO_3, and 0.1 mg $Na_2MoO_4 \cdot 2H_2O$.

Methanothermobacter marburgensis (formerly *Methanobacterium thermautotrophicum* strain Marburg-OCM 82; Wasserfallen et al., 2000) was used in studies of CO_2/H_2 methanogenesis. The organism was originally isolated from a thermophilic waste digestor 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_2 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_4) 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_4 (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
δ^{13}C-Acetate (whole molecule-‰)	N/A	−25	−25
δ^{13}C-Acetate (methyl position-‰)	−31.0	−29.1	−28.3
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 CH_4 concentration, acetate concentration, δ^{13}C-CH_4, δ^{13}C-Ac_{bulk}, and δ^{13}C-Ac_{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 CH_4 concentration, acetate concentration, δ^{13}C-CH_4, δ^{13}C-Ac_{bulk}, and δ^{13}C-Ac_{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 CH_4 mixing ratio. Then, a known overpressure of N_2 was added (to avoid drawing a vacuum) and a large volume gas sample (20–240 mL) was removed for analysis of δ^{13}C-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 CH_4 after each sampling. The bottle was purged for 4 min at a temperature of 51–61°C (5–10 L-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 CO_2 was added as an overpressure. The partial pressure of residual 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 CH_4 production.

A second strain of *M. thermophila*, strain 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 CH_4 reached 6% so as to avoid significant isotopic enrichment of the residual acetate.

2.2.2. Carbon isotope fractionation from H_2/CO_2

Four experiments (C-1 through C-4) were performed using an H_2-controlling bioreactor (Valentine et al., 2000a) to assess the impact of H_2 partial pressure, temperature, media organic content, and light level on carbon isotope fractionation. Studies were designed to test the hypothesis that 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, $OD_{598} \cong 0.3$) in sealed 1 L bottles under a mixture of 10^5 Pa H_2, 3×10^4 Pa CO_2, and 7×10^4 Pa N_2. After inoculation into the bioreactor, the total gas flow rate, the partial pressure of CO_2 and δ^{13}C-CO_2 were held constant for the duration of each experiment. 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. The downstream 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 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 CH_4. Inoculum for experiments with *M. thermophila* was obtained from late stationary phase cultures that had been thoroughly purged of 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 δD-H_2O 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 CH_4 concentration (<0.1 Pa), δD-H_2O, and δD-Ac. Liquid and gas samples were then taken every other day for 4 d for analysis of CH_4 concentration, δD-CH_4, δD-Ac, and δ^{13}C-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 P_T, was also used to study hydrogen isotopic fractionation from H_2O/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 CH_4 reached 6% so as to avoid significant isotopic enrichment of the residual acetate. The initial δD-H_2O of the culture media was −71 ± 16.6‰.

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

Experiment	Gas flow rate (mL-min^{-1})	CO_2 upstream (%)	H_2 upstream (Pa)	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	8×10^4	0.03	65	L	8.3	N/A	200	Defined
D-1	50/15	30	7×10^4	0	65	L	7.8	6	260	Defined
D-2	15	30	7×10^4	0	65	L	7.6	6	250	Defined
D-3	15	30	7×10^4	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 δD-H_2 on δD-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, 3×10^4 Pa CO_2, and 7×10^4 Pa N_2. The total gas flow rate was set to 55 mL min^{-1} (normalized to STP) at the beginning of Experiment D-1, and was changed to 15 mL min^{-1} after the 31st hour of experimentation due to analytical difficulties in quantifying δD-CH_4 at relatively low mixing ratios. Experiments D-2 and D-3 were performed with a flow rate of 15 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 α or ε notation. In the case where isotopic equilibrium is achieved, α is defined as:

$$\alpha = R_a/R_b \tag{3}$$

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, α is defined as:

$$\alpha = r_l/r_h \tag{4}$$

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, ε, can be used:

$$\varepsilon = (\alpha - 1) \times 1000 \tag{5}$$

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 aceticlastic 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, α. 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 aceticlastic methanogenesis (Experiment A) can be determined as a closed system using the approach of Gelwicks et al. (1989, 1994):

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

where f is the fractional yield based on the consumption of acetate, δ_{af} is the δ^{13}C of the methyl position of acetate at any f, δ_{ai} is the initial δ^{13}C of the methyl position of acetate, and ε_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:

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

where δ_{wf} is the δ^{13}C of the whole acetate molecule at any f, δ_{wi} is the initial δ^{13}C of the whole acetate molecule, and ε_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:

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

where δ_{mf} is the δ^{13}C of pooled CH_4 at any f. The isotopic fractionation factor, ε_m, can be determined by regressing δ_{af} against $\ln(1 - f)$, or δ_{mf} against $(1 - f)$ $[\ln(1 - f)]/f$. Similarly, ε_w can be determined by regressing δ_{wf} against $\ln(1 - f)$. In each case ε 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, δ_{af} and δ_{wf} at any given time are equal to the δ_{af} and δ_{wf} values measured at that time. However, in the case of Eqn. 8, δ_{mf} represents the δ^{13}C of all CH_4 produced, and is thus dependent on gas removed from the system. The value of δ_{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 aceticlastic 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, ε_m, is used here for aceticlastic 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$3%) and the rapid gas flow through the culture system. In addition, the upstream δ^{13}C-CO_2 was assumed to be equal to the downstream δ^{13}C-CO_2, an assumption supported by the distribution of δ^{13}C-CO_2 in the various experiments, and by the results of Chidthaisong et al. (2002). The measured δ^{13}C-CO_2 was assumed to be the same as that available for methanogenesis. Although fractionation occurs during CO_2 dissolution (thermodynamic) and during gas transfer (kinetic), the fractionations are small ($\cong$ 1–2‰; Zhang et al., 1995) and were not quantified for the experimental conditions employed. Stable isotope fractionation factors (α-factors) for coexisting CO_2-CH_4 pairs were

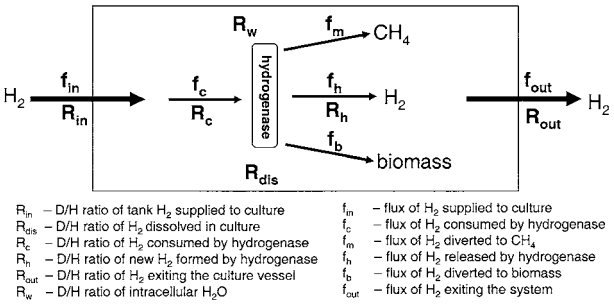

Fig. 1. Schematic diagram for the calculation of hydrogenase efficiency in *M. marburgensis*.

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

2.4.3. Hydrogen isotope fractionation in M. marburgensis

During H_2/CO_2 methanogenesis the ultimate source of carbon-bound hydrogen in CH_4 (e.g., CH_4-bound hydrogen) is thought to be H_2O (Daniels et al., 1980; Spencer et al., 1980), though the immediate precursors are not completely understood. There are several potential sources of CH_4 hydrogen, including H_2O, H_2, and methyl-bound hydrogen (e.g., from acetate, methyl amines, methyl thiols, and methanol). Furthermore, CH_4 hydrogen may cycle through the proton pool, through biologic reductants such as coenzyme F_{420} 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_4. Because δD varies by several hundred per mil in these studies, and because the δ notation is nonlinear in this range (O'Neil, 1986), D/H ratios (r = D/H) are needed to calculate fractionation factors between H_2O and CH_4:

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

and between H_2O and H_2:

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

The α notation, as applied here, quantifies the difference in isotopic composition between two hydrogen-containing molecules linked biochemically. For example, in Eqn. 10, α only applies to the H_2 produced by the hydrogenase system (see section 2.4.4), not to the H_2 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_2 besides hydrogenase in the system. The gross H_2 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:

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

The D/H ratio of H_2 consumed (activated) by hydrogenase (R_c) can be expressed in terms of the D/H ratio of dissolved H_2 (R_{dis}), given the fractionation factor (α_c) associated with H_2 uptake by hydrogenase (e.g., $R_{dis} = \alpha_c \times R_c$).

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

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 α_c), the flux of reducing equivalents diverted to CH_4 is assumed to be equal to four times the rate of CH_4 production (see Eqn. 1). We further assume $R_{dis} = R_{out}$, R_w = D/H of the culture media, R_h and α_c remain constant over time, and each H_2 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 δ^{13}C-CH_4 and δD-CH_4 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 δ^{13}C-CH_4 and δ^{13}C-CO_2 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_4 (δ^{13}C, δD), CO_2 (δ^{13}C), H_2 (δD), and H_2O (δD) 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: δ^{13}C-CO_2 ($\pm$ 0.4‰), δ^{13}C-CH_4 ($\pm$ 0.3‰), δD-H_2 ($\pm$ 7.3‰), δD-CH_4 ($\pm$ 4.6‰), and δD-H_2O ($\pm$ 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_2 and the methyl carbon to CH_4 (Blair et al., 1985). The CH_4 was separated from the CO_2 in a vacuum line, combusted to CO_2, 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_2 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 δ^{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_2 until dry, and were then stored in an airtight jar containing anhydrous $CaSO_4$. Isoto-

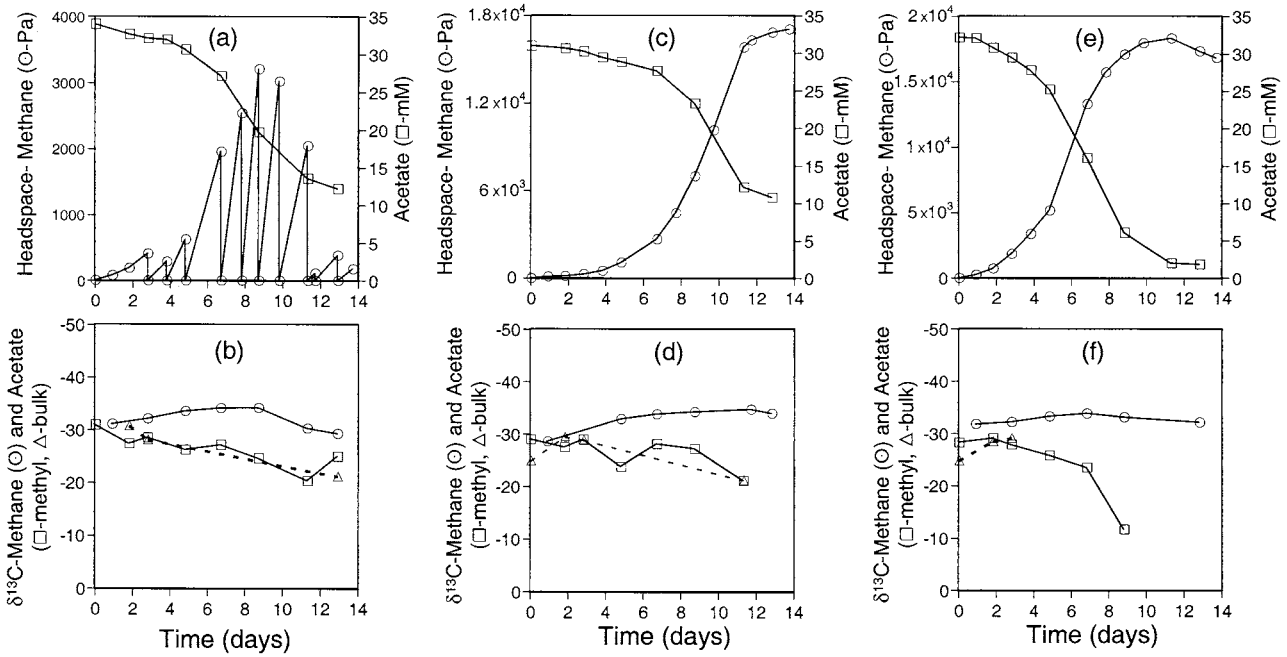

Fig. 2. Variations in the partial pressure (Pa) and $\delta^{13}C$ of headspace methane (○) as well as the concentration (mM) and $\delta^{13}C$ of aqueous acetate (methyl position, □; whole molecule, △) during growth of *M. thermophila* strain CALS-1 in sealed-bottle Experiments. (a, b) Experiment A-1; (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 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 ($^{13}C/^{12}C$) 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, 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 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 CH_4 quantified in these bottles and the 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 (CH_4 is underestimated by 30–40% relative to acetate consumption), presumably because of calculation errors associated with frequent purging of CH_4 from the headspace.

In each experiment the cultures grew at different rates. Based on the rate of 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 ~ 10 mM. *M. thermophila* consumes acetate to a threshold value near 20 μM (Min and Zinder, 1989), therefore another factor must have inhibited growth at the end of the experiment.

The $\delta^{13}C$ of the ether lipid archaeol, which constituted ~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 ε_m. Isotopic fractionation factors (ε_m) for the conversion of the acetate methyl to CH_4 were determined using a closed-system approach (Eqn. 6) by comparing the $\delta^{13}C$ of the residual acetate to the fractional yield (Fig. 3). The fractionation factors calculated for conversion of methyl acetate to CH_4 range from 6.1 to 10‰ (CH_4 depleted relative to acetate).

Isotopic fractionation factors (ε_m) for the conversion of the acetate methyl to CH_4 were determined independently using a closed-system approach by comparing the $\delta^{13}C$ of the pooled CH_4 (calculated by mass balance) to the fractional yield of 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 $\delta^{13}C$ of pooled acetate yields a slope of ε_m. Results indicate two distinctive trends, one corresponding to the early

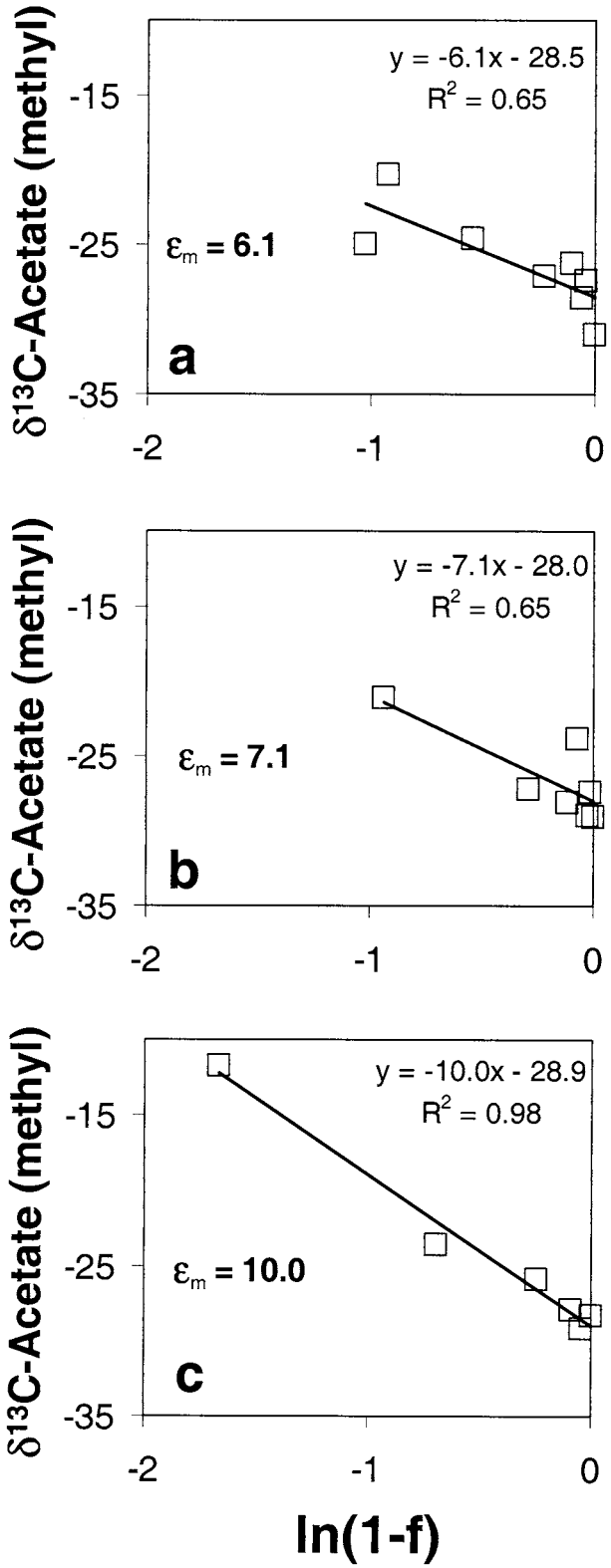

Fig. 3. Determination of ε_m for Experiments A-1 (a), A-2 (b) and A-3 (c) using a closed-system approach. Both δ^{13}C-acetate (methyl) and f were measured directly.

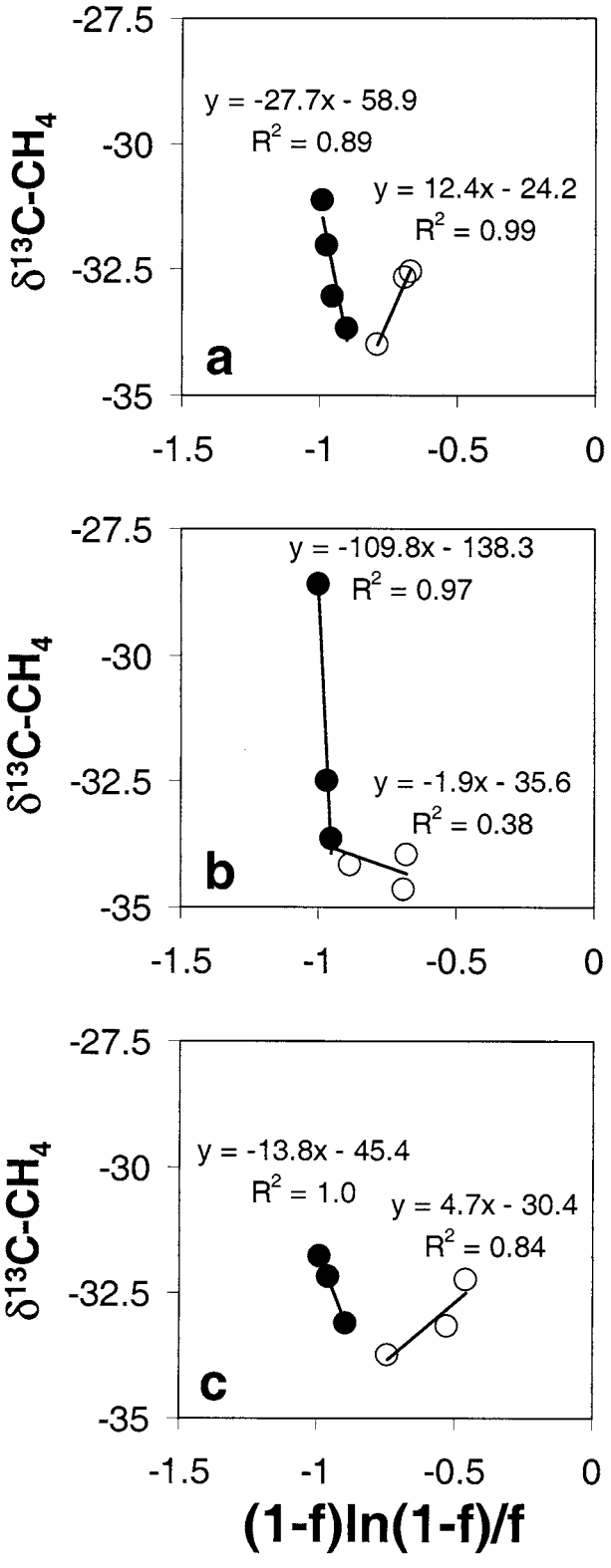

Fig. 4. Independent determination of ε_m for Experiments A-1 (a), A-2 (b) and A-3 (c) using a closed-system approach. Both δ^{13}C-CH_4 and f were back-calculated from experimental data using mass balance. Closed circles represent samples taken between days 1 and 7 while open circles represent samples taken between days 7 and 14.

Table 4. Results of isotope fractionation experiments with *M. thermophila* strain P_T (Experiments A-4 and B-2).

	PCH_4 (Pa)		δ^{13}–CH_4 (‰)	δD–CH_4 (‰)	Acetate (mM)		δ^{13}C–Ac (methyl)	δ^{13}C–Ac (bulk)	δD–H_2O (‰)
Culture	Initial	Final	Final	Final	Initial	Final	Initial	Initial	Initial
P_T(1)	1	7000	−29.28	−295.06	50	44	−22.18	−24.71	−73 ± 17
P_T(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 ε_m. Fractionation factors calculated for only the latter stages of growth from Experiments A-1 and A-3 yield ε_m values of 12.4 and 4.7‰, respectively.

In addition to a closed-system approach, ε_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 δ^{13}C-CH_4 and δ^{13}C-Ac_{methyl} is indicative of the instantaneous isotopic fractionation. The average ε_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 ^{13}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 ε_m of 6.5 and 5.9‰ respectively. The average ε_m from these experiments is 6.1‰, and compares well with the average ε_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_T. 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, ε_m averaged 4.4‰ in these experiments, and is similar to strain CALS-1.

By equally considering each approach and each experiment, an average ε_m of 7.0 ± 2.6‰ is calculated, corresponding to an α of 1.007.

3.1.1.2. Determination of ε_w. Isotopic fractionation factors (ε_w) for the conversion of the whole acetate molecule to CH_4 and CO_2 were determined using a closed-system approach (Eqn. 7) by comparing the δ^{13}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_4 and CO_2 depleted relative to acetate). ε_w represents the average fractionation against the methyl and carboxyl positions of acetate. The similar values determined for ε_w and ε_m seemingly indicate there is no intramolecular ordering of acetate during aceticlastic 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_2 (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_2 and the carboxyl po-

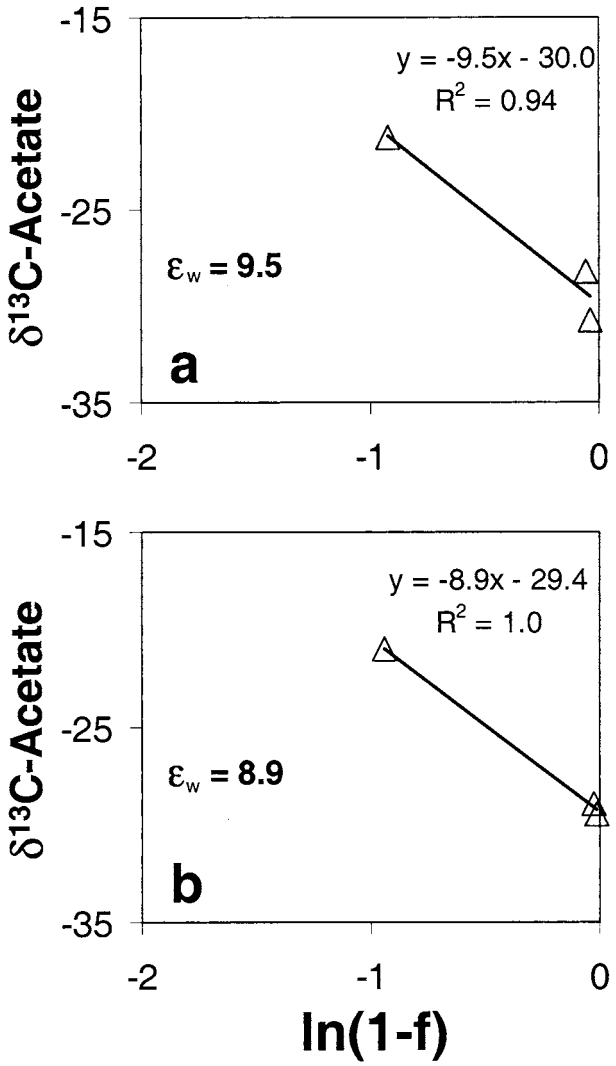

Fig. 5. Determination of ε_w for Experiments A-1 (a) and A-2 (b) using a closed-system approach.

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

Organism	Substrate[a]	Strain number DSMZ/OCM[b]	Temp (°C)	H_2 (Pa)	Growth phase	System type	Reference	Max α Substrate $-CH_4$
Methanothermobacter marburgensis	H_2/CO_2	2133/82	65	8×10^4	log	open	Fuchs et al. (1979)	1.034
Methanosarcina barkeri (MS)	H_2/CO_2	800/38	36	8×10^4	log	open	Krzycki et al. (1987)	1.049
Methanobacterium ivanovii (Ivanov)	H_2/CO_2	2611/140	37	8×10^4	log	open	Belyaev et al. (1983)	1.037
Methanobacterium thermautotrophicum (ΔH)	H_2/CO_2	1053/[c]	65	2.4×10^5	early log	closed	Games et al. (1978)	1.025
Methanosarcina barkeri	H_2/CO_2	[d]	40	8×10^4	mid-log	open	Games et al. (1978)	1.045
Methanobacterium bryantii (M.o.H.)	H_2/CO_2	863/110	40	8×10^4	mid-log	open	Games et al. (1978)	1.061
Methanococcus thermolithotrophicus (SN-1)	H_2CO_2	2095/138	55[e]	8×10^4	stationary	open	Botz et al. (1996)	1.069
Methanococcus vannielii	H_2/CO_2	1224/148	35[e]	8×10^4	stationary	open	Botz et al. (1996)	1.079
Methanococcus igneus	H_2/CO_2	5666/[c]	85[e]	8×10^4	late log	open	Botz et al. (1996)	1.068
Methanobacterium formicicum (MF)	H_2/CO_2	1535/55	34	2.4×10^5	[f]	closed	Balabane et al. (1987)	<1.05[g]
Methanothermobacter marburgensis	H_2/CO_2	2133/82	65	310	SL[h]	open	Present study	1.064
Methanothermobacter marburgensis	H_2/CO_2	2133/82	65	8×10^4	log	open	Present study	1.031
Aceticlastic and Methylotrophic Methanogenesis								
Methanosarcina barkeri strain MS	Ac	800/38	37	N/A		closed	Krzycki et al. (1987)	1.022
Methanosarcina barkeri strain 227	Ac	1538/35	37	N/A		closed	Gelwicks et al. (1994)	1.022
Methanosaeta concilii	Ac	N/A	25	N/A		closed	Chidthaisong, unpublished	1.017
Methanosaeta thermophila strain CALS-1	Ac	3870/N/A	61	N/A	all	both[i]	Present study	1.007[i]
Methanosaeta thermophila strain P_T	Ac	6194/778	60	N/A	early log	closed	Present study	1.007
Methanosarcina barkeri strain MS	Me	800/38	37	N/A		closed	Krzycki et al. (1987)	1.079
Methanococcoides burtonii	TMA	6242/468	20	N/A		closed	Summons et al. (1998)	1.076
Methanosarcina barkeri	TMA	800/38	37	N/A		closed	Summons et al. (1998)	1.053

[a] Ac (acetate), Me (methanol), TMA (trimethylamine).
[b] DSMZ = German Collection of Microorganisms and Cell Cultures, OCM = Oregon Collection for Methanogens.
[c] Strain not currently available through OCM.
[d] Authors unable to determine which strain was used (likely strain MS).
[e] Cultures grown in titanium fermentor.
[f] Samples taken from sealed vials over 3 days of growth.
[g] Maximum value estimated using $\Delta\delta^{13}C(CO_2\text{-}CH_4)$ assuming influence of biomass to be negligible.
[h] SL = substrate limited.
[i] Both open- and closed-system approaches used to calculate an average value for α.

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 ε_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, *Mathanosarcina* 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 ε_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 μM, whereas the acetate threshold for the *Methanosarcina* is near 500 μM (Min and Zinder, 1989). The difference in catabolic pathways is consistent with

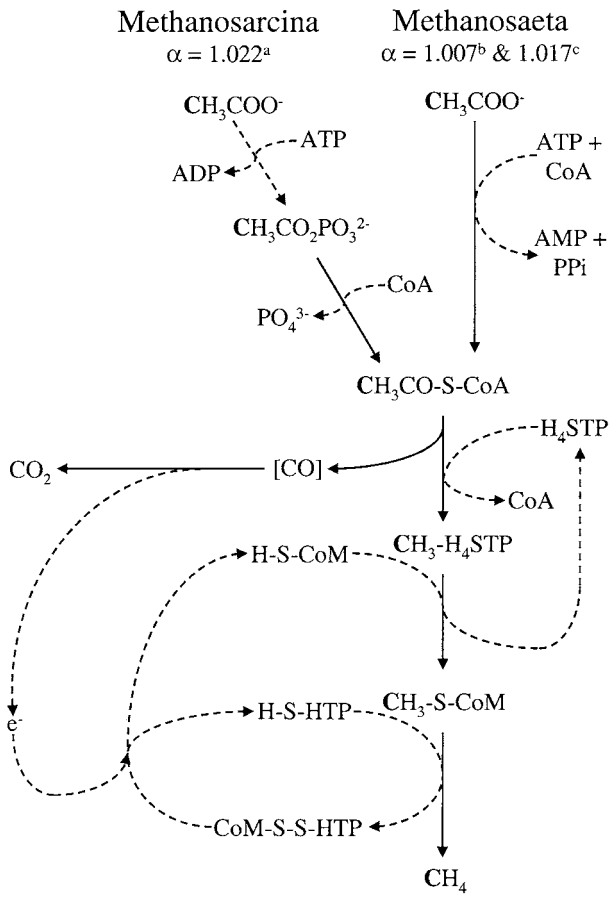

Fig. 6. Catabolic pathways of aceticlastic methanogens. The *Methanosarcina* utilize a two step pathway to fix acetate to acetyl CoA, while the *Methanosaeta* utilize only one step. The pathways converge at acetyl CoA. The solid lines represent steps where bonds to acetate carbon (carbon originating 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 CO_2 while the methyl position goes to CH_4. Key to abbreviations: ATP (adenosine triphosphate), PPi (inorganic pyrophosphate), CoA (coenzyme A), H_4STP (tetrahydrosarcinapterin), CoM (coenzyme M), and H-S-HTP (*N*-7-mercaptoheptanoyl-*O*-phospho-L-threonine). [a]Krzycki et al. (1987), Gelwicks et al. (1994). [b]This study. [c]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 ε_m of ~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 ε_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 CO_2-utilizing methanogens (Valentine, 2000).

3.1.1.4. Environmental implications. Geologic CH_4 is typically categorized as thermogenic if δ^{13}C-CH_4 is heavier than −50‰ or biogenic if δ^{13}C-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 CH_4 produced by *Methanosaeta* at elevated temperature is likely to appear isotopically enriched relative to other sources of 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, CH_4 produced at elevated temperatures by the aceticlastic reaction is likely to appear thermogenic in origin. The true environmental importance of acetate-derived 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 CH_4.

The apparent isotopic equilibration between the carboxyl position of acetate and 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 (200–360°C) leads directly to ^{13}C-enrichment of the carboxyl position (Dias et al., 2002). Microbially catalyzed exchange could further act on organic acids after cooling, altering the initial δ^{13}C-$Ac_{carboxyl}$ by way of exchange with ambient CO_2. Isotope exchange of ^{13}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 ~1.0027 (carboxyl position is enriched in ^{13}C relative to CO_2) at 25°C and pH 7.5 (O'Leary and Yapp, 1978).

3.1.2. Methanogenesis from H_2/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_2 up[a] (Pa)	H_2 down[b] (Pa)	CH_4 down[b] (Pa)	Light/dark	$\delta^{13}C$–CO_2 down[b] (‰)	$\delta^{13}C$–CH_4 down[b] (‰)	α CO_2–CH_4
				Results from Experiment C-1				
4	65	310	226[c]	21	L	−43.50	−94.10	1.056
7	55	310	218[c]	23	L	−44.09	−91.47	1.052
13	45	310	242[c]	17	L	−44.04	−90.32	1.051
16	75	310	286[c]	6	L	−43.43	−96.75	1.059
19	40	310	282[c]	7	L	−44.39	−92.33	1.053
				Results from Experiment C-2				
7.5	65	290	193[c]	25	L	−43.54	−94.21	1.056
13	55	290	178[c]	29	L	−43.95	−93.47	1.055
24.5	45	290	182[c]	26	L	−43.81	−93.45	1.055
32	75	290	276[c]	3	L	−44.32	−97.73	1.059
				Results from Experiments C-3 and C-4				
13	65	350	345[c]	1.3	L	−43.99	−96.23	1.058
24	65	350	342[c]	2.1	D	−43.90	−97.70	1.060
35	65	350	344[c]	4.1	L	−44.23	−73.15	1.031
48	65	350	316[c]	8.5	D	−44.18	−99.97	1.062
78	65	350	270[c]	20	L	−44.52	−102.17	1.064
80	65	8.0×10^4	7.9×10^4[d]	210	L	[e]	−68.06	1.026
83	65	8.0×10^4	7.9×10^4[d]	300	L	−43.98	−70.59	1.029
86	65	8.0×10^4	7.8×10^4[d]	400	L	−43.76	−71.99	1.030
97	65	8.0×10^4	7.8×10^4[d]	510	L	[e]	−65.87	1.023

[a] Upstream of the culture vessel.
[b] Downstream of the culture vessel.
[c] Measured directly.
[d] Calculated from CH_4 assuming 4:1 stoichiometry.
[e] Sample lost: value assumed to be average of $\delta^{13}C$–CO_2 for Experiments C-3 and C-4.

such as temperature (Experiments C-1 and C-2), H_2 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_2 and CH_4. 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_2 partial pressure. Low H_2 partial pressures (Experiments C-1 through C-3) yield large fractionation factors between CO_2 and CH_4. Increasing the H_2 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_2 (290–350 Pa H_2); the average α-factor was 1.027 for experiments with elevated levels of H_2 (8.0×10^4 Pa H_2). Previous studies of H_2/CO_2 methanogenesis with other strains demonstrated a growth-phase dependence in carbon isotope fractionation. The "H_2 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_2/CO_2 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_2 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_2/CO_2 methanogenesis (Eqn. 2) is approximately -131 kJ mol^{-1} under standard conditions, including 10^5 Pa H_2. 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^{-1}. Furthermore, catabolism is coupled to energy conservation, by way of a chemiosmotic potential (equivalent to ¼ – ⅓ 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_2-replete) conditions (Hoehler et al., 1998; Hoehler et al., 2001).

$$CO_2 + 4H_2 + ¼ADP + ¼Pi \rightarrow CH_4 + 2H_2O + ¼ATP \quad (16)$$

Under conditions of excess H_2 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 ($\Delta G'$) for catabolism is calculated to be -97 kJ mol^{-1}

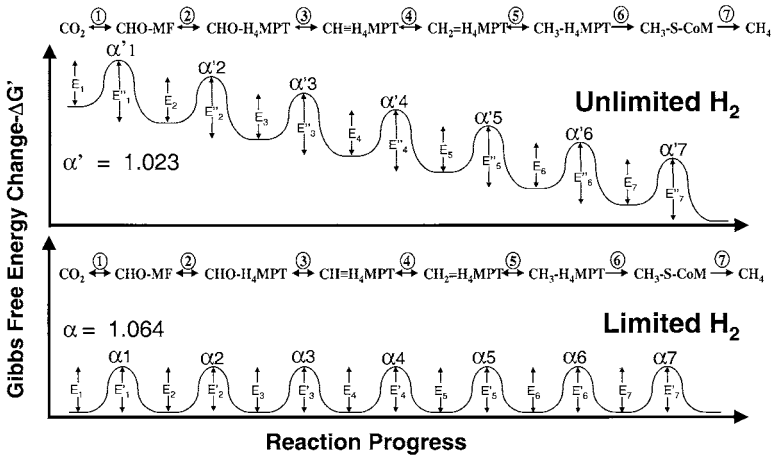

Fig. 7. Schematic representation of the Gibbs Free Energy change associated with catabolism during H_2/CO_2 methanogenesis (Eqn. 16), focusing on the activation energies for the forward and reverse reactions. Each of the seven catabolic reactions (shown at bottom and in Fig. 8) are outlined for conditions of excess H_2 (top) and limited H_2 (lower). Under conditions of excess 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 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 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 H_2 conditions because rapid H_2 consumption by dense cultures draws the dissolved and intracellular H_2 lower, and the $\Delta G'$ experienced by the organism is decreased (e.g., much of the $\Delta G'$ is lost to the H_2 concentration gradient; Sorenson et al., 2001). MF = methanofuran, H_4MPT = 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 H_2 and -42 kJ mol^{-1} (assuming reaction by Eqn. 16 and conditions from Experiment C-3, t = 78 h; $\alpha = 1.064$) for conditions of limiting H_2. Both calculations assume the formation of ¼ ATP requires 20 kJ mol^{-1}.

This "differential reversibility" hypothesis requires that the first five steps of the methanogenic pathway be fundamentally reversible under low 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 H_4MPT 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 H_2/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 H_2 levels (Fig. 8). The reduction of CH ≡ H_4MPT to CH_2 = H_4MPT (Fig. 8, step 4) is linked to the oxidation of H_2 under condition of excess H_2, and is linked to the oxidation of coenzyme F_{420} under limited H_2 (Nolling et al., 1995; Nolling and Reeve, 1997; Reeve et al., 1997). The H_2-linked reduction of CH ≡ H_4MPT to CH_2 = H_4MPT proceeds with a free energy yield 11 kJ mol^{-1} more exergonic (Thauer, 1998) than the equivalent F_{420}-linked reduction. Although both reactions are reversible, the F_{420}-linked reaction may be "more" reversible than the 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 H_2-linked reaction (e.g., the oxidation of CH_2 = H_4MPT to CH ≡ H_4MPT) could create an isotopic bottleneck, allowing only steps 1–4 to express their full fractionation. In contrast, greater reversibility of the 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 CO_2 (e.g., a steady-state isotopic enrichment in the intracellular 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 CO_2, effectively creating a situation of irreversible flux into the cell (once CO_2 enters the cell there is no fate but conversion to 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.

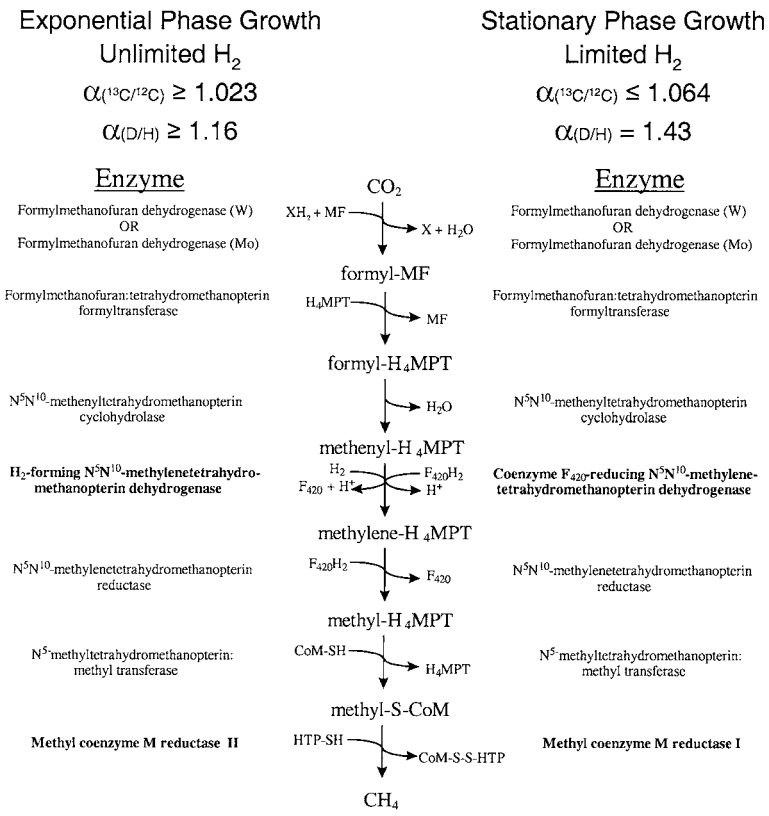

Fig. 8. The central catabolic pathway of methanogenesis in *M. marburgensis* and the enzymes which catalyze each step. Factors which differ between H_2 limited and H_2 unlimited conditions are given in bold. The catabolic pathway is based on Thauer (1998) while the enzymatic information is taken from 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 H_2/CO_2 methanogenesis, including enzyme expression patterns, cell-specific catabolic rate, cell density, kinetics of H_2 transport, energy conservation mechanisms, and the $\Delta G'$ of catabolism. Furthermore, the fractionation associated with H_2/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 H_2/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 aceticlastic methanogenesis, homoacetogenesis, anaerobic CH_4 oxidation, iron (III) reduction and fermentation.

3.1.2.2. Relation to previous studies. Observations of H_2-dependent fractionation of carbon isotopes during H_2/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 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 aceticlastic reaction were designed to quantify the impact of δD-Acetate, δD-H_2O and the isotope effect on the δD of the resulting CH_4. However, technical problems with the quantification of δD in acetate only allow for comparison of δD-H_2O with δD-CH_4. Results comparing the δD-CH_4 produced by *M. thermophila* (Experiments B-1 through B-4) with the δD-H_2O 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 CH_4-bound hydrogen originates from a source in isotopic equi-

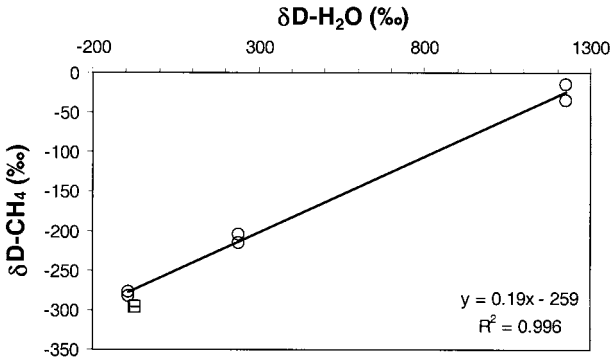

Fig. 9. The correlation between δD-CH_4 produced by *M. thermophila* strains CALS-1 (circles) and P_T (squares) and the δD-H_2O of corresponding culture media (Experiments B-1 through B-4).

librium with H_2O, which is slightly lower than the expected value of 25%. This calculation assumes all acetate has the same initial δD, 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 CH_4-bound hydrogen (Fig. 6; 3 hydrogen atoms), and other sources of hydrogen (not in isotopic equilibrium with H_2O) are not certain. The Y intercept of the curve (−259‰) is representative of both the acetate substrate (δD-Ac) and the kinetic isotope effect associated with the aceticlastic reaction. Given the uncertainties in quantification of δD-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 δD in acetate is that of isotope exchange. Hydrogen atoms bound to a carbon adjacent to a carboxyl group are able to exchange with H_2O (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 δD-CH_4 and δD-H_2O (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 H_2/CO_2*

Hydrogen isotope experiments with *M. marburgensis* (D-1, D-2) were performed to determine whether varying δD-H_2 would impact δD-CH_4 at conditions with a constant δD-H_2O. In Experiments D-1 and D-2, H_2 gas was supplied with two distinctive values of δD-H_2, −692 and −190‰, respectively. Results of these two experiments are presented in Figures 10 and 11, respectively. The isotopic fractionation between H_2O and CH_4 was constant at 270–275‰ (α = 1.43) in the latter half of Experiment D-1. The δD-H_2 remained constant throughout Experiment D-1 and did not deviate measurably from the value of the source H_2 (−692‰). The difference between H_2O

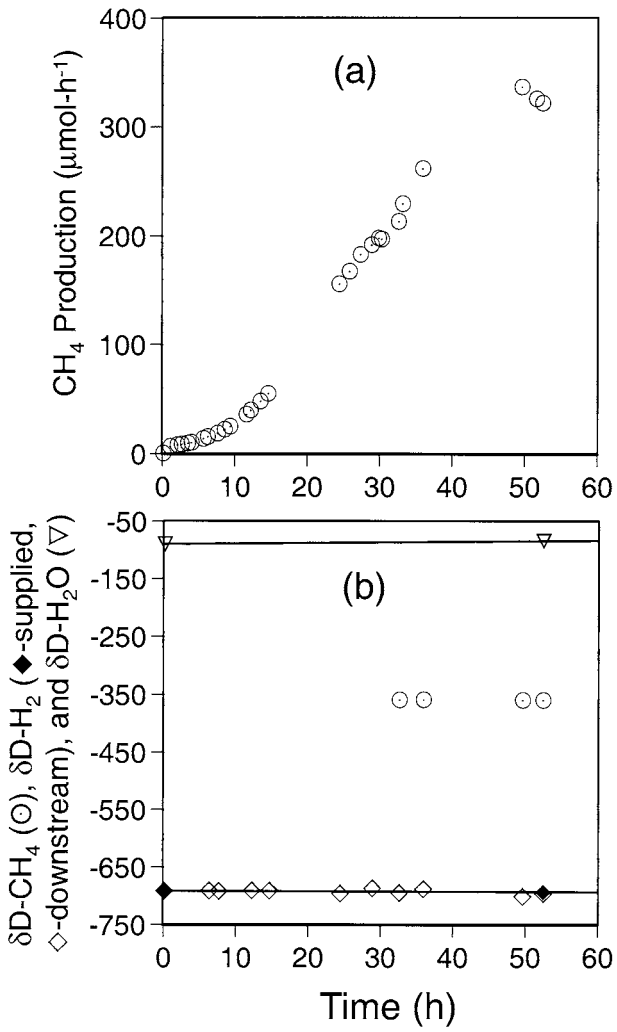

Fig. 10. Methane production and hydrogen isotope variations during growth of *M. marburgensis* in a flowthrough bioreactor (Experiment D-1). The rapid and continuous supply of substrate, and the constant removal of product, approximates an open system. Variations in the rate of methane production (⊙) are given in (a). Variations in δD-CH_4 (⊙), δD-H_2 (◆, supplied; ◇, downstream) and δD-H_2O (▽) are given in (b). The initial flow rate of gas entering the vessel was 55 mL min^{-1}, and was decreased to 15 mL min^{-1} after 31 h due to analytical considerations. The α(H_2O to CH_4) was identical for each of the (4) sampling times, 1.43.

and CH_4 was more variable in Experiment D-2, ranging from −127 to −275‰ (α = 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‰ during the stationary phase. The δD-H_2 of the residual H_2 exiting the vessel varied throughout Experiment D-2, following the same pattern as the δD-CH_4 produced during the experiment. The δD-H_2 of the residual H_2 was initially similar to the source H_2 (−190‰), and became more depleted over the course of the experiment to a final δD-H_2 of −263‰. At the maximum rate of metabolism, during stationary phase, less than 8% of the total H_2 supplied to the vessel was consumed. The time scale for isotopic exchange for these experiments can

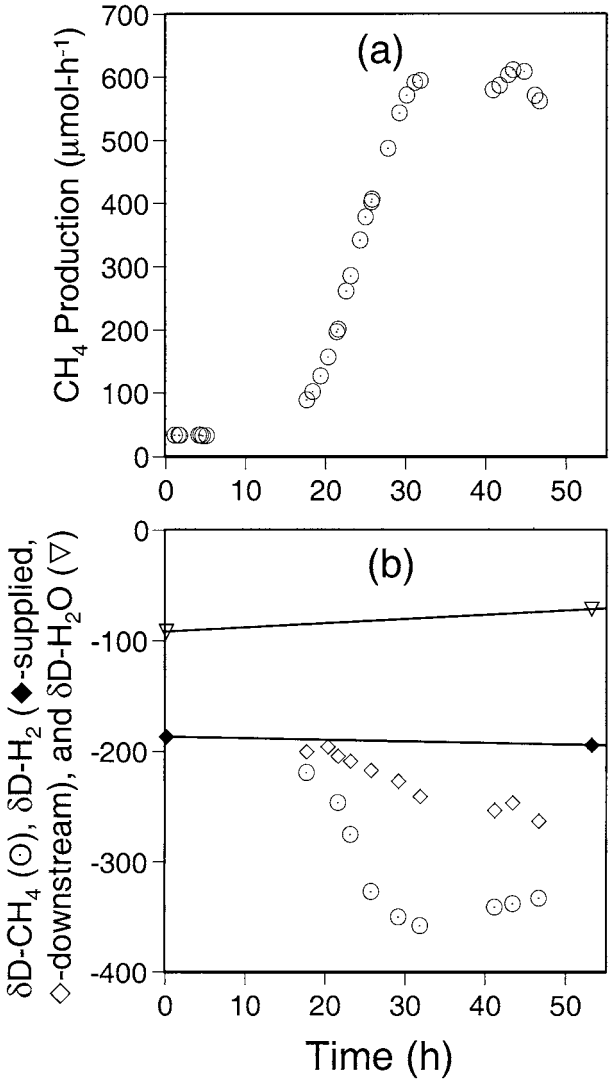

Fig. 11. Methane production and hydrogen isotope variations during growth of *M. marburgensis* in a flowthrough bioreactor (Experiment D-2). Conditions approach those of an open system. Variations in the rate of methane production (⊙) are given in (a). Variations in δD-CH_4 (⊙), δD-H_2 (◆, supplied; ◇, downstream) and δD-H_2O (▽) are given in (b). The flow rate of gas entering the vessel was maintained at 15 mL min^{-1} over the course of the experiment. The α(H_2O to CH_4) ranged from 1.16 to 1.43 over the course of the experiment. The H_2 exiting the vessel is depleted by as much as 71‰ relative to the H_2 entering the vessel, and displays a growth-phase dependence.

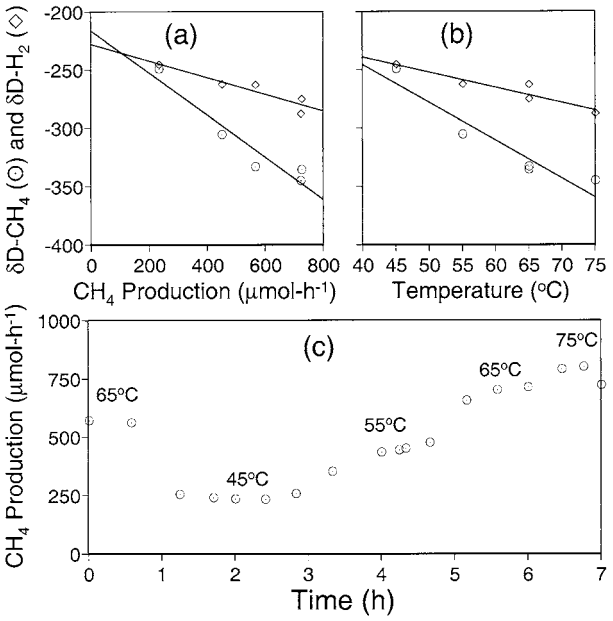

Fig. 12. The impact of temperature and metabolic rate on hydrogen isotope fractionation during growth of *M. marburgensis* in a flowthrough bioreactor. Variations in δD-CH_4 (⊙) and δD-H_2 (◇) measured downstream from the bioreactor are shown as a function of CH_4 production rate (a) and temperature (b). The methane production rate is shown as a function of time in (c), with temperature included. The flow rate was maintained at 15 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°C) indicates slight growth may have occurred. (a) δD-CH_4 (⊙) vs. CH_4 production rate (R^2 = 0.92); δD-H_2 (◇) vs. CH_4 production rate (R^2 = 0.88). (b) δD-CH_4 (⊙) vs. temperature (R^2 = 0.90); δD-H_2 (◇) vs. temperature (R^2 = 0.89). The α(H_2O to CH_4) ranged from 1.24 to 1.42 over the course of the experiment.

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 δD-CH_4 at a constant δD-H_2 (supplied) and δD-H_2O. Results are presented in Figure 12. The δD of CH_4 produced by the culture is strongly correlated with both the CH_4 production rate and the temperature (R^2 of 0.92 and 0.90, respectively). The residual δD-H_2 exiting the vessel also correlates strongly with both the 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 fractionations 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 CH_4 production rates at the beginning of the experiment and after 6 h (both at 65°C).

3.2.2.1. The source of CH_4-bound hydrogen. Experiments on H_2/CO_2 methanogenesis (Experiment D) were designed to test whether the δD-H_2 supplied to methanogens impacts the δD-CH_4 produced, at constant δD-H_2O. The maximal observed fractionation factor (α) between CH_4 and H_2O was 1.43, and was independent of the δD-H_2 provided. These results suggest, but do not prove, δD-H_2O is the primary factor controlling δD-CH_4 produced by *M. marburgensis* during the stationary phase of growth. Our results are consistent with those or Daniels et al. (1980) who used 81 atom% deuterated H_2O to determine the ultimate source of CH_4-bound hydrogen during methanogenesis; results indicated the hydrogen source was H_2O, with a corresponding α(H_2O-CH_4) of 1.5 ± 0.2. However, results from several biochemical studies indicate 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_2 as a source of CH_4-bound hydrogen, due to the rapid isotopic equilibration between H_2O and H_2. The possibility remains hydrogen from H_2 is incorporated into CH_4– if the intracellular H_2 isotopically equilibrates with H_2O before being incorporated into CH_4.

3.2.2.2. δD-CH_4 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 δD-CH_4 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_4MPT to methylene-H_4MPT, 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_2 availability and/or growth phase (Nolling et al., 1995; Nolling and Reeve, 1997; Reeve et al., 1997).

The first enzyme (H_2-forming N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase) receives reducing power directly from H_2 and is preferentially expressed at early stages of growth and under conditions of excess H_2. This enzyme catalyzes the heterolytic cleavage of H_2 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_2 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_{420}-dependent N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase) receives reducing power from the reduced form of coenzyme F_{420}, and is expressed under conditions of H_2 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 $\alpha(H_2O/CH_4)$.

One possible mechanism by which these enzymes could impact the δD-CH_4 involves distinctive fractionations associated with the H_2- and F_{420}-linked enzymes. Greater fractionation associated with hydrogen addition by way of F_{420} is consistent with experimental observations, and could be related to the two-step reduction, first from H_2 to $F_{420}H_2$ (Nambiar et al., 1983; Yamazake et al., 1985; Schauer et al., 1986; Klein and Thauer, 1995) and then to methylene-H_4MPT. During the early stages of Experiment D-2 the observed fractionation during CH_4 production was rather small ($\alpha = 1.16$), as might be expected if the H_2-linked enzyme is dominant and expresses no fractionation. The experimental results are consistent with the F_{420}-linked enzyme expressing an $\alpha(H_2O/CH_4)$ of ~ 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_2 activation and release by the action of hydrogenase. The isotopic evidence clearly indicates the hydrogenase system is not completely efficient, as the H_2 leaving the culture vessel is depleted in deuterium relative to the H_2 entering the vessel; hydrogenase enzymes are well known for rapid reversibility and for catalysis of D-H exchange between H_2 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_2 from H_2O, $\alpha(H_2O\text{-}H_2)$, and a fractionation for the uptake of H_2 by hydrogenase (α_c). The mass balance is detailed in section 2.4.4. Although one uptake hydrogenase (H_2-forming N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase, as in section 3.2.2.2) displays no fractionation during H_2 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_2 production, $\alpha(H_2O\text{-}H_2)$, and the efficiency of the hydrogenase system (E), at three different values of α_c. Results indicate the hydrogenase system in *M. marburgensis* is highly inefficient, even as $\alpha(H_2O\text{-}H_2)$ approaches 10. Likely values of $\alpha(H_2O\text{-}H_2)$ are at least 3 and possibly higher. Assuming an $\alpha(H_2O\text{-}H_2)$ of 3.1 (which also corresponds to the equilibrium isotope fractionation factor for H_2/H_2O at 65°C; Horibe and Craig, 1995), *M. marburgensis* (as observed in this study) would activate 8 H_2 molecules for each CH_4 produced; one H_2 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 δD-H_2O and δD-CH_4 in an environment where both H_2/CO_2 and acetate are important CH_4 precursors. These authors attributed temporal variations in δD-CH_4 to changes in the relative contributions of acetate versus H_2/CO_2 methanogenesis, and used such variations to estimate the fraction of CH_4 derived from H_2/CO_2 versus acetate. Sugimoto and Wada (1995) observed increasing fractionation between $\delta^{13}C\text{-}CH_4/\delta^{13}C\text{-}CO_2$ and between δD-CH_4/δD-H_2O 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 δD-CH_4 (Chidthaisong et al., 2002).

Few culture studies have considered hydrogen isotope fractionation during H_2/CO_2 methanogenesis. Calculations using data presented by Balabane et al. (1987) indicate a hydrogen isotope fractionation of 1.67 ± 0.05 between source H_2O and the resulting CH_4 for pure cultures of *Methanobacterium for-*

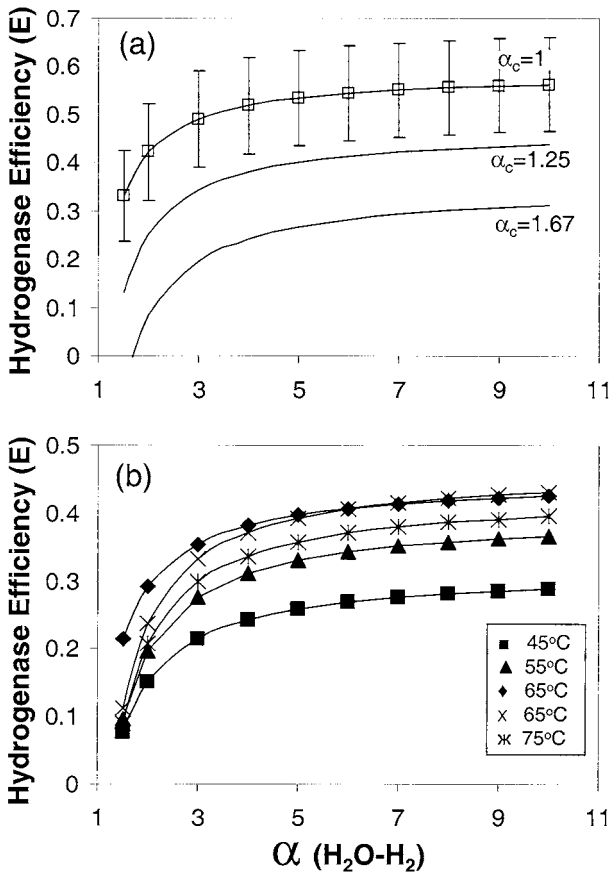

Fig. 13. The calculated efficiency (E) of the hydrogenase uptake system expressed as a function of the fractionation between intracellular H_2O and H_2 produced, $\alpha(H_2O/H_2)$. (a) All data from Experiments D-2 and D-3 were used to calculate an average efficiency (E), which is plotted as a function of $\alpha(H_2O/H_2)$. Error bars represent ± one standard deviation from the mean. Efficiency is plotted for three different values of α_c (fractionation associated with H_2 uptake by hydrogenase) (b) Individual data points from Experiment D-3, corresponding to different temperatures, are plotted as a function of $\alpha(H_2O/H_2)$. 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 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 H_2/CO_2 experiments (Experiment D) may help to explain a long-standing discrepancy between the δD-CH_4 in biogenic natural gas reservoirs and the δD-CH_4 from terrestrial environments and ruminants. Biogenic natural gas (CH_4) tends to be depleted by only ~160‰ relative to source H_2O (Schoell, 1980), whereas CH_4 produced in wetlands and ruminants tends to be depleted by 300–400‰ relative to source H_2O (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 themophilic H_2/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 H_2/CO_2 methanogenesis in nature is controlled by ambient H_2 levels, and that high H_2 levels are correlated to deuterium-depleted CH_4. The proposed mechanism behind this hypothesis is the depletion of the intracellular H_2O pool by H_2 uptake under high 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 δD of CH_4 produced. These results further demonstrate large variations in δD-CH_4, at constant extra-cellular H_2 concentration.

4. CONCLUDING REMARKS

Methane produced from acetate by *M. thermophila* at elevated temperatures is fractionated only slightly in ^{13}C (~7‰) from the methyl position of acetate. Given the potential importance of thermophilic *Methanosaeta* in nature, biogenic CH_4 produced from acetate at elevated temperature may be easily mistaken for thermogenically derived CH_4 based on carbon isotopes alone.

Methane produced by H_2/CO_2 methanogenesis at elevated temperature displays a broad range of carbon isotope fractionation (α) factors, ranging from 1.023 to 1.064. Low levels of 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 H_2/CO_2 methanogenesis is commonly referred to as a kinetic isotope effect. If the differential reversibility hypothesis holds true, the fractionation arising from H_2/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 δD of CH_4 derived from H_2/CO_2 at elevated temperature is highly variable, even within one species, with fractionation (α) 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 H_2O and 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 CH_4 produced at moderate temperatures.

Hydrogen isotopic fractionation between H_2O and H_2 occurs rapidly, on the order of seconds, in the presence of 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 H_2 molecules (reducing equivalents) taken up by *M. marburgensis* are converted to

CH_4, 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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