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Carbon isotopic fractionations associated with thermophilic bacteria *Thermotoga maritima* and *Persephonella marina*

Chuanlun L. Zhang,[1]* Qi Ye,[1] Anna-Louise Reysenbach,[2] Dorothee Götz,[2] Aaron Peacock,[3] David C. White,[3] Juske Horita,[4] David R. Cole,[4] Jon Fong,[5] Lisa Pratt,[5] Jiasong Fang[6] and Yongsong Huang[7]

[1]*Department of Geological Sciences, University of Missouri, Columbia, MO 65211, USA.*
[2]*Department of Biology, Portland State University, Portland, OR 97201, USA.*
[3]*Institute for Applied Microbiology, The University of Tennessee, Knoxville, TN 37932–2575, USA.*
[4]*Chemical and Analytical Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831–6110, USA.*
[5]*Department of Geological Sciences, Indiana University, Bloomington, IN 47405, USA.*
[6]*Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, MI 48109–2099, USA.*
[7]*Department of Geological Sciences, Brown University, Providence, RI 02912, USA.*

Summary

Stable carbon isotopes can provide insight into carbon cycling pathways in natural environments. We examined carbon isotope fractionations associated with a hyperthermophilic fermentative bacterium, *Thermotoga maritima,* and a thermophilic chemolithoautotrophic bacterium *Persephonella marina.* In *T. maritima*, phospholipid fatty acids (PLFA) are slightly enriched in ^{13}C relative to biomass (ε = 0.1–0.8‰). However, PLFA and biomass are depleted in ^{13}C relative to the substrate glucose by ~8‰. In *P. marina*, PLFA are 1.8–14.5‰ enriched in ^{13}C relative to biomass, which suggests that the reversed tricarboxylic acid (TCA) cycle or the 3-hydroxypropionate pathway may be used for CO_2 fixation. This is supported by small fractionation between biomass and CO_2 (ε = –3.8‰ to –5.0‰), which is similar to fractionations reported for other organisms using similar CO_2 fixation pathways. Identification of the exact pathway will require biochemical assay for specific enzymes associated with the reversed TCA cycle or the 3-hydroxypropionate pathway.

Received 14 August, 2001; revised 13 November, 2001; accepted 27 November, 2001. *For correspondence. E-mail zhangcl@missouri.edu; Tel. (+1) 573 884 2677; Fax (+1) 573 8825458.

Introduction

In situ microbial production is an important source of organic matter in hydrothermal vents (McCollom and Shock, 1997), yet the mechanisms of carbon cycling by vent microorganisms, especially by thermophiles, are poorly understood. Isotopic fractionations of elements such as carbon have traditionally been used to differentiate between biotic and abiotic processes because biologically mediated chemical reactions preferentially incorporate lighter isotopes (i.e. ^{12}C versus ^{13}C) into products. Little is known about isotopic fractionations associated with thermophiles in vent ecosystems (Karl, 1995; Des Marais, 1996). Limited carbon isotope data are available for deep-sea vent invertebrates that host mesophilic, chemolithoautotrophic symbionts (Cavanaugh *et al*., 1992; Jahnke *et al*., 1995; Kennicutt and Burke, 1995; Robinson *et al*., 1998). For example, low $\delta^{13}C$ values (–32‰ to –65‰) are associated with bivalves or mussel gills hosting methanotrophic bacteria, and values as high as –10‰ are associated with vestimentiferan worms hosting sulphur-oxidizing chemolithoautotrophic bacteria (Cavanaugh *et al*., 1992; Jahnke *et al*., 1995; Kennicutt and Burke, 1995).

Laboratory studies of carbon isotope fractionation by thermophiles can provide insight into the natural variation of carbon isotopes in hydrothermal environments. House *et al.* (1999) reported isotopic fractionations for a wide array of chemolithoautotrophs under laboratory conditions, some of which are hyperthermophiles growing at temperatures above 100°C. Most of these chemolithoautotrophs do not significantly fractionate carbon isotopes among different CO_2 fixation pathways except for methanogens, which partition carbon between biomass and methane product. Thus, it can be speculated that

Table 1. Fatty acid profiles of *Thermotoga maritima* at different growth phases[a].

	Mole percentage of total fatty acids		
Fatty acid	12 h	36 h[b]	60 h
14:0	1.5	5.2 ± 0.5	5.5
15:1	0.0	0.0 ± 0.0	0.2
15:0	0.0	0.6 ± 0.1	0.2
16:1	0.4	0.1 ± 0.0	0.1
16:0	87.3	77.3 ± 13.6	59.0
17:1ω8c	0.2	0.3 ± 0.0	0.1
i17:0	0.1	0.0 ± 0.0	0.0
a17:0	0.2	0.1 ± 0.0	0.1
17:0	0.6	0.2 ± 0.1	0.2
3OH16:0	0.0	0.7 ± 0.1	0.5
18:2ω6	0.7	0.1 ± 0.0	0.0
18:1ω9c	2.2	0.2 ± 0.0	0.0
18:1ω7c	0.6	0.1 ± 0.0	0.1
18:0	5.8	1.3 ± 0.3	1.6
20:0	0.1	0.0 ± 0.0	0.0
22:1ω9c	0.1	0.1 ± 0.0	0.1
unknown-1	0.0	3.8 ± 3.4	14.8
unknown-2	0.0	2.3 ± 1.6	9.9
unknown-3	0.0	0.0 ± 0.0	2.4
unknown-4	0.0	4.4 ± 5.8	3.0
unknown-5	0.0	3.1 ± 3.5	2.1
Total	100	100	100

a. Average cell counts (n = 2) were 1.2 ± 0.2 × 10^8 cells ml^{-1}, 4.9 ± 2.1 × 10^8 cells ml^{-1} and 2.3 ± 0.9 × 10^8 cells ml^{-1} at 12, 36 and 60 h respectively. These cell abundances corresponded to the early log phase, late log phase and early stationary phase based a detailed time-course experiment (data not shown). Average H_2 production (n = 2) was 0.44 ± 0.09 mmol l^{-1}, 0.51 ± 0.13 mmol l^{-1} and 1.04 mmol l^{-1} at 12, 36 and 60 h respectively. *T. maritima* converts 1 mol glucose into 2 mol acetate, 2 mol CO_2, about 0.5 mol H_2 and 3.5 mol H_2S in the presence of elemental sulphur (Schroder *et al.*, 1994). In this study, elemental sulphur was replaced with $Na_2S_2O_3$, which was also reduced to H_2S.
b. Mean ± one standard deviation (n = 2).

the common ancestor of all extant species, if indeed autotrophic thermophiles as suggested (Stetter, 1996; Barns and Nierzwick-Bauer, 1997; Reysenbach *et al.*, 2000a;b), may exhibit relatively low carbon isotope fractionation (House *et al.*, 1999). To test this, we have examined carbon isotope fractionations associated with two metabolically distinct thermophilic bacteria: organotrophic *Thermotoga maritima* and chemolithoautotrophic *Persephonella marina*. Results show that *T. maritima* has fractionations (>8‰) greater than aerobic organotrophs (Monson and Hayes, 1982; Blair *et al.*, 1985). *Persephonella marina* has small fractionation (≤5‰) between biomass and CO_2 during CO_2 fixation, which is consistent with the reversed tricarboxylic acid (TCA) cycle or the 3-hydroxypropionate pathway (Preuβ *et al.*, 1989; van der Meer *et al.*, 1998; 2001; C. H. House, personal communication). The carbon isotopic fractionations between lipid biomarkers and biomass are also distinct for *T. maritima* and *P. marina* as a result of the different biosynthetic pathways utilized by these bacteria.

Results and discussion

Fatty acids and isotopic compositions of T. maritima

Sixteen fatty acids in the range of C_{14}-C_{22} were identified. Five other fatty acids >C_{22} were observed but could not be identified (Table 1). The fatty acid profiles were compared at 12, 36 and 60 h incubations, which corresponded to early log growth phase, late log growth phase and early stationary phase respectively. The purpose was to determine whether fatty acid and isotopic compositions changed during different growth phases of *T. maritima*.

Among the known fatty acids, 16:0 was most abundant and decreased from 87.3% at 12 h to 59% at 60 h. Other known fatty acids were less than 6%. Notable changes are 18:0, which decreased from 5.8% at 12 h to 1.6% at 60 h, and 14:0, which increased from 1.5% at 12 h to 5.5% at 60 h (Table 1). The five unidentified fatty acids were produced after 36 h; the unidentified peaks 1 and 2 increased by fourfold at 60 h (Table 1). Increases in 14:0 and the unidentified peaks may be at the expense of 16:0 and 18:0 because abundances of the latter compounds decreased with time (Table 1).

Fatty acids of *T. maritima* have been reported by Huber *et al.* (1986), De Rosa *et al.* (1988), and Carballeira *et al.* (1997); the latter two studies also identified 16:0, 14:0, and 18:0 (in decreasing order) as major fatty acids. In addition, De Rosa and colleagues (De Rosa *et al.*, 1988) identified a novel 15,16-dimethyl-30-glyceryloxytriacontanoic acid (30% of CH_3Cl-soluble fraction) and Caraballeira and colleagues (Carballeira *et al.*, 1997) identified a novel 13,14-dimethyloctacosanedioic acid (0.6% of total fatty acids) and a novel 15, 16-dimethyltriacontanedioic acid (2.9% of total fatty acids). The unidentified peaks in this study may belong to some of these unique fatty acids. However, this needs to be confirmed by comparison with standards of these unusual compounds.

Carbon isotope compositions of biomass and fatty acids (represented by 16:0) were similar and changed little during the growth of *T. maritima* (Table 2). The $\delta^{13}C$

Table 2. Carbon isotopic ratios of biomass and fatty acid 16:0 at different growing stages of *T. maritima* (80°C).

	$\delta^{13}C$ (‰)			
Time (h)	Biomass[a]	16:0	ε^1	ε^2
12	−18.6 ± 0.06	−17.8	−8.4	−7.6
36	−17.6 ± 0.65	−17.5	−7.4	−7.3
60	−17.6 ± 0.59	−16.9	−7.4	−6.7

The substrate glucose (22 mM) has an average $\delta^{13}C$ of −10.3 ± 0.2‰ (n = 3). The fractionation factor (ε) between substances A and B is $\varepsilon = (1000 + \delta^{13}C_A)/[(1000 + \delta^{13}C_B) - 1] \times 1000$.
[1]Carbon isotope fractionation between biomass and glucose.
[2]Carbon isotope fractionation between fatty acid and glucose.
a. Mean ± one SD (n = 2–4).

Table 3. Carbon isotope fractionation (ε) between biomass and substrate for aerobic heterotrophic bacteria.

Bacteria	Temp. (°C)	Substrate	$\delta^{13}C_{substrate}$ (‰)	$\delta^{13}C_{biomass}$ (‰)	ε (‰)	References
Escherichia coli	25	glucose	–10.0	–9.7	0.3	Monson and Hayes (1982)
Escherichia coli	25	glucose	–9.0	–9.6	–0.6	Blair et al. (1985)
Escherichia coli	25	glucose	–10.2	–11.9	–1.7	Zhang et al., unpublished data
Pseudomonas aeruginosa	37	glucose	–13.3	–10.3	3.0	Coffin *et al.* (1990)
Pseudomonas aeruginosa	37	glutamate	–27.4	–25.4	2.1	Coffin *et al.* (1990)
Shewanella putrefaciens	25	lactate	–27.8	–26.5	1.3	Teece *et al.* (1999)

ε (‰) = $(1000 + \delta^{13}C_{biomass})/[1000 + \delta^{13}C_{substrate}) - 1] \times 1000$.

values of some of the unidentified fatty acids (36 and 60 h) were close to that of 16:0 (data not shown). Other fatty acids were too low in abundance to be measured isotopically under the same conditions as 16:0 was measured. The results were consistent with other observations that isotope ratios of biomass or lipids during heterotrophic growth remain relatively constant (Blair *et al*., 1985; Teece *et al*., 1999).

Thermotoga maritima uses the Embden–Meyerhof pathway for glucose fermentation with acetate, H_2 and CO_2 being major by-products (Schroder *et al*., 1994). Isotopic analysis of the metabolic products of *Escherichia coli* indicates that acetate is significantly enriched in ^{13}C relative to substrate and all other metabolic products (Blair *et al*., 1985). This suggests that depletion in ^{13}C of cellular materials may be balanced by enrichment in ^{13}C of the acetate by-product. Compared with *E. coli* and other heterotrophs grown aerobically (Table 3), *T. maritima* resulted in greater depletion in ^{13}C (Table 2). This may be explained by greater production of ^{13}C-enriched acetate during glucose fermentation than during aerobic glucose oxidation (N. Blair, personal communication). This speculation, however, needs to be validated by actual measurement of the $\delta^{13}C$ of acetate during glucose fermentation. Another cause for this observation may be temperature. DeNiro and Epstein (DeNiro and Epstein, 1977) observed that increases in temperature resulted in greater fractionation between acetaldehyde and substrate ($\varepsilon = -0.19 \times T(°C) - 3.32$, calculated using data in table 2 of DeNiro and Epstein, 1977). This equation, however, gives a fractionation of –19.5‰ between fatty acids and glucose for *T. maritima* grown at 85°C, which is greater than the measured fractionations (–7.2 ± 0.5‰, $n = 3$) (Table 2). No other studies have examined temperature effect on the kinetic isotopic effect during the oxidation of pyruvate to acetyl Co-A. It is uncertain whether and how temperature may affect carbon isotopic fractionation during lipid biosynthesis.

Fatty acids and isotopic compositions of P. marina

Fatty acid compositions of *P. marina* were dominated by 18:1ω9c/t, 18:0, and 20:1ω9t (Table 4). These fatty acids are similar to those observed in *Hydrogenobacter thermophilus*, a chemolithoautotrophic, hydrogen-oxidizing bacterium isolated from hot springs located in Izu and Kyushu, Japan (Kawasumi *et al*., 1984), and *Desulphurobacterium thermolithotrophum*, a chemolithoautotrophic, sulphur-reducing bacterium isolated from a deep-sea hydrothermal vent (L'Haridon *et al*., 1998). The predominance of the C_{18}-C_{20} fatty acids appears to be unique to these thermophilic, chemolithoautotrophic bacteria (Kawasumi *et al*., 1984).

Whole cells of *P. marina* were depleted in ^{13}C by about 5‰ relative to the substrate CO_2 (Table 4). All chemolithoautotrophs discriminate against ^{13}C during CO_2 assimilation; however, the magnitude of discrimination varies among different CO_2 fixation pathways. Four CO_2 assimilation pathways are known: the Calvin cycle, the reductive acetyl-CoA pathway, the reversed TCA cycle, and the 3-hydroxypropionate pathway (Preuβ *et al*., 1989; van der Meer *et al*., 1998; 2000). Among these pathways, the reversed TCA cycle and the 3-hydroxypropionate pathway produce small and similar ranges of fractionation between biomass and CO_2 (Table 5). Small fractionations

Table 4. Isotopic ratios of fatty acids, biomass and CO_2 in cultures of *P. marina* grown at 70°C.

Fatty acid	Mole (%)	$\delta^{13}C$ (‰)	ε^a
16:1ω7c	0.3 ± 0.0		
16:0	0.8 ± 0.1	–43.38	2.3
17:0	0.2 ± 0.0		
18:1ω9c/t[b]	26.9 ± 0.6	–41.7 ± 0.2	4.1
18:0	23.2 ± 0.1	–43.9 ± 0.7	1.8
19:1ω12c	0.1 ± 0.1		
20:1ω9t	47.7 ± 0.3	–42.7 ± 0.5	3.0
20:0	0.9 ± 0.1		
Biomass		–45.6 ± 0.1	–5.0[c]
Head-gas CO_2		–40.8 ± 0.2	

Headspace CO_2 in control experiments had an average $\delta^{13}C$ of –38.9 ± 0.0‰ ($n = 2$). The ± indicates one standard deviation of the mean ($n = 2$–4).

a. $\varepsilon = (1000 + \delta^{13}C_{fatty\ acid})/[(1000 + \delta^{13}C_{biomass}) - 1] \times 1000$.

b. 18:1ω9c and 18:1ω9t could not be baseline resolved and were combined as 18:1ω9c/t.

c. Fractionation between biomass and Headspace CO_2.

Table 5. Carbon isotope fractionation between biomass and CO_2 for autotrophic bacteria.

Bacteria	Temp. (°C)	$\delta^{13}C_{CO2}$ (‰)	$\delta^{13}C_{biomass}$ (‰)	(‰)	References
Calvin cycle					
Alkaligenes eutrophus	28	–49.5	–75.6	–27.5	Preuβ *et al.* (1989)
Chromatium vinosum	30	–13.0	–32.6	–19.8	Quandt *et al.* (1977)
Rhodospirilum rubrum	30	–11.3	–23.6	–12.5	Quandt *et al.* (1977)
Rhodopseudomonas capsulata	30	–11.3	–21.9	–10.7	Quandt *et al.* (1977)
Chromatium				–22.5	Sirevag *et al.* (1977)
Rhodospirilum rubrum				–20.5	Sirevag *et al.* (1977)
Chromatium tepidum	50			–20.5	Madigan *et al.* (1989)
Reductive acetyl CoA					
Desulfobacterium autotrophicum	28	–49.5	–85.4	–37.8	Preuβ *et al.* (1989)
Acetabacterium woodii	28	–49.8	–70.4	–21.7	Preuβ *et al.* (1989)
Acetabacterium woodii	28	–49.5	–76.3	–28.2	Preuβ *et al.* (1989)
Acetabacterium woodii	28	–47.6	–62.4	–15.5	Preuβ *et al.* (1989)
Desulfobacter hydrogenophis	28	–49.8	–59.2	–9.9	Preuβ *et al.* (1989)
Desulfobacter hydrogenophis	28	–49.5	–57.9	–8.8	Preuβ *et al.* (1989)
Desulfobacter hydrogenophis	28	–47.6	–60.9	–14.0	Preuβ *et al.* (1989)
Reversed TCA cycle					
Thermoproteus neutrophilus	85	–47.6	–55.8	–8.6	Preuβ *et al.* (1989)
Chlorobium phaeobibrioides	30	–13.0	–16.5	–3.5	Quandt *et al.* (1977)
Chlorobium phaeobibrioides	30	–13.0	–15.5	–2.5	Quandt *et al.* (1977)
Chlorobium phaeobibrioides	30	–13.0	–15.6	–2.6	Quandt *et al.* (1977)
Chlorobium limicola	30	–13.0	–18.2	–5.3	Quandt *et al.* (1977)
Chlorobium vibrioforme	30	–12.9	–16.8	–4.0	Quandt *et al.* (1977)
Chlorobium vibrioforme	30	–12.9	–16.7	–3.9	Quandt *et al.* (1977)
Chlorobium limicola				–12.2	Sirevag *et al.* (1977)
3-hydroxypropionate					
Chloroflexus aurantiacus				–13.7[a]	van der Meer *et al.* (2000)
Chloroflexus aurantiacus	55	–41.	–48.9	–7.6	van der Meer *et al.* (2001)
Not determined					
P. marina	70	–36.2	–39.9	–3.8	This study
P. marina	70	–40.8	–45.6	–5.0	This study

ε(‰) = (1000 + $\delta^{13}C_{biomass}$)/[1000 + δ^{13}CCO2) − 1] × 1000.
van der Meer and colleagues (van der Meer *et al.*, 1998) studied isotopic fractionations between biomass and lipids associated with the reversed TCA cycle utilized by *Chlorobium limicola* and *Thiocapsa roseopersicina*. However, fractionations between biomass and CO_2 were not reported.
a. Biomass was reported to be ~14‰ lighter than the inorganic carbon (possibly HCO_3^-) from which it was formed.

(2.7–8‰) have also been observed for the reductive acetyl-CoA pathway in some hyperthermophilic bacteria (C. H. House, personal communication). Isotopic behaviour of *P. marina* is consistent with these pathways (Table 5). Further identification of the exact pathway of *P. marina* metabolism will require isolation of enzymes mediating CO_2 fixation.

Fractionation between fatty acids and total biomass in *P. marina* supports the use of the reversed TCA cycle. Green and purple sulphur bacteria using the reversed TCA cycle for CO_2 fixation produce lipids that are 2–16‰ enriched in ^{13}C relative to biomass (van der Meer *et al.*, 1998). This is consistent with the use of the reversed TCA cycle for the biosynthesis of polysaccarides via acetyl-CoA, pyruvate, and phosphoenolpyruvate (Evans *et al.*, 1966), which become increasingly depleted in ^{13}C along the carbon flow (van der Meer *et al.*, 1998). As a result, straight-chain fatty acids from acetyl-CoA can be enriched in ^{13}C compared with the total biomass, which is the sum of cellular materials synthesized from acetyl-CoA, pyruvate, and phosphoenolpyruvate (van der Meer *et al.*, 1998). Fatty acids of *P. marina* are 1.8–4.1‰ enriched in ^{13}C relative to biomass (Table 4), consistent with fractionations of the reversed TCA cycle. Fractionation between fatty acids and biomass associated with the 3-hydroxypropionate pathway is reported for a culture of *Chloroflexus aurantiacus*, which ranges from 0.21‰ to –1.89‰ (van der Meer *et al.*, 2001).

Implication for microbial interactions

Production of H_2 and CO_2 in addition to organic acids is a common feature of fermentative microorganisms. Studies show that such fermentors grow better when they are in syntrophy with hydrogen-utilizing species such as autotrophic methanogens (Bonch-Osmolovskaya and Stetter, 1991; Muralidharan *et al.*, 1997). For example, biomass of *T. maritima* increased 10-fold when co-cultured with *Methanococcus jannaschii* (Muralidharan *et al.*, 1997). This is as a result of the removal of H_2, which

inhibits the growth of *T. maritima* at high concentrations. Syntrophy may be an important mechanism for microbial coexistence and co-evolution. For example, thermophilic sulphur-reducing heterotrophs are able to grow at the expense of organic matter such as protein and carbohydrates in the absence of S° (Bonch-Osmolovskaya and Stetter, 1991). Because heterotrophs and chemolithoautotrophs use different biosynthesis pathways, stable carbon isotopes may be a powerful tool for elucidating the syntrophic activities in such environments. Extensive research needs to be performed to document the isotopic signatures of both heterotrophs and chemolithoautotrophs grown in co-cultures. The expected results may shed light on the microbial co-evolution and biogeochemical dynamics in hydrothermal systems.

Experimental procedures

Sources of bacteria and growth conditions

Thermotoga maritima was isolated from geothermally heated marine sediments (Huber *et al*., 1986). It is a strictly anaerobic and fermentative hyperthermophile and grows on various sugars or complex organic substrate at 55–90°C (optimal at 85°C) and pH between 6 and 9 (optimal at 7). *Persephonella marina* was isolated from a hydrothermal vent at 9°N on the East Pacific Rise (Reysenbach *et al*., 2000b). The isolate is a hydrogen-oxidizing, microaerophilic chemolithoautotroph and grows optimally at pH 6 and 70°C. It can also grow anaerobically using nitrate as the sole electron acceptor.

The medium for growing *T. maritima* (modified from Huber *et al*., 1986) contained (in g l^{-1} distilled water): NaCl (20.0), NH_4Cl (1.1), KCl (2.0), $MgSO_4$ (1.7), $MgCl_2$ (1.4), $CaCl_2$ (0.1), $Na_2S_2O_3$ (1.6), glucose (4.0), yeast extract (0.5), and an organic buffer 1,4 piperazinediethanesulphonic acid (6.7), trace minerals (10 ml) and vitamins (1 ml). The medium was transferred into 160 ml serum bottles in 50 ml aliquots or into 1 l bottles in 500 ml aliquots. N_2 was used to strip inorganic CO_2 from the medium and used as the head gas during sterilization (120°C for 20 min). Final pH was adjusted to 7.0. A strict anaerobic procedure (Zhang *et al*., 1996) was followed during all stages of experimentation.

The medium for growing *P. marina* consisted of MH (333 ml) and MS (666 ml) buffer solutions. The MH buffer contained (in g l^{-1} distilled water): NaCl (87.8), NaOH (6.0), KCl (1.5), and $MgCl_2 \cdot 6H_2O$ (5.1). The MS buffer was a 0.1 M NaOH solution. $MgSO_4$ (7.0 g), $Na_2S_2O_3$ (2.0 g), $CaCl_2 \cdot 2H_2O$ (0.4 g), solution A (2.0 ml), solution B (1.5 ml), and solution D (10.0 ml) were added sequentially to the medium. Solution A (pH 4 with HCl) contained (in g l^{-1} distilled water): NH_4Cl (100), $MgCl_2 \cdot 6H_2O$ (100), and $CaCl_2 \cdot 2H_2O$ (40). Solution B was a 0.9 M $K_2HPO_4 \cdot 3H_2O$. Solution D was a trace element solution prepared according to a recipe listed in http://methanogens.pdx.edu. The medium was bubbled with CO_2 for approximately 20 min before dispensing into 1 l bottles and autoclaved at 120°C for 20 min. Nitrate (20 mM) was used as the electron acceptor and H_2 was used as the electron donor.

A 10% inoculum from a freshly grown culture was used. *Thermotoga maritima* was grown at 85°C and *P. marina* at 70°C. Incubation was conducted in the dark without shaking. Control experiments were performed using the growth media without inocula.

For *T. maritima*, six identical bottles in three pairs were used to examine isotopic variation as a function of time. Gas and liquid samples were collected at 12, 36 and 60 h using each pair of bottles respectively. Two additional experiments (each using duplicate bottles) were performed at 36 and 60 h as replicates. For *P. marina*, samples were collected when growth reached the stationary phase (about 24 h).

In all cases, gas samples were collected using a needle and syringe and injected into 160 ml vacuum containers for chemical and isotopic analysis of H_2 and CO_2. About 1 ml of the liquid culture was fixed with 2.5% gluteraldehyde and saved for cell counts. The rest of liquid culture was centrifuged at 18 000 *g* for 10 min to collect biomass. About 20% of the biomass was used for isotopic measurement of whole-cell carbon, wherein each sample was treated with 1.0 N HCl for 2 h followed by a distilled-water wash and a centrifugation step to remove any inorganic carbon. The remaining biomass was used for lipid extraction and carbon isotope analysis of derivatized fatty acids.

Gas measurement and cell counts

Concentration of headspace H_2 was measured on a Varian Aerograph Model 90-P gas chromatograph using a Series 550P thermoconductivity detector. Bacterial cell numbers were enumerated using an epifluorescence light microscope (Zhang *et al*., 1996).

Extraction of cellular lipids

Extraction of cellular lipids followed the procedure of Zhang and colleagues (Zhang *et al*., 2002). Freeze-dried biomass was extracted by a single-phase organic solvent system (White *et al*., 1979). The organic (lipid-containing) phase was collected and fractionated on a silicic acid column into neutral lipids, glycolipids and polar lipids (Guckert *et al*., 1985). The polar phospholipid fatty acids were treated using a mild alkaline methanolysis to produce fatty acid methyl esters (FAME), which were analysed by gas chromatography/mass spectrometry (Zhang *et al*., 2002).

Carbon isotopic analyses

Carbon isotope compositions of the FAME were determined on a Finnigan MAT 252 (Zhang *et al*., 2002) or Delta Plus-XL mass spectrometer (Finnigan MAT). The Finnigan MAT 252 was interfaced with a HP 5890A gas chromatograph (Hewlett Packard) (Hayes *et al*., 1990; Merritt *et al*., 1994; Ricci *et al*., 1994). The Finnigan MAT Delta Plus–XL was interfaced with a HP6890 gas chromatograph. Measured isotopic ratios of the methylated fatty acids were corrected for the methyl moiety (Abrajano *et al*., 1994) using the following equation:

$$\delta^{13}C_{FA}(‰) = [(C_n + 1) \times \delta^{13}C_{FAME} - \delta^{13}C_{MeOH}]/C_n$$

where $\delta^{13}C_{FA}$ is the $\delta^{13}C$ of the fatty acid, C_n is the number of carbons in the fatty acid, $\delta^{13}C_{FAME}$ is the $\delta^{13}C$ of the methylated fatty acid, and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C$ of the methanol used for the methylation reaction. Precision of an internal fatty acid standard (19:0) was ±1.01‰ (n = 5) for the FAME. Carbon isotope ratios of total biomass and CO_2 were determined according to Zhang and colleagues (Zhang *et al*., 2002). Precision of an organic standard (acetanilide) was ±0.03‰ (n = 3). All carbon isotope values were reported against the Vienna Peedee belemnite (PDB) standard.

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