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Carbon isotopic fractionation by Archaeans and other thermophilic prokaryotes

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Abstract

This study of carbon isotopic fractionation in a wide array of 21 phylogenetically diverse microbial species provides an opportunity to correlate carbon isotopic fractionations with a biochemical pathway. These carbon isotopic fractionation experiments included two members of the Aquificales and two members of the Thermoproteales using the reductive TCA cycle, three members of the Sulfolobales using the 3-hydroxypropionate cycle, as well as three Archaeoglobales and seven methanogens using the acetyl-CoA pathway. In these experiments, microorganisms using the reductive tricarboxylic acid cycle (with ε values between 2.0 and 5.5‰) and the 3-hydroxypropionate cycle (with ε values between 0.2 and 3.6‰) demonstrated significantly less carbon isotopic fractionation than methanogens using the acetyl-CoA pathway. The results reported here for the acetyl-CoA pathway-utilizing microbes, however, vary over a remarkably wide range with ε values of 2.7 to 8.0‰ for the Archaeoglobales and ε values of 4.8 to 26.7‰ for the methanogens. The magnitude of carbon isotopic fractionation observed in species of *Methanococcus* were related to the particular growth status that had been attained by the various cultures, with increasing isotopic fractionation as growth proceeded.

1. Introduction

Because autotrophic microorganisms preferentially incorporate $^{12}CO_2$ into their biomass, specific carbon fixation pathways may each leave an isotopic signature in the geologic record of ^{13}C-depleted sedimentary organic matter. Among the Bacteria and the Archaea, the two prokaryotic domains of life, there are four known carbon-fixation pathways: (1) the reductive tricarboxylic acid (TCA) cycle; (2) the 3-hydroxypropionate cycle; (3) the reductive pentose phosphate cycle (Calvin cycle); and (4) the reductive acetyl-CoA pathway. Preuß and co-workers (1989) have shown in diverse prokaryotes that three of the four autotrophic pathways exhibit systematic differences in their degree of carbon isotopic discrimination during carbon fixation. Their observations, along with other published studies of carbon isotopic fractionation to biomass, indicate that the reductive TCA cycle and 3-hydroxypropionate cycle exhibit lower fractionations than do the pentose-phosphate cycle and the acetyl-CoA pathway.

In this paper, we report new data on the magnitude of carbon isotopic fractionation from a diverse set of 21 chemolithoautotrophic Archaea and Bacteria. These data allow for the comparisons of carbon isotopic fractionation between the four known carbon fixation biochemical

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pathways and reveal a previously unreported growth-dependent effect for carbon isotopic fractionation to the biomass of methanogens.

2. Experimental methods

2.1. General procedure

Unless otherwise specified, growth experiments were conducted in 575 mL glass bottles under autotrophic conditions in an atmosphere of 300 kPa, of which 20% was CO_2. Approximately 0.5 ml of preculture were used to inoculate each experiment. For methanogens, 50 ml of medium were used; for all other microbes, 75 ml of medium were used. Less medium was used for the methanogens for two reasons: first, methanogens grow rapidly to large cell densities; and second, a larger headspace was desired in order to avoid large corrections due to isotopic distillation of inorganic carbon in each culture bottle. Care was taken to stop growth before $\sim$10% of the inorganic carbon was consumed with the exception of three experiments grown until 10 – 20% of the inorganic carbon was consumed. These later experiments were grown longer to more fully document the fractionation trend observed as a function of growth. All cultures (except that of *Pyrodictium occultum*) were shaken continuously at 150 rpm during incubation.

Precultures were grown in 20 ml of medium under autotrophic conditions in 120-ml serum bottles and were transferred several times before inoculation of the experiment (with the exception of *Thiobacillus novellus*, which was transferred from a preculture containing 0.03% yeast extract). Thus, for each experiment, the inoculum used had a cell density similar to that of the grown culture and was grown using the same CO_2 and the same media, reducing the impact of the inoculum on the isotopic composition of the grown culture. Further, $\delta^{13}C$ measurements on several cultures of *Methanococcus thermolithotrophicus* grown in 120-ml serum bottles confirmed that the $\delta^{13}C$ of the cells from the precultures was similar to that obtained in the growth experiments.

2.2. Experiment set-up

One l of the appropriate growth medium was prepared without adding sodium bicarbonate. Instead, the required amount of 2.5 N NaOH was added to the medium to make each solution alkaline, which was later neutralized by dissolved CO_2 from the gas mix during pressurization (and thus acting as a buffer during microbial growth). If being prepared for growth under anaerobic conditions, the solution was first degassed with N_2 for 5–10 min after which 0.5 g $Na_2S{\cdot}H_2O$/l was added to maintain reducing conditions. The prepared solutions were dispensed into culture bottles (in a glove box for anaerobic solutions). Either 50 or 75 ml of medium was added to the 575 ml glass bottles. All bottles were sealed with rubber stoppers. The bottles were then flushed three times with N_2 gas, evacuated, and then pressurized with gas mixture (80% N_2/20% CO_2 or 80% H_2/20% CO_2), which had been passed over a 250 °C Cu column to remove O_2. For experiments carried out under aerobic conditions, oxygen was then injected by syringe. After the bottles were filled and pressurized and the pH of the solution was checked with pH indicator paper, the bottles were sterilized at 120 °C for 20 min (unless the medium contained elemental sulfur, in which case they were sterilized at 100 °C for 1 h). The pH of each medium was again measured after sterilization. Later, experiments were inoculated and incubated either in New Brunswick water shakers (for temperatures up to 65 °C) or in large dry incubators equipped with shaking platforms (65–105 °C). Incubation times for non-methanogens allowed the cultures to be harvested during late log phase or early stationary phase. Incubation times for the methanogens were originally set to stop growth before $\sim$10% of the inorganic carbon was consumed. Later *Methanococcus* experiments were grown longer to more fully document the fractionation trend observed as a function of growth. After incubation, the bottles were allowed to cool to room temperature overnight. Gases in the headspaces were then sampled by use of a double-headed needle to draw gas into an evacuated bottle. The dry gas samples were sterilized at 120 °C for 30 min to ensure that microbial processes, including methanogenesis, did not alter the gas composition during the time between sampling and analysis. The cells were isolated by centrifugation (under vacuum) and then dried under vacuum. Selected cell samples were acidified by adding 1 ml of 0.1 N HCl to the cell pellet and then dried under vacuum. This was done, in part, to remove carbonate minerals that may have precipitated during growth, although the results do not suggest that significant carbonate precipitation occurred.

2.3. Elemental, isotopic, and cell density analyses

Elemental and isotopic analyses of gas and isolated cells were carried out by Global Geochemistry Corporation (now Zymax Forensics). Parameters measured included the carbon isotopic composition of CO_2, CH_4, and the cultured biomass, as well as the relative percentages of H_2 and CO_2 in the headspace gas mixtures. Cell pellets for $\delta^{13}C$ analysis were dried under vacuum before shipping. $\delta^{13}C$ analysis was performed by standard gas source isotope ratio mass spectrometry on the CO_2 cryogenically purified from the combustion (850 °C) of the biomass in the presence of cupric oxide in evacuated

quartz tubes. The analytical error for $\delta^{13}C$ analysis was $\leqslant 0.12‰$ (2σ, based on the repeated analysis of the NBS-22 oil standard) and the analytical error for the gas compositional analyses was $<1\%$. The analyses of cell culture densities were done by visual cell counts using either phase-contrast microscopy alone or using phase-contrast microscopy coupled with a cell counting chamber. The reproducibility of these measurements was $\pm 10^6$ cells per ml for cultures with cell densities below 10^7 cells per ml and $\pm 5x10^6$ cells per ml for cultures with cell densities above 10^7 cells per ml.

2.4. Data reduction and calculations

Carbon isotopic fractionation from CO_2 to cell biomass was estimated from the measured data. Because a significant portion of available inorganic carbon was metabolically consumed in each experiment in which methanogenesis occurred, the calculations used to estimate such fractionations for methane-producing microbes necessarily differed from those for non-methanogenic prokaryotes. Although some species of *Archaeoglobus* produce a small amount of methane during growth, the concentration was too low for our detection and analysis, so both species of *Archaeoglobus* have been treated as non-methanogens.

Because many of the experiments were incubated at temperatures greater than 25 °C, the fractionation values were calculated from an estimation of the initial $\delta^{13}C$ of the dissolved inorganic carbon and of the CO_2 at the temperature of growth using measured parameters, data on the solubility of CO_2 (Weiss, 1974), the carbonic acid dissociation constants (Goyet and Poisson, 1989), and data on carbon isotopic fractionation during dissolution of CO_2 (Mook et al., 1974; Zhang et al., 1995). The steps used and the estimations required to calculate fractionation values are delineated in the next paragraph. The fractionation values calculated, however, are little affected ($<1‰$ for methanogens and $<0.5‰$ for non-methanogens) by the various assumptions and estimations used, and, therefore, the conclusions drawn from these results are not dependent on the data reduction. Table 1 shows the measured data, estimations of the initial $\delta^{13}C$ of the dissolved inorganic carbon and of the CO_2 at the temperature of growth, as well as the carbon isotopic fractionations in ‰ for the production of biomass and methane in these growth experiments.

For all experiments, measured parameters included incubation temperature, pH at 25 °C (before and after incubation), incubation time, the $\delta^{13}C$ of CO_2 after incubation, and $\delta^{13}C$ of biomass after incubation. First, from the pCO_2 of each bottle and the pH of each solution, the concentrations of dissolved CO_2, bicarbonate, and carbonate in each solution at 25 °C and the alkalinity of each solution was calculated using the carbonic acid dissociation constants (Goyet and Poisson, 1989). The $\delta^{13}C$ of the total carbon in the bottle was estimated based on the carbon isotopic composition of CO_2 gas in the bottle and the equilibrium fractionations between dissolved inorganic phases (Mook et al., 1974; Zhang et al., 1995). These last two steps assume that equilibria are approximately maintained in the carbonate buffer system during the experiments in both the concentrations of inorganic phases and in the carbon isotopic composition of each phase. If these assumptions are not met, the calculated isotopic fractionations will be smaller in magnitude than their true values as carbon would be more limiting than the model predicts. Next, the concentrations of CO_2, bicarbonate, and carbonate at the incubation temperature were calculated based on the alkalinity, the pCO_2 for the closed bottle at its incubation temperature (Weiss, 1974), and the carbonic acid dissociation constants (Goyet and Poisson, 1989). Next, the equilibrium fractionations between dissolved inorganic carbon phases (Mook et al., 1974; Zhang et al., 1995) were used to estimate the carbon isotopic composition of CO_2, bicarbonate, and carbonate at the incubation temperature. Finally, because experiments for non-methanogens had a great excess of CO_2 relative to the amount of carbon converted to cell biomass, the carbon isotopic fractionation factor (α) for these experiments was determined without considering isotopic distillation using the $\delta^{13}C$ of the CO_2 at the incubation temperature and the $\delta^{13}C$ of the cells after growth. The equation for this calculation is shown as a note in Table 1.

Experiments on methanogens required the additional measurements of the $\delta^{13}C$ of methane after incubation and the concentration of methane, CO_2, and H_2 after growth (from which the fraction of inorganic carbon converted to methane was deduced). In addition to the calculations required for the non-methanogens, the experiments on methanogens required corrections for the isotopic distillation of the inorganic carbon reservoir. Because the amount of inorganic carbon converted to cell biomass is very small relative to the amount of inorganic carbon converted to methane, a standard isotopic distillation model (Melander and Saunders, 1980) was applied when calculating the isotopic fractionation during methanogenesis, while the fractionation to cell biomass was modeled as fractionation from an inorganic carbon reservoir continually changing in isotopic composition as a result of the distillation process. The equations for these models are shown as a note in Table 1.

3. Discussion

Fig. 1 shows the carbon isotopic fractionation from CO_2 to biomass by cultured microbes under conditions

Table 1
Results of new growth experiments estimating carbon-isotopic fractionation, ‰, for a diverse set of prokaryotes. The carbon fixation pathways are: the reductive TCA cycle (TCA), the 3-hydroxypropionate cycle (3-HP), the reductive pentose phosphate cycle (PP), and the acetyl-CoA pathway (AP)

Exp. #	Taxon (suspected pathway)	T_{growth} (°C)	pH at 25 °C	Initial atmosphere	Time (h)	Final cell density (cells/ml)	$\delta^{13}C_{CO_2}$ at 25 °C (after growth)	$\delta^{13}C_{CH_4}$ (after growth)	Initial $\delta^{13}C_{CO_2}$ gas (at T growth)	Initial $\delta^{13}C_{DIC}$ (at T growth)	$\delta^{13}C_{cells}$ (after growth)	F (fraction of CO_2 converted to CH_4)	ε (‰) from CO_2 to CH_4 (1000 in α)	ε (‰) from CO_2 to cells (1000 in α)
KC4	*Ammonifex degensii* (?)	70	6.3	300 kPa (80%H2/20%CO_2)	48	2.50E+07	−26.2	N/A	−25.7	−22.9	−29.7	N/A	N/A	4.1
KC4*	*A. degensii*, HCl treated	*	*	*	*	*	*	N/A	*	*	−29.8	N/A	N/A	4.2
VF5	*Aquifex aeolicus* (TCA)	85	7.0	300 kPa (80%H_2/20%CO_2 + 5.75 ml O_2)	10	5.0E+07	−26.8	N/A	−24.4	−21.9	−29.6	N/A	N/A	5.4
VF5*	*A. aeolicus*, HCl treated	*	*	*	*	*	*	N/A	*	*	−29.3	N/A	N/A	5.1
TK6	*Hydrogenobacter thermophilus* (TCA)	70	6.9	300 kPa (80%H_2/20%CO_2 + 20 ml O_2)	65	ND	−27.0	N/A	−25.4	−21.9	−30.7	N/A	N/A	5.5
JM2	*Pyrobaculum aerophilum* (TCA)	100	7.0	300 kPa (80%H_2/20%CO_2)	48	2.50E+06	−26.3	N/A	−23.3	−21.9	−26.2	N/A	N/A	2.9
TN	*Thermoproteus neutrophilus* (TCA)	85	6.0	300 kPa (80%H_2/20%CO_2)	26	2.80E+07	−26.1	N/A	−25.7	−24.2	−27.7	N/A	N/A	2.0
PL19	*Pyrodictium occultum* (TCA?)	102	5.5	300 kPa (80%H_2/20%CO_2)	21	ND	−26.2	N/A	−26.1	−26.0	−28.4	N/A	N/A	2.3
1A	*Pyrolobus fumarii* (TCA?)	105	5.5	300 kPa (80%H_2/20%CO_2)	21	3.00E+07	−26.7	N/A	−26.6	−26.5	−30.3	N/A	N/A	3.8
Acb	*Acidianus brierleyi* (3-H)	65	2.0	300 kPa (80%H_2/20%CO_2)	143	1.00E+07	−24.3	N/A	−24.4	−25.3	−27.9	N/A	N/A	3.6
TH2	*Metallosphaera sedula* (3-H)	65	2.0	300 kPa (80%H_2/20%CO_2 + 20 ml O_2)	40	5.00E+07	−25.8	N/A	−25.9	−26.9	−28.9	N/A	N/A	3.1
RON	*Sulfolobus solfataricus* RON 12 III (3-H)	85	2.0	300 kPa (80%H_2/20%CO_2 + 20 ml O_2)	120	2.50E+07	−25.8	N/A	−25.9	−26.8	−26.1	N/A	N/A	0.2
Tbn	*Thiobacillus novellus* (PP)	30	7.5	300 kPa (80%N_2/20%CO_2 + 115 ml O_2)	160	5.00E+07	−8.8	N/A	−8.4	−1.3	−13.5	N/A	N/A	5.1
VC16	*Archaeoglobus fulgidus* (AP)	85	6.3	300 kPa (80%H_2/20%CO_2)	40	1.60E+07	−26.1	N/A	−25.4	−23.5	−28.1	N/A	N/A	2.7
VC162*	*A. fulgidus*, HCl treated	85	6.3	200 kPa (80%H_2/20%CO_2)	40	7.5E+06	−25.4	N/A	−24.7	−22.8	−30.4	N/A	N/A	5.8
TF2	*A. lithotrophicus* (AP)	85	6.5	300 kPa (80%H_2/20%CO_2)	48	2.50E+07	−26.4	N/A	−25.4	−23.3	−33.2	N/A	N/A	8.0
AED	*Ferroglobus placidus* (AP)	85	7.0	300 kPa (80%H_2/20%CO_2)	48	7.50E+07	−27.3	N/A	−24.8	−22.4	−28.3	N/A	N/A	3.5
USA	*Methanobacterium thermoauto* (AP)	65	6.5	300 kPa (80%H_2/20%CO_2)	23	6.8E+07	−24.8	−49.4	−26.3	−23.2	−40.2	0.05	24.6	15.0
KoL5	*Methanococcus igneous* (AP)	85	6.3	300 kPa (80%H_2/20%CO_2)	4	8.80E+07	−22.8	−51.6	−25.1	−23.2	−43.8	0.06	28.4	20.2
MJ	*M. jannaschii* (AP)	85	6.3	300 kPa (80%H_2/20%CO_2)	6	1.00E+07	−25.7	−49.3	−25.5	−23.5	−31.3	0.01	25.0	6.2
SN11.5	*M. thermolithotrophicus* (AP)	65	6.3	150 kPa (80%H_2/20%CO_2)	4	6.80E+07	−23.0	−53.6	−27.0	−23.9	−47.6	0.09	29.0	22.7
SN12	*M. thermolithotrophicus* (AP)	65	6.3	200 kPa (80%H_2/20%CO_2)	4	6.00E+07	−23.1	−53.8	−26.4	−23.3	−50.2	0.07	29.6	25.8
SN12.5	*M. thermolithotrophicus* (AP)	65	6.3	250 kPa (80%H_2/20%CO_2)	4	7.50E+07	−24.0	−53.8	−26.3	−23.2	−51.2	0.05	29.5	26.7
SN141	*M. thermolithotrophicus* (AP)	41	6.3	300 kPa (80%H_2/20%CO_2)	6	2.50E+07	−26.3	−51.4	−26.2	−21.9	−33.3	0.00	26.2	7.4
SN151	*M. thermolithotrophicus* (AP)	51	6.3	300 kPa (80%H_2/20%CO_2)	4.5	2.50E+07	−26.0	−51.9	−26.0	−22.1	−38.9	0.01	27.0	13.5
SN160	*M. thermolithotrophicus* (AP)	60	6.3	300 kPa (80%H_2/20%CO_2)	4	3.00E+07	−25.8	−51.3	−26.1	−22.7	−44.9	0.02	26.4	19.7
SN165A	*M. thermolithotrophicus* (AP)	65	6.3	300 kPa (80%H_2/20%CO_2)	4	7.50E+07	−23.3	−54.1	−26.5	−23.4	−49.4	0.07	29.8	24.8
SN165A*	*M. thermolithotrophicus*, HCl treated	*	*	*	*	*	*	*	*	*	−44.2	*	*	19.4
SN165B	*M. thermolithotrophicus* (AP)	65	6.3	300 kPa (80%H_2/20%CO_2)	4	6.00E+07	−23.2	−53.6	−24.6	−21.5	−49.1	0.04	30.8	26.0
SN170	*M. thermolithotrophicus* (AP)	70	6.3	300 kPa (80%H_2/20%CO_2)	6	6.00E+07	−23.0	−52.2	−26.5	−23.7	−49.4	0.08	27.9	24.9
AV19A	*Methanopyrus kandleri* (AP)	100	6.5	300 kPa (80%H_2/20%CO_2)	12	7.50E+07	−22.9	−53.3	−25.7	−24.4	−44.1	0.08	30.0	20.3
AV19A*	*Methanopyrus kandleri*, HCl treated	*	*	*	*	*	*	*	*	*	−41.5	*	*	17.7
AV19B	*M. kandleri* (AP)	100	6.4	300 kPa (80%H_2/20%CO_2)	12	1.50E+07	−25.4	−51.7	−25.5	−24.3	−37.4	0.03	27.6	12.7
MS	*Methanosarcina barkeri* (AP)	37	6.5	300 kPa (80%H_2/20%CO_2)	80	ND	−25.7	−42.3	−25.7	−20.5	−44.5	0.01	17.2	19.5
MS*	*Methanosarcina barkeri*, HCl treated	*	*	*	*	*	*	*	*	*	−33.4	*	*	7.9
V24S	*Methanothermus fervidus* (AP)	85	6.5	300 kPa (80%H_2/20%CO_2)	12	2.50E+07	−24.9	−54.7	−26.0	−24.0	−38.1	0.05	30.6	13.1
99MJ.1*	*M. jannaschii*, HCl treated	85	6.3	300 kPa (80%H_2/20%CO_2)	7	7.0E+07	−22.0	−43.6	−28.0	−26.0	−43.6	0.16	17.7	17.7
99MJ.2*	*M. jannaschii*, HCl treated	85	6.3	300 kPa (80%H_2/20%CO_2)	4.5	1.0E+07	−26.0	−45.3	−26.7	−24.8	−36.7	0.04	19.6	10.7
99MJ.3*	*M. jannaschii*, HCl treated	85	6.3	300 kPa (80%H_2/20%CO_2)	6	2.0E+07	−25.1	−45.3	−26.9	−25.0	−39.6	0.06	19.6	13.7
99SN1.1*	*M. thermolithotrophicus*, HCl treated	65	6.3	300 kPa (80%H_2/20%CO_2)	10.5	5.0E+06	−26.0	−49.9	−26.2	−23.1	−34.4	0.02	24.9	8.7

(*continued on next page*)

Table 1 (*continued*)

Exp. #	Taxon (suspected pathway)	T_{growth} (°C)	pH at 25 °C	Initial atmosphere	Time (h)	Final cell density (cells/ml)	$\delta^{13}C_{CO_2}$ at 25 °C (after growth)	$\delta^{13}C_{CH_4}$ (after growth)	Initial $\delta^{13}C_{CO_2}$ gas (at T growth)	Initial $\delta^{13}C_{DIC}$ (at T growth)	$\delta^{13}C_{cells}$ (after growth)	F (fraction of CO_2 converted to CH_4)	ε (‰) from CO_2 to CH_4 (1000 in α)	ε (‰) from CO_2 to cells (1000 in α)
99SN1.2*	*M. thermolithotrophicus*, HCl treated	45	6.3	300 kPa (80%H_2/20%CO_2)	19	1.0E+08	−22.6	−47.0	−27.9	−23.7	−46.4	0.12	21.1	20.5
99SN1.3*	*M. thermolithotrophicus*, HCl treated	45	6.3	300 kPa (80%H_2/20%CO_2)	19	2.0E+07	−26.1	−48.2	−26.2	−21.9	−39.1	0.01	23.0	13.4
99SN1.4*	*M. thermolithotrophicus*, HCl treated	45	6.3	300 kPa (80%H_2/20%CO_2)	10	1.0E+06	−26.4	−42.1	−26.1	−21.9	−30.8	0.00	16.5	4.8
99SN1.5*	*M. thermolithotrophicus*, HCl treated	65	6.3	300 kPa (80%H_2/20%CO_2)	19	1.0E+08	−19.3	−47.8	−27.8	−24.7	−46.7	0.19	23.2	22.0

Notes = $\delta^{13}C = [(R_{sample} - R_{PDB})/R_{PDB}] * 1000$. α (the fractionation factor) is the rate of reaction for ^{12}C divided by the rate of reaction for ^{13}C. For non-methanogens, α was calculated as = $(\delta_A + 1000)/(\delta_B + 1000)$. For methane produced by methanogens, α was modeled as = $[\log(1-F)/\log(1-F*R_m/R)]$ and, for methanogen cells, α was modeled as = $[R_m*\log(1-F)]/[R_c*\log(1-F*R_m/R)]$ where F is the fraction of CO_2 converted to methane, R is the initial isotopic ratio of unreacted CO_2, R_m is the isotopic ratio of the methane produced, and R_c is the isotopic ratio of the methanogen cells. These equations are modified from Melander and Saunders (1980); see also House (1999). 99MJ experiments had methanogen vitamin solution added, all other experiments did not.

of elevated CO_2, as reported in published results (Fig. 1a), and as observed in this present study (Fig. 1b), ordered according to the carbon fixation pathway probably used. Fig. 1c shows a generalize summary of published and present results of carbon isotopic fractionation. Table 2 shows the published carbon isotopic fractionation results used to make Fig. 1a (Belyaev et al., 1983; Calder and Parker, 1973; Fuchs et al., 1979; Holo and Sirevåg, 1986; Jahnke et al., 2001; Mirkin and Ruby, 1991; Mizutani and Wada, 1982; Pardue et al., 1976; Popp et al., 1998; Preuß et al., 1989; Quandt et al., 1977; Ruby et al., 1987; Sirevåg et al., 1977; Wong et al., 1975). However, the published results were originally reported as fractionation either from CO_2 or from total dissolved inorganic carbon. At neutral pH and 25 °C, there would be about a 7‰ difference between the isotopic composition of CO_2 and that of total dissolved inorganic carbon. Because the data shown in Fig. 1a is based on fractionation from CO_2 to biomass, data originally reported as fractionation from total dissolved inorganic carbon have been reduced by 7‰ before inclusion in Fig. 1a, with the original data still preserved in Table 2.

Because the published carbon isotopic fractionation results are generally from well studied species or from species belonging to groups for which diversity in carbon fixation pathways is presently unknown (such as the Cyanobacteria), the assignment of carbon fixation pathways for taxa in Table 2 were based on the known carbon fixation biochemistry of closely related or identical microorganisms. The assignments of carbon fixation pathways for the organisms in this present study (Table 3) were based on reported enzymatic studies that detected carbon fixation activity, the published results of genome sequencing efforts, and/or published sequences for key carbon fixation genes. For *Pyrodictium* and *Pyrolobus*, the tenuous assignment of the reductive TCA cycle (noted with a "?") was based on their phylogenetic position in the Crenarchaeota (for which reductive TCA cycle-utilizing *Thermoproteus* is also a member), considered along with their significant physiological and genetic difference from the Sulfolobales, members of the Crenarchaeota that use the 3-hydroxypropionate cycle.

The differences in carbon isotopic fractionation between the four different carbon isotopic fixation pathways are apparent in the published data shown in Fig. 1a. Of the four autotrophic pathways, the published data show the reductive TCA cycle results in the least isotopic discrimination (~2–13‰), while carbon isotope fractionation by the 3-hydroxypropionate cycle (exhibited by *Chloroflexus* and represented by only a single measurement) is also low at 6.7‰ (Sirevåg et al., 1977).

The published data also show that microorganisms utilizing the pentose phosphate cycle and CO_2-fixation by ribulose 1,5-bisphosphate carboxylase oxygenase

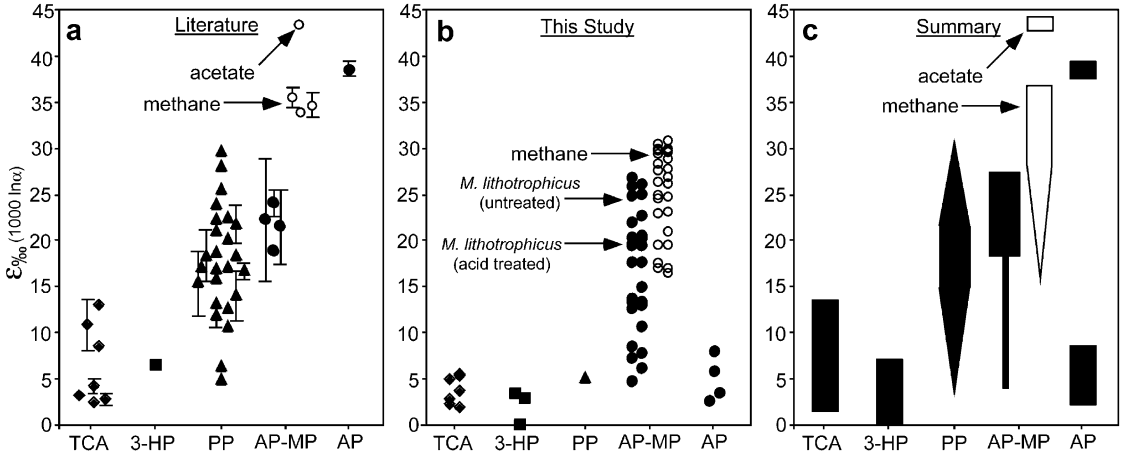

Fig. 1. Carbon-isotopic fractionation from carbon dioxide to biomass by prokaryotes grouped by their known or suspected carbon-fixation pathway based on: (a) published results of laboratory growth experiments, see Table 2, and (b) the results of the present study. (c) A generalized summary of published and present results of carbon isotopic fractionation. The carbon fixation pathways are: the reductive TCA cycle (TCA), the 3-hydroxypropionate cycle (3-HP), the reductive pentose phosphate cycle (PP), and the acetyl-CoA pathway in species that form a metablic product from carbon dioxide such as methane or acetate (AP–MP), or in species that do not (AP). Analyses of the same genus reported in the same publication (under similar conditions) have been averaged with 1 SD shown. Isotopic compositions of cell masses are shown as solid symbols. Isotopic compositions of organic metabolic products produced from CO_2 during cultivation (acetate and methane) are shown as open symbols. The changing carbon isotopic fractionation observed for methanogens (listed in AP–MP) is shown in (c) as a thin soild line extending up to the values expected for a culture at stationary growth.

(RuBisCO) generally demonstrate greater fractionation than those having the reductive TCA or 3-hydroxypropionate cycles. The mean carbon isotopic fractionation by this pathway is 18‰ (Fig. 1a). In vivo studies of RuBisCO Forms I and II place the maximum fractionation (verses CO_2) by RuBisCO Form I (isolated from spinach) at about 29‰ and the maximum fractionation of RuBisCO Form II (from *Rhodospirillum*) at about 18‰ (Robinson and Cavanaugh, 1995; Roeske and O'Leary, 1984; Roeske and O'Leary, 1985). These results are in reasonable agreement with the data from cultured cells, considering that effects, such as limited mass transport, inhibit the fixation pathway from attaining the maximum possible discrimination (Goericke et al., 1994).

The reductive acetyl-CoA pathway produces the most carbon isotopic fractionation observed (with ε values up to 39‰) (Preuß et al., 1989), reported from a sulfur-reducing proteobacterium utilizing the acetyl-CoA pathway for carbon fixation, but does not produce methane or acetate. Cell masses of methanogenic and acetogenic prokaryotes using the acetyl-CoA pathway are not as ^{13}C-depleted, probably due to the production and excretion of extremely ^{13}C-depleted methane or acetate.

This present study of carbon isotopic fractionation yielded lower overall fractionations than results reported in the literature (Fig. 1). While this difference may be due, in part, to high growth rates in some cases (Laws et al., 1995), lower fractionations were observed both in slowly growing cultures, as well as in cultures which grew quickly.

Although the fractionations between biomass and CO_2 observed here are generally smaller than those reported previously, the relationships between pathways of carbon assimilation are consistent with earlier reports. Specifically, isotopic depletions associated with the reductive TCA cycle and the 3-hydroxypropionate cycle generally produce less carbon isotopic fractionation than the other two pathways. Results for the reductive TCA cycle (values between 2.0 and 5.5‰) and the 3-hydroxypropionate cycle (values between 0.2 and 3.6‰) are smaller on average than those associated with the reductive pentose-phosphate (value of 5.1‰) and acetyl-CoA pathways (values between 2.7 and 26.7‰). However, *Thiobacillus novelus*, the only pentose phosphate cycle utilizing-prokayote studied here, was less ^{13}C-depleted than expected, falling just within the range of values reported for this cycle (Fig. 1a).

These carbon isotopic fractionation experiments yielded results that were in some ways expected, but in other ways surprising. As expected, the reductive TCA cycle and 3-hydroxypropionate cycle produced biomass less ^{13}C-depleted on average than the reductive pentose phosphate cycle or the acetyl-CoA pathway. The experiments involving species of the order Archaeoglobales (Fig. 2b), however, did not yield as high a magnitude of fractionation as expected for these acetyl-CoA pathway-using taxa.

Table 2
Published results of growth experiments estimating carbon-isotopic fractionation to cell biomass, ‰. The carbon fixation pathways are: the reductive TCA cycle (TCA), the 3-hydroxypropionate cycle (3-HP), the reductive pentose phosphate cycle (PP), and the acetyl-CoA pathway (AP). Carbon-isotopic fractionation from carbon dioxide to methane or acetate is shown for species that produce such a metablic product (MP)

TAXON	Pathway "MP"	T (°C)	ε (‰) from CO_2 to Cells	Reference
Desulfobacter hydrogenophilus	TCA	28	10.0	Preuß et al., 1989
		28	8.9	
		28	14.2	
Thermoproteus neutrophilus	TCA	85	8.7	
Alkaligenes eutrophus	PP	28	28.2	
Thermocrinis ruber	TCA	85	3.3	Jahnke et al., 2001
Coccochloris elebens	PP	39	12.3	Calder and Parker, 1973
		39	16.0	
Agmenelium quadrupicatum	PP	39	15.9	
Osillatoria williamsii	PP	39	5.0	
Anacystis nidulans	PP	39	13.1	
		39	18.0	
Agmenelium quadrupicatum	PP	39	22.2	Pardue et al., 1976
		39	23.9	
		39	19.6	
Synechococcus lividus	PP	47	12.8	
		70	11.1	
Schizothrix calcicola	PP	39	13.2	
Oscillatoria williamsii	PP	39	17.3	
Microcoleus chthonoplastes	PP	39	17.1	
Chorella sorokiniana	PP	39	22.6	
Rhodospirillium rubrum	PP	20	21.1	Sirevåg et al., 1977
Rhodospirillum rubrum	PP		12.7	Quandt et al., 1977
Rhodopseudomonas capsulata	PP		10.8	

TAXON	Pathway "Product"	T (°C)	ε (‰) from DIC to cells	Reference
Synechococcus (continued)			15.6	Popp et al., 1998
			16.9	
			18.9	
			18.1	
Desulfobacterium autotrophicum	AP	28	39.3	Preuß et al., 1989
		28	38.2	
Acetobacterium woodii	AP "Acetate"	28	22.2, 43.6	
		28	29.0, ND	
		28	15.8, 43.4	
Methanobacterium thermoauto	AP "Methane"	65	19.1, 34.1	Fuchs et al. , 1979
Methanobacterium sp.	AP "Methane"	37	25.1, 36.4	Belyaev et al., 1983
		37	23.2, 34.9	
		46	18.8, 33.7	
		46	24.5, 35.7	

TAXON	Pathway "Product"	T (°C)	ε (‰) from DIC to cells	Reference
Chlorobium thiosulfatophilum	TCA	20	20.1	Sirevåg et al., 1977
Chlorobium phaeovibrioides	TCA	30	10.4	Quandt et al., 1977
	TCA	30	9.5	
Chlorobium limicola	TCA	30	9.5	
Chlorobium vibrioforme	TCA	30	12.2	
	TCA	30	10.8	
	TCA	30	10.7	
Chloroflexus aurantiacus	3-H	55	13.7	Holo and Sirevåg, 1986

(*continued on next page*)

Table 2 (*continued*)

TAXON	Pathway "MP"	*T* (°C)	ε (‰) from CO_2 to Cells	Reference	TAXON	Pathway "Product"	*T* (°C)	ε (‰) from DIC to cells	Reference
Anacystis nidulans	PP	33	16.0	Mizutani and Wada et al. 1982	*Thiomicrospira* sp. L-12	PP		25.5	Ruby et al., 1987
		33	21.5		*Thiobacillus neapolitanus*	PP		25.8	
		33	18.0		*Chromatium vinosum*	PP	35	32.7	Wong et al., 1975
Synechococcus	PP	57	22.5		*Thiomicrospira crunogena*	PP		24.5	Mirkin and Ruby, 1991
Synechococcus	PP		15.5	Popp et al., 1998		PP		23.3	
			16.4		*Nitrosomonas europaea*	PP		13.8	
			18.6			PP		13.2	
			18.6		*Chromatium strain* D	PP	20	31.0	Sirevåg et al., 1977
			17.2		*Chlamydomonas reinhardii*	PP	20	36.8	
			17.1		*Chromatium vinosum*	PP	30	27.3	

Table 3
Known or suspected carbon fixation pathways for organisms used in this study shown along with the method by which the carbon fixation pathway is known. "Biochem" indicates that enzymatic studies have been reported giving strong evidence of which pathway is operating. "Genome" indicates that a full genome sequence is available that suggests the indicated pathway is operating. "Indirect" means that the carbon fixation pathway suspected is based on the operation of that pathway in the most phylogentically related microorganisms for which carbon fixation has been studied. The two cases for which the carbon fixation pathway assignments are most tenuous are *Pyrodictium* and *Pyrolobus*, for which a question mark has been indicated next to the assignment of carbon fixation. Further, *Ammonifex* has not been included in this analysis because of the inability to assign a "suspected" carbon fixation pathway to this taxon

Taxon	Pathway (method)	Reference
Aquifex aeolicus	TCA (biochem)	Beh et al., 1993
Hydrogenobacter thermophilus	TCA (biochem)	Ishii et al., 1998
Thermoproteus neutrophilus	TCA (biochem)	Beh et al., 1993
Pyrobaculum aerophilum	TCA (indirect, genome)	Based on *Thermoproteus*, also see Fitz-Gibbon et al., 2002
Pyrodictium occultum	TCA? (indirect)	Based on *Thermoproteus*
Pyrolobus fumarii	TCA? (indirect)	Based on *Thermoproteus*
Acidianus brierleyi	3-H (biochem)	Ishii et al., 1996
Metallosphaera sedula	3-H (biochem)	Menendez et al., 1999
Sulfolobus solfataricus RON 12 III	3-H (biochem)	Menendez et al., 1999
Thiobacillus novellus	PP (sequence)	Meijer et al., 1991
Archaeoglobus fulgidus	AP (genome, indirect)	Klenk et al., 1997 also based on *A. lithotrophicus*
A. lithotrophicus	AP (biochem)	Vorholt et al., 1995
Ferroglobus placidus	AP (biochem)	Vorholt et al., 1997
Methanogens	AP (biochem)	Fuchs and Stupperich, 1982; Sprott et al., 1993

The magnitude of carbon isotopic fractionation observed for HCl-untreated methanogen cells occasionally reached within ~3‰ of the magnitude of carbon isotopic fractionation observed for methane generated by the organism (see Table 1, Figs. 1b and 2a). In fact, the results from HCl-treated and HCl-untreated experiments on methanogens differed substantially, in contrast to non-methanogens. The HCl-untreated methanogens were more depleted in ^{13}C (~5‰) than their acid

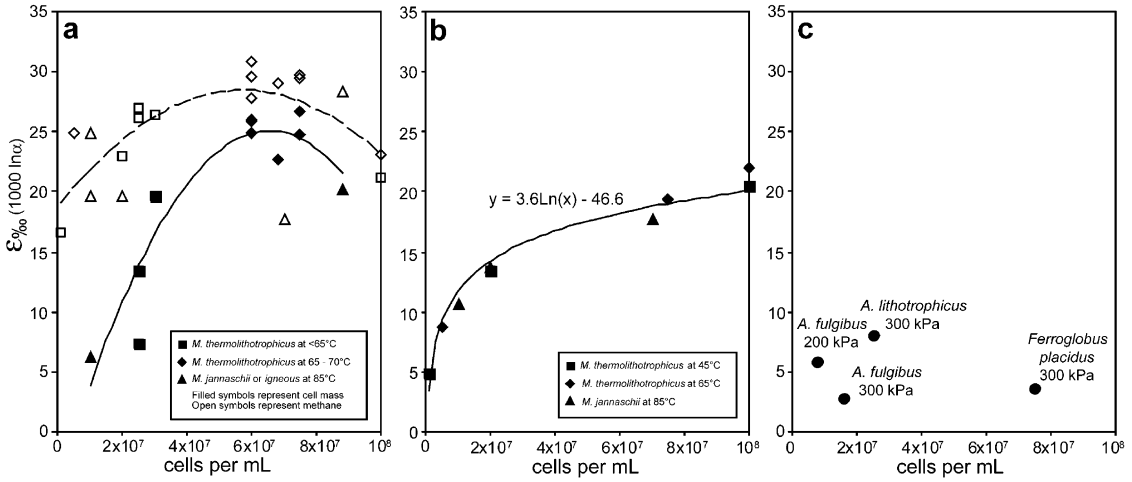

Fig. 2. Carbon isotopic fractionation plotted against the final cell density obtained experimentally during laboratory growth experiments on (a) species of the genus *Methanococcus* with isolated cells not treated with acid, (b) species of the genus *Methanococcus* with isolated cells treated with 0.1 M HCl before combustion, and (c) species of the order Archaeoglobales. Fractionation results to cells are shown as solid symbols, while open symbols refer to fractionation to methane produced during growth. The curves are polynomial approximations of the data (a) and a logarithmic regression to the data (b). While members of the genus *Methanococcus* were grown at different temperatures, all of the Archaeoglobales experiments were at 85 °C.

treated counterparts (indicated in Fig. 1b), a result that suggests that acid treatment may have liberated a substantially ^{13}C-depleted mix of volatiles. These volatiles may be unstable intermediates in the methanogenesis pathway or perhaps acetic acid liberated from the hydrolysis of acetyl-CoA.

Another unexpected result was the wide range of values obtained for methanogens using the acetyl-CoA pathway, with ε values of 4.8–26.7‰. This surprising result is evidently related to the final cell densities attained by the various cultures when the experiments were stopped. The various methanogens were harvested over a range of different cell densities, and the data show that as the culture grew increasingly dense, the amount of carbon isotopic fractionation increased from low values to the most fractionated observed.

Fig. 2a and b clearly demonstrates this effect. They show the carbon isotopic fractionation (in ‰) from CO_2 found for species of *Methanococcus* as a function of the cell growth in the culture. In experiments in which the isolated cells were not treated with acid before combustion, shown in Fig. 2a, the observed carbon isotopic fractionations from carbon dioxide to biomass and from carbon dioxide to methane increase as a function of the growth of the culture reaching a maximum before dipping at the higher cell densities investigated. Fig. 2b shows the results of *Methanococcus* experiments in which the isolated cells were treated with 0.1 M HCl before combustion as a function of the cell growth in the culture. The results from acid-treated *Methanococcus thermolithotrophicus* and *Methanococcus jannaschii* cells, regardless of the temperature of growth, fall on the same logarithmic curve. As discussed in Section 2 (Experimental methods), these data have been corrected for the effect of isotope distillation. For two reasons, these results cannot be attributed to general decreasing external CO_2 limitation. First, the cell densities in these experiments have not yet reached stationary growth (which would be nearly 10^9 cells per ml for these species). Second, in these experiments, the rate of consumption of CO_2 by the cell culture (and, therefore, CO_2 limitation in general) is greatest at the end of the experiment when cell densities are highest because the cells are always participating in active methanogenesis, irrespective of growth phase.

Fig. 2c shows the results of experiments involving species of the Archaeoglobales as a function of the amount of growth of the culture. No trend is apparent, suggesting that the effect observed in the methanogens is not operating in the Archaeoglobales.

The logarithmic relationship shown in Fig. 2b between the final cell density in *Methanococcus* cultures and the amount of carbon isotopic fractionation observed suggests that, under these conditions, the amount of fractionation possible by these species is not constrained by a theoretical limit, but rather by the maximum cell density obtainable (i.e., stationary growth, nearly 10^9 cells per ml).

A similar phenomenon was observed by Botz et al. (1996) for the methane produced by species of *Methanococcus* grown in a flow-through fermentor. Botz et al. did not measure the carbon isotopic composition of the biomass produced in their experiments, but observed carbon isotopic fractionation values (α) for the methane

produced during methanogenesis as low as 1.002 and as high as 1.079 (Botz et al., 1996). In these flow-through experiments, at 55 °C and below, carbon isotopic fractionation during methanogenesis remained low until stationary growth phase was achieved, whereas at 65 and 85 °C, fractionation was evidently independent of growth phase. In contrast, our results for biogenic methane, using a closed system for cultivation, reveal only a minor dependence on growth phase (see Fig. 2a). The experiments reported by Botz et al. show both less fractionation (at low cell densities), and significantly more fractionation [at very high cell densities (almost 10^9 cells per ml)] than the results reported here. These differing experimental results evidently stem from the different growth conditions (use of a flow-through fermentor versus closed culture bottles) in the two sets of experiments.

In summary, Botz et al. observed a strong dependence between carbon isotopic fractionation and growth phase for biogenic methane produced by *Methanococcus* cultured at temperatures of 55 °C and below in flow-through fermentors; whereas the results of the experiments reported here indicate that in a closed system carbon isotopic fractionation for biomass-producing carbon fixation by methanogens is dependent on the final cell density of the culture at all temperatures investigated (41–85 °C). Furthermore, the results of the present study suggest the absence of an interdependence between carbon isotopic fractionation and final cell density for the non-methane producing or micro-methanogenic Archaeoglobaleans investigated. Further study may elucidate the molecular bases of these results and demonstrate whether methanogens in nature exhibit variable isotopic compositions or a relatively constant maximum amount of carbon isotopic fractionation resulting from "perpetual" growth at the stationary phase.

While changes in observed isotopic fractionation during logarithmic growth is surprising, it has also been observed in other microbial systems, which do not involve methanogenesis. A decrease in fractionation linked to changes in carbon assimilation biochemistry has been observed in logarithmic growing cultures of methanotrophs (Summons et al., 1994; Jahnke et al., 1999), and an increase in the magnitude of Se isotopic fractionation during microbial selenate and selenite reduction has been observed as logarithmic growth proceeds (Herbel et al., 2000).

To account fully for the results reported here, there must be a mechanism by which the overall isotopic fractionation for biomass production changes throughout the logarithmic growth phase of the methanogen cultures. Although further work will be necessary to elucidate such a mechanism, it could be explained by either a change of carbon fixation biochemistry during growth, or by an increase in the amount of CO_2 being converted to biomass relative to CO_2 being converted to methane in each cell as logarithmic growth proceeds. More CO_2 being converted to methane for each cell division could produce lower observed fractionation to biomass because of increased residual $^{13}CO_2$ in the cell.

In conclusion, we report here new data on carbon isotopic fractionation from a diverse set of 21 chemolithoautotrophic prokaryotes from both the Archaea and the bacteria. This new data, taken with that from published reports, allow for the comparisons of the carbon isotopic fractionation between the four known carbon fixation biochemical pathways. The summary of carbon isotopic fractionation to biomass (Fig. 2c) shows that the reductive pentose phosphate cycle and acetyl-CoA pathway can yield higher fractionations than the reductive TCA cycle and 3-hydroxypropionate cycle. These systematic differences in carbon isotopic fractionation between biochemical pathways suggest that carbon isotopic analysis can reveal metabolic aspects of the ancient organic fossil material (House et al., 2000). However, this study also indicates that the acetyl-CoA pathway does not always produce large fractionations. Smaller fractionations were observed in the Archaeoglobales, and the data show a growth-dependent wide range of carbon isotopic fractionations in methanogen cultures. Thus, the use of carbon isotopic fractionation as a tool for inferring prokaryotic metabolism must be pursued with caution.

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