Supplementary Materials for

Controls on the isotopic composition of microbial methane

Jonathan Gropp*, Qusheng Jin, Itay Halevy

*Corresponding author. Email: jonathan.gropp@weizmann.ac.il

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Supplementary Materials

Carbon isotope fractionation between CO$_2$ and a biomass precursor

The carbon isotope composition of biomass of the cell’s biomass ($\delta^{13}C_B$) can be described by the isotopic mass balance:

$$\delta^{13}C_B = \frac{1}{3} \left( \delta^{13}C_{CO_2} - 13\varepsilon_{CO_2-CH_3} - 13\varepsilon_{CH_3-CH_3(Ac)} \right) + \frac{1}{3} \left( \delta^{13}C_{CO_2} - 13\varepsilon_{CO_2-Z} \right) + \frac{1}{3} \left( \delta^{13}C_{CO_2} - 13\varepsilon_{CO_2-COO} \right), \tag{S1}$$

where $\delta^{13}C_{CO_2}$ is the carbon isotope composition of CO$_2$, and the $\varepsilon$ terms are the net carbon isotope fractionations between CO$_2$ and CH$_3$-H$_4$MPT ($13\varepsilon_{CO_2-CH_3}$), between CH$_3$-H$_4$MPT and the methyl moiety in acetyl-CoA ($13\varepsilon_{CH_3-CH_3(Ac)}$), between CO$_2$ and the carbonyl moiety in acetyl-CoA ($13\varepsilon_{CO_2-CO}$), and between CO$_2$ and the additional (third) carboxyl group added to acetyl-CoA to form pyruvate ($13\varepsilon_{CO_2-COO}$). We found a relatively constant offset of $\approx 20 \pm 10\%e$ between model $13\varepsilon_{CO_2-CH_3}$ and observed CO$_2$-biomass carbon isotope fractionations ($13\varepsilon_{CO_2-B}$) at a given $13\varepsilon_{CO_2-CH_4}$ (fig. S6), which implies that:

$$\delta^{13}C_B \approx \delta^{13}C_{CO_2} - 13\varepsilon_{CO_2-CH_3} - 20\%e. \tag{S2}$$

Combining Eqns. S1 and S2 yields:

$$13\varepsilon_{CO_2-CO} + 13\varepsilon_{CO_2-COO} + 13\varepsilon_{CH_3-CH_3(Ac)} \approx 2 \times 13\varepsilon_{CO_2-CH_3} + 60\%e. \tag{S3}$$

If, for example, $13\varepsilon_{CO_2-CH_3}$ is 30\%e, as in the extreme right of fig. S6, the sum of the three fractionations on the left side of Eq. S3 is 120\%e, while if $13\varepsilon_{CO_2-CH_3}$ is $-20\%e$, as in the extreme left of fig. S6, the sum of the three fractionations is 0\%e.

Sensitivity analysis

We conducted a sensitivity analysis to the tunable model parameters by examining the effect of a 3-fold increase or decrease in the value of these parameters on the reversibility of the enzymatically-catalyzed reactions (fig. S13) and on $13\varepsilon_{CO_2-CH_4}$ (S14) and $2\varepsilon_{CH_4-H_2O}$ (S15). We find that the values
of $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ and $^2\varepsilon_{\text{CH}_4-\text{H}_2\text{O}}$ are sensitive only to some of the model parameters, but that the overall trajectories of the isotopic fractionation dependence on $\Delta G_{\text{net}}$ are preserved. We identified three types of sensitivity of $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ and $^2\varepsilon_{\text{CH}_4-\text{H}_2\text{O}}$ to some of the model parameters, which are typical to three $\Delta G_{\text{net}}$ ranges:

(i) At small-negative $\Delta G_{\text{net}}$ ($\lesssim -60 \text{ kJ mol}^{-1}$) the Mtr- and Mcr-catalyzed reactions depart from equilibrium, and consequently, the combination of these reactions’ equilibrium and kinetic fractionation factors (EFFs and KFFs, respectively) determines the magnitude of $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ over this $\Delta G_{\text{net}}$ range. Thus, changes to parameters that affect the departure from equilibrium of these reactions manifest as a change in the maximal $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ value reached at $\Delta G_{\text{net}}$ of $\approx -40 \text{ kJ mol}^{-1}$ (a vertical movement of the $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ peak, as in panels 13–15, 26, 27, 31, and 35 of fig. S14). These parameters include the $K_M$ and $V^+$ values, the exact $\Delta G_r^0$ values of the Mtr- and Mcr-catalyzed reactions ($R_{\Delta G_r^0}$, see table S1), and the initial concentration of HS-CoB and CoM-S-S-CoB ($C_i$ CoB). Over the same $\Delta G_{\text{net}}$ range, changes in some parameters drive a shift of the $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ and $^2\varepsilon_{\text{CH}_4-\text{H}_2\text{O}}$ trajectories along the $\Delta G_{\text{net}}$ axis, with minimal effect on the peak $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ and $^2\varepsilon_{\text{CH}_4-\text{H}_2\text{O}}$ values (as in panels 19, 20, and 29 in fig. S14 and panels 13, 19, 26, 29, 35, and 36 in fig. S15). This is mostly evident in $K_M$ and $V^+$ values of the Hdr-catalyzed reaction, which is irreversible ($J_i^-/J_i^+ \rightarrow 0$) already at small-negative $\Delta G_{\text{net}}$. Changes in the kinetic parameters of this reaction affect the steady-state concentration of HS-CoB and CoM-S-S-CoB, and consequently shifts the departure from equilibrium of the Mtr- and Mcr-catalyzed reactions along the $\Delta G_{\text{net}}$ axis. In contrast to changes that directly affect Mtr- and Mcr-catalyzed reactions, here both reactions shift together along the $\Delta G_{\text{net}}$ axis (e.g., when forward $K_M$ values of Hdr decrease, both Mtr- and Mcr-catalyzed reactions depart from equilibrium at smaller $\Delta G_{\text{net}}$ values) and thus the position of the $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ value changes accordingly on the $\Delta G_{\text{net}}$ axis, but its magnitude remains similar. Lastly, over the same $\Delta G_{\text{net}}$ range, $Y_{\text{ATP}}$ values affect both the peak $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ values, and the location of the $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ and $^2\varepsilon_{\text{CH}_4-\text{H}_2\text{O}}$ trajectories of departure from equilibrium along the $\Delta G_{\text{net}}$ axis (panel 36 in figs. S14 and S15). The $Y_{\text{ATP}}$ parameter, which sets the minimal energetic requirement for ATP production, affects the Gibbs free energy of the Mtr-catalyzed reaction.
(\Delta G_{I,Mtr}', see Methods), which in turn affects the relative order of departure from equilibrium of the Mtr- and Mcr-catalyzed reactions, and the relative expressions of their respective KFFs. As the \(Y_{\text{ATP}}\) also sets the minimal threshold on methanogenic activity, increasing or decreasing its value shifts the departure from equilibrium along the \(\Delta G_{\text{net}}\) axis.

\(\text{(ii)}\) At \(\Delta G_{\text{net}}\) between \(\approx -50\) and \(\approx -100\) kJ mol\(^{-1}\), \(^{13} \varepsilon_{\text{CO}_2-\text{CH}_4}\) values are dominated by departure from equilibrium of the Ftr-catalyzed reaction. Thus, changes to the kinetic parameters of this reaction result in a shift in the location of the decrease of \(^{13} \varepsilon_{\text{CO}_2-\text{CH}_4}\) values from the larger-than-equilibrium peak to their minimal values at large negative \(\Delta G_{\text{net}}\) (panels 3, 4, and 22 in fig. S14). The Ftr-catalyzed reaction is not directly involved in hydrogen atom exchange, and the departure from hydrogen isotope equilibrium is controlled mostly by departure from equilibrium of the Mcr- andHdr-catalyzed reactions, which occurs at less negative \(\Delta G_{\text{net}}\) values. Consequently, over the intermediate \(\Delta G_{\text{net}}\) range, \(^{2} \varepsilon_{\text{CH}_4-\text{H}_2\text{O}}\) is insensitive to essentially all model parameters (fig. S15).

\(\text{(iii)}\) At large-negative \(\Delta G_{\text{net}}\) (\(\lesssim -100\) kJ mol\(^{-1}\)), several parameters affect the minimal values of \(^{13} \varepsilon_{\text{CO}_2-\text{CH}_4}\) and \(^{2} \varepsilon_{\text{CH}_4-\text{H}_2\text{O}}\). Over this \(\Delta G_{\text{net}}\) range, \(^{13} \varepsilon_{\text{CO}_2-\text{CH}_4}\) is controlled mostly by the relative expression of the EFFs and KFFs of the Fmd- and Mtr-catalyzed reactions. Therefore, \(^{13} \varepsilon_{\text{CO}_2-\text{CH}_4}\) is sensitive to the kinetic parameters of these enzymes, most notably to their \(V^+\) values of the Fmd- and Mtr-catalyzed reactions. Mtr has the smallest \(V^+\) value and as \(\Delta G_{\text{net}}\) increases and the enzyme is saturated with substrates, its rate comes to limit the overall methanogenesis rate. A sufficient increase in the \(V^+\) of Mtr results in colimitation of net methanogenesis rates at large \(\Delta G_{\text{net}}\) values with other enzymes, e.g., the Fmd-catalyzed reaction that completely departs from equilibrium (fig. S13), thus maximizing the expression of the KFF of Fmd (panel 26 in fig. S14). A similar effect emerges also in \(^{2} \varepsilon_{\text{CH}_4-\text{H}_2\text{O}}\), mostly as sensitivity to the values of \(K_M\) and \(V^+\) of the Mtd-, Hmd, and Frh-catalyzed reactions, which control the degree of hydrogen atom mixing between \(\text{H}_2\) and \(\text{H}_2\text{O}\), and to the \(V^+\) value of the Mtr-catalyzed reaction, which is rate limiting as explained above.
Data for model calibration and validation

Model calibration to laboratory culture results

We curated net carbon and hydrogen isotopic fractionations and their relations to the Gibbs free energy of the net methanogenic reaction ($\Delta G_{\text{net}}$) from culture experiments. Among other experimental parameters, this dataset consists of experiments with varying temperature, growth phases, substrates used and microbial strains. We screened the data according to the following criteria: (i) including data from stationary growth phases where growth was reported; (ii) excluding data that may have been affected by Rayleigh (isotopic) distillation; (iii) excluding data that were ignored in the original publications due to known measurement errors. For each sample we used the reported $\Delta G_{\text{net}}$, where available, or calculated it based on the reported concentrations of $H_2$, $CO_2$ and $CH_4$ using: $\Delta G_{\text{net}} = \Delta G_r^0 + R T \ln Q$, where $\Delta G_r^0$ is the standard Gibbs free energy of the reaction (corrected for the temperature with the Van’t Hoff equation), $R$ is the ideal gas constant, $T$ is the temperature and $Q = [CH_4]/([CO_2][H_2]^4)$ is the reaction quotient. Isotopic data from experiments with small negative $\Delta G_{\text{net}}$ values were mostly obtained from co-cultures, which complicated measurements of headspace $H_2$ concentrations. We corrected for the concentrations of $H_2$ in these samples as explained in the Methods. All the samples that were used are listed in table S9.

The $^{2}$$\varepsilon_{CH_4-H_2O}-\Delta G_{\text{net}}$ relation at small negative $\Delta G_{\text{net}}$ values shows contradicting trajectories between two similar experiments, conducted by Yoshioka et al., 2008 (39) and by Okumura et al., 2016 (20) (henceforth Yos08 and Oku16). While both reports used the same thermophilic co-culture of $S. lipocalidus$ and $M. thermoautotrophicus$ strain $\Delta H$, $^{2}$$\varepsilon_{CH_4-H_2O}$ values inferred in the two studies differ by $\approx 100\%$ at similar $\Delta G_{\text{net}}$ values (fig. S4). These differences are at least in part due to the analytical methods used. While Yos08 oxidized $CH_4$ to $H_2O$ and then reduced the $H_2O$ to $H_2$, which was then introduced into an isotope-ratio mass spectrometer (IRMS), Oku16 pyrolyzed $CH_4$ directly to $H_2$, which was then introduced into an IRMS. The latter approach is considered more accurate due to a lower risk of contamination during sample processing and lesser exposure to humid air. This is one reason for which we chose to use the Oku16 over the Yos08 data. A second reason is that the Oku16 data also include measurements of both carbon and hydrogen
isotopes over a larger range of $\Delta G_{\text{net}}$ values.

There are considerable differences between $\Delta^{13}CH_3D$ values in hydrogenotrophic methanogens with and without membrane-embedded electron carriers (e.g., methanophenazine), the latter of which are the focus of our model. While $^2\varepsilon_{\text{CH}_4-H_2O}$ is of a similar range for these two groups, $\Delta^{13}CH_3D$ for methanogens with methanophenazines is between $-6$ and $0\%e$, whereas for methanogens without methanophenazines the observed range is $-2$ to $3\%e$. The laboratory culture data collected to date is insufficient to determine whether this difference is statistically significant, though there may be a physiological basis for it, as methanogens with methanophenazines have distinct metabolic characteristics that may affect the dynamics of departure from equilibrium.

**Cell-specific methanogenesis rates**

There are currently limited data on cell-specific methanogenesis rates in natural environments. We bridge this gap by comparing compiled bulk methanogenesis rates (bMR) and estimates of cell density. bMR values were obtained from the results of either ratiotracer experiments or reaction-diffusion models, both of which carry uncertainties. In radiotracer experiments, the rate of methanogenesis is assumed to be equal to the rate of CO$_2$ reduction to methane, but in fact the measured rate of CO$_2$ reduction serves as an upper limit on methanogenesis rates. If the reaction is close to equilibrium, then the net rate of methanogenesis will be lower than the radiotracer-based estimate, possibly by orders of magnitude if the reversibility between methane and CO$_2$ is higher than 0.9. Moreover, some of the radiotracer experiments are conducted under conditions that may favor higher methanogenesis rates (e.g., increased partial pressure of H$_2$ in the headspace), resulting in overestimation of the in-situ rates. Models of in-situ methanogenesis rates provide an estimate for the net methanogenesis rates based on the concentration and isotopic gradients of methane and DIC, but carry uncertainties due to the choice of model parameters, such as the net fractionation of carbon and hydrogen isotopes associated with methanogenesis.

To estimate the abundance of cells, where no measurements exist, we used a general relation between cell density and depth within the sediment in marine environments (90, 91): $y = 7.73 \cdot$
$10^7 \times z^{-0.6332}$, where $z$ is the depth in meters and $y$ is the number of cells per cubic centimeter of sediment. We assume that of these cells 12% are Archaea in open-ocean sites and 40% in ocean margin sites (92), and that 50% of Archaea are methanogens (91).
Figure S1: Intracellular metabolite concentrations. (A-I) $\log_{10}$ of concentrations in mM (see panel color legends for each row in the right-most panel). (J-L) Percentage of electron carriers in reduced form.
Figure S2: Prior and posterior distributions of metabolic model parameters. (A–H) $K_m$ values and (I) $U_{(viv/vit)}$, for $10^6$ simulations. An overview of the prior and posterior distributions is in table S5.
Figure S3: Posterior distributions of kinetic isotope fractionation factors (KFFs). The posterior KFF distributions were generated by weighting $10^6$ individual KFF value combinations drawn from uniform prior distributions by the inverse of the square of the sum of squared model-measurement mismatches ($1/SSE^2$). Here we assumed that KFFs are similar in both mesophilic and thermophilic conditions. Values are plotted in permil (‰), where $\varepsilon = \alpha - 1$. Carbon isotope KFFs (top row) were drawn from uniform prior distributions with $\alpha^+ \in (0.935, 1)$. The KFF of Mcr was assigned a normal prior distribution with a mean of 0.9615 and a standard deviation of 0.01 (42). Secondary hydrogen isotope KFFs (middle row) were drawn from uniform prior distributions with $\alpha^+ \in (0.6, 1)$, and primary hydrogen isotope KFFs (bottom row) were drawn from prior uniform distributions with $\alpha^+ \in (0.4, 1)$ ($\alpha^+ \in (0.2, 1)$ for the Mvh/Hdr-catalyzed reaction). The primary hydrogen isotope KFFs of Mcr were drawn from normal prior distributions with a mean of 0.41 and a standard deviation of 0.04 (42). The secondary hydrogen isotope KFFs of Mcr were drawn from normal prior distributions with a mean of 0.85 and a standard deviation of 0.035. The values in the upper right corner of the panels represent the median posterior KFF values. In simulations where the posterior distribution is similar to the prior (uniform) distribution we do not show these median values. Primary and secondary hydrogen isotope KFFs are denoted by superscripted ‘p’ and ‘s’ respectively.
Figure S4: Isotopic fractionation in laboratory cultures depends on the thermodynamic drive. A compilation of (A) carbon isotope fractionation between CO$_2$ and CH$_4$ ($^{13}$ε$_{CO_2}$-CH$_4$), and (B) the hydrogen isotope fractionation between CH$_4$ and H$_2$O ($^{2}$ε$_{CH_4}$-H$_2$O). The dashed lines represent the EFFs for the temperatures that are noted next to the lines, calculated at the M06-L/def2-TZVP level of theory with the SMD implicit solvation model (43). The data (16–21, 39) are listed in table S9.
Figure S5: Calibration of hydrogen isotope kinetic fractionation factors. Top: model results for calibration against data (circles) from Yoshioka et al., 2008 (39), Hattori et al., 2012 (19), and Okumura et al., 2016 (20). The diamonds are the binned averages that the model was calibrated against. Middle and lower rows are the posterior KFF distributions for the data from Okumura et al., (red histograms) and for the three data sets combined (black line). The respective median values of the KFFs (where the posterior distribution is not uniform) are noted in the upper left corner of each panel.
Figure S6: Carbon isotope fractionation between CO$_2$, CH$_3$-H$_4$MPT, and biomass. Lines show the median model results for the CO$_2$–CH$_3$-H$_4$MPT carbon isotope fractionation ($\varepsilon_{\text{CO}_2\text{-CH}_3}$), at 40 and 60°C, and [CO$_2$] of 1 mM. The circles are experimentally measured values of the CO$_2$–biomass carbon isotope fractionation, as previously compiled (49, 51). The colors of the circles correspond to the experimental temperature, and the dashed lines represent 10, 20 and 30‰ offsets from $\varepsilon_{\text{CO}_2\text{-CH}_3}$ on the y-axis.
Figure S7: Clumped isotopologue abundances. Model clumped isotope results of 200 simulations at 30°C (blue) and 60°C (red) against $\Delta G_{\text{net}}$. The thick red and blue lines show the median, and the envelopes show 68% of the model results with Hmd activity. The dashed red and blue lines and the envelopes denoted by thin lines show the median and 68% of the model results without Hmd activity. The gray lines represent temperature-dependent isotopic equilibrium. (A-B) $\Delta^{13} \text{CH}_3\text{D}$. (C-D) $\Delta^{12}\text{CH}_2\text{D}_2$.

Figure S8: A combinatorial effect in hydrogenotrophic methanogenesis due to activity of Hmd. Methane $\Delta^{12}\text{CH}_2\text{D}_2$ values against $\text{H}_2$ δD values calculated at 60°C, at [H$_2$] of 50 mM. The δD values of H$_2$O in all the calculations are 0‰. The black and orange lines are medians of $10^4$ simulations with and without the Hmd-catalyzed reaction, respectively, and the envelopes contain 68% of the results. The variability in the $\Delta^{12}\text{CH}_2\text{D}_2$ values is due to the hydrogen isotope KFFs, which were randomly drawn from the posterior distributions presented in fig. S3, with a constant KFF of 0.95 for Hmd.
**Figure S9: Clumped isotopologue abundances for thermophilic conditions.** Experimental data (gray circles) and model results (lines and envelopes) of 200 simulations at 60°C. The dark gray lines represent temperature-dependent isotopic equilibrium at 0-350°C, and the thick red lines show the median of the simulations, with red circles at $\Delta G_{\text{net}}$ values of $-20$, $-30$, $-80$, and $-120$ kJ mol$^{-1}$. (A) $\Delta^{13}\text{CH}_3\text{D}$ against $\Delta^{12}\text{CH}_2\text{D}_2$. (B) $\Delta^{13}\text{CH}_3\text{D}$ against $\varepsilon\text{CH}_4-\text{H}_2\text{O}$. (C) $\Delta^{13}\text{CH}_3\text{D}$ against $^{13}\varepsilon\text{CO}_2-\text{CH}_4$. Laboratory culture samples are from hydrogenotrophic methanogens that do not have membrane-associated methanophenazines.

**Figure S10: The dependence of cell-specific methanogenesis rate (csMR) on $\Delta G_{\text{net}}$.** The solid lines show the default laboratory-calibrated model results for the same temperatures with $Q_{10}^{V^+}$ of 1, and the dotted lines show the results for $Q_{10}^{V^+}$ of 1.5. The calculations are for $[\text{CO}_2]$ and $[\text{CH}_4]$ of 1 mM. The cell volume is 1 µm$^3$. 
Figure S11: Reversibility against the thermodynamic drive ($\Delta G_{\text{net}}$) of methanogenesis under energy-limiting conditions. Mcr II results simulate growth under optimal conditions in lab cultures (black), and Mcr I is for growth in energy-limited conditions (orange). The solid lines are the median of 100 model simulations. The reversibility, defined as the ratio of the backward to forward reaction rates of an individual reaction ($J_i^- / J_i^+$) ranges from 1 (full reversibility) to 0 (unidirectional reaction).
Figure S12: Isotopic fractionation during methanogenesis in energy-limited conditions. The dependence on csMR of (A) $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$, (B) $^{2}\varepsilon_{\text{CH}_4-\text{H}_2\text{O}}$, (C) $\Delta^{13}\text{CH}_3\text{D}$, and (D) $\Delta^{12}\text{CH}_2\text{D}_2$. The dashed gray lines in panels A-D represent the equilibrium isotopic fractionations between 0°C and 60°C. (E) Co-variation of $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ and $^{2}\varepsilon_{\text{CH}_4-\text{H}_2\text{O}}$. The black lines indicate the co-variation of $^{13}\varepsilon_{\text{CO}_2-\text{CH}_4}$ and $^{2}\varepsilon_{\text{CH}_4-\text{H}_2\text{O}}$ with KFFs from posterior distributions that were calibrated against a dataset including results from Yoshioka et al., 2008 (39). (F) Co-variation of $\Delta^{12}\text{CH}_2\text{D}_2$ and $\Delta^{13}\text{CH}_3\text{D}$. The contours in panels E-F are the log10 of the csMR as predicted by our metabolic model between 0°C and 60°C, and the circles are biogenic environmental samples (table S10). In panels A-D, the dotted lines show the default laboratory-calibrated model results for the same temperatures (Mcr II). The calculations are for $[\text{H}_2]$ in the range of 1 nM to 5 µM, and $[\text{CO}_2]$ and $[\text{CH}_4]$ of 1 mM. The cell volume is 1 µm³.
Figure S13: Sensitivity of reaction reversibility to model parameters. Black lines represent results with the default parameters, purple and green lines represent a decrease or an increase by a factor of three to a default parameter value, respectively. The parameter names are written next to their respective reversibility trajectories. For $K_M$ values, the ‘f’ and ‘r’ notations denote forward and reverse direction of the reaction, respectively. The ‘$C_i$’ denote initial concentrations of a metabolite. The gray trajectories are for parameters that had little or no effect on the reversibility.
Figure S14: Sensitivity of $\text{CO}_2$–$\text{CH}_4$ carbon isotope fractionation to model parameters. Black lines represent results with the default parameters, purple and green lines represent a decrease or an increase by a factor of three to the default parameter, respectively, unless otherwise noted in the panel. For $K_M$ values, the ‘f’ and ‘r’ denote forward and reverse direction of the reaction, respectively. The ‘C_i’ denote initial concentrations of a metabolite. The KFFs used are the median values from the posterior distributions in fig. S3.
Figure S15: Sensitivity of hydrogen isotope fractionation to model parameters. Black lines represent results with the default parameters, purple and green lines represent a decrease or an increase by a factor of three to the default parameter, respectively, unless otherwise noted in the panel. For $K_M$ values, the ‘f’ and ‘r’ denote forward and reverse direction of the reaction, respectively. The ‘$C_i$’ denote initial concentrations of a metabolite. The KFFs used are the median values from the posterior distributions in fig. S3.
Figure S16: Posterior distributions of reverse kinetic isotope fractionation factors (KFFs). The posterior reverse KFF distributions are derived from the ratio $\alpha^- = \alpha^{\text{eq}} \times \alpha^+$, where $\alpha^{\text{eq}}$ is the temperature-dependent EFF of each reaction, and $\alpha^+$ is the forward KFF from distributions in fig. S3, for mesophilic (blue) and thermophilic (red) conditions. The values in the upper-left of the panels represent the median posterior KFF values for mesophilic (blue) and thermophilic (red) conditions. In simulations where the posterior distribution is similar to the prior (uniform) distribution we do not show these median values. Primary and secondary hydrogen isotope KFFs are denoted by superscripted ‘p’ and ‘s’ respectively.
Table S1: The Gibbs free energy of the reactions in hydrogenotrophic methanogenesis. We used the transformed Gibbs free energy ($\Delta G_r^0$), which is the Gibbs free energy at a particular pH and ionic strength (here at pH 7) (27). For redox reactions, we calculated $\Delta G_r^0$ of the half-reactions using the Nernst equation with $\Delta G_r^0 = -nF\Delta E^0$, where $n$ is the numbers of electrons transferred, $F$ is the Faraday constant and $\Delta E^0$ is the standard redox potential (table S4). We note that the temperature dependence of $\Delta G_r^0$ values is not available, other than that of the net methanogenic reaction.

| Enzyme | Reaction | $\Delta G_r^0$ (kJ mol$^{-1}$) | Ref./Source |
|--------|----------|-------------------------------|-------------|
| Fmd    | $4\text{H}_2(\text{aq}) + \text{CO}_2(\text{aq}) \rightleftharpoons \text{CH}_4(\text{aq}) + 2\text{H}_2\text{O}$ | $-188.8$ | † |
| Ftr    | $\text{CHO-MFR} + \text{H}_4\text{MPT} \rightleftharpoons \text{CHO-H}_4\text{MPT} + \text{MFR}$ | $-3.5$ | (95) |
| Mch    | $\text{CHO-H}_4\text{MPT} \rightleftharpoons \text{CH}≡\text{H}_4\text{MPT} + \text{H}_2\text{O}$ | $-4.2$ | (95) |
| Mtd    | $\text{CH}≡\text{H}_4\text{MPT}^+ + \text{F}_4\text{H}_2 \rightleftharpoons \text{CH}_2=\text{H}_4\text{MPT} + \text{F}_4\text{H}_2$ | $2.0$ | (95) |
| Mtr    | $\text{CH}_2=\text{H}_4\text{MPT}^+ + \text{H}_2(\text{aq}) \rightleftharpoons \text{CH}_3\text{H}_4\text{MPT} + \text{F}_4\text{H}_2$ | $-1.7$ | (95) |
| Mcr    | $\text{CH}_3\text{H}_4\text{MPT} + \text{HS-CoM} \rightleftharpoons \text{CH}_3\text{S-CoM} + \text{H}_4\text{MPT}$ | $-26.9$ | ¶ |
| Mvh/Hdr| $\text{H}_2(\text{aq}) + \text{CoM-S-S-CoB} + \text{F}_4\text{H}_2 \rightleftharpoons \text{HS-CoM} + \text{HS-CoB}$ | $-90.8$ | (93, 96) |

Abbreviations: F$_{420}$, coenzyme F$_{420}$; Fd, ferredoxin; H$_4$MPT, tetrahydromethanopterin; HS-CoB, coenzyme B; HS-CoM, coenzyme M; MFR, methanofuran; Fmd, formyl-MFR dehydrogenase; Ftr, formyltransferase; Mch, methenyl cyclohydrolase; Mtd, methylene-H$_4$MPT dehydrogenase; Mtr, H$_2$-dependent methylene-H$_4$MPT dehydrogenase; Mer, methylene-H$_4$MPT reductase; Mtr, methyl transferase, Mcr, methyl-CoM reductase; Frh, F$_{420}$ reducing hydrogenase; Mvh/Hdr, methylviologen hydrogenase/heterodisulfide reductase.

† Net methanogenesis at 60°C, calculated by Van’t Hoff’s Equation. Whenever possible, $\Delta G_r^0$ for individual reactions were at 60°C.

‡ Internally consistent value, inferred from summation of reactions 4 and 8 in this table.

¶ Reactions 6 and 7 each have an estimated $\Delta G_r^0$ of $-30 \pm 10$ kJ mol$^{-1}$ (with CH$_4$ in the gaseous phase). We allocate the exact $\Delta G_r^0$ of reactions 6 and 7 by splitting the remaining energy (i.e., the $\Delta G_r^0$ of the total pathway minus the sum of $\Delta G_r^0$ of reactions 1–5 and 8–9, denoted by $\Delta G_{\text{res}}^0$), by a factor $R_{\Delta G_r^0}$ with a default value of 0.4, such that $\Delta G_{\text{Mcr}}^0 = \Delta G_{\text{res}}^0 \times R_{\Delta G_r^0}$ and $\Delta G_{\text{Mtr}}^0 = \Delta G_{\text{res}}^0 \times (1 - R_{\Delta G_r^0})$. 
Table S2: Metabolic model kinetic parameters.

| Metabolite | \(V^*\) (mM s\(^{-1}\)) | \(K_M\) (mM) | Ref/Source Notes |
|------------|--------------------------|--------------|-----------------|
| **1. formyl-MFR dehydrogenase (Fmd): \(\text{CO}_2 + \text{MFR} + \text{Fd}_{\text{red}} \rightleftharpoons \text{CHO-MFR} + \text{Fd}_{\text{ox}}\)** |
| \(\text{CO}_2, \text{MFR}, \text{Fd}_{\text{red}}\) | 6.09 | (97) | Inferred from \(V^+/V^- = 25\) |
| \(\text{CHO-MFR}\) | 6.80 | (93) | Harmonic mean of two values |
| \(\text{Fd}_{\text{ox}}\) | 0.030 | (97, 98) | Estimate based on methylviologen as electron carrier |
| **2. formyl transferase (Ftr): \(\text{CHO-MFR} + \text{H}_4\text{MPT} \rightleftharpoons \text{CHO-H}_4\text{MPT} + \text{MFR}\)** |
| \(\text{CHO-MFR}\) | 28.53 | (99) | Harmonic mean of two values |
| \(\text{H}_4\text{MPT}\) | 0.050 | (100) | Estimate based on \(M. \text{barkeri}\) |
| \(\text{CHO-H}_4\text{MPT}, \text{MFR}\) | 0.005 | ‡ | |
| **3. methenyl cyclohydrolase (Mch): \(\text{CHO-H}_4\text{MPT} \rightleftharpoons \text{CH-H}_4\text{MPT}\)** |
| \(\text{CHO-H}_4\text{MPT}\) | 4.03 | (101) | |
| \(\text{CH-H}_4\text{MPT}\) | 0.148 | ‡ | Estimate based harmonic mean of two values from \(M. \text{barkeri} \) and \(M. \text{kandleri}\) |
| **4a. methylene dehydrogenase (Mtd): \(\text{CH-H}_4\text{MPT} + \text{F}_4\text{H}_2 \rightleftharpoons \text{CH}_2\text{-H}_4\text{MPT} + \text{F}_4\text{H}_2\)** |
| \(\text{CH-H}_4\text{MPT}\) | 14.69 | (104, 105) | Harmonic mean of two values |
| \(\text{F}_4\text{H}_2\) | 0.050 | (106) | |
| \(\text{CH}_2\text{-H}_4\text{MPT}\) | 0.016 | ‡ | |
| \(\text{F}_4\text{H}_2\) | 0.033 | (105) | |
| **4b. \(\text{H}_2\)-producing methylene dehydrogenase (Hmd): \(\text{CH-H}_4\text{MPT} + \text{H}_2 \rightleftharpoons \text{CH}_2\text{-H}_4\text{MPT}\)** |
| \(\text{CH-H}_4\text{MPT}\) | 45.65 | (107) | |
| \(\text{H}_2\) | 0.050 | (106) | |
| \(\text{CH}_2\text{-H}_4\text{MPT}\) | 0.150 | (108) | |
| **5: methylene reductase (Mer): \(\text{CH}_2\text{-H}_4\text{MPT} + \text{F}_4\text{H}_2 \rightleftharpoons \text{CH}_3\text{-H}_4\text{MPT} + \text{F}_4\text{H}_2\)** |
| \(\text{CH}_2\text{-H}_4\text{MPT}\) | 6.09 | (109) | Estimate based on \(M. \text{marburgensis}\) |
| \(\text{F}_4\text{H}_2\) | 0.300 | (109) | Estimate based on \(M. \text{marburgensis}\) |
| \(\text{CH}_3\text{-H}_4\text{MPT}\) | 0.003 | (109) | |
| \(\text{F}_4\text{H}_2\) | 0.024 | ‡ | |
| **Table continued on next page.**
| Metabolite | $K_M$ (mM) | Ref/Source | Notes |
|------------|------------|------------|-------|
| $\text{CH}_3\text{-H}_4\text{MPT}$ | 0.135 | (111) | Estimate based on $M.\ acetivorans$ |
| $\text{HS-CoM}$ | 0.277 | (112) | Estimate based on $M.\ acetivorans$ |
| $\text{CH}_3\text{-S-CoM}$ | 0.559 | ‡ | |
| $\text{H}_2\text{MPT}$ | 0.098 | ‡ | |
| $\text{CH}_3\text{-S-CoM}$ | 0.821 | (0.280 †) | Harmonic mean of three values |
| $\text{HS-CoB}$ | 0.204 | (0.075 †) | Harmonic mean of three values |
| $\text{CH}_4$, $\text{CoM-S-S-CoB}$ | 0.110 | | |
| $\text{H}_2$ | 0.012 | (117) | |
| $\text{F}_{420}$ | 0.036 | (117) | |
| $\text{F}_{420}\text{H}_2$ | 0.012 | ‡ | |

| Metabolite | $K_M$ (mM) | Ref/Source | Notes |
|------------|------------|------------|-------|
| $\text{H}_2$ | 0.030 | (28, 118) | |
| $\text{CoM-S-S-CoB}$ | 0.145 | (118) | Estimate based on $M.\ barkeri$ |
| $\text{Fd}_{\text{ox}}$ | 0.010 | (119) | |
| $\text{HS-CoM}$ | 0.200 | (120) | |
| $\text{HS-CoB}$ | 0.200 | (120) | |
| $\text{Fd}_{\text{red}}$ | 0.075 | (119) | |

‡ Median values drawn from the posterior distributions (see also table S5).
† Values that were for simulating methanogenesis in $\text{H}_2$-limited conditions.
Table S3: Metabolic model results compared to available measurements of metabolite concentrations and the oxidation state of electron carriers (represented as percent reduced). The oxidation state of CoB is calculated by $\frac{[\text{HS-CoB}]}{[\text{CoM-SS-CoB}] [\text{HS-CoB}]}$, and the oxidation state of $F_{420}$ by $\frac{[F_{420}H_2]}{[F_{420}H_2][F_{420}]}$. The data were obtained at $[H_2] \approx 10 \mu M$, and the experiments were conducted on M. thermoautotrophicus at 60 °C in batch cultures, unless otherwise noted.

| Parameter | Observations | Model results | Figure | Ref. |
|-----------|--------------|---------------|--------|------|
| CoB % reduced | 0.5%–55% | 2%–20% | S1L | (34) |
| CHO-MFR/MFR | 1–10 | 0.1–2.6 | S1A,G | (34) |
| HS-CoM | 4–200 µM | 3–200 µM | S1I | (34) |
| $F_{420}$ % reduced | 61%–90% | 90% | S1K | (30) |
| (H$_2$ ≈ 40 µM) | | | | |
| $F_{420}$ % reduced | 17% at [H$_2$] = 0.7 µM | 17% at [H$_2$] = 7 µM | | |
| (cell suspension) | 29% at [H$_2$] = 2 µM | 29% at [H$_2$] = 20 µM | S1K | (30) |
| | 50% at [H$_2$] = 3 µM | 50% at [H$_2$] = 40 µM | | |
| | 67% at [H$_2$] = 7 µM | 67% at [H$_2$] = 80 µM | | |

Table S4: The standard redox potentials ($E^0$) of reactions that were used to determine $\Delta G_r^0$ (see table S1).

| Reaction | $E^0$ (mV) | Ref. |
|----------|------------|------|
| $2H^+ + 2e^- \rightarrow H_2(aq)$ | $-505$ | (27) |
| $F_{420} + 2e^- \rightarrow F_{420}H_2$ | $-360$ | (32) |
| CoM-S-S-CoB + 2e$^-$ $\rightarrow$ HS-CoB + HS-CoM | $-143$ | (96) |
| $\text{Fd}_{\text{ox}} + 2e^- \rightarrow \text{Fd}_{\text{red}}$ | $-400$ | (27) |
| $\text{CO}_2 + \text{MFR} + 2e^- \rightarrow \text{CHO-MFR} + \text{H}_2\text{O}$ | $-497$ | (98) |
Table S5: Statistics of prior distributions for missing $K_M$ parameters. Each $K_M$ parameter was drawn from a normal distribution with a mean and standard deviation on a logarithmic scale. The prior distributions are based on the observation that $K_M$ values of specific compounds from different enzymes are normally distributed (78). For each compound we compiled the available $K_M$ in the BRENDA database (79), and used their associated distributions. In some cases ($K_M$ for MFR, CHO-H$_4$MPT, CH$_3$-H$_4$MPT, CH$_4$ and CoM-S-S-CoB) there were insufficient data to generate a prior distribution, and we used instead the distribution of all $K_M$ values (78). The prior distributions were used to estimate the model-observation mismatch, and were weighted by the the inverse of the squared sum of squared errors ($1/SSE^2$) to generate the posterior distributions (see Methods). The full prior and posterior distributions are in fig. S2.

| Enzyme | Metabolite(s) | Prior Median ($\mu$M) | Prior Standard deviation log$_{10}$(µM) | Posterior Median ($\mu$M) |
|--------|---------------|------------------------|-----------------------------------------|--------------------------|
| Ftr    | CHO-H$_4$MPT, MFR | 123$^a$ | 1.24 | 5 |
| Mch    | CHO-H$_4$MPT | 123$^a$ | 1.24 | 148 |
| Mtd    | $F_{420}H_2$ | 15 | 0.47 | 16 |
| Mer    | CH$_3$-H$_4$MPT | 123$^a$ | 1.24 | 23 |
| Mtr    | CH$_3$-SCoM | 692 | 2.02 | 560 |
| Mtr    | H$_4$MPT | 105 | 0.60 | 98 |
| Mcr    | CH$_4$, CoM-SS-CoB | 123$^a$ | 1.24 | 110 |
| Frh    | $F_{420}H_2$ | 15 | 0.47 | 11 |

$^a$ Distributions that are based on all available $K_M$ measurements (78).

Table S6: Ranges of H$_2$ concentrations reported in natural environments.

| Environment   | [H$_2$] (nM) | Ref. |
|---------------|--------------|------|
| Freshwater sediments | 20 | (67, 121) |
| Marine sediments | | |
| Shallow | 0.5–5 | (122–124) |
| Deep | 100 | (125) |
| Estuaries | 10–100 | (126) |
| Rice paddies | 15–60 | (127) |
Table S7: Estimated environmental csMR based on the relations between measured cell densities and bulk methanogenesis rates in shallow and deep marine sediments.

| Source                          | Max. sediment depth (m) | Temp. (°C) | Cell density (cm\(^{-3}\)) | bMR (nmol cm\(^{-3}\) d\(^{-1}\)) | csMR (fmol cell\(^{-1}\) d\(^{-1}\)) | Ref. |
|--------------------------------|-------------------------|------------|-----------------------------|-----------------------------------|-------------------------------------|------|
| Hoehler et al., 1994 (W)       | 0.4                     | 6          | 4.3 × 10\(^7\)             | 1.1\(^{\dagger}\)§               | 2.6 × 10\(^{-2}\)                   | (128) |
| Hoehler et al., 1994 (S)       | 0.4                     | 28         | 4.3 × 10\(^7\)             | 32\(^{\dagger}\)§               | 7.5 × 10\(^{-1}\)                   | (128) |
| Claypool et al., 2006          | 120                     | 10         | 2.9 × 10\(^5\)             | 7.2 × 10\(^{-4}\)               | 2.4 × 10\(^{-3}\)                   | (129) |
| Sivan et al., 2007             | 200                     | 10         | 1.3 × 10\(^5\)             | 2.3 × 10\(^{-4}\)               | 1.8 × 10\(^{-3}\)                   | (130) |
| Parkes et al., 2007            | 4                       | 16         | 5.4 × 10\(^7\)¶            | 4.7 × 10\(^{-1}\)\(^{\dagger}\) | 8.7 × 10\(^{-3}\)                   | (131) |
| Beulig et al., 2018            | 1.4                     | 8          | 9.5 × 10\(^5\)¶            | 4.8 × 10\(^{-1}\)\(^{\dagger}\) | 5.0 × 10\(^{-1}\)                   | (132) |
| Chuang et al., 2018            | 40                      | 5          | 6.8 × 10\(^5\)¶            | 4.1 × 10\(^{-4}\)               | 6.0 × 10\(^{-4}\)                   | (133) |
| Zhuang et al., 2018‡           | 6                       | 13         | 7.9 × 10\(^6\)             | 7.6 × 10\(^{-1}\)               | 9.5 × 10\(^{-2}\)                   | (124) |
| Zhuang et al., 2018§           | 5                       | 13         | 7.9 × 10\(^6\)             | 1.0 × 10\(^{-3}\)               | 1.3 × 10\(^{-4}\)                   | (124) |

Where not measured directly, cell densities were estimated based on their dependence on depth (90, 91) and assuming that of these cells 12% are Archaea in open-ocean sites and 40% in ocean margin sites (92), and that 50% of Archaea are methanogens (91).

\(^{\dagger}\)14\(^{\circ}\)C tracer measurements.

\(^{\dagger}\) Tracer measurements were done for both gross methane production and consumption, and the results are shown as a net rate, which is calculated as the difference between the measured forward and backward rates.

\(^{\dagger}\) Direct cell counts.

\(^{\dagger}\) Rhone River pro-delta.

\(^{\dagger}\) Gulf of Lion shelf.
### Table S8: Physical parameters used in the bio-isotopic model.

| Parameter                          | Value    | Units          | Ref. | Notes                      |
|------------------------------------|----------|----------------|------|----------------------------|
| Cell volume                        | 2.3      | fL/cell        | (134)| *M. thermoautotrophicus*   |
| Cell mass                          | $10^{-12}$ | gdw/cell      | (89) | *M. thermoautotrophicus*   |
| Dry weight protein content         | 41       | %              | (135)| *M. bryantii*              |
| Membrane thickness                 | 0.5      | nm             |      |                            |
| Diffusivity constant               | $2.9\times10^{-9}$ | m$^2$ s$^{-1}$ | (75) |                            |
| $K_H$ (H$_2$)                      | $6.46\times10^{-9}$ | mol L$^{-1}$ Pa$^{-1}$ | (136)| Henry’s Law constant at 60$^\circ$C |
| $K_H$ (CO$_2$)                     | $1.42\times10^{-7}$ | mol L$^{-1}$ Pa$^{-1}$ | (136)| Henry’s Law constant at 60$^\circ$C |
| $K_H$ (CH$_4$)                     | $7.97\times10^{-9}$ | mol L$^{-1}$ Pa$^{-1}$ | (136)| Henry’s Law constant at 60$^\circ$C |
| Total ferredoxin (Fd)$^\dagger$   | 5        | mM             |      |                            |
| Total coenzyme F$_{420}$           | 0.5      | mM             | (35, 135, 137)|                          |
| Total coenzyme B (HS-CoB)$^\dagger$| 6        | mM             | (34) |                            |
| Total methanofuran (MFR)$^\dagger$ | 1.8      | mM             | (34) |                            |

$^\dagger$ Total Fd and F$_{420}$ include the oxidized and reduced forms, total coenzyme B includes HS-CoB and CoM-S-S-CoB, and total MFR includes MFR and CHO-MFR. The model is insensitive to initial concentrations of non-coupled metabolites.
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