Stable carbon isotope fractionation by methylotrophic methanogenic archaea

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ABSTRACT

In natural environments methane is usually produced by aceticlastic and hydrogenotrophic methanogenic archaea. However, some methanogens can use C-1 compounds such as methanol as substrate. To determine the contribution of individual substrates to methane production, the stable isotope values of the substrates and the released methane are often used. Additional information can be obtained by using selective inhibitors (e.g. methyl-fluoride a selective inhibitor of acetoclastic methanogenesis). We studied stable carbon isotope fractionation during the conversion of methanol to methane in *Methanosarcina acetivorans*, *Methanosarcina barkeri*, and *Methanolobus zinderi*, and generally found large fractionation factors (-83‰ to -72‰). We further tested whether methyl-fluoride impairs methylotrophic methanogenesis. Our experiments showed that even though a slight inhibition occurred, the carbon isotope fractionation was not affected. Therefore, the production of isotopically light methane observed in the presence of methyl fluoride may be due to the strong fractionation by methylotrophic methanogens and not only by hydrogenotrophic methanogens as previously assumed.
INTRODUCTION

Biogenic methane release into the atmosphere is based on methane production by methanogenic archaea. The main substrates for methanogenesis are either acetate (acetoclastic methanogenesis) or hydrogen plus carbon dioxide (hydrogenotrophic methanogenesis). To a minor extent C-1 compounds such as methanol, trimethylamines or dimethyl sulfide can also serve as methanogenic substrates (35). A number of studies indicate that less than 5-10% of total methanogenesis originates from methanol (24-25, 29, 31, 38, 44). Therefore most studies concerning environmental methane production just focus on the two main methanogenic pathways (acetoclastic and hydrogenotrophic). To distinguish them in environmental studies, methyl fluoride is widely used as a selective inhibitor of acetoclastic methanogenesis (23, 27). However, it is presently unclear how methylotrophic methanogens would react to methyl fluoride inhibition. If not affected by methyl fluoride, methylotrophic methanogens may contribute to the isotopic signal of methane, erroneously believed to be produced exclusively from CO₂ reduction.

Instead of specific inhibition, an alternative technique to differentiate between the substrates of methanogenesis is the determination of the difference between the stable carbon isotopes in the methanogenic substrates and the methane in environmental settings. It is believed that the so called isotope fractionation factor (sometimes also called enrichment factor) ε, is a rather characteristic value of the individual pathways involved in carbon transformation (8, 16). A number of recent studies concerning the isotopic signature in methane production focused on the two main pathways. These studies showed a rather small fractionation range of -35‰ to about -5‰ for acetoclastic methanogenesis (21, 33, 41) and a comparatively broad range of fractionation of -79‰ to about -28‰ for hydrogenotrophic methanogenesis (34, 41). However, the very strong fractionation during hydrogenotrophic methanogenesis is probably caused by the restricted metabolism of methanogens in the late-logarithmic or stationary growth phase (4, 41) or by the low energy status of the cells (34).
Compared to aceticlastic and hydrogenotrophic methanogenesis little is known about the carbon isotope fractionation during methylotrophic methanogenesis. A comparative study of *Methanosarcina barkeri* grown on different substrates found the strongest carbon isotope fractionation when cells were grown on methanol, i.e., for acetate \( \epsilon = -22\%_o \), for \( \text{H}_2/\text{CO}_2 \) \( \epsilon = -49\%_o \) and for methanol \( \epsilon = -79\%_o \) (26). In accordance with that, a strong fractionation of \( \epsilon = -65\%_o \) to \(-81\%_o \) was reported for a methylotrophic enrichment culture (36).

The occurrence of methanol in the environment is mainly based on the turnover of methylated compounds of the plant cell wall, the degradation of pectin and lignin. While around 100 Tg y\(^{-1}\) of methanol are released into the atmosphere from leaves of plant vegetation, the potential source of methanol from pectin degradation of dead plants in soil was estimated to be 800 Tg y\(^{-1}\) (18). Therefore, methanol may be expected to be a common metabolite in soil environments. Especially in anoxic environments, where the degradation of plant litter is a concerted process of several bacterial guilds, methanol was found to be produced during the degradation of pectin (15, 37) and lignin (43). However methanol can be rapidly consumed by many different microorganisms, methylotrophic methanogens being only one of them.

Even though the conversion of methanol may contribute only a relatively small percentage to total methane production, the rather large isotopic fractionation may nevertheless strongly affect the carbon isotopic readings of the produced methane. Since carbon isotope fractionation has so far only been studied in few methylotrophic methanogens (only *Methanosarcina barkeri*) involving only a few data points, we decided to investigate three methylotrophic methanogens: *Methanosarcina barkeri*, *Methanosarcina acetivorans* and *Methanolobus zinderi* (an obligate methylotroph). Our determination of the fractionation factor was independently based on substrate and product values. Likewise it has been argued (but never tested), that the isotopic signal of methane under methyl fluoride inhibition can exclusively be assigned to the hydrogenotrophic methanogens (13). Therefore we further
investigated how methyl fluoride affects the carbon isotope fractionation of M. barkeri and M. zinderi.

MATERIALS AND METHODS

Cultures and growth conditions. Pure cultures of Methanosarcina acetivorans (Type Strain, DSM 2834), Methanosarcina barkeri (Type Strain, DSM 800) and Methanolobus zinderi (Type Strain, DSM 21339) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). All cultures were grown under N2/CO2 (80:20) in 120 ml serum bottles (Ochs, Bovenden-Lengeln, Germany) filled with 50 ml medium and incubated without shaking at 37°C.

Methanosarcina acetivorans was grown using a medium with the following composition (in g l⁻¹): NaCl, 23.4; MgSO₄ • 7H₂O, 9.4; Na₂CO₃, 5.0; yeast extract, 1.0; NH₄Cl, 1.0; KCl, 0.9; CaCl₂ • 2H₂O, 0.14; Na₃HPO₄, 0.6; cysteine-HCl • H₂O, 0.5; Na₂S • 9H₂O, 0.045. In addition 12.5 ml methanol as substrate and 1 ml trace element solution was added. The trace element solution contained (in g l⁻¹): Nitrilotriacetic acid, 1.5; FeSO₄ • 7H₂O, 0.1; CoCl₂ • 6H₂O, 0.1; ZnSO₄ • 7H₂O, 0.1; CuSO₄ • 5H₂O, 0.0087; AlCl₃ • 6H₂O, 0.01; Na₂MoO₄ • 2H₂O, 0.01; NiCl₂ • 6H₂O, 0.03; Na₂SeO₄, 0.019.

Methanosarcina barkeri was grown using a modified medium with the following composition (in g l⁻¹ unless otherwise noted): K₂HPO₄, 0.25; KH₂PO₄, 0.23; NH₄Cl, 0.5; MgSO₄ • 7H₂O, 0.5; NaCl, 2.25; FeSO₄ • 7H₂O, 0.002; yeast extract, 2.0; casitone, 2.0; NaHCO₃, 0.85; cysteine-HCl • H₂O, 0.5; Na₂S • 9H₂O, 0.045. In addition 10.0 ml methanol as substrate, 1.0 ml trace element solution SL-10 (6) and 10 ml vitamin solution (45) was added. The medium used for cultivation of Methanolobus zinderi contained the following (in g l⁻¹): KCl, 0.33; MgCl₂ • 6H₂O, 4.0; MgSO₄ • 7H₂O, 3.5; NH₄Cl, 0.25; K₂HPO₄, 0.14; NaCl, 18.0; Fe(NH₄)₂(SO₄)₂ • 6H₂O, 0.002; Na-acetate, 1.0; yeast extract, 2.0; trypticase, 2.0; NaHCO₃, 5.0; cysteine-HCl • H₂O, 0.5; Na₂S • 9H₂O, 0.045. In addition 5 ml methanol as
substrate, 1.0 ml of the for *M. acetivorans* used trace element solution and 10 ml vitamin solution (45) was added.

For the experiments, the culture bottles were inoculated with 1 ml of a growing bacterial culture. Samples from the headspace were removed by a gas-tight syringe to determine concentration and carbon isotopic signature of methane and carbon dioxide. The liquid phase was analyzed for concentration and carbon isotopic signature of methanol. The pH of the culture liquid was also analyzed. Experiments were usually performed in triplicate. Methyl fluoride was added to the headspace as percentage (v/v) of the bottle volume (120ml).

**Chemical and isotopic analysis.** The concentration of CH₄ and CO₂ in gas samples was analyzed during the stable isotope analysis of ^13^C/^12^C using a gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) system (Thermo Fisher Scientific, Bremen, Germany). The principle of operation was described by Brand (5). The CH₄ and CO₂ in the gas samples (20-100 μL) were first separated in a Trace GC Ultra gas chromatograph using a Pora PLOT Q column (27.5 m length, 0.32 mm i.d.; 10 μm film thickness; Varian, Palo Alto, CA, USA) at 30 °C and Helium (99.996% purity; 2.6 ml/min) as carrier gas. After conversion of CH₄ to CO₂ in the GC Isolink 1030, the isotope ratio of ^13^C/^12^C was analyzed in the IRMS (Delta V Advantage). The isotope reference gas was CO₂ (99.998% purity; Air Liquide, Duesseldorf, Germany), calibrated with the working standard methylstearate (Merck). The latter was intercalibrated at the Max Planck Institute for Biogeochemistry, Jena, Germany (courtesy of W. A. Brand) against NBS 22 and USGS 24, and reported in the delta notation versus Vienna Pee Dee Belemnite:

\[
\delta^{13}C = 10^3 \left( \frac{R_{sa}}{R_{st}} - 1 \right) \text{ [%o]} \quad (1)
\]

with \( R = ^{13}C/^{12}C \) of sample (sa) and standard (st), respectively.
Isotopic analysis and quantification of methanol was performed in liquid samples (1 µl) using a second gas chromatograph combustion isotope ratio mass spectrometer (GC-C-IRMS) system (Thermo Electron, Bremen, Germany). A similar method was used by Conrad and Claus (10). The liquid sample was first evaporated in the injector at 240°C. Then, the methanol was separated in a Hewlett Packard 6890 GC using a forte BP20 column (25 m length, 0.32 mm i.d.; 0.5 µm film thickness; SGE, Ringwood, Victoria, Australia) with the following temperature program: 50°C, 10°C min^-1 to 140°C, 20°C min^-1 to 220°C, 220°C for 1 min. Helium (99.996% purity; 2.6 ml/min) was used as carrier gas. After conversion of methanol to CO₂ in the Standard GC Combustion Interface III, the isotope ratio of ^13C/12C was analyzed in the IRMS (Finnigan MAT Delta plus). Isotope reference gas was CO₂ calibrated as described above.

Calculations. Fractionation factors for a reaction $A \rightarrow B$ are defined after Hayes (22) as follows:

$$\alpha_{A/B} = (\delta_A + 1000)/(\delta_B + 1000)$$  \hspace{1cm} (2)

also expressed as $\epsilon_{A/B} = 10^2(1 - \alpha_{A/B})$. The isotope enrichment factor $\epsilon$ was determined as described by Mariotti et al. (30) from the residual reactant, calculated as

$$\delta_r = \delta_i + \epsilon \ln(1-f)$$  \hspace{1cm} (3)

and from the product formed, calculated as

$$\delta_p = \delta_i - \epsilon (1-f)[\ln(1-f)]/f$$  \hspace{1cm} (4)
where δ_r is the isotope composition of the reactant at the beginning, δ_r and δ_p are the isotope compositions of the residual methanol and the pooled CH₄, respectively, at the instant when f was determined. f is the fractional yield of the products based on the consumption of methanol (0 < f < 1). An alternative way to calculate the fractional yield purely on the base of the measured δ-values was promoted by Gelwicks et al. (20):

\[ f_{\text{delta}} = (\delta_{\text{r}} - \delta_{\text{r}})/(\delta_{\text{p}} - \delta_{\text{r}}) \]  

(5)

Linear regression of δ_r against ln(1-f) and of δ_p against (1-f)[ln(1-f)]/f gives ε for substrate and product data as the slope of best-fit lines.

RESULTS

Methylo trophic methanogenesis

Even though the time needed for growth and complete consumption of methanol was different in all three methylotrophic archaea (M. acetivorans, M. barkeri and M. zinderi).

Methanol was finally completely consumed during production of methane and CO₂. The stoichiometry of equation (6) was observed in all three strains (Fig. 1, Table 1).

\[ 4 \text{CH}_3\text{OH} \rightarrow 3 \text{CH}_4 + \text{CO}_2 + 2 \text{H}_2\text{O} \]  

(6)

Figure 1 shows the CO₂ measured in the headspace of the culture vessel, since it has been shown that CO₂ rather than bicarbonate is the active substrate of methanogenesis (17, 40, 42). The total amount of inorganic carbon was higher than that in the headspace due to dissolved CO₂ and bicarbonate in the medium (initial total inorganic carbon: M. barkeri: 1.1 mmol; M. acetivorans: 3.0 mmol; M. zinderi: 3.6 mmol). The growth was paralleled by a slight decrease
of the pH in *M. zinderi* (7.0 → 6.7) and *M. acetivorans* (7.1 → 6.4). By contrast, there was a relative large decrease of the pH in *M. barkeri* (6.9 → 5.2).

Consumption of $^{12}$C-methanol was preferred causing an enrichment of the heavier isotope $^{13}$C in the residual methanol (Fig. 1). Consequently, the initial CH$_4$ produced from methanol was relatively depleted in $^{13}$C, but then increased with time. The initially high $^{13}$C value of CH$_4$ in the cultures may have resulted from the inoculation by transfer of dissolved CH$_4$ or from methane produced from intracellularly stored carbon. Carbon dioxide first became slightly depleted in $^{13}$C but then became enriched with time resulting in relatively heavy CO$_2$ at the end of the reaction. However, CO$_2$ was not used for determination of isotope fractionation due to the high bicarbonate background.

The isotopic signatures recorded in the cultures of the three methanogenic strains are summarized in Fig. 2, where they are plotted as a function of the fractional yield $f$ (values taken from supplementary Table S1). The data showed good agreement between the different strains. For regression analysis data of all replicates of each strain were plotted together in Mariotti plots (Fig. 3). Fractionation factors were determined from the fractional regression of the $\delta^{13}$C of both, the substrate ($\delta$methanol) and the product ($\delta$CH$_4$). All three strains showed similar fractionation factors, ranging between -83.4‰ and -71.6‰ (the isotopic fractionation factors of the individual replicates can be found in supplementary Table S1). Lacking the isotopic signatures for low methanol concentrations (detection limit: ~2.5mM, 125µmol), we could not cover the whole range of substrate turnover. Assuming complete conversion of methanol to CH$_4$ and CO$_2$, the regression of methane was forced through $\delta_{\text{CH}_3\text{OH}}$ at time zero ($t_0$). The initial isotopic signatures of methane, which were apparently affected by methane carried over during inoculation, were not taken into account for the regression analysis (gray values in supplementary Table S1).

**Effect of methyl fluoride**
M. barkeri and M. zinderi were grown on methanol in the presence of 0% to 3% methyl fluoride. For all M. barkeri incubations the overall growth performance (supplementary Fig. S1A), maximal methane production rates (Table 2), carbon flow and isotopic signature of substrate and product (Fig. 4A) were similar to the uninhibited samples. A slightly different picture was obtained for the incubations of M. zinderi: These incubations showed a prolonged lag phase under increasing methyl-fluoride concentrations (up to five days; supplementary Fig. S1B) paralleled by a reduced maximal methane production rate (Table 2). However the isotopic signature was not impaired by the presence of methyl-fluoride (Fig.4B).

Most importantly, the isotopic fractionation of both strains was unaffected by the presence of methyl fluoride (Table 2, details in Table S2A/B). The average stable isotope fractionation factors during methanogenic methanol conversion ranged between -73.6‰ and -79.9‰ for M. barkeri and -74.2‰ and -82.4‰ for M. zinderi.

DISCUSSION

Methylotrophic methanogenesis

It is generally accepted that only a minor portion of the methane released from the environment originates from methylotrophic methanogenesis. However, this pathway might significantly contribute to the isotopic signature of total methane, since carbon isotopes seem to be strongly fractionated in this pathway. Krzycki et al. (26) obtained an fractionation of $\varepsilon = -74.8‰$ to -72.5‰ in Methanosarcina barkeri. Londry et al. (28) found a slightly lower value of $\varepsilon = -83.4‰$ in Methanosarcina barkeri. Both studies were based on initial and end point measurements and did not monitor substrate consumption over time. However, our results using a closed system confirmed the previous results for M. barkeri by recording $\varepsilon_{\text{methanol}} = -73.5‰$ and $\varepsilon_{\text{CH}_4} = -76.1‰$. In another Methanosarcina species, M. acetivorans, we recorded similar isotopic fractionation factors, $\varepsilon_{\text{methanol}} = -72.0‰$ and $\varepsilon_{\text{CH}_4} = -71.6‰$, and the fractionation values for Methanolobus zinderi were with $\varepsilon_{\text{methanol}} = -83.4‰$ and $\varepsilon_{\text{CH}_4} = -77.9‰$. 
only slightly lower than in the two _Methanosarcina_ spp. In summary, we found that methylotrophic methanogens indeed fractionate carbon isotopes very strongly during the methanogenic conversion of methanol, and have ε values covering a relatively narrow range of around -83‰ to -72‰.

Among methanogenic archaea utilization of methylated substrates is restricted to members of the family _Methanosarcinaceae_. The only exception are species of the genus _Methanosphaera_, which can use H₂ to reduce methanol to CH₄ (3). While both _Methanosarcina_ species used in this study have a broad substrate range and can produce methane from many different substrates (e.g., from acetate, H₂/CO₂, methanol, methylamines and methylated sulfides) (28), _Methanolobus zinderi_ is an obligate methylotroph able to use only methylated compounds (14). This difference in substrate usage may in part be responsible for the observed differences in the fractionation factor.

Comparing the fractionation factors expressed during CH₄ production from the three methanogenic substrates acetate, H₂/CO₂ and methanol, it is obvious that they each cover a different range (Table 3). While acetoclastic methanogenesis is generally associated with the weakest fractionation of ε = -35‰ to -9‰ (21, 33, 41), hydrogenotrophic methanogenesis exhibits a broad range of fractionation factors, ε = -79‰ to -28‰ (41). However, methylotrophic methanogenesis has the strongest fractionation, ε = -83‰ to -72‰. These differences in the fractionation factors may hence be useful to discriminate the different methanogenic pathways in environmental studies.

The biochemical processes underlying the three methanogenic pathways (Fig. 5) show that the conversion of methanol to methane involves only two enzymes (methanol:coenzyme M methyltransferase and methyl-coenzyme M reductase) while the cleavage of acetate depends on three and the reduction of CO₂ on seven enzymes. All three pathways share the final step (methyl-coenzyme M reductase). The only distinctive enzyme in methanogenic conversion of methanol is the methanol:coenzyme M methyltransferase, which must be responsible for the
very strong fractionation, if the fractionation is a matter of enzyme function. However, it is
more likely that the strong fractionation originates in the branching of the methanol pathway.
The electrons needed to reduce methanol to methane originate from the concomitant oxidation
of methanol to CO$_2$ by reverting the hydrogenotrophic pathway. Therefore it is possible that
mostly the light $^{12}$C is converted to CH$_4$, while relatively heavy carbon is converted to CO$_2$ or
is left as residual methanol. Indeed, the CO$_2$ produced from methanol was not as depleted in
$^{13}$C as the CH$_4$. If we extrapolate the isotopic signature of the produced CO$_2$ a signature as
low as $\delta_{\text{CO}_2 \text{new formed}} = -60\%$ and an apparent fractionation of $\epsilon_{\text{CO}_2-\text{methanol}} \approx -20 \%$ can be
obtained.

Likewise the strong fractionation (ranging from $-73\%$ to $-53\%$) of other methylated
compounds like trimethylamines (Table 3) which differ just in the first enzyme needed to
activate the methyl-group may be explained by the disproportionation of the methyl
compound to CO$_2$ and CH$_4$.

**Methyl fluoride and environmental implications**

Theoretically, acetoclastic methanogenesis should account for 67% of total
methanogenesis, when polysaccharides are completely degraded to CO$_2$ and CH$_4$ (7-8). The
residual CH$_4$ production would be due to hydrogenotrophic methanogenesis and the isotopic
signature of the produced CH$_4$ would suggest the relatively strong fractionation factors
involved in CH$_4$ production from H$_2$/CO$_2$. In many studies a concentration of 2% methyl
fluoride is used to inhibit acetoclastic methanogenesis (9, 11-13, 27). Applying this technique
to various methanogenic aquatic sediments, fractionation factors for hydrogenotrophic
methanogenesis were found to be in a range of $-85\%$ to $-57\%$ (9, 11, 13). Compared to these
data, the apparent fractionation factors under methyl-fluoride inhibition are lower by $-33$ to $-9\%$ when compared to the uninhibited samples. Even the uninhibited samples fractionate in
general stronger than the fractionation factors reported for pure cultures of hydrogenotrophic methanogens (compare Table 3).

One possible reason for this observation is that methylotrophic methanogenesis contributes to CH$_4$ production in the presence of methyl fluoride, thus causing a stronger apparent fractionation. However, this option can only be relevant if methylotrophic methanogenesis is not inhibited by methyl fluoride, and if stable carbon isotope fractionation is not affected by methyl fluoride. Our experiments proved that methyl fluoride had indeed no effect on the isotopic fractionation of CH$_4$ production from methanol. Although a certain inhibition of methane production was found for *M. zinderi*, inhibition was not observed in *M. barkeri*. It is worth noting that for *Methanolobus tylorii* a growth limiting effect of methyl fluoride has been observed for 3.4% but not for 1.7% methyl fluoride (32).

It therefore is possible that contribution of methylotrophic methanogenesis in the presence of methyl fluoride may affect the resulting isotopic signature of CH$_4$. Let us assume that 33% of methanogenesis originate from H$_2$/CO$_2$ (7) and that methanol contributes up to 10% (10). In the presence of methyl fluoride methylotrophic methanogenesis could contribute roughly 30% to total CH$_4$ production. Under these conditions the released methane would be on average 10‰ lighter when methylotrophic methanogens are active. Nevertheless due to the large range of fractionation reported for hydrogenotrophic methanogens the contribution of the methylotrophic pathway to the released methane would still be hard to judge in an environmental system. Future studies with methylotrophic archaea grown mixotrophically on varying rations of H$_2$/CO$_2$ and methanol could be used to further constrain the contribution of methanol to the isotopic signature of methane.

**Conclusion**

Our results showed that three different species of methylotrophic methanogenic archaea exhibited similar fractionation factors for the methanogenic conversion of methanol, and that
these fractionation factors were much stronger than those reported for aceticlastic or hydrogenotrophic methanogenesis pure cultures grown under optimal substrate conditions. Hence, even though the contribution of methanol to total methane production may be limited in the environment, methanol may nevertheless significantly affect the carbon isotopic signature of the produced CH₄. Since our study showed that methyl fluoride did not affect the fractionation of methane produced from methanol, methylotrophic methanogenesis may affect the carbon isotopic signature of the produced CH₄ even in the presence of methyl fluoride when acetoclastic methanogenesis is inhibited. The carbon isotopic signature of CH₄ under these conditions may thus not only be due to hydrogenotrophic methanogenesis but may in addition be affected to a larger extent by methylotrophic methanogens than previously anticipated.

ACKNOWLEDGEMENTS

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38. **Singh, N., M. M. Kendall, Y. T. Liu, and D. R. Boone.** 2005. Isolation and characterization of methylotrophic methanogens from anoxic marine sediments in


Table 1: Carbon recovery (based on measured initial methanol) and isotope enrichment factors for methanol and methane during methylotrophic Methanogenesis by Methanosarcina acetivorans, Methanosarcina barkeri and Methanolobus zinderi (values for isotopic calculation taken from supplementary Table S1). Note that carbon recovery of M. zinderi was highest. This may be due to the acetate in the medium (1g l⁻¹) which is not needed for energy metabolism but for cell growth (14).

<table>
<thead>
<tr>
<th></th>
<th>used H₃COH [mmol]</th>
<th>produced CH₄ [mmol]</th>
<th>carbon recovery [%]</th>
<th>εCH₃OH [%]</th>
<th>εCH₄ [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. acetivorans</td>
<td>17.14 ± 0.5</td>
<td>9.94 ± 1.2</td>
<td>77.32 ± 6.7</td>
<td>-72.0 ± 1.5</td>
<td>-71.6 ± 1.2</td>
</tr>
<tr>
<td>M. barkeri</td>
<td>12.57 ± 0.5</td>
<td>7.55 ± 0.3</td>
<td>80.07 ± 1.0</td>
<td>-73.5 ± 0.3</td>
<td>-76.1 ± 1.1</td>
</tr>
<tr>
<td>M. zinderi</td>
<td>5.85 ± 0.2</td>
<td>3.93 ± 0.3</td>
<td>89.69 ± 2.6</td>
<td>-83.4 ± 0.5</td>
<td>-77.9 ± 1.8</td>
</tr>
</tbody>
</table>
Table 2: Highest CH₄ production rates (maximal slope of methane concentration over time) and calculated fractionation factors for Methanosarcina barkeri and Methanolobus zinderi with and without methyl fluoride (values for isotopic calculation taken from supplementary Table S2).

<table>
<thead>
<tr>
<th>M. barkeri</th>
<th>M. zinderi</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeF</td>
<td>max. prod. [mmol d⁻¹]</td>
</tr>
<tr>
<td>0%</td>
<td>1.89</td>
</tr>
<tr>
<td>1%</td>
<td>1.83</td>
</tr>
<tr>
<td>2%</td>
<td>1.92</td>
</tr>
<tr>
<td>3%</td>
<td>1.68</td>
</tr>
</tbody>
</table>
Table 3: Compilation of carbon isotope fractionation factors (ε) for mesophilic methanogenic pure cultures grown on different carbon substrates. The calculation of the fractionation factor is either based on initial and end point measurements (εCH4-substrate) or on regression analysis of substrate and product data (εsubstrate and εCH4).

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strain number</th>
<th>Substrate</th>
<th>Temp. [°C]</th>
<th>Fractionation factor [%]</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanobacterium ivanovii</td>
<td>2611</td>
<td>H2/CO2</td>
<td>37</td>
<td>-34</td>
<td>Belyaev et al. (1983)</td>
</tr>
<tr>
<td>Methanosarcina barkeri</td>
<td>a</td>
<td>H2/CO2</td>
<td>40</td>
<td>-41</td>
<td>Games et al. (1978)</td>
</tr>
<tr>
<td>Methanosarcina barkeri</td>
<td>804</td>
<td>H2/CO2</td>
<td>37</td>
<td>-45b</td>
<td>Londry et al. (2008)</td>
</tr>
<tr>
<td>Methanobacterium formicicum</td>
<td>1535</td>
<td>H2/CO2</td>
<td>34</td>
<td>-48</td>
<td>Babakhan et al. (1987)</td>
</tr>
<tr>
<td>Methanosarcina barkeri</td>
<td>800</td>
<td>H2/CO2</td>
<td>36</td>
<td>-49</td>
<td>Krzycki et al. (1987)</td>
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<tr>
<td>Methanobacterium bryantii</td>
<td>863</td>
<td>H2/CO2</td>
<td>40</td>
<td>-56</td>
<td>Games et al. (1978)</td>
</tr>
<tr>
<td>Methanococcus vanieli</td>
<td>1224</td>
<td>H2/CO2</td>
<td>35</td>
<td>-66f</td>
<td>Botz et al. (1996)</td>
</tr>
</tbody>
</table>

Methanoseta concilii 3671 acetate 37 -14 -9 Penning et al. (2006)
Methanosarcina barkeri 1538 acetate 37 -24 -12 Gelwicks et al. (1994)
Methanosarcina acetivorans 2834 acetate 37 -35 -24 Goever et al. (2009)
Methanosarcina barkeri 804 acetate 30 -31 -27 Goever et al. (2009)
Methanosarcina barkeri 800 acetate 37 -22 Krzycki et al. (1987)
Methanosarcina barkeri 804 acetate 37 -35 Londry et al. (2008)
Methanosarcina acetivorans 2834 methanol 37 -72 -72 this study
Methanobulbus zinderi 23339 methanol 37 -83 -78 this study
Methanosarcina barkeri 800 methanol 37 -79 Krzycki et al. (1987)
Methanosarcina barkeri 804 methanol 37 -83 Londry et al. (2008)
Methanosarcina barkeri 800 trimethylamine 37 -53 Summons et al. (1998)
Methanosarcina barkeri 804 trimethylamine 38 -67 Londry et al. (2008)
Methanococcoides burtonii 6242 trimethylamine 29 -73 Summons et al. (1998)

a not given, presumably DSM 800
b under substrate saturation; -80‰ under substrate limitation
c glass fermenter; -69‰ for titanium fermenter
Figure 1: Catabolism of methanol in pure cultures of *M. acetivorans* (A and B), *M. barkeri* (C and D) and *M. zinderi* (E and F). (A, C and E) Methanol consumption, CH₄ production and concentration of CO₂ in the headspace. (B, D and F) Isotope values of methanol, CH₄ and CO₂. ■, methanol; ●, CH₄; ●, CO₂. The concentrations are given as mmol per bottle; values are means standard errors (n = 3 for *Methanosarcina* spp., n = 2 for *Methanolobus zinderi*).
Figure 2: Carbon isotope signatures in cultures of the three methylotrophic methanogens as a function of the fractional yield $f_{\Delta \delta}$ (showing values of all replicates). □, *M. acetivorans*; ▲, *M. barkeri*; ×, *M. zinderi*. Methanol (black symbols); CH$_4$ (grey symbols); CO$_2$ (light grey symbols). (Values are taken from supplementary Table S1.)
Figure 3: Mariotti plots of the substrate methanol (A) and the product methane (B) of all three methylotrophic methanogens. Linear regression gives the respective fractionation factors ($\varepsilon$) ± standard deviation of regression. □, *M. acetivorans* ($\varepsilon_{\text{methanol}} = -72.0 \pm 1.5\%o$ and $\varepsilon_{\text{CH}_4} = -71.6 \pm 1.2\%o$); ▲, *M. barkeri* ($\varepsilon_{\text{methanol}} = -73.5 \pm 0.3\%o$ and $\varepsilon_{\text{CH}_4} = -76.1 \pm 1.1\%o$); ×, *M. zinderi* ($\varepsilon_{\text{methanol}} = -83.4 \pm 0.5\%o$ and $\varepsilon_{\text{CH}_4} = -77.9 \pm 1.8\%o$).
Figure 4: Effect of different methyl fluoride concentrations on the carbon isotope signatures in cultures of *M. barkeri* (A) and *M. zinderi* (B) as function of the fractional yield $f_{\text{Delta}}$. □, 0% methyl fluoride; ●, 1% methyl fluoride; ▲, 2% methyl fluoride; ×, 3% methyl fluoride. Methanol (black symbols); CH$_4$ (grey symbols); CO$_2$ (light grey symbols). (Values are taken from supplementary Table S2.)
Figure 5: Schematic pathways of methanogenesis from acetate (A), H₂/CO₂ (B), and methanol (C). Steps not required by each pathway are shaded gray. CH₃-CoM, methyl-coenzyme M. Redox-reactions involving H₂ as electron donor are indicated by 2 e⁻. The different ranges for the fractionation factors of stable carbon isotopes obtained from methanogenic pure cultures are given (compare Table 3).