Stable Carbon Isotope Fractionation by Methylotrophic Methanogenic Archaea

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In natural environments methane is usually produced by aceticlastic and hydrogenotrophic methanogenic archaea. However, some methanogens can use C1 compounds such as methanol as the substrate. To determine the contributions 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 aceticlastic 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.

Biogenic methane release into the atmosphere is based on methane production by methanogenic archaea. The main substrates for methanogenesis are either acetate (aceticlastic methanogenesis) or hydrogen plus carbon dioxide (hydrogenotrophic methanogenesis). To a minor extent, C1 compounds such as methanol, trimethylamines, or dimethyl sulfide can also serve as methanogenic substrates (35). A number of studies indicate that less than 5 to 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 (aceticlastic and hydrogenotrophic). To distinguish them in environmental studies, methyl fluoride is widely used as a selective inhibitor of aceticlastic 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 aceticlastic 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: for acetate, ε = −22‰; for H2/CO2, ε = −49‰; and for methanol, ε = −79‰ (26). In accordance with that, a strong fractionation with ε of −94‰ to −81‰ was reported for a methylotrophic enrichment culture (36).

The occurrence of methanol in the environment is based mainly on the turnover of methylated compounds of the plant cell wall, the degradation of pectin and lignin. While around 100 Tg year−1 of methanol is 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 year−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 lignins (43). However, methanol can be rapidly consumed by many different microorganisms, with 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 been studied in only few methylotrophic methanogens (only Methanosarcinaarkeri), involving only a few data points, we decided to investigate three methylotrophic methanogens: Methanosarcina barkeri, Methanosarcina acetivorans, and Methanolobus zinderi (an obligate methy-
lotroph). 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 be assigned exclusively 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-Lenglern, 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 liter⁻¹): NaCl, 23.4; MgSO4 · 7H2O, 9.4; Na2CO3, 5.0; yeast extract, 1.0; NH4Cl, 0.9; CaCl2 · 2H2O, 0.14; NaH2PO4 · 0.6; cysteine-HCl · H2O, 0.5; and Na2S · 9H2O, 0.045. In addition, 12.5 ml methanol as the substrate and 1 ml trace element solution were added. The trace element solution contained (in g liter⁻¹): 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; and Na2SeO3, 0.019.

Methanosarcina Barkeri was grown using a modified medium with the following composition (in g liter⁻¹ unless otherwise noted): KH2PO4, 0.25; KH2PO4, 0.23; NaHCl, 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; and Na2S · 9H2O, 0.045. In addition, 10.0 ml methanol as the substrate, 1 ml trace element solution (6), and 10 ml vitamin solution (45) were added.

Methanolobus zinderi was grown using a medium with the following composition (in g liter⁻¹): KCl, 0.33; MgCl2 · 6H2O, 4.0; MgSO4 · 7H2O, 3.5; NaHCl, 0.23; 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; and Na2S · 9H2O, 0.045. In addition, 5 ml methanol as the substrate, 1.0 ml of the trace element solution used for M. acetivorans, and 10 ml vitamin solution (45) were added.

For the experiments, the culture bottles were inoculated with 1 ml of a growing bacterial culture. Samples from the headspace were removed with a gas-tight syringe to determine the concentrations and carbon isotopic signatures of methane and carbon dioxide. The liquid phase was analyzed for the 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 a percentage (vol/vol) of the bottle volume (120 ml).

Chemical and isotopic analysis. The concentrations of CH4 and CO2 in gas samples were analyzed during the stable-isotope analysis of 13C/12C 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 were first separated in a Trace GC Ultra gas chromatograph using a Pora Plot Q column (27.5-m length, 0.32-mm inner diameter [i.d.], 10-μm film thickness; Varian, Palo Alto, CA) at 30°C with helium (99.996% purity; 2.6 ml/min) as the carrier gas. After conversion of CH4 to CO2 in the GC Isolink 1030, the 13C/12C isotope ratio was analyzed in the IRMS (Delta V Advantage). The isotope reference gas was CO2 (99.998% purity; Air Liquide, Dusseldorf, 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, δ13C = 10⁶ (Rsample / Rstd - 1) (%), with Rsample and Rstd the 13C/12C ratio for the sample and standard, respectively.

Isotopic analysis and quantification of methanol were 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. The methanol was then 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:

FIG 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. (A, C, and E) M. acetivorans; (B, D, and F) M. Barkeri; (A, C, and E) M. zinderi. The concentrations are given as mmol per bottle; values are means and standard errors (n = 3 for Methanosarcina spp., n = 2 for Methanolobus zinderi).
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*.

<table>
<thead>
<tr>
<th>Organism</th>
<th>H$_2$COH used (mmol)</th>
<th>CH$_4$ produced (mmol)</th>
<th>Carbon recovery (%)</th>
<th>ε$^{13}$CH$_3$OH (%δ)</th>
<th>ε$^{13}$CH$_4$ (%δ)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. acetivorans</em></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><em>M. barkeri</em></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>−71.6 ± 1.1</td>
</tr>
<tr>
<td><em>M. zinderi</em></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>

*Values are means ± standard deviations (values for isotopic calculation were taken from Table S1 in the supplemental material). Note that carbon recovery for *M. zinderi* was highest. This may be due to the acetate in the medium (1 g liter$^{-1}$), which is not needed for energy metabolism but is needed for cell growth (14).*

50°C, 10°C min$^{-1}$ to 140°C, 20°C min$^{-1}$ to 220°C, and 220°C for 1 min. Helium (99.996% purity; 2.6 ml/min) was used as the carrier gas. After conversion of methanol to CO$_2$, the isotopic composition of the reactants was analyzed in the IRMS (Finnigan MAT DeltaPlus). The isotopic reference gas was CO$_2$ calibrated as described above.

**Calculations.** Fractionation factors for a reaction $A \rightarrow B$ are defined as described by Hayes (22), $\alpha_{A/B} = (\delta_A + 1,000)/(\delta_B + 1,000)$, also expressed as $\varepsilon_{A/B} = 10^{(1 - \alpha_{A/B})}$. The isotope enrichment factor $\varepsilon$ was determined as described by Mariotti et al. (30) from the residual reactant, calculated as $\delta_{r} = \delta_{o} + \varepsilon[ln(1 - f)]$, and from the product formed, calculated as $\delta_{p} = \delta_{o} - \varepsilon[ln(1 - f)]/f$, where $\delta_{i}$ is the isotope composition of the reactant at the beginning and $\delta_{o}$ and $\delta_{r}$ are the isotope compositions of the residual methanol and the pooled CH$_4$, 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 basis of the measured $\delta$ values was promoted by Gelwicks et al. (20): $f_{\Delta} = (\delta_{o} - \delta_{r})/(\delta_{p} - \delta_{o})$. Linear regression of $\delta_{r}$ against $ln(1 - f)$, and of $\delta_{p}$ against $ln(1 - f)/f$, gives $\varepsilon$ for substrate and product data as the slopes of best-fit lines.

**RESULTS**

**Methylotrophic methanogenesis.** Even though the times needed for growth and complete consumption of methanol were different in all three methylotrophic archaea (*M. acetivorans*, *M. barkeri*, and *M. zinderi*), methanol was finally completely consumed during production of methane and CO$_2$. The following stoichiometry was observed in all three strains (Fig. 1; Table 1): 4CH$_3$OH $\rightarrow$ 3CH$_4$ + CO$_2$ + 2H$_2$O.

Figure 1 shows the CO$_2$ measured in the headspace of the culture vessel, since it has been shown that CO$_2$ rather than bicarbonate is the active substrate of methanogenesis (17, 40, 42). The total amount of inorganic carbon was larger than that in the headspace due to dissolved CO$_2$ and bicarbonate in the medium (initial amounts of total inorganic carbon were as follows: *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* (from 7.0 to 6.7) and *M. acetivorans* (from 7.1 to 6.4). In contrast, there was a relatively large decrease of the pH in *M. barkeri* (from 6.9 to 5.2).

Consumption of [12C]methanol was preferred, causing an enrichment of the heavier isotope [13C] in the residual methanol (Fig. 1). Consequently, the initial CH$_4$ produced from methanol was relatively depleted of [13C], but this then increased with time. The initially high [13C] 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 of [13C] 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 $f_{\Delta}$ (values are taken from Table S1 in the supplemental material). 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 $\delta$[13C] of both the substrate ($\varepsilon_{\text{methanol}}$) and the product ($\varepsilon_{\text{CH4}}$). 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 Table S1 in the supplemental material). Lacking the isotopic signatures for low methanol concentrations (detection limit, −2.5 mM [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 $\delta_{\text{CH3OH}}$ 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 Table S1 in the supplemental material).

**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 (see Fig. S1A in the supplemental material), maximal methane production rates (Table 2), and carbon flow and isotopic signature of substrate and product (Fig. 4A) were similar to those in the uninhibited samples. A slightly different picture was obtained for
was unaffected by the presence of methyl fluoride (Table 2; for every, the isotopic signature was not impaired by the presence of (acetivorans $\varepsilon$ (‰)

### TABLE 2

<table>
<thead>
<tr>
<th>Methyl fluoride (%)</th>
<th>Maximum CH₄ production (mmol day⁻¹)</th>
<th>$\varepsilon_{\text{CH}_3\text{OH}}$ (%)</th>
<th>$\varepsilon_{\text{CH}_4}$ (%)</th>
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</thead>
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<tr>
<td>0</td>
<td>1.89</td>
<td>−79.6 ± 0.1</td>
<td>−76.9 ± 1.7</td>
</tr>
<tr>
<td>1</td>
<td>1.93</td>
<td>−73.6 ± 0.7</td>
<td>−77.3 ± 1.7</td>
</tr>
<tr>
<td>2</td>
<td>1.92</td>
<td>−79.9 ± 0.1</td>
<td>−75.0 ± 2.5</td>
</tr>
<tr>
<td>3</td>
<td>1.68</td>
<td>−76.0 ± 0.0</td>
<td>−75.4 ± 0.6</td>
</tr>
</tbody>
</table>

$\varepsilon$ values are means ± standard deviations (values for isotopic calculation were taken from Table S2 in the supplemental material).

### 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 $\varepsilon = −74.8%o$ to −72.5%o for Methanosarcina barkeri. Londry et al. (28) found a slightly lower value of $\varepsilon = −83.4%o$ for Methanosarcina barkeri. Both studies were based on initial and endpoint 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 $\varepsilon_{\text{methanol}} = −73.5%o$ and $\varepsilon_{\text{CH}_4} = −76.1%o$; $\varepsilon$ M. zinderi ($\varepsilon_{\text{methanol}} = −83.4%o$ and $\varepsilon_{\text{CH}_4} = −77.9%o$, only slightly lower than those for 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 $\varepsilon$ values covering a relatively narrow range of around $−83%o$ to $−72%o$.

Among methanogenic archaebacteria, utilization of methylated substrates is restricted to members of the family Methanosarcinaceae. The only exceptions are species of the genus Methanoplaera, 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), Methanobalbus zinderi is an obligate methylotrophic 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 ($\varepsilon = −35%o$ to $−9%o$) (21, 33, 41), hydrogenotrophic methanogenesis exhibits a broad range of fractionation factors ($\varepsilon = −79%o$ to $−28%o$) (41). However, methylotrophic methanogenesis has the strongest fractionation ($\varepsilon = −83%o$ to $−72%o$). These

![FIG 3 Mariotti plots of the substrate methanol (A) and the product methane (B) for all three methylotrophic methanogens. Linear regression gives the respective fractionation factors ($\varepsilon$) $\pm$ standard deviation of regression. △, M. acetivorans ($\varepsilon_{\text{methanol}} = −72.0 ± 1.5%o$ and $\varepsilon_{\text{CH}_4} = −71.6 ± 1.2%o$); ▲, M. barkeri ($\varepsilon_{\text{methanol}} = −73.5 ± 0.3%o$ and $\varepsilon_{\text{CH}_4} = −76.1 ± 1.1%o$); ×, M. zinderi ($\varepsilon_{\text{methanol}} = −83.4 ± 0.5%o$ and $\varepsilon_{\text{CH}_4} = −77.9 ± 1.8%o$).]
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 CO2 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 CO2 or is left as residual methanol. Indeed, the

![FIG 4](image-url) Effects of different methyl fluoride concentrations on the carbon isotope signatures in cultures of M. barkeri (A) and M. zinderi (B) as a function of the fractional yield $f_{\text{delta}}$. (Values are taken from Table S2 in the supplemental material.)

### TABLE 3 Compilation of carbon isotope fractionation factors (ε) for mesophilic methanogenic pure cultures grown on different carbon substrates

<table>
<thead>
<tr>
<th>Organism</th>
<th>DSMZ strain no.</th>
<th>Substrate</th>
<th>Temp (°C)</th>
<th>$\varepsilon_{\text{substrate}}$</th>
<th>$\varepsilon_{\text{CH}_4}$</th>
<th>$\varepsilon_{\text{CH}_4-\text{substrate}}$</th>
<th>Reference</th>
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<tr>
<td>Methanobacterium ivanovii</td>
<td>2611</td>
<td>H$_2$-CO$_2$</td>
<td>37</td>
<td>$-34$</td>
<td>$-$</td>
<td>$-19$</td>
<td>2</td>
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<tr>
<td>Methanosarcina barkeri</td>
<td>—</td>
<td>H$_2$-CO$_2$</td>
<td>40</td>
<td>$-41$</td>
<td>$-41$</td>
<td>$-41$</td>
<td>19</td>
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<td>$-42$</td>
<td>$-43$</td>
<td>28</td>
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<td>37</td>
<td>$-53$</td>
<td>$-53$</td>
<td>$-53$</td>
<td>39</td>
</tr>
<tr>
<td>Methanosarcina barkeri</td>
<td>804</td>
<td>Trimethylamine</td>
<td>37</td>
<td>$-67$</td>
<td>$-67$</td>
<td>$-67$</td>
<td>28</td>
</tr>
<tr>
<td>Methanococcoides burtonii</td>
<td>6242</td>
<td>Trimethylamine</td>
<td>20</td>
<td>$-73$</td>
<td>$-73$</td>
<td>$-73$</td>
<td>39</td>
</tr>
</tbody>
</table>

$a$ The calculation of the fractionation factor is based either on initial and endpoint measurements ($\varepsilon_{\text{CH}_4-\text{substrate}}$) or on regression analysis of substrate and product data ($\varepsilon_{\text{substrate}}$ and $\varepsilon_{\text{CH}_4}$).

$b$ —, not given, but presumably DSM 800.

$c$ Value under substrate saturation; $-80\%$ under substrate limitation.

$d$ Value in a glass fermentor; $-69\%$ for a titanium fermentor.
CO₂ produced from methanol was not as depleted of ¹³C as the CH₄. If we extrapolate the isotopic signature of the produced CO₂, a signature as low as δ¹³C of newly formed CO₂ would be inferred. Likewise, the strong fractionation (ranging from −73‰ to −53‰) of other methylated compounds, such as trimethylamines (Table 3), which differ in just the first enzyme needed to activate the methyl group may be explained by the disproportionation of the methyl compound to CO₂ and CH₄.

Methyl fluoride and environmental implications. Theoretically, acetoclastic methanogenesis should account for 67% of total methanogenesis, when polysaccharides are completely degraded to CO₂ and CH₄ (7, 8). The residual CH₄ production would be due to hydrogenotrophic methanogenesis, and the isotopic signature of the produced CH₄ would suggest the relatively strong fractionation factors involved in CH₄ production from H₂-CO₂. In many studies, a concentration of 2% methyl fluoride is used to inhibit acetoclastic methanogenesis (9, 11–13). 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‰ than those for the uninhibited samples. Even the uninhibited samples fractionate in general more strongly than the fractionation factors reported for pure cultures of hydrogenotrophic methanogens (Table 3).

One possible reason for this observation is that methylotrophic methanogenesis contributes to CH₄ 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 indeed had no effect on the isotopic fractionation of CH₄ 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 the contribution of methylotrophic methanogenesis in the presence of methyl fluoride may affect the resulting isotopic signature of CH₄. Let us assume that 33% of methanogenesis originates from H₂-CO₂ (7) and that methanol contributes up to 10% (10). In the presence of methyl fluoride, methanolotrophic methanogenesis could contribute roughly 30% to total CH₄ 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 various ratios of H₂-CO₂ 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 acetoclastic or hydrogenotrophic methanogenesis in pure cultures grown under optimal substrate conditions. Hence, even though the contribution of methane 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 thus may not only be due to hydrogenotrophic methanogenesis but may in addition be affected to a larger extent by methylotrophic methanogens than previously anticipated.

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REFERENCES


