Carbon Isotope Fractionation during Catabolism and Anabolism in Acetogenic Bacteria Growing on Different Substrates

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Acetogenic bacteria are widespread microbial groups unified by their ability to use the reductive acetyl coenzyme A (acytel-CoA) pathway, which allows chemolithoautotrophic growth on hydrogen and carbonate and is the only known pathway that combines carbon dioxide fixation with energy conservation (1–6). Most acetogens can use the reductive acetyl-CoA pathway for autotrophic growth, reducing 2 carbon dioxide molecules with 4 hydrogen molecules to liberate 1 acetic acid. Under these conditions, CO₂ reduction with H₂ yields a ΔG’’ value of −95 kJ/mol. This is barely enough to generate 1 ATP molecule using energy conservation (7). On the other hand, the acetyl-CoA pathway can be accompanied by the fermentation of carbon substrates. Under these autotrophic conditions, the energy yield for, e.g., glucose fermentation to 3 acetate molecules is expressed by the equation ΔG’’ = −310.9 kJ/mol. In contrast to fermentations of glucose by most other anaerobes, which yield only 1 to 3 ATP molecules via substrate-level phosphorylation during glycolysis, acetogens can redirect the generation of an additional ATP molecule as described above (3, 8).

One approach to evaluating the contributions of different bacterial pathways to certain compound pools in environmental systems is to use the natural abundance of stable carbon isotopes to track the metabolic activity of certain microorganisms in the environment (9, 10). In principle, each metabolic pathway is characterized by a specific preference for a certain carbon isotope (usually the lighter ¹³C) during catalysis, resulting in a characteristic depletion of ¹³C between the substrate and the end product. Mathematically, this depletion is characterized by a so-called apparent fractionation factor (α) or enrichment factor (ε); ε is defined as ¹³C/¹²C and is calculated as (α⁺¹³C/α⁻¹²C)² (see equation 6) (11). There are two types of isotope effects: the equilibrium isotope effect and the kinetic isotope effect. In the first case, equilibrium is attained in a closed, mixed system in which a bidirectional reaction occurs. The associated isotope effects are typically small, e.g., the dissolution of inorganic carbon (DIC) in seawater results in unequal isotope distribution between atmospheric CO₂, which is −7.9‰ depleted of ¹³C, and dissolved bicarbonate (12, 13). In contrast, biological reactions usually display a kinetic isotope effect, which generally discriminates against the heavy isotope, so that the isotopic value of the product is lower than that of the substrate. Biochemical pathways usually consist of a sequence of irreversible or unidirectional reactions that influence the overall fractionation factor to various extents (14–16). Most biochemical reactions that interconvert C₃ compounds have large fractionation effects; in contrast, most heterotrophic reactions involving complex organic substrates have small fractionation effects (see below). Usually, the fractionation factors are determined in pure microbial cultures and can be derived from either substrate depletion or product accumulation (17). A well-documented example of carbon isotope fractionations is methanogenesis from different substrates: fractionation factors are −25% to −69‰ with CO₂ (18–21), −73‰ to −83‰ with methanol (22, 23), and −7‰ to −35‰ with acetate (21, 24–27). In environmental samples, fractionation factors can be used to distinguish different pathways if the difference in fractionation is large enough. For example, the fractionation factor for hydrogenotrophic methanogenesis can be determined by the incubation of soil samples in the presence of CH₄/F (28–31), which specifically inhibits aceticlastic methanogenesis (32, 33).

The acetyl-CoA pathway imparts strong isotopic enrichment of −55‰ to −60‰ during acetate formation with H₂/CO₂ (80/
As shown in Fig. 1, a pathway used by acetogenic bacteria has so far been evaluated only for the reduction of CO₂ to either the methyl or the carboxyl group is shown in black; the reverse reaction (oxidation) is shown in gray. For simplicity, reducing equivalents are given as H₂. The entry points of different C₁ substrates are shown in dark gray. Note that the carboxyl group can be formed by the reduction of CO₂ or indirectly, by the oxidation of the respective C₁ compound. Numbers in parentheses represent the enzymes acting on the different C₁ compounds, as follows: 1, methanol-cobalamin methyltransferase; 2, CO dehydrogenase/acetyl-CoA synthase; 3, formate dehydrogenase; 4, formyl-tetrahydrofolate synthetase. Details on the reactions are given in equations 1 to 6 and in the text.

FIG 1 Schematic representation of the reductive acetyl-CoA pathway and entry points of possible substrates. The reduction of CO₂ to either the methyl or the carboxyl group is shown in black; the reverse reaction (oxidation) is shown in gray. For simplicity, reducing equivalents are given as H₂. The entry points of different C₁ substrates are shown in dark gray. Note that the carboxyl group can be formed by the reduction of CO₂ or indirectly, by the oxidation of the respective C₁ compound. Numbers in parentheses represent the enzymes acting on the different C₁ compounds, as follows: 1, methanol-cobalamin methyltransferase; 2, CO dehydrogenase/acetyl-CoA synthase; 3, formate dehydrogenase; 4, formyl-tetrahydrofolate synthetase. Details on the reactions are given in equations 1 to 6 and in the text.

However, the fractionation of the reductive acetyl-CoA pathway used by acetogenic bacteria has so far been evaluated only for pure cultures grown on H₂/CO₂. As shown in Fig. 1, in this pathway, 1 CO₂ molecule is fixed by formate dehydrogenase and is then reduced stepwise to the methyl group of acetate (methyl branch), while a second molecule of CO₂ is directly used by the CO dehydrogenase/acetyl-CoA synthase (CODH/ACS) to form the carboxy group of acetate (carbonyl branch) (41, 42). It has been shown that both branches exhibit the same strong fractionation when H₂/CO₂ is used as a substrate (35).

We hypothesized that other C₁ compounds that can be incorporated either alone or in the presence of H₂/CO₂ may affect the overall fractionation of the resulting acetate. In this study, we tested two substrates (formate and methanol) entering at different sites of the methyl branch. In addition, we grew Thermoaerobacter kivui mixotrophically on H₂/CO₂ together with different formate concentrations.

In a second project, we evaluated the fractionation of pure acetogenic cultures during fermentation. As a model substrate, we used glucose, which is oxidized by many acetogens to pyruvate acetogenic cultures during fermentation. As a model substrate, we used glucose, which is oxidized by many acetogens to pyruvate during glycolysis and oxidative decarboxylation (37–40) and the respective C₁ compound. Numbers in parentheses represent the enzymes acting on the different C₁ compounds, as follows: 1, methanol-cobalamin methyltransferase; 2, CO dehydrogenase/acetyl-CoA synthase; 3, formate dehydrogenase; 4, formyl-tetrahydrofolate synthetase. Details on the reactions are given in equations 1 to 6 and in the text.

As a third objective, we evaluated the fractionation of acetogenic bacteria during anabolism. Usually, calculations of the fractionation factor rely on a mass balance equation focusing on the catabolic processes and neglecting the anabolic reactions. Since we observed a higher optical density for T. kivui when it was grown on glucose than when it was grown on H₂/CO₂, we wondered if the fractionation during anabolic reactions could be neglected for the calculations of stable carbon isotope fractionation. So far, the fractionation into biomass has been determined mainly at the end of incubation for several CO₂-fixing pathways (26, 47, 48) and in methanogens grown on different substrates (22) and at several time points during aerobic mineralization of 1,2-dichloroethane by Xanthobacter autotrophicus as reported by Hunkeler and Arenal (49). In all cases, the isotopic signal of anabolism was lower than the fractionation during catabolism. We hypothesized, therefore, that the fractionation of pure acetogenic cultures into biomass would be lower than the fractionation into acetate. In contrast to the procedures in most previous studies, we performed a time series experiment, which allows precise calculation of the fractionation factors during anabolism and catabolism.

Altogether, we intended to estimate how strongly the fractionation of stable carbon isotopes associated with the acetyl-CoA pathway is affected by the substrate usage of pure acetogenic cultures.
Cultures were grown in 120-ml serum bottles, which contained 50 ml medium. At the times indicated in the figures, 100–μl gas samples were taken with a gastight syringe (Vici, Baton Rouge, LA, USA) for analysis of the concentrations and δ13C values of both CO2 and CH4; 2-ml liquid samples were taken for analysis of the concentrations and δ13C values of glucose and short-chain fatty acids (SCFA).

**Chemical and isotopic analyses.** CH4 was analyzed by gas chromatography (GC) using a flame ionization detector (Shimadzu, Kyoto, Japan). CO2 was analyzed after conversion to CH4 with a methanizer (Ni catalyst at 350°C; Chrompack, Middelfarb, Netherlands). Isotope ratio (13C/12C) measurements in gas samples were performed on a gas chromatography combustion isotope ratio mass spectrometer (GC-C–IRMS) system (Thermo Fisher Scientific, Bremen, Germany). The principle of operation has been described by Brand (51). The gaseous compounds were separated using a PorapLOT Q column (length, 27.5 m; internal diameter, 0.32 mm; film thickness, 10 μm; Chrompack, Frankfurt, Germany) at 30°C with He (purity, 99.996%; 2.6 ml/min) as the carrier gas. The sample was run through the Finnigan Standard GC Combustion Interface III, and the 13C/12C isotope ratio was measured against that of Vienna Pee Dee Belemnite and is reported in the delta notation as follows:

\[ \delta^{13}C = 1000 \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \] (6)

where \( R \) is the 13C/12C ratio.

Isotopic analysis and quantification of glucose and SCFA were performed on a high-pressure liquid chromatography (HPLC) system (SpectraSYSTEM P1000; Thermo Fisher Scientific, San Jose, CA, USA) with a Mistral thermostaat (Spark, Emmen, Netherlands), equipped with an ion exclusion column (Aminex HPX-87H; Bio-Rad, Munich, Germany) and coupled to the Finnigan LC IsoLink interface (Thermo Fisher Scientific, Bremen, Germany) as described previously (52). Isotope ratios were determined on an IRMS (Finnigan MAT Delta Plus Advantage). The precision of the GC-IRMS was ±0.2‰, and that of the HPLC-IRMS was ±0.3‰. For further details on the determination of acetate concentrations via HPLC-IRMS, see Fig. S9 and Table S2 in the supplemental material.

The isotopic fractionation into biomass was evaluated by harvesting three replicate samples (120-ml serum bottles), each containing 50 ml of culture, for each time point. The cells were harvested by centrifugation (10 min at 6,000 rpm [9,700 × g]). The resulting pellet was dried in tin capsules and was sent to the isotope center in Göttingen, Germany. There the isotopic values were determined using an elemental analyzer coupled to an IRMS (Kompetenzzentrum Stabile Isotope, University of Göttingen). Pure acetate was used as a reference to compare our HPLC-IRMS measurement with the results of the elemental analyzer.

**Calculations.** The apparent fractionation factor (\( \alpha \)) for an A → B reaction is defined according to reference 11 as

\[ \alpha_{A/B} = \left( \frac{\delta_A + 1,000}{\delta_B + 1,000} \right) \] (7)

and the isotope enrichment factor is defined as \( \varepsilon = 10^{\alpha(1-\alpha)} \). The isotope enrichment factor associated with homoaacetogenesis was determined as described by Mariotti et al. (17) from the residual reactant

\[ \delta_f = \delta_i + \varepsilon \ln(1 - f) \] (8)

and from the product formed

\[ \delta_f = \delta_i - \varepsilon (1 - f) \ln(1 - f) / f \] (9)

where \( \delta_i \) is the isotope composition of the reactant (CO2) at the beginning, and \( \delta_i \) and \( \delta_f \) are the isotope compositions of the residual CO2 and the pooled acetate, respectively, at the instant when \( f \) was determined. Linear regression of \( \delta \) against ln(1–f) and linear regression of \( \delta \) against (1–f)[ln(1–f)]/f gives \( \varepsilon \) as the slope of best-fit lines. The fractional yield of a reaction (\( f \)) is usually based on the consumption of the substrate CO2 (0 < f < 1); in a closed system, the amount of substrate used (\( f_{\text{substrate}} \)) can be derived from the measured isotopic composition (35) as follows:

\[ f_{\text{substrate}} = (\delta_i - \delta_f) / (\delta_f - \delta_p) \] (10)

In contrast to the isotope enrichment factor (\( \varepsilon \)) derived from pure microbial cultures as described above, many environmental studies calculate the environmental fractionation (\( \Delta \)) as follows:

\[ \Delta = \delta_{\text{prod}} - \delta_{\text{sub}} \] (11)

where \( \delta_{\text{sub}} \) is the δ13C value of the substrate and \( \delta_{\text{prod}} \) is the δ13C value of the product.

While this estimated fractionation is valid for open systems, such as environmental samples, it is valid in a closed system with limited substrate concentrations only at the very beginning of a reaction (17, 53, 54). Nevertheless, we used this formula for the mixotrophic experiments to estimate the environmental fractionation into acetate versus biomass for cells grown on formate or H2/CO2.

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**RESULTS**

**Growth on C1 compounds.** In order to evaluate the fractionation factor in the acetyl-CoA pathway, we measured the production of acetate during the consumption of different C1 compounds that either are direct intermediates (CO2, formate) or can be easily incorporated into the acetyl-CoA pathway (methanol) (Fig. 1). These experiments were conducted using *Sporomusa sphaeroides*. Substrate depletion and product formation were analyzed over time, and the δ13C values of all carbon-containing compounds were measured during the reaction (Fig. 2). Despite the different 13C values of the initial substrates (δmethanol = −38‰, formate (δformate = −31‰), and H2/CO2 (δCO2 = −17‰), the acetate released with all three substrates eventually reached the same value (δacetate = −67‰). The δ13C values of all three substrates became enriched in heavy carbon at roughly the same rate. When the apparent fractionation factors were calculated on the basis of the substrate data, the values obtained were —56.9‰ for methanol, −56.5‰ for formate, and −63.8‰ for CO2 (with H2/CO2 as the substrate). The δ13C of CO2 did not differ much whether methanol or formate served as the substrate, due to the high background level of bicarbonate (4.5 mmol NaHCO3/bottle) in the culture medium. Details on acetate formation for the individual substrates are given in Fig. S2 in the supplemental material. Details and further discussion of the isotopic values of acetate are given in Fig. S3 and the accompanying discussion in the supplemental material.

In a different approach, the influence of different C1 compounds was tested using the thermophilic acetogen *Thermoanaerobacter kivui* grown under a headspace of N2/CO2 on formate (1 mmol per bottle). In parallel, *T. kivui* was inoculated into a headspace of H2/CO2 (2.5 mmol H2 and 0.6 mmol CO2 per bottle [70 ml headspace in 120 ml]) either alone or under mixotrophic conditions in the presence of a low (0.25 mmol per bottle [50 ml medium per 120-ml bottle]) or a high (1 mmol per bottle [50 ml medium per 120-ml bottle]) concentration of formate (Fig. 3; Table 1). The bicarbonate concentration was 4.5 mmol per bottle in all incubations. Details on carbon turnover are given in Fig. S4 in the supplemental material. Using hydrogen consumption (equation 1) and formate consumption (equation 3) as proxies for
substrate turnover into acetate, almost-complete turnover could be documented for all experiments (Table 1). This substrate consumption was used to calculate the relative contributions of the two substrates under mixotrophic conditions and to estimate the apparent fractionation ($\delta^{13}C$) between the substrate (at time zero $[t_0]$) and the product, acetate (at the end of the reaction $[t_{\text{end}}]$), or between the substrate (at $t_0$) and biomass (at $t_{\text{end}}$). A statistically nonsignificant trend toward $^{13}$C depletion of acetate as well as biomass could be observed for increasing contributions of formate relative to CO$_2$ (Table 1). Calculation of enrichment factors using the linear regression given in equation 8 gave a $\epsilon_{\text{CO}_2}$ of $-48.7‰$ for incubations under H$_2$/CO$_2$ and a $\epsilon_{\text{form}}$ of $-51.9‰$ for formate incubations under N$_2$/CO$_2$.

Growth on glucose and nitrate inhibition. In a further set of experiments, fractionation was determined when pure acetogenic cultures were grown on complex carbon substrates. As an example, Moorella thermoacetica grown on glucose alone (200 $\mu$mol), glucose (200 $\mu$mol) plus nitrate (300 $\mu$mol), or H$_2$/CO$_2$ (2.5 mmol/625 $\mu$mol) was used. Indeed, our isotopic measurements revealed almost no fractionation with glucose and nitrate ($\epsilon_{\text{Gluc}+\text{NO}_3} = -0.4‰$), strong fractionation with H$_2$/CO$_2$ ($\epsilon_{\text{H}_2/\text{CO}_2} = -55.8‰$), and intermediate fractionation with glucose ($\epsilon_{\text{Gluc}} = -18.5‰$), reflecting the mixing of the two acetate-generating pathways (Fig. 4). Additional data on nitrate depletion are given in Fig. S5 in the supplemental material. Similar results could be obtained for T. kivui, where the fractionation under glucose ($\epsilon_{\text{Gluc}} = -14.1‰$) was roughly one-third of the fractionation under H$_2$/CO$_2$ ($\epsilon_{\text{H}_2/\text{CO}_2} = -53.0‰$) (see Fig. S6 in the supplemental material), and also for Acetobacterium carbinolicum ($\epsilon_{\text{Gluc}} = -18.8‰$; $\epsilon_{\text{H}_2/\text{CO}_2} = -54.0‰$) (data not shown).

Fractionation into biomass. In contrast to aerobic bacteria, anaerobic bacteria can use only a small amount of the available energy for anabolism (usually around 10%), and hence only a small portion of the available carbon is fixed into biomass. However, it was observed that T. kivui grew to dense cultures on glucose, but only faint turbidity was observed for cells grown on H$_2$/CO$_2$. Therefore, we questioned how substrate usage affects the $\delta^{13}$C values of the microbial biomass. Thermoanaerobacter kivui was grown on glucose (200 $\mu$mol under N$_2$/CO$_2$) or on H$_2$/CO$_2$ (2.5 mmol/625 $\mu$mol) in the bicarbonate-buffered minimal medium described above. We expected that under these two conditions, roughly the same amount of acetate would be produced. The $\delta^{13}$C values of the substrates and the product, as well as those of biomass, were followed over time (Fig. 5). The fractionation during catabolism was calculated with equation 8 using the $^{13}$C values of the substrate together with the $^{13}$C values of acetate; the fractionation during anabolism was calculated using the $^{13}$C values of the substrate together with the $^{13}$C values of the biomass. While growth on glucose resulted in weak positive fractionation during catabolism ($\epsilon_{\text{catabol.}} = +4.2‰$) as well as during anabolism ($\epsilon_{\text{anabol.}} = +2.9‰$), growth on H$_2$/CO$_2$ was accompanied by stronger fractionation during catabolism ($\epsilon_{\text{catabol.}} = -48.6‰$) than during anabolism ($\epsilon_{\text{anabol.}} = -28.6‰$).

FIG 2 Sporomusa sphaeroides DSM 2875 grown on different C$_1$ compounds. (The substrate [H$_2$/CO$_2$, formate, or methanol] is given in parentheses in the key after the analyte measured.) Shown are the $\delta^{13}$C values of the substrate (filled symbols) and the product (acetate) (open symbols); $\delta^{13}$C values of CO$_2$ for incubations with methanol or formate are represented by open symbols with gray borders. Values are averages for three independent replicates ± standard deviations. Individual plots for the different substrates are given in Fig. S1 in the supplemental material.
DISCUSSION

Growth on C1 compounds. The data presented in this report confirmed the overall strong fractionation of acetogens using the acetyl-CoA pathway when H2/CO2 was the substrate (34,35); in addition, it could be shown that the fractionation of cells grown on different C1 compounds likewise resulted in very strong fractionation and was largely independent of substrate usage (different C1 compounds). This is in contrast to the findings of several published reports where the rate-limiting step (usually the initial step of a reaction cascade) primarily determined the overall fractionation (20–23, 25, 27, 55, 58).

One prominent example of the initial step of a reaction cascade determining the overall fractionation can be found in plant systems, where the initial CO2-fixing reactions of C3 and C4 plants (Rubisco versus phosphoenolpyruvate carboxylase) dictate strong differences in the resulting plant material (55), a pattern that is largely unchanged throughout the food chain (56, 57). In the microbial world, similar behavior is well documented for pure

![FIG 3 Mixotrophic growth of Thermoanaerobacter kivui DSM 2030 on formate and H2/CO2 leads to the incorporation of both substrates. The analytes measured—CO2, formate (Form), and acetate (Ac)—are given in parentheses in the key. H2/CO2 concentrations were 2.5 mmol/bottle (H2) and 0.6 mmol/bottle (CO2) in the H2/CO2 treatment as well as in the low- and high-mix treatments (incubations in the presence of a low or a high concentration of formate, respectively, under a headspace of H2/CO2). Formate concentrations were 0.25 mmol/bottle in the low-mix treatment and 1 mmol/bottle in the high-mix and formate treatments. The Δ13C value of the released acetate is a direct result of the mixing of the Δ13C values of the substrates, while the fractionation of the individual substrates is quite constant. Values are averages for three independent replicates ± standard deviations.]

### TABLE 1 Mixotrophic growth of T. kivui on H2/CO2 and different amounts of formate

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Amt (mmol/bottle) of:</th>
<th>Formate used</th>
<th>H2 used</th>
<th>Acetate formed</th>
<th>Recovery (%)</th>
<th>% of acetate from H2</th>
<th>Δ13C (%)</th>
<th>Acetate (t&lt;sub&gt;end&lt;/sub&gt;)</th>
<th>Biomass (t&lt;sub&gt;end&lt;/sub&gt;)</th>
<th>Δ(substrate-acetate)</th>
<th>Δ(substrate-biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2/CO2</td>
<td>Low mix</td>
<td>0.3</td>
<td>2.1</td>
<td>0.8</td>
<td>102</td>
<td>53</td>
<td>-30.4</td>
<td>-17.7</td>
<td>-67.3</td>
<td>-37.1</td>
<td>-17.1</td>
</tr>
<tr>
<td></td>
<td>High mix</td>
<td>1.1</td>
<td>2.1</td>
<td>0.7</td>
<td>93</td>
<td>80</td>
<td>-30.6</td>
<td>-19.1</td>
<td>-64.1</td>
<td>-40.0</td>
<td>-12.6</td>
</tr>
<tr>
<td>Formate</td>
<td></td>
<td>1.2</td>
<td>0.5</td>
<td>0.7</td>
<td>95</td>
<td>100</td>
<td>-30.2</td>
<td>-17.6</td>
<td>-67.3</td>
<td>-37.1</td>
<td>-17.1</td>
</tr>
</tbody>
</table>

a Low mix, H2/CO2 and a low concentration of formate; high mix, H2/CO2 and a high concentration of formate.
b Calculated using equations 1 and 3 and assuming substrate limitation (either H2 or formate). Since the cells were grown in a bicarbonate-buffered system (4.5 mmol NaHCO3/bottle), CO2 was never limiting.
c The percentages of acetate from H2 have been calculated assuming complete turnover of H2 and formate into acetate using the reactions given in equations 1 and 3.
d Derived from the Δ13C values of the substrate and product assuming the percentages of acetate from H2 given in this table.
methanogenic cultures on different substrates, where each substrate is accompanied by a unique fractionation factor. For example, pure methanogenic cultures of *Methanosarcina barkeri* grown on different carbon substrates show highly distinct carbon isotope fractionations between substrate and product when grown on H₂/CO₂ (Δ¹³C = 45.4‰ to 46.3‰), methanol (Δ¹³C = 72.5‰ to 83.4‰), or acetate (Δ¹³C = 21.2‰ to 34.8‰) (22,58). These results have been independently validated for other pure methanogenic cultures on H₂/CO₂ (20, 21), methanol (23), and acetate (25, 27). Further, it was shown for hydrogenotrophic methanogens (using H₂/CO₂) that fractionation is greatly affected by the hydrogen (energy) level of the incubation, with stronger fractionation under H₂-limiting conditions (20–22). For a sulfate-reducing *Desulfovibrio* sp., the fractionation of sulfur isotopes was also affected by the electron donors and was negatively correlated with the cell-specific sulfate reduction rate (59). Recent evidence also suggests that for sulfate-reducing bacteria, the overall isotope fractionation during sulfate reduction is influenced by all steps in the dissimilatory pathway (16, 60).

In contrast, differences in the substrate ratios of H₂ and CO₂ for acetogenic cultures did not affect fractionation (61). Furthermore, it has been shown that there is no intramolecular fractionation during acetate formation: the isotope values of the methyl group were similar to the δ¹³C values of the overall acetate signal (35). The range of fractionations for different C₁ compounds to acetate suggest similar discrimination against ¹³C irrespective of the individual C₁ compound.

Our results for the *S. sphaeroides* incubations can be interpreted in two ways. (i) One explanation is that the initial fraction-
ations of all possible initial enzymes are highly similar. (ii) Alternatively, our results can be explained by assuming that the rate-limiting step determining overall fractionation is the formation of a C—C bond by the CODH/ACS complex. We will discuss both possibilities:

(i) The individual entry points for the different C1 compounds are schematically given in Fig. 1. The following four enzymes are involved: methanol-cobalamin methyltransferase, CO dehydrogenase/acetyl-CoA synthase, formate dehydrogenase, and formyl-tetrahydrofolate synthetase. Methanol is incorporated using methanol-cobalamin methyltransferase (62). While 1 molecule of CO2 is reduced via CO to the carboxyl group of acetate by the enzyme complex CO dehydrogenase/acetyl-CoA synthase, a second molecule of CO2 is reduced to formate by formate dehydrogenase. Hence, formate is a direct intermediate and can be either oxidized to CO2 or further reduced in an ATP-dependent reaction catalyzed by formyl-tetrahydrofolate synthetase (3). In principle, it is possible that all four of these entry points could exhibit the same fractionation. However, fractionation of methanol and CO2 in pure methanogenic cultures renders completely different frac-

FIG 5 Fractionation into biomass. (A) δ13C values for *Thermoanaerobacter kivui* grown on either H2/CO2 or glucose. Three replicate samples were harvested for each time point. In the key, the substrates are given in parentheses. (B) Regression analysis of CO2 conversion into acetate □ or biomass △ for cells grown on H2/CO2 and regression analysis of glucose conversion into acetate ◇ or biomass △ for cells grown on glucose.
Fractionation factors when homologous enzymes are used for the initial incorporation. Therefore, we think that in acetogenic bacteria, fractionation is controlled mainly by a later step in the reductive acetyl-CoA pathway, presumably the condensation of the 2 carbon molecules in the CODH/ACS system:

(ii) All the substrates we used were incorporated into the methyl branch of the acetyl-CoA pathway (Fig. 1), yielding the methyl group of the released acetate. However, the reduction of formate is accompanied by the release of CO₂ (equation 2); while one part of the methanol is oxidized to CO₂ to provide reducing equivalents, 3 molecules of methanol and 3 molecules of CO₂ are reduced to 3 molecules of acetate, resulting in a net consumption of 2 molecules of CO₂ (equation 4). In any case, our experimental setup does not allow us to determine whether the carboxyl group is directly formed from external CO₂ reduction (e.g., carbonate buffer) or originates from an intracellular CO₂ pool generated through the oxidation of methanol or formate.

The acetyl-CoA pathway can operate in both directions; while most acetogens use it for carbon fixation from CO₂ and energy conservation via an Ech or Rnf system (7), others can operate in the reverse direction together with a syntrophic partner to perform syntrophic acetate oxidation (63, 64). As a result, most reactions of the acetyl-CoA pathway can be seen as highly reversible and hence can easily equilibrate different isotopic δ¹³C values. Therefore, it is plausible that the first condensing step, not the fractionation of the catabolic reactions.

When *T. kivui* is grown at the expense of a methylated aromatic compound (vanillic acid), the initial step (O-demethylation of vanillic acid) may be less reversible. Indeed, initial trials resulted in less depleted acetate for *T. kivui* grown at the expense of vanillic acid (δ_{acet} = -50‰) (see Fig. S7 in the supplemental material) than for cultures grown on C₃ compounds (δ_{acet} = -67‰) (Fig. 1). These data could not be further evaluated, however, since the ¹³C value of the substrate vanillic acid could not be measured using our HPLC-IRMS approach.

**Growth on glucose and nitrate inhibition.** Both cultures investigated (*M. thermoacetica* and *T. kivui*) showed strong fractionation (ca. -50‰ to -35‰) during acetate formation when the acetyl-CoA pathway was the only operative pathway (34). As soon as glucose was used as a substrate, the apparent fractionation was due to a mixture of fermentation and the acetyl-CoA pathway and was less than -20‰.

These findings have environmental implications. The strong fractionation during acetate production via the acetyl-CoA pathway can easily be tracked in natural environments. Ideally, it can be used to estimate the contribution of acetogens to the acetate pool when the fractionations of all other acetate-generating and -depleting reactions are known (9). The mixed fractionation of acetogens grown on molecules larger than C₃, such as glucose (less than -20‰), may be difficult to differentiate in environmental systems, where the acetate signal deviates by ±10‰ from the 6¹³C value of total organic carbon (65). Part of this variation may originate from the contribution of acetylogenically formed ¹³C-depleted acetate. Indeed, incubations of lake sediments resulted in ¹³C-depleted acetate only under conditions of H₂/CO₂ addition, not in control experiments with N₂ (66), suggesting that the acetogens in the latter samples either are less active or grow on complex substrates.

**Fractionation into biomass.** Many studies use published pure culture fractionation factors to deduce the importance of a certain reaction in environmental settings. Most of these fractionation factors have been calculated using a mass balance equation based on the substrate-to-product conversion but neglecting the buildup of biomass. Hence, we aimed to ascertain if this approach is reasonable. Comparison of the fractionations during catabolism and anabolism shows that fractionation into biomass is usually lower than fractionation during catabolism. For example, the fractionation into biomass is roughly one-third of the fractionation into methane for *M.arkeri* grown on H₂/CO₂ (-13.9‰ versus -45.3‰), acetate (-7.3‰ versus -34.4‰), methanol (-31.1‰ versus -83.4‰), or trimethylamine (-24.8‰ versus -66.5‰) (22).

So far, the fractionation of pure bacterial cultures into biomass has been documented mainly using endpoint measurements of the microbial biomass (see Table S1 in the supplemental material). In contrast, we wanted to calculate the apparent fractionation into biomass following a time series experiment. Indeed, we found that the fractionation during anabolism was roughly two-thirds of the fractionation during catabolism. We consider some possible reasons for this ratio in the discussion accompanying Fig. S8 in the supplemental material.

In general, we would conclude that the fractionation into biomass is usually weaker than the fractionation during catabolism. Oxidation of glucose gives a high energy yield (ΔG°' = -2,870 kJ mol⁻¹), while anaerobic fermentation to CO₂ and CH₄ liberates much less energy (ΔG°' = -390 kJ mol⁻¹) (67). Hence, it can be assumed that under anaerobic conditions, most of the available carbon is used for catabolism and only a small portion can be used for anabolism. Therefore, it is unlikely that the bias caused by the fractionation into biomass will have an impact on the apparent fractionation of the catabolic reactions.

Another way to rationalize the fractionation into biomass becomes apparent when we compare different substrates (Table 2). The strongest fractionation can be found for C₁ compounds (CO₂, methanol) (Δ_{substrate-biomass} = -12.9‰ to -23‰). In comparison, the fractionation into biomass for organisms using acetate is much weaker (Δ_{substrate-biomass} = 1.4‰ to -6.5‰), while organisms utilizing larger molecules, such as glucose, have almost no discriminatory power with regard to the different carbon isotopes (Δ_{substrate-biomass} = 0.4‰ to -3.3‰).

<table>
<thead>
<tr>
<th>Organismic group</th>
<th>Substrate</th>
<th>Δ_{substrate-biomass} (avg ± SD)</th>
<th>No. of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetogens</td>
<td>CO₂</td>
<td>-12.9 ± 5.0</td>
<td>18</td>
</tr>
<tr>
<td>Methanogens</td>
<td>CO₂</td>
<td>-18.5 ± 6.3</td>
<td>23</td>
</tr>
<tr>
<td>Sulfate reducers</td>
<td>CO₂</td>
<td>-20.4 ± 13.9</td>
<td>5</td>
</tr>
<tr>
<td>Phototrophs</td>
<td>CO₂</td>
<td>-23.0 ± 8.4</td>
<td>8</td>
</tr>
<tr>
<td>Methanogens</td>
<td>Methanol</td>
<td>-20.5 ± 11.9</td>
<td>5</td>
</tr>
<tr>
<td>Methanogens</td>
<td>Acetate</td>
<td>+1.4 ± 6.7</td>
<td>4</td>
</tr>
<tr>
<td>Sulfate reducers</td>
<td>Acetate</td>
<td>-6.5 ± 1.3</td>
<td>2</td>
</tr>
<tr>
<td>Fermenting bacteria</td>
<td>Glucose</td>
<td>-0.4 ± 1.9</td>
<td>6</td>
</tr>
<tr>
<td>Acetogens</td>
<td>Glucose</td>
<td>-3.3 ± 3.0</td>
<td>6</td>
</tr>
</tbody>
</table>

* Details can be found in Table S1 in the supplemental material.
The isotope signal of organic carbon in environmental samples is rather constant, as is the signal in important carbon pools (65). Usually, the small differences observed can be explained by the small fractionation factors reported for fermentative processes (catabolic as well as anaerobic). On the other hand, strong fractionation into biomass has been reported for methanogens by use of endpoint measurements (22) and has been observed for acetogens grown on H2/CO2 (this study). Indeed, our results suggest that the fractionation of autotrophic organisms into their respective biomass is relatively strong and may, in principle, affect the organic fractionation of autotrophic organisms into their respective bio-
tionation during aceticlastic methanogenesis by Methanosa
pone.0053656.