Chapter 7

Quantification of Methanogenic Pathways Using Stable Carbon Isotopic Signatures

Quan Yuan

Abstract

In many anaerobic environments methane (CH_4) is produced by methanogens, with either H_2/CO_2 or acetate (i.e., the methyl group) as precursors, through what are referred to as hydrogenotrophic and acetoclastic methanogenic pathways respectively. Their relative contribution to total CH_4 production can be quantified by determining the stable carbon isotopic fractionation factors for both pathways as well as the isotopic signatures of CO_2, CH_4, and the methyl group in acetate of the sample. The procedures for measuring carbon isotopic fractionation factors of both methanogenic pathways and isotopic composition of these compounds by isotope ratio mass spectrometry are described in this chapter. The results are very helpful in evaluating the activity of the methanogens involved in each methanogenic pathway as well as those of other biological pathways with different fractionation factors.

Key words Stable carbon isotope, Isotopic fractionation factor, Methane, Methanogenic pathway, GC-C-IRMS

1 Introduction

While some information on microbial functioning can be obtained by using stable isotope probing techniques [1, 2] or combining genomic and metaproteomic approaches [3, 4], the in situ functions of the microbial communities usually can only be analyzed by incubation and measurement of the temporal change of biomarkers including DNA, RNA, and protein. However, analysis of stable isotope signatures in soil samples might overcome this problem, since the isotopic signatures partially reflect the microbial functioning [5].

Just under 99% of all carbon on earth consists of the stable isotope ^{12}C and approximately 1.11% of the stable isotope ^{13}C. The ^{13}C isotopic signature of a particular carbon compound is given by its ratio $R = {}^{13}C/{}^{12}C$ and is usually denoted relative to a standard (st) as $\delta^{13}C = 10^3\ (R/R_{st} - 1)$ [6]. The reactions in a

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biochemical pathway, especially those involving the cleavage of carbon bonds, often discriminate against the heavy ^{13}C isotope (kinetic isotope effect), because the reaction rate constants are larger for substrates with ^{12}C than ^{13}C [7]. As a result, the $\delta^{13}C$ of the product is always lower than that of the substrate. The fractionation factors (α) have been applied to quantify how much a given biochemical reaction (or pathway) discriminates against the substrate molecules containing the ^{13}C. For a reaction A → B the fractionation factor is defined as $\alpha_{A/B} = (\delta A + 1000)/(\delta B + 1000)$ [8], sometimes also expressed as an isotopic enrichment factor $\varepsilon \equiv 10^3 \times (1 - \alpha)$.

If two biochemical pathways display sufficiently different fractionation factors, reflected in the difference of $\delta^{13}C$ between substrate and product, these pathways can be differentiated by stable carbon isotope signatures [5, 9]. Indeed, fractionation factors are sufficiently different for some key biochemical pathways in anaerobic biodegradation of organic substrate. Therefore, it is possible to quantify the relative contribution of hydrogenotrophic and acetoclastic methanogenic pathways to CH_4 production, and of chemolithotrophic (acetyl-CoA synthase) and heterotrophic (fermentation) pathways to acetate formation.

In addition, stable carbon isotope analysis may allow partitioning the contribution of different organic substrates to end products of degradation, for example, the relative contribution of root exudation versus soil organic matter to CH_4 production in rice field soil [10, 11], provided the different substrates have substantial difference in $\delta^{13}C$ values (e.g., a mixture of C_3 and C_4 plants), and the carbon conversion pathways have negligible fractionation factors or the fractionation factors could be solved [10].

Here we present the methods of using stable carbon isotopic signatures for elucidating the microbial functional pathways of methane production.

2 Materials

1. Soil or sediment samples.
2. 26-mL borosilicate glass pressure tubes with crimp top.
3. Butyl rubber stoppers, aluminum crimps, and a crimping tool.
4. N_2 gas.
5. CH_3F (methyl fluoride) (*see* **Note 1**).
6. 1 mmol/L H_2SO_4.
7. 0.42 mol/L sodium peroxodisulfate.
8. 1.35 mol/L phosphoric acid.
9. NaOH.
10. Gas-tight pressure lock syringe.

11. 0.2-μm polytetrafluoroethylene (PTFE) membrane filters.
12. Gas chromatograph (GC) with flame ionization detector and methanizer (Ni catalyst at 350 °C).
13. High-performance liquid chromatograph (HPLC) with ion exclusion column, refractive index and UV detectors.
14. Isotope ratio mass spectrometer (IRMS).
15. Finnigan Standard GC Combustion Interface III, Finnigan LC IsoLink.
16. Pyrolytic oven.

3 Methods

3.1 Incubation Experiments

Set up all batches of anoxic rice field soil in multiple replicates, of which triplicates will be opened at different time points during the incubation, and analyzed as described below.

1. Prepare anoxic microcosms by adding 5 g soil + 5 mL of deionized water into 26-mL pressure tubes. Close the tubes with butyl rubber stoppers, and exchange the gas phase with N_2.
2. Add CH_3F to the headspace of half the treatments to a final concentration of 2%. Leave the remaining tubes without added CH_3F.
3. At regular intervals take gas samples from the headspace of the tubes and analyze for CH_4 and CO_2 as well as $\delta^{13}C$ value of CH_4 and CO_2, as described below.

3.2 Analyses of Gas and Liquid Samples

1. After vigorously shaking the bottles by hand, take gas samples (200 μL) with a gas-tight pressure lock syringe, and analyze immediately using gas chromatography (GC). CH_4, CH_3F, and CO_2 are analyzed using GC with a flame-ionization detector. CO_2 is detected after conversion to CH_4 with a methanizer.
2. Take liquid samples with a sterile syringe, membrane-filtered (0.2 μm) and store frozen (−20 °C) until analysis. Acetate is measured using high-performance liquid chromatography (HPLC) with refractive index and UV detectors.

3.3 Stable Carbon Isotope Analysis of CH_4 and CO_2 with Gas Chromatograph Combustion Isotope Ratio Mass Spectrometry (GC-C-IRMS)

1. The CH_4 and CO_2 in the gas samples are first separated by GC; after conversion of CH_4 to CO_2 in the Finnigan Standard GC Combustion Interface III, the $^{13}C/^{12}C$ is determined by the IRMS instrument.

3.4 Carbon Isotopic Measurements of Acetate Using an HPLC-LC-IRMS System

1. Load the acetate on an ion exclusion column with 1 mmol/L of H_2SO_4 at 0.3 mL/min as eluent, and coupled to a Finnigan LC IsoLink for oxidation of the separated compounds to CO_2 at 99.9 °C with 0.42 mol/L sodium peroxodisulfate and 1.35 mol/L phosphoric acid [12].
2. Detect the isotope ratios on an IRMS; the analysis results in determination of $\delta^{13}C$ of total acetate.

3.5 Measuring $\delta^{13}C$ of the Methyl Group of Acetate ($\delta_{ac\text{-}methyl}$) by Off-Line Pyrolysis

1. Purify the acetate in the liquid sample with HPLC by collecting the acetate fraction from each run.
2. Add the purified sample to a strong NaOH solution (final molar ratio of acetate to NaOH of 1:200), and dry in a Pyrex tube under vacuum.
3. Pyrolyze the dried reactants under vacuum at 400 °C, so as to convert the carboxyl carbon to CO_2 and the methyl carbon to CH_4 [13].
4. Take the gas samples and analyze the $\delta^{13}C$ of the produced CH_4 by GC-C-IRMS (*see* Subheading 3.3). This is identical to the $\delta^{13}C$ of the methyl carbon.

3.6 Calculations

3.6.1 Determination of the δCH_4

Calculate the isotopic signature for newly formed CH_4 (δn) from the isotopic signatures at two time points $t = 1(\delta_1)$ and $t = 2(\delta_2)$ with the following mass balance equation:

$$\delta_2 = f_n \delta_n + (1 - f_n)\delta_1 \tag{1}$$

with f_n the fraction of the newly formed C compound relative to the total at $t = 2$.

3.6.2 Calculation of Fractionation Factor for Conversion of CO_2 to CH_4 (α_{mc})

The apparent fractionation factor for conversion of CO_2 to CH_4 is given by

$$\alpha_{app} = (\delta_{CO_2} + 1000)/(\delta_{CH_4} + 1000) \tag{2}$$

the term "apparent" is used, since the isotope signature of CH_4 might be determined by acetoclastic plus hydrogenotrophic methanogenesis, while for the calculation only the isotope signature of the methanogenic substrate CO_2 is used. While in the presence of CH_3F, the α_{app} will be taken as αmc since the acetoclastic methanogenesis is inhibited (*see* **Note 2**).

3.6.3 Calculation of Fractionation Factor for Conversion of Acetate to CH_4 (α_{ma})

The isotopic effect $\varepsilon_{ac\text{-}methyl/CH4}$ associated with acetoclastic methanogenesis is calculated according to the Mariotti equation [14]:

$$\delta_r = \delta_{ri} + \varepsilon[\ln(1 - f)] \tag{3}$$

where δ_{ri} is the isotopic composition of the reactant (ac-methyl) at the beginning, which in our case is the maximum accumulation of acetate in the incubation; δ_r is the isotope composition of the residual reactant, when f was determined; and f is the fractional yield of the products based on the consumption of acetate ($0 < f < 1$). Linear regression of δ_r against $\ln(1 - f)$ gives ε as the slope. The enrichment factor could be converted to the fractionation factor according to $\varepsilon \equiv 10^3 \times (1 - \alpha)$.

When the acetate concentration approaches threshold values (200 μM), no fractionation occurs during conversion of fermentatively produced acetate to CH_4 ($\alpha_{ma} = 0$). In that case, $\delta^{13}C$ of CH_4 derived from acetate equals to $\delta_{ac\text{-}fermentation}$, which is the $\delta^{13}C$ value of acetate methyl produced by fermentation and is equal to $\delta_{ac\text{-}methyl}$ in the presence of CH_3F.

3.6.4 Determination of Relative Contribution of Hydrogenotrophic and Acetoclastic Methanogenic Pathways

Determine the relative contribution of H_2/CO_2-derived CH_4 to total CH_4 with the following mass balance equation [5]:

$$\delta_{CH_4} = f_{H_2}\delta_{mc} + \left(1 - f_{H_2}\right)\delta_{ma} \tag{4}$$

solved for f_{H_2}

$$f_{H_2} = (\delta_{CH_4} - \delta_{ma})/(\delta_{mc} - \delta_{ma}) \tag{5}$$

where f_{H_2} is the fraction of CH_4 formed from H_2/CO_2, δ_{CH_4} the $\delta^{13}C$ of total produced CH_4, and δ_{ma} and δ_{mc} are the isotope ratios of CH_4 derived from acetate and H_2/CO_2, respectively. The relative contribution of acetoclastic methanogenic pathway equals to $1 - f_{H_2}$ (*see* **Note 3**).

4 Notes

1. CH_3F is a specific inhibitor of acetoclastic methanogens, which does not affect hydrogenotrophic methanogens [15].
2. Fractionation factors have to be determined under well-defined conditions, which are usually only met by assaying defined microbial cultures or biochemical reactions in which the desired pathway operates.
3. Use of carbon isotopic signatures in CH_4 emitted from a production site (e.g., a wetland) requires even more complex models, since isotopic discrimination in addition occurs during transport and oxidation of the produced CH_4.

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