Geochemical Perspectives Letters

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Published by the European Association of Geochemistry

Carbon and hydrogen isotope fractionation in lipid biosynthesis by *Sporosarcina* sp. DSK25

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Supplementary Information

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- Materials and Methods
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Explanation for Graphical Abstract

The lambda index plot was constructed based on different groups of fatty acids (FAs) synthesised by gram-positive piezotolerant bacterium *Sporosarcina* sp. DSK25 under different growth pressures. The vertical coordinate represents bacterial growth pressure, while the horizontal coordinates are the two modified isotope fractionation factors, ($\alpha_C^{-1} - 1$) and ($\alpha_H^{-1} - 1$), computed based on Elsner *et al*. (2007). We divide the FAs into 4 groups: branched-FAs (BrFAs), saturated FAs (SFAs), and monounsaturated FAs (MUFAs) among the total FAs (TFAs), identified by different colors. Each group of FAs under each pressure has its ($\alpha_C^{-1} - 1$) and ($\alpha_H^{-1} - 1$) value (shown by red cubes). We then project the corresponding red cubes into the three-dimensional space. The red cubes in the three-dimensional space are the spatial distribution of the $\alpha_C^{-1} - 1$ and $\alpha_H^{-1} - 1$ values, for each group of fatty acids under all pressures. The bold lines and the coloured areas show the predictive relationships between the modified isotope discrimination factors and growth pressure for different groups of microorganisms (indicated by the four groups of fatty acid biomarkers)

Materials and Methods

Growth of *Sporosarcina* sp. DSK25

Sporosarcina sp. DSK25 is a piezotolerant bacterium isolated from a sediment sample taken from the Japan Trench (40'06.8'N 144'11.0"E) at a depth of 6500 m (Kato *et al*., 1995). This strain can grow at pressures ranging from 0.1 to 60 MPa (megapascal), with an optimal growth pressure of 0.1 MPa and growth temperature of 35 °C (Kato *et al*., 1995). Enzymatic activity assay showed that the protease produced by strain DSK25 exhibited optimal activity at 40 ° C and pH 9. It is interesting to note that pressure enhanced the activity of the protease, and the protease activity of strain DSK25 nearly doubled at pressure of 60 MPa relative to at atmospheric pressure (Kato *et al*., 1995).

Sporosarcina is a genus in the phylum of Firmicutes (class Bacilli). The Firmicutes are widely distributed in the pressured environments, *i.e.* the deep ocean and the deep biosphere (*e.g*., Kawai *et al*., 2015; Filippidou *et al*., 2016; Fang *et al*., 2017 and references cited therein). Therefore, *Sporosarcina* spp. is a species well representing the deep ocean and the deep biosphere and a model organism for studying carbon and hydrogen isotopic fractionation in lipid biosynthesis under high pressures.

In this study, strain DSK25 was grown in a custom-built high-pressure vessel (Fang *et al*., 2006, 2014). Growth medium was prepared using sterile-filtered natural seawater (δD = 6.2 ‰; Sigma Chem. Co.) with glucose (50 mM) as the sole carbon source, supplemented with yeast extract (0.04 %). The media was distributed into airtight pouches. Twenty milliliters of Florinert™ FC-40 (Sigma-Aldrich. Co.), pre-saturated by bubbling with high-purity oxygen at 4 °C for 12 h was added to the media to provide oxygen for the bacterium. FC-40 was filtered through a 0.22 μm sterilised membrane prior to use. The media was inoculated with DSK25 that was initially grown on agar plates (Difco™ Marine Broth 2216, Becton, Dickinson and Company, USA) at atmospheric pressure. Strain DSK25 was then incubated in high-pressure vessels at 35 °C and pressures of 0.1, 10, 20, 30, 40 and 50 MPa. Cultures were removed at early stationary phase (based on optical density measurements at 600 nm). Cell pellets were collected after centrifugation at 10, 000 g for 20 min for fatty acid and isotope analyses.

Lipid Extraction and Separation

Total bacteria lipids were extracted by using a one-phase solvent system containing dichloromethane (DCM), methanol, and phosphate buffer (potassium phosphate, dibasic, 50 mM, pH = 7.4, 2 : 1 : 0.8 by volume, Fang and Findlay, 1996). Crude lipids were obtained after phase partitioning by adding DCM and deionised water to test tube to the final ratio of methanol/ dichloromethane/ water 1 : 1 : 0.9. The total lipid was separated into hydrocarbons, neutral lipids, glycolipids, and phospholipids using miniature columns (Supelco, Inc., Bellefonte, PA) containing 100 mg silicic acid. Hydrocarbons, neutral lipids, glycolipids, and phospholipids were obtained by sequential elution with 5 mL aliquots of hexane, chloroform, acetone, and methanol, respectively.

Analysis of Fatty Acid by Gas Chromatography-Mass Spectrometry (GC-MS)

Phospholipids experienced a trans-methylation procedure to produce fatty acid methyl ester (FAMEs) before analysing (Fang and Findlay, 1996). Fatty acids were analysed on an Agilent 7890B Gas Chromatograph coupled with an Agilent 5977A Mass Spectrometer. Analytical separation of the compounds was accomplished using a 30 m ×0.25 mm×0.25 μm i.d. DB-5ms fused-silica capillary column (Agilent Technologies, Santa Clara, CA, USA). The column temperature was programmed from 50 to 120 °C at 10 °C/min and maintained at 120 °C for 5 min , and then heated to 300 °C at 5 °C /min and kept isothermally for 20 min. individual compounds were identified based on their mass spectra by comparison with the standard or published spectra and from the relative retention times. Concentration of the compound was determined according to the GC-MS response relative to that of an internal standard (Methyl stearate, Sigma-Aldrich. Inc.).

Carbon and Hydrogen Isotope Analysis by Isotope-Ratio Mass Spectrometer (IR-MS)

Hydrogen isotope analysis was done at the State Key Laboratory of Biogeology and Environmental Geology, China University of Geosciences, Wuhan, China. The δD of FAMEs was determined on a ThermoFinnigan Trace GC coupled with a Delta plus XP mass spectrometer via a ThermoFinnigan GC/TC pyrolysis interface operated at 1440 °C. Squalane with known hydrogen isotopic composition was used as reference peaks for calibrating isotopic accuracy. Instrument system error was corrected by using a standard mixture of $C_{16\sim30}$ even carbon numbered *n*-alkanes inserted every four analyses in the sequence. The δD value of FAMEs was corrected for the added methyl hydrogen by isotopic mass balance, with the δD value of methyl hydrogen derived from analysis of methanol. Each sample was measured three times, and the standard deviation was controlled within 5 ‰. The reported δD value is the average of triplicate measurements.

Hydrogen isotope analysis of glucose and seawater was done at the Third Institute of Oceanography, State Oceanic Administration (Xiamen, China) and the Institute of Earth Environment, Chinese Academy of Sciences (Xi'an, China), respectively. δD of glucose was determined using a Finnigan Thermal Conversion/-Elemental Analyzer (TCEA) interfaced to Finnigan Delta Plus XL isotope-ratio mass spectrometer. δD of the seawater was analysed using a 6890 gas chromatograph coupled with Finnigan Mat Delta Plus isotope ratio mass spectrometer. Two reference water samples were used to verify accuracy and reproducibility. Precisions for δD measurements are ±2 %.

Carbon isotope ratios analysis was performed in the Key Laboratory of Geological Processes and Mineral Resources, China University of Geosciences (Wuhan, China). $\delta^{13}C$ was determined using a HP 6890 gas chromatograph coupled with a Finnigan Mat Delta Plus isotope ratio mass spectrometer *via* a Finnigan Mat combustion furnace containing Cu and Ni wires that were doped with oxygen and maintained at a temperature of 850 °C. The GC was equipped with a DB-5ms fused silica capillary column (30 m × 0.25 mm × 0.25 μm). The initial temperature of the GC oven and the injection port was 50 °C, it was programmed from 50 to 120 °C at 10 °C /min after maintained at 50 °C for 1 min, and from 120 to 310 °C at 5 °C /min, then maintained at 310 °C for 20 min, with injection port temperature of 3 °C higher than oven temperature at all times. The carrier gas was helium with a flow speed of 1.2 ml/min. Two microliters of a sample solution spiked with squalane as an internal standard of known isotopic composition (-19.8 ‰ of $\delta^{13}C$) were injected into an injection port.

Isotope values were determined by averaging duplicate analyses. For compounds with a peak height of 0.5 ~ 5 V, precision was typically ≤0.5 ‰. The carbon isotopic composition of glucose (-10.8 ‰) was determined using bulk stable isotope analysis. Hydrogen and carbon isotopic composition of fatty acids is reported in the delta notation relative to the Vienna Standard Mean Ocean Water (VSMOW) and the Pee Dee Belemnite (PDB) standard, respectively. δ notion was given by: $\delta = \frac{R_{sample} - R_{std}}{R_{std}} \times 1000$ ‰, where R is the ratio of $^{13}C/^{12}C$ or $^{2}H/^{1}H$.

Calculation of Isotope Enrichment, Fractionation Factors and the Kinetic Isotope Effect, ε_{PDH}

Calculation of Isotope Enrichment and Fractionation Factors

The isotope fractionation factor, α, between compound a and b is defined as $\alpha_{a/b} = (1000 + \delta_a) / (1000 + \delta_b)$, and isotope enrichment factor, ε, was calculated by $\varepsilon_{a/b} = (\alpha_{a/b} - 1) * 1000$ (Sakata *et al.*, 1997; Sessions *et al.*, 1999). The detected δ values are the bulk isotope compositions subjected to correction by mass balance protocol (Hayes, 2001, Fang *et al.*, 2006, 2014). The isotopic composition of individual fatty acids were obtained after the correlation for the additional methyl group from methanol during derivatization of fatty acids, using a mass balance equation (Fang *et al.*, 2006, 2014).

Calculation of the Kinetic Isotope Effect, ε_{PDH}

The pressure-dependent isotope fractionation in biosynthesis of fatty acids by gram-negative piezophilic bacteria has been attributed to the kinetics of a series of enzymatic reactions (ε_{PDH}), and can be calculated using equation: $2 * \varepsilon_{FA/g} = (1 - f)\ \varepsilon_{PDH}$ (Sakata *et al.*, 1997), where *f* is the portion of pyruvate converting to acetyl-CoA (Monson and Hayes, 1982).

Generally, when glucose is the sole carbon source, carbon isotope fractionation during the biosynthesising of bacterial fatty acids by *Escherichia coli* will happen in carboxyl carbon rather than methyl carbon when pyruvate flows to acetyl-CoA catalysed by dehydrogenase, and no other mechanisms control this fractionation (Monson and Hayes, 1982; Sakata *et al.*, 1997). Based on these concepts, we have the following equations:

$$\delta^{13}C_{FA} = (\delta^{13}C_M + \delta^{13}C_C) / 2 \qquad \text{Eq. S-1}$$

$$\varepsilon_{C/g} = (1-f)\ \varepsilon_{PDH} \qquad \text{Eq. S-2}$$

where $\delta^{13}C_{FA}$ is the measured carbon isotope composition of overall fatty acids by IR-MS, $\delta^{13}C_M$ is the $\delta^{13}C$ value of methyl carbon, while $\delta^{13}C_C$ is the $\delta^{13}C$ value of carboxyl carbon in acetyl-CoA, $\varepsilon_{C/g}$ designates the fractionation between carboxyl carbon of acetyl-CoA and glucose, *f* is the fraction of pyruvate flowing to acetyl-CoA and ε_{PDH} represents the kinetic isotope effect of carbon by this enzymatic reaction.

Because methyl carbon doesn't fractionate, leading $\varepsilon_{M/g}$ to be zero, where $\varepsilon_{M/g}$ represents the fractionation between methyl carbon and glucose. Therefore, from Eq. S-1, we have the equation Eq. S-3: $\varepsilon_{FA/g} = \varepsilon_{C/g} / 2$, where $\varepsilon_{FA/g}$ is the fractionation between

overall fatty acids and glucose.

The kinetic isotope effect (ε_{PDH}) in fatty acid biosynthesis by *Escherichia coli* was approximately 23‰ (Monson and Hayes, 1982), however, if we assume that the ε_{PDH} were 23 ‰ in this study, then *f* will be negative under high growth pressures, this is apparently unreasonable (otherwise fatty acids wouldn't have been biosynthesised). Considering the low biomass and growth rate of DSK25 under high hydrostatic pressure, we then assume that *f* were as low as 0.1, the same value as reported in Fang *et al*. (2006)'s study. Subsequently, we can calculate the ε_{PDH} by (2 * $\varepsilon_{FA/g}$) / 0.9. Because ε_{PDH} are always expressed in positive values (Monson and Hayes, 1982; Sakata *et al*., 1997; Fang *et al*., 2006), thus here we adopted the absolute values of $\varepsilon_{FA/g}$.

In this study, we calculate the ε_{PDH} assuming that: 1) *Sporosarcina* sp. DSK25 biosynthesises fatty acids by the same biosynthetic pathway as do surface gram-negative bacteria, 2) ε_{PDH} for strain DSK25 was 23 ‰ at atmospheric growth pressure, the same as that for *E.coli* (Monson and Hayes, 1982), *3) f* was as low as 0.1 under high growth pressure (Fang *et al*., 2006). The corresponding ε_{PDH} thus can be calculated as: 25.2, 30.4, 34.5, 49.2 and 56.0 ‰ based on the average $\delta^{13}C$ values of all even carbon-numbered fatty acids, -22.0, -24.4, -26.2, -32.7 and 35.7 ‰ at 10, 20, 30, 40 and 50 MPa, respectively. Notice that the ε_{PDH} is supposed to be calculated from fatty acids with even number of carbon atoms (Sakata *et al*., 1997).

It is worthwhile to note that the average $\delta^{13}C$ values of all even carbon-numbered fatty acids at 0.1 MPa was -20.4 ‰, based on the ε_{PDH} value of 23 ‰, we then calculated the f value as 0.17, significantly lower than that (f = 0.76) for a well-studied gram-negative piezophilic bacterium *Moritella japonica* DSK1 (Fang *et al*., 2006).

Biosynthesis Pathways for Straight- and Branched-Chain Fatty Acids

Bacterial biosynthesis of BrFAs started by utilising branched short-chain carboxylic acids and α-keto acids as primer sources in a soluble/dissociated system known as the fatty acid synthase type Ⅱ (Fig. S-2, Brindley *et al*.,1969; Kaneda, 1991). Acyl-CoA : ACP transacylase can convert acetyl-CoA to acetyl-ACP by the reaction: Acyl-CoA + ACP ⇌ acyl-ACP + CoA in straight-chain fatty acid synthetase. However, the branched-chain fatty acid synthetase cannot efficiently catalyse this reaction, thus acetyl-acyl carrier protein (ACP) must be provided when a bacterium uses branched short-chain carboxylic acids as primer source, and this step is supposed to involve an enzyme that can convert the branched short-chain acyl-CoA to the corresponding ACP. On the other hand, when a bacterium uses α-keto acids as primer source, branched-chain α-keto acid decarboxylase, which does not exist in straight-chain fatty acid synthesising process will functionally catalyse the reaction from α-keto acids to the relative aldehyde (Fig. S-2), this step is followed by an enzyme-catalysed condensing reaction (Kaneda, 1991). The rest of the reactions are shared by two different fatty acid biosynthetic pathways using branched short-chain carboxylic acids and α-keto acids as primer sources, respectively. Fatty acids are finally produced through multiple repeating enzymatic reactions. In the entire biosynthetic process, hydrogenation reaction happens exactly in the β-ketoacyl ACP reductase reaction (KR) and the enoyl ACP reductase reaction (ER), while dehydration reactions take place in the β-hydroxyacyl-ACP dehydratase reaction (DH).

Calculation of Lambda Index

Two equations used to calculate the Λ values were (Elsner *et al*., 2007; Feisthauer *et al*., 2011):

$$(\Lambda) \approx \Delta(\delta D) / \Delta(\delta^{13}C) = (\delta_t D - \delta_0 D) / (\delta_t{}^{13}C - \delta_0{}^{13}C) \qquad \text{Eq. S-4}$$

$$(\Lambda) = (\alpha_H^{-1} - 1) / (\alpha_C^{-1} - 1) \qquad \text{Eq. S-5}$$

where $\delta_t D$ and $\delta_0 D$ are the measured hydrogen isotope values of fatty acids at time t and time 0, $\Delta(\delta D)$ is the difference between $\delta_t D$ and $\delta_0 D$, and α_H and α_C refer to the fractionation factors of hydrogen and carbon isotopes, respectively. Eq. S-4 gives the lambda value from the slope value of the linear expression of δD and $\delta^{13}C$. For cases with high hydrogen isotope fractionation (*e.g*., >-100 ‰, Feisthauer *et al*., 2011), Eq. S-5 is used to calculate lambda values. In this work, values of the lambda index were obtained by, first plotting the $(\alpha_H^{-1} - 1)$ and $((\alpha_C^{-1} - 1)$ of fatty acids at different pressures, then making the best linear fitting for different groups of fatty acids, the slope of the best-fit curve is the lambda value for each group of fatty acids (*e.g*., SFAs, BrFAs, etc). We set δ_0 in this study as the δD and $\delta^{13}C$ values at 0.1 MPa and δ_t as the corresponding values at higher pressures, 10, 20, 30, 40 and 50 MPa. Consider that hydrogen isotope fractionation is much higher than 100 ‰, we used Eq. S-5 for Λ calculation.

Statistical Analysis for Correlation Coefficient

We applied the t-test for the significance testing of correlation coefficient. The formula was given by: $t = \frac{r\sqrt{n-2}}{\sqrt{1-r^2}}$, where r refers to the

correlation coefficient, n is the sample size. The degree of freedom in our data is 2. The calculated t values were compared to the corresponding values in t-test table, P values were then obtained based on t and n. The difference in δD among BrFAs, MUFAs and SFAs was tested using independent-samples T test performed on IBM SPSS 20.0. We recalculated the abundance-weighted average δD values for BrFAs, MUFAs and SFAs. Calculations for $\delta^{13}C$ were done similarly.

Supplementary Figures

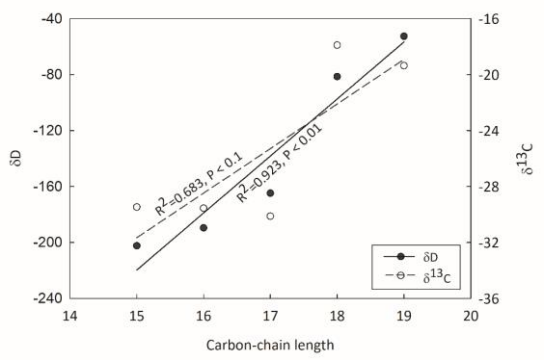

Figure S-1 Changes in carbon ($\delta^{13}C$) and hydrogen isotope compositions (δD) relative to the carbon-chain length of fatty acids biosynthesised by *Sporosarcina* sp. DSK25. Isotope ratios were obtained by averaging the delta values of all fatty acids that have the same carbon-chain length regardless of growth pressure.

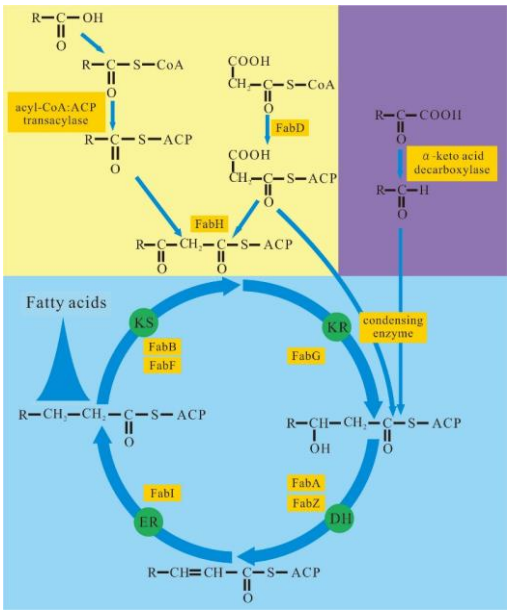

Figure S-2 A schematic diagram showing bacterial biosynthetic pathways of branched- and straight-chain fatty acids in a soluble system (modified from Wakil *et al.*, 1983; Kaneda, 1991; Heath *et al.*, 2001). The light yellow area shows the biosynthetic pathway that uses branched short-chain carboxylic acids as primer sources, while purple area displays the pathway that utilises α-keto acids as primer sources for branched-chain fatty acid biosynthesis. Biosynthetic pathway within the blue area is shared by both branched- and straight-chain fatty acids. Enzymes participated in individual reactions are tabbed in yellow. FabA: β-hydroxyacyl-ACP dehydratase/isomerase, FabB: β-ketoacyl-ACP synthase Ⅰ, FabD: malonyl CoA carbonxylase, carboxyltransferase β-subunit, FabF: β-ketoacyl-ACP synthase Ⅱ,FabG: β-ketoacyl-ACP reductase, FabH: β-ketoacyl-ACP synthase Ⅲ, FabI: trans-2-enoyl-ACP reductase Ⅰ, FabZ: β-hydroxyacyl-ACP dehydratase, KS: condensation, KR: reduction of β-keto group, DH: dehydration, ER: reduction of double bond.

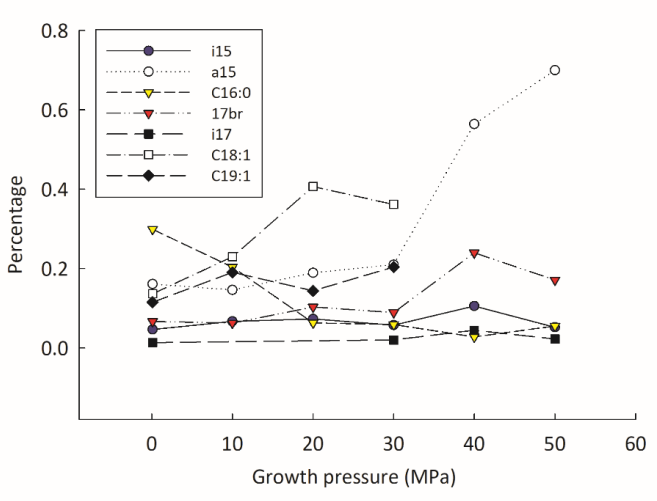

Figure S-3 Abundance of the phospholipids extracted from *Sporosarcina* sp. DSK25 grown under different hydrostatic pressures (expressed in percentage).

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