

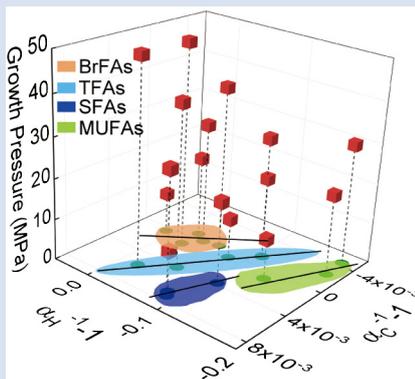
Carbon and hydrogen isotope fractionation in lipid biosynthesis by *Sporosarcina* sp. DSK25

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Abstract



bacterial growth pressure, can serve as a diagnostic tool for delineating microbially-driven biogeochemical processes in the deep ocean and deep biosphere.

Pressure dependent fractionation (PDF) of carbon and hydrogen isotopes in lipid biosynthesis by gram-negative piezophilic bacteria has been reported thus far, however, such PDF for gram-positive piezophilic bacteria remains to be characterised. Here, we report for the first time simultaneous quantification of stable carbon and hydrogen isotopic fractionations in lipid biosynthesis by the deep sea gram-positive piezotolerant bacterium *Sporosarcina* sp. DSK25. DSK25 was grown on glucose at pressures of 0.1, 10, 20, 30, 40 and 50 MPa, and δD and $\delta^{13}C$ of bacterial intact polar membrane lipids (IPLs) were determined. Carbon isotope fractionation between IPLs and glucose showed a positive correlation with bacterial growth pressure, whereas hydrogen isotope fractionation between IPLs and growth water exhibited an inverse correlation with growth pressure, suggesting that fractionation of both carbon and hydrogen isotopes is pressure dependent. We further examined the effects of pressure on the isotope enrichment factors of carbon and hydrogen isotopes and propose that the dual isotope derived lambda index, calculated based on

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Introduction

$\delta^{13}C$ and δD of lipids have been widely utilised as proxies in studying marine biogeochemical cycles (Li *et al.*, 2009), apportioning sources of marine organic matter (Chikaraishi *et al.*, 2005), determining microbial metabolisms (Kümmel *et al.*, 2016; Wijker *et al.*, 2019), reconstructing palaeoenvironments (Meyers, 1997) and tracing ancient biogeochemical processes in the geologic record (Sessions *et al.*, 2004). However, using δD or $\delta^{13}C$ values alone may be inconclusive or cause confusion or even errors because of the large variability in $\delta^{13}C$ and δD of microbial lipids. The latter, for example, can vary up to 400 ‰ for microbial lipids (Wijker *et al.*, 2019), depending on the type of microbial metabolism (Zhang *et al.*, 2009), lipid biosynthetic pathway (Fang *et al.*, 2006, 2014) and environmental factors. For instance, Fang *et al.* (2006) showed that fatty acid biomarkers with highly negative $\delta^{13}C$ values may be derived from marine bacterial secondary metabolism, not from bacterial utilisation of a ^{13}C -depleted carbon source (*e.g.*, methane), because carbon isotope fractionation in the biosynthesis of lipids is correlated with bacterial growth pressure. In a recent

study, Miller *et al.* (2018) observed large variations in isotope fractionation during methanogenesis under alkaline conditions, where carbon isotope fractionation increased with pH, reaching a maximum ^{13}C depletion of -85 ‰, whereas hydrogen isotope fractionation did not vary with pH. Thus, the two dimensional compound specific isotope analysis approach, employing $\delta^{13}C$ and δD simultaneously, has been favourably used in distinguishing biotic from abiotic processes, or identifying aerobic and anaerobic metabolism (Feisthauer *et al.*, 2011; Kümmel *et al.*, 2016; Musat *et al.*, 2016), among other applications. For example, the combined use of carbon and hydrogen isotope analyses allowed Feisthauer *et al.* (2011) to monitor and assess the occurrence of microbial methane oxidation in the marine and terrestrial environments.

Additionally, the current stable isotope geochemical theory, established based on surface bacteria rather than deep sea piezophilic bacteria, is not applicable to studying deep ocean biogeochemistry because of the pressure induced changes in microbial physiology and isotope fractionation (Fang *et al.*, 2006). However, previous studies focused mainly on investigating carbon and hydrogen isotope fractionations in lipid

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biosynthesis by gram-negative piezophilic bacteria (Fang *et al.*, 2006, 2014), and there have been no reports of similar studies on gram-positive piezophilic bacteria. Yet, gram-positive bacteria likely play a similarly important role as gram-negative bacteria in mediating biogeochemical processes, especially in the deep ocean and the deep biosphere (Fang *et al.*, 2017).

Motivated by these concerns, we designed experiments aiming to quantify carbon and hydrogen isotope fractionation simultaneously in lipid biosynthesis by the gram-positive piezotolerant bacterium *Sporosarcina* sp. DSK25. Results from this study allow us to derive the lambda index for deciphering microbially mediated biogeochemical processes in pressured environments of the deep ocean and the deep biosphere.

Materials and Methods

Sporosarcina sp. DSK25 is a piezotolerant bacterium isolated from the Japan Trench at a depth of 6500 m (Kato *et al.*, 1995). DSK25 was grown in custom-built high pressure vessels (Fang *et al.*, 2006). Growth medium was prepared using sterile filtered natural seawater (Sigma-Aldrich Co.) with glucose as the sole carbon source, supplemented with yeast extract (0.04 %, Supplementary Information, SI). The medium was distributed into airtight pouches, and then incubated in high pressure vessels at 35 °C (optimal growth temperature of the strain) and pressures of 0.1, 10, 20, 30, 40 and 50 MPa (SI). Cultures were removed at the stationary phase (Fig. 1). Cell pellets were collected after centrifugation.

Bacterial lipids were extracted using a one phase solvent system containing dichloromethane, methanol, and phosphate buffer (Fang and Findlay, 1996). Intact polar membrane lipids (IPLs) were obtained from the total lipids by using miniature columns containing 100 mg silicic acid, eluted with 5 mL of methanol, followed by a trans-methylation procedure to produce fatty acid methyl esters (FAMES) (Fang and Findlay, 1996). FAMES were analysed using gas chromatography mass spectrometry. The δD and $\delta^{13}C$ of FAMES were determined by an isotope ratio mass spectrometer (Fang *et al.*, 2006, 2014). δ notation was given by: $\delta = \frac{R_{\text{sample}} - R_{\text{std}}}{R_{\text{std}}} \times 1000 \text{‰}$, where R is the ratio of $^{13}C/^{12}C$ or $^2H/^1H$. Significance testing of the correlation coefficient was performed using a t-test (SI).

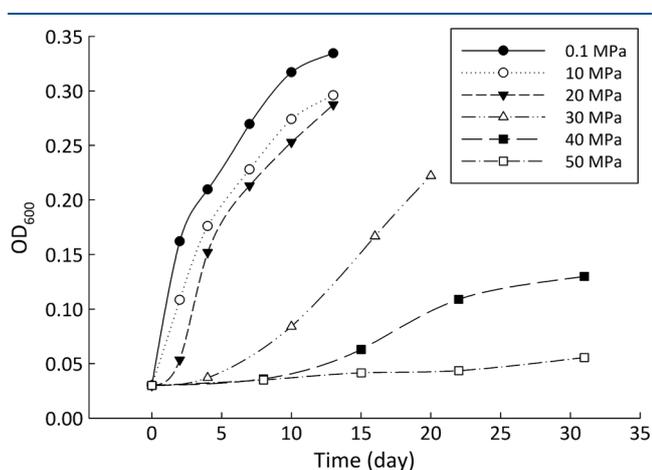


Figure 1 Growth curve of *Sporosarcina* sp. DSK25 under different pressures.

Results

DSK25 synthesised saturated (SFAs), monounsaturated (MUFAs) and branched chain fatty acids (BrFAs). The three groups of FAs constitute 35.6, 38.0 and 26.4 % of the total fatty acids (TFAs), respectively (Table 1a; Fig. S-2). This lipid profile is similar to that observed in gram-positive non-piezophilic bacteria grown at atmospheric pressure, except that higher proportions of BrFAs were observed in DSK25 (*e.g.*, Kaneda, 1991). BrFAs had the most depleted $\delta^{13}C$ values, -29.9‰ , abundance weighted averaging (awa) over all growth pressures (Table 1b), whereas straight chain fatty acids (SCFAs) had relatively higher $\delta^{13}C$ values (awa): -24.2 and -19.3‰ for SFAs and MUFAs (Table 1b), respectively. Yet C16:0 and C16:1 were more depleted in ^{13}C than BrFAs at high pressures (*e.g.*, 40 and 50 MPa). The calculated carbon isotopic enrichment factor, ϵ , showed a strong correlation with pressure, with R^2 of 0.75 ($p < 0.1$) and 0.84 ($p < 0.02$) for SFAs and MUFAs, respectively (Fig. 2). A progressive increase in ϵ with pressure was observed, as evidenced by $\epsilon_{\text{FA/gy}}$ -12.6 , -16.2 , -15.3 , -16.2 , -21.0 and -22.6‰ at 0.1, 10, 20, 30, 40 and 50 MPa, respectively. A linear correlation was observed between $\delta^{13}C$ and FAs carbon chain length ($p < 0.1$, Fig. S-1).

Similarly, BrFAs were more depleted in D compared to MUFAs and SFAs under all pressures (Table 1c) ($p < 0.05$ between BrFAs and SFAs), except that C16:0 and C16:1 were more depleted in D than i15:0 under atmospheric pressure. The δD values (awa) for SFAs, MUFAs and BrFAs were -173.2 , -76.9 and -196.1‰ , respectively. Average hydrogen isotope fractionation for SFAs, MUFAs and BrFAs was -165.7 , -72.8 , and -212.2‰ relative to growth water, and -155.3 , -61.1 , and -212.2‰ relative to glucose. A strong negative linear correlation was observed between hydrogen isotope fractionation and bacterial growth pressure (Fig. 2), with R^2 values of 0.99 ($p < 0.01$), 0.95 ($p < 0.002$), 0.79 ($p < 0.05$), 0.75 ($p < 0.5$) and 0.70 ($p < 0.5$) for 17:0br, a15:0, i15:0, C19:1 and C18:1, respectively. A strong linear correlation between δD values and FAs carbon chain length was also observed ($P < 0.01$, Fig. S-1).

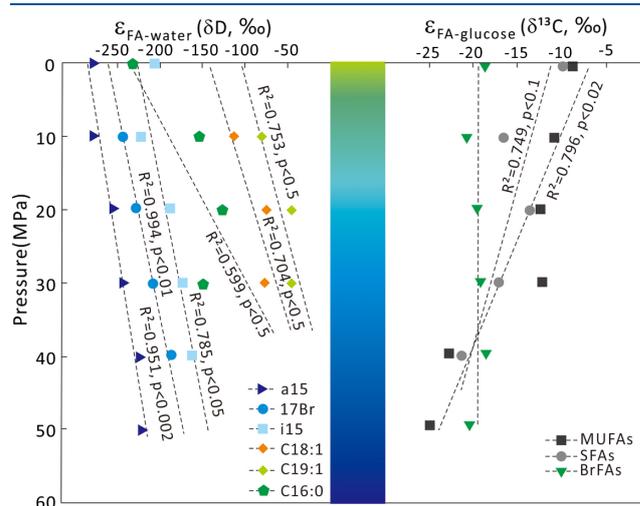


Figure 2 Carbon and hydrogen isotope fractionation of *Sporosarcina* sp. DSK25 changes with different growth pressures.

Discussion

Pressure dependent carbon and hydrogen isotope fractionation in lipid biosynthesis. The deep ocean and the deep biosphere are the most extensive pressured habitat on Earth, hydrostatic

Table 1 (a) Abundance (in percentage) of fatty acids extracted from *Sporosarcina* sp. DSK25 grown under different pressures. (b) $\delta^{13}\text{C}$ values (‰) of fatty acids isolated from *Sporosarcina* sp. DSK25 grown under different pressures. (c) δD values (‰) of fatty acids isolated from *Sporosarcina* sp. DSK25 grown under different pressures.

(a)						
Compound	Pressure (MPa)					
	0.1	10	20	30	40	50
<i>i</i> 15:0 ^a	4.63	6.69	7.30	5.74	10.54	5.19
<i>a</i> 15:0	16.09	14.60	18.93	20.96	56.41	69.99
C16:0	29.93	20.36	6.28	5.86	2.75	5.49
C16:1	7.10	-	-	-	-	-
C17:0	-	1.22	-	-	-	-
C17:1	9.15	8.90	-	-	-	-
<i>i</i> 17:0	1.33	-	-	2.00	4.43	2.24
<i>a</i> 17:0	-	-	2.13	-	1.92	-
17:0 <i>br</i> *	6.65	6.26	10.29	8.90	23.95	17.09
C18:1	13.63	22.94	40.69	36.17	-	-
C19:1	11.49	19.03	14.38	20.37	-	-
(b)						
Compound	Pressure (MPa)					
	0.1	10	20	30	40	50
<i>i</i> 15:0	-29.3	-29.7	-28.5	-29.0	-28.2	-30.1
<i>a</i> 15:0	-29.9	-30.5	-29.2	-30.0	-28.7	-30.7
C16:0	-20.6	-27.3	-24.3	-27.7	-32.0	-
C16:1	-21.2	-19.4	-31.6	-35.0	-33.4	-35.7
<i>i</i> 16:0	-	-30.1	-	-	-	-
<i>a</i> 16:0	-	-32.4	-	-	-	-
C17:1	-20.1	-26.9	-	-	-	-
<i>a</i> 17:1	-	-	-	-	-	-34.7
<i>i</i> 17:0	-30.8	-34.2	-31.8	-31.8	-30.7	-
<i>a</i> 17:0	-27.8	-32.0	-31.8	-28.6	-29.7	-32.7
C18:1	-19.4	-19.4	-17.1	-15.7	-	-
C19:1	-18.4	-20.6	-20.6	-17.9	-	-
(c)						
Compound	Pressure (MPa)					
	0.1	10	20	30	40	50
<i>i</i> 15:0	-188.6	-203.6	-174.1	-158.8	-150.5	-
<i>a</i> 15:0	-254.9	-255.5	-234.7	-224.1	-205.7	-203.1
C16:0	-213.4	-141.6	-117.0	-137.3	-	-
C16:1	-226.9	-	-	-	-	-
C17:1	-	-127.8	-	-	-	-
<i>i</i> 17:0	-	-	-	-	-167.1	-
17:0 <i>br</i>	-	-223.2	-210.0	-191.9	-173.2	-
C18:1	-	-104.7	-68.8	-70.9	-	-
C19:1	-	-74.2	-41.9	-41.7	-	-

^aFatty acids are designated by the total number of carbon atoms: numbers of double bond, *a* is anteiso-branched, and *i* is iso-branched.

Fatty acids that were either absent or at a too low concentration for a reliable determination of δD or $\delta^{13}\text{C}$ values.

*Methyl branched fatty acid. The position of the methyl group was not identified on GC-MS.

pressure ranges over four or more orders of magnitude (Bartlett and Welch, 1995). Pressure affects microbial physiology, metabolism and carbon isotope fractionation in the biosynthesis of lipids (Fang *et al.*, 2006, 2014). Thus, Fang *et al.* (2010) suggested establishing a new field of geochemistry: high pressure microbial isotope geochemistry.

Our results show that, similar to their gram-negative counterpart (Fang *et al.*, 2006), the deep sea gram-positive piezotolerant bacterium DSK25 exhibited pressure dependent carbon isotope fractionation between fatty acids and glucose, with ϵ increasing with bacterial growth pressure. The kinetic carbon isotope effect, ϵ_{PDH} is calculated as 25.2, 30.4, 34.5, 49.2 and



56.0 ‰ at 10, 20, 30, 40 and 50 MPa, respectively (see calculations presented in SI), suggesting a consistent pressure dependent carbon isotopic fractionation. However, the magnitude of carbon isotope fractionation observed here is much lower than that found in the gram-negative piezophilic bacterium DSK1, by 2.5 and 1.9 ‰ at 10 and 20 MPa, respectively (Fang *et al.*, 2006). Such differences might be related to the physiological differences between the two groups of bacteria (gram-positive and -negative bacteria). We hypothesise that bacterial cell wall physiology and properties of the cell envelope affect isotope fractionation. Specifically, gram-positive bacteria, lacking the restrictive outer membrane, experience less substrate mass transfer limitation at cell membranes than gram-negative bacteria and therefore, less isotope fractionation. Additionally, microbial stress response and fitness optimisation may also play a role on carbon isotope fractionation (Fang *et al.*, 2006). These hypotheses await to be tested in future studies.

Compared with SCFAs, BrFAs have the most depleted $\delta^{13}\text{C}$ and δD values (awa), except for the $\delta^{13}\text{C}$ values of C16:0 and C16:1 under high pressures and δD values of the two fatty acids at atmospheric pressure (Tables 1b and 1c). It seems plausible that microbial growth pressure affects the biosynthesis and isotopic composition of BrFAs and SCFAs differently. One hypothesis is that, under high pressures, biosynthesis of BrFAs may compete with that of SCFAs for D-depleted substrates and may be biosynthesised prior to SCFAs, leading to the remaining substrate being enriched in D, and thus SCFAs enriched in D. Alternatively, this resulted from microbial utilisation of different energy metabolic pathways in growth and metabolism (Wijker *et al.*, 2019). Despite the fact that both BrFAs and SCFAs are synthesised by the same biosynthetic pathway with almost the same enzymes (Fig. S-3) and that the mechanisms of lipid biosynthesis are strongly conserved across most bacteria phyla (Rock and Jackowski, 2002), the reducing power NADPH produced by different metabolic pathways retain different isotopic compositions, and consequently differing δD of fatty acids, as NADPH provides the largest source of hydrogen in microbial biosynthesis of fatty acids (Zhang *et al.*, 2009; Wijker *et al.*, 2019; see discussion below).

Hydrogen isotopic fractionations relative to growth water, $\epsilon_{\text{FA}/\text{w}}$ and to glucose, $\epsilon_{\text{FA}/\text{g}}$ are also pressure dependent, but in an inverse pattern (Table 1c, Fig. 2). Findings from this and previous works (Fang *et al.*, 2006, 2014) suggest that the pressure dependent fractionation of carbon and hydrogen isotopes may be a general trait for both gram-positive and -negative piezophiles in the deep ocean. Hydrogen isotope fractionation may be governed by pressure regulated kinetic isotope effects during lipid biosynthesis. Fang *et al.* (2014) suggested that lipid biosynthetic pathways (the PKS *vs.* the FAS pathway) and the pressure affected hydrogen tunneling are the determining factors in controlling δD of lipids biosynthesised by the piezophilic gram-negative bacterium DSK1 under high pressures. Zhang *et al.* (2009) showed that the δD signal of NADPH derived from different metabolic pathways may control the δD of microbial lipids, with NADPH from heterotrophic growth on the TCA-cycle yielding the most enriched lipid δD . Recently, Wijker *et al.* (2019) found that the flux of NADPH through the TCA-cycle was strongly positively correlated with $\epsilon_{\text{L}/\text{w}}$, producing the most positive fractionations. We hypothesise that the up-regulation of the TCA-cycle may occur at the expense of the PP and EMP pathways under high pressures, leading to D-enrichment of lipids. The exchange of hydrogen between NADPH and growth water, which could result in D enrichment in lipids, was studied in previous works (Zhang *et al.*, 2009; Wijker *et al.*, 2019). For example, more H exchange between NADPH and extracellular water would happen in the TCA cycle than in other metabolic

pathways (Sachs *et al.*, 2016 and references therein). In addition, bacterial growth rate was quite low at high pressures (Fig. 1). Therefore, an alternative hypothesis is that the exchange of hydrogen between NADPH and growth water could result in D-enrichment in lipids under high growth pressures, where bacteria doubling time is prolonged.

The $\delta^{13}\text{C}$ and δD -derived lambda index. It is apparent that both carbon and hydrogen isotope fractionations are pressure dependent and that different groups of FAs exhibit distinct isotopic characteristics. Our results lead us to propose the lambda index (Λ), ratio of hydrogen *versus* carbon discrimination which can be calculated based on α_{H}^{-1} and α_{C}^{-1} values of FAs (SI; Feisthauer *et al.*, 2011). Λ values of 11.3 ± 1.1 , 7.9 ± 3.3 and 6.7 ± 2.0 were obtained for TFAs, MUFAs and SFAs, respectively (Fig. 3). Previous studies showed that the Λ index differed in aerobic and anaerobic microbiological processes (Dorer *et al.*, 2014). Feisthauer *et al.* (2011) reported that the lambda values ranged from 7.3 ± 0.3 to 10.5 ± 0.5 for aerobic methane oxidising bacteria, significantly different from that for sulfate and nitrate reducing bacteria (Dorer *et al.*, 2014). Musat *et al.* (2016) used the Λ values to differentiate aerobic and anaerobic degradation processes of saturated and alkylated aromatic hydrocarbons.

Conceptually, carbon and hydrogen are inseparable atoms of lipid biomarkers, and would experience the same enzymatic reactions, but suffer from different hydrostatic pressure effects (*e.g.*, hydrogen tunneling). An important observation of the present study is that bacterial growth pressure affects both α_{C}^{-1} and α_{H}^{-1} . Hence, simultaneous measurement of $\delta^{13}\text{C}$ and δD and the derived α_{C}^{-1} and α_{H}^{-1} provides a two dimensional isotopic characterisation of the lipids. We suggest that the lambda index, calculated as such, provides new insights into hydrostatic pressure related, microbially-driven biogeochemical processes. For one, the lambda index may serve as a useful tracer for determining the source of marine organic matter in the deep ocean. For the other, it has the potential to differentiate biogeochemical processes mediated by different groups of microorganisms. For instance, Λ for BrFAs, biomarkers for gram-positive bacteria (Kaneda, 1991; White *et al.*, 1997) and certain sulfate reducing bacteria (Taylor and Parkes, 1983), is different from that for MUFAs, abundant in gram-negative bacteria (White *et al.*, 1997; Fang *et al.*, 2006). However, it must be noted that there may be overlap in lipid

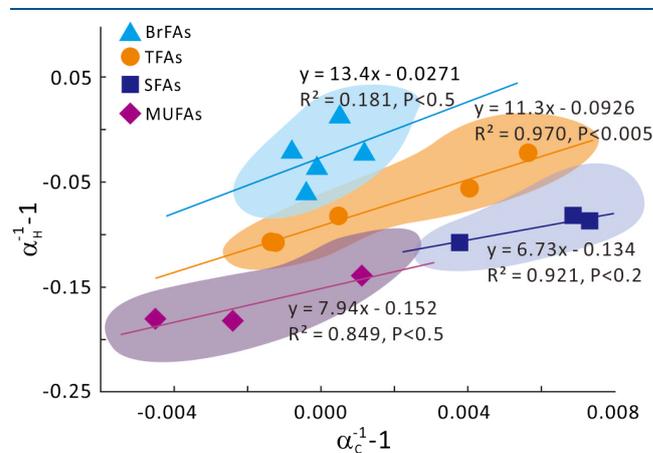


Figure 3 Plot of hydrogen versus carbon isotope fractionation factors observed in lipid biosynthesis by *Sporosarcina* sp. DSK25 grown at pressures of 0.1, 10, 20, 30, 40 and 50 MPa. The slope of the regression curves gives the lambda (Λ) values. TFAs: total fatty acids; MUFAs: monounsaturated fatty acids; SFAs: saturated fatty acids; BrFAs: branched-chain fatty acids; α_{H} : fractionation factor of hydrogen isotope, α_{C} : fractionation factor of carbon isotope.

biomarkers and lambda values between different groups of microbes. Therefore, caution is necessary when the lambda index is used to constrain microbes with different metabolisms. Nevertheless, the lambda values in this study are derived from bacteria grown at different pressures, fundamentally different from those reported in previous studies, and can therefore, be used for deciphering microbial metabolism and biogeochemical processes in pressured environments of the deep ocean and the deep biosphere. Future studies using $\delta^{13}\text{C}$ and δD in investigating deep ocean microbial biogeochemical processes should take into consideration of hydrostatic pressure-induced changes in $\delta^{13}\text{C}$ and δD .

Conclusions

This study for the first time simultaneously quantified carbon and hydrogen isotope fractionations in the biosynthesis of fatty acids by a gram-positive piezotolerant bacterium, influenced by microbial physiology and growth pressure. Our results confirm the consistent pressure dependent fractionation of carbon and hydrogen isotopes, in both direction and magnitude. Our results suggest that pressure dependent isotope fractionation is a general feature of deep sea pressure-loving bacteria. We propose the dual isotope derived lambda index, calculated based on bacterial growth pressure for different groups of fatty acids biomarkers, can serve as a useful tool for constraining microbially-driven biogeochemical processes in the deep ocean and the deep biosphere. We hope that our approach will contribute to establishing high pressure isotope biogeochemistry, towards understanding of natural microbial biogeochemistry in pressured environments today and those in the geological past. Future culture-based research should be directed to studying the changing $\delta^{13}\text{C}$, δD and the lambda index of lipids for more deep sea microorganisms, associated with the shifting microbial metabolism and production of isotopically different NADPHs, under different hydrostatic pressures.

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Additional Information

Supplementary Information accompanies this letter at <http://www.geochemicalperspectivesletters.org/article2016>.



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

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■ Supplementary Information

The Supplementary Information includes:

- Explanation for Graphical Abstract
- Materials and Methods
- Calculation of Isotope Enrichment, Fractionation Factors and the Kinetic Isotope Effect, ϵ_{PDH}
- Biosynthesis Pathways for Straight- and Branched-Chain Fatty Acids
- Calculation of Lambda Index
- Statistical Analysis for Correlation Coefficient
- Figures S-1 to S-3
- Supplementary Information References

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 ($\delta D = 6.2$ ‰; 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 \times 0.25 mm \times 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 C₁₆₋₃₀ 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 $\pm 2\%$.

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 \times 0.25 mm \times 0.25 μ m). 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 $\leq 0.5\%$. 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 $^2H/^1H$.

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 $\epsilon_{a/b} = (\alpha_{a/b} - 1) \times 1000$ (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: $2 * \epsilon_{FA/g} = (1 - f) \epsilon_{PDH}$ (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 biosynthesis 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:

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

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

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, $\epsilon_{C/g}$ 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 $\epsilon_{M/g}$ to be zero, where $\epsilon_{M/g}$ represents the fractionation between methyl carbon and glucose. Therefore, from Eq. S-1, we have the equation Eq. S-3: $\epsilon_{FA/g} = \epsilon_{C/g} / 2$, where $\epsilon_{FA/g}$ 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 $(2 * \epsilon_{\text{FA/G}}) / 0.9$. 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 $\epsilon_{\text{FA/G}}$.

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 $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ 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: $\text{Acyl-CoA} + \text{ACP} \rightleftharpoons \text{acyl-ACP} + \text{CoA}$ 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):

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

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

where $\delta_t\text{D}$ and $\delta_0\text{D}$ are the measured hydrogen isotope values of fatty acids at time t and time 0, $\Delta(\delta\text{D})$ is the difference between $\delta_t\text{D}$ and $\delta_0\text{D}$, 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 $\delta^{13}\text{C}$. 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 $\delta^{13}\text{C}$ 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

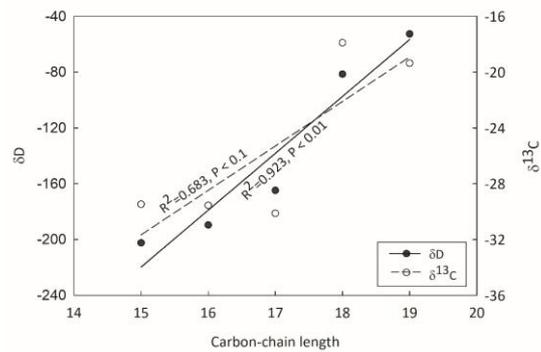


Figure S-1 Changes in carbon ($\delta^{13}C$) and hydrogen isotope compositions (δD) relative to the carbon-chain length of fatty acids biosynthesised by *Sporosarcina* sp. DSK25. Isotope ratios were obtained by averaging the delta values of all fatty acids that have the same carbon-chain length regardless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

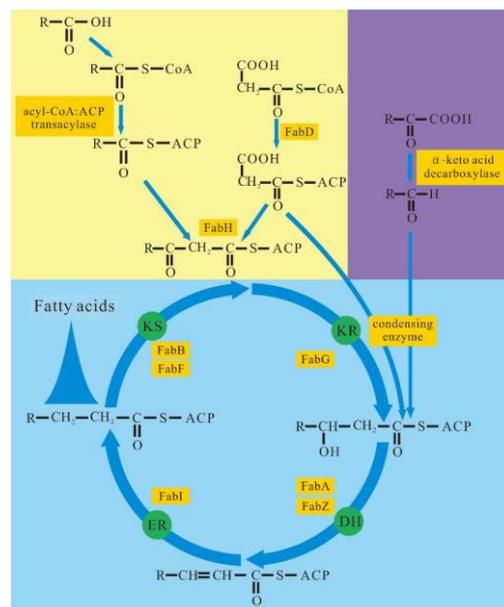


Figure S-2 A schematic diagram showing bacterial biosynthetic pathways of branched- and straight-chain fatty acids in a soluble system (modified from Wakil *et al.*, 1983; Kaneda, 1991; Heath *et al.*, 2001). The light yellow area shows the biosynthetic pathway that uses branched short-chain carboxylic 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 carboxylase, 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.



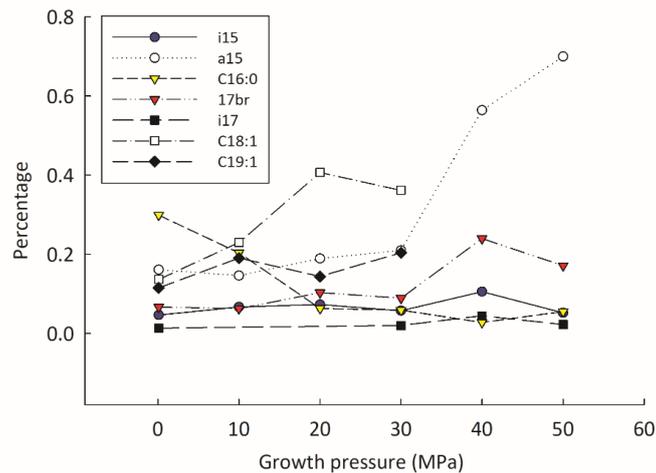


Figure S-3 Abundance of the phospholipids extracted from *Sporosarcina* sp. DSK25 grown under different hydrostatic pressures (expressed in percentage).

Supplementary Information References

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