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Tracing carbon assimilation in endosymbiotic deep-sea hydrothermal vent Mytilid fatty acids by ^{13}C-fingerprinting

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Abstract. *Bathymodiolus azoricus* mussels thrive at Mid-Atlantic Ridge hydrothermal vents, where part of their energy requirements are met via an endosymbiotic association with chemolithotrophic and methanotrophic bacteria. In an effort to describe phenotypic characteristics of the two bacterial endosymbionts and to assess their ability to assimilate CO_2, CH_4 and multi-carbon compounds, we performed experiments in aquaria using ^{13}C-labeled $NaHCO_3$ (in the presence of H_2S), CH_4 or amino-acids and traced the incorporation of ^{13}C into total and phospholipid fatty acids (tFA and PLFA, respectively). $14:0$; $15:0$; $16:0$; $16:1(n-7)c+t$; $18:1(n-13)c+t$ and $(n-7)c+t$; $20:1(n-7)$; $20:2(n-9,15)$; $18:3(n-7)$ and $(n-5,10,13)$ PLFA were labeled in the presence of $H^{13}CO_3^-$ (+H_2S) and $^{13}CH_4$, while the $12:0$ compound became labeled only in the presence of $H^{13}CO_3^-$ (+H_2S). In contrast, the $17:0$; $18:0$; $16:1(n-9)$; $16:1(n-8)$ and $(n-6)$; $18:1(n-8)$; and $18:2(n-7)$ PLFA were only labeled in the presence of $^{13}CH_4$. Some of these symbiont-specific fatty acids also appeared to be labeled in mussel gill tFA when incubated with ^{13}C-enriched amino acids, and so were mussel-specific fatty acids such as $22:2(n-7,15)$. Our results provide experimental evidence for the potential of specific fatty acid markers to distinguish between the two endosymbiotic bacteria, shedding new light on C_1 and multi-carbon compound metabolic pathways in *B. azoricus* and its symbionts.

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1 Introduction

Deep-sea hydrothermal vents host peculiar ecosystems fueled by methane, sulfide, iron or even hydrogen (e.g., Perner et al., 2009). The symbiotic association of bacteria with marine invertebrate hosts provides the former with access to the chemical substrates necessary for their metabolism and the latter with a source of organic carbon (and nitrogen). One of the two species of endosymbiotic bathymodioline Mytilids occurring along the Mid-Atlantic Ridge (MAR), *Bathymodiolus azoricus*, is found at the northernmost sites Menez Gwen, Lucky Strike and Rainbow (Desbruyères et al., 2001). Transmission electron microscopy has demonstrated the presence of two distinct Gram-negative bacterial endosymbionts inside specialized gill epithelial cells (bacteriocytes). One of these symbionts has centrally stacked intracytoplasmic membranes characteristic for methane-oxidising gamma-proteobacteria (Fiala-Médioni et al., 2002). Analysis of 16S rRNA sequences followed by fluorescence in situ hybridization (FISH) evidenced that *B. azoricus* endosymbiotic 16S rRNA phylotypes cluster with natural symbiotic and cultured sulfide-oxidising (SOX) and methane-oxidising gamma-proteobacteria (Won et al., 2003; Duperron et al., 2006; Spiridonova et al., 2006). Enzymatic and physiological assays using gills of *B. azoricus* from the Lucky Strike and Menez Gwen vent sites revealed the presence of active enzymes of the metabolic cycles of inorganic C assimilation and sulfide oxidation and of the C_1 carbon assimilation pathway (Fiala-Médioni et al., 2002). Furthermore, Riou et al. (2008) report active ^{13}C incorporation from bicarbonate

in the presence of sulfide as well as from methane within the gills of *B. azoricus*. This incorporation was followed by carbon transfer to the host's aposymbiotic muscle tissue.

In order to understand the modes of matter and energy transfer to the host, the phenotypes of *B. azoricus* symbionts' need to be characterized. Classification of phospholipid ester-linked fatty acids (PLFA) profiles has proven useful to clarify bacterial genus and species interrelationships established by DNA-based phylogeny (Bodelier et al., 2009). PLFA profiles thus allow to distinguish between different methane-oxidising bacteria (MOB) species as evidenced for example by the fact that methane-oxidising gamma-proteobacteria (formerly named after "Type I MOB") mainly contain fatty acids with 14 and 16 carbon atoms, while methane-oxidising alpha-proteobacteria (formerly "Type II MOB") PLFA are mainly composed of 18 carbon atoms (Nichols et al., 1985). In addition, MOB bacteria possess fatty acids that are not found in any other known microorganism (methane-oxidising gamma-proteobacteria: $16:1(n-8)$ and $16:1(n-5)t$; alpha-proteobacteria: $18:1(n-8)$) and these compounds therefore represent valuable biomarkers (e.g. Nichols et al., 1985).

The main difficulty consists in the impossibility to grow *B. azoricus*' symbionts in pure cultures. Tracer experiments represent powerful tools enabling the detection of the bacterial fatty acid signal inside the host. However, in a tissue total lipid extract, the symbionts' fatty acid molecules are "diluted" by the fatty acids of the eukaryotic cells since the bacterial symbionts are hosted inside mussel cells. Unlike plant and animal cells, bacteria do not contain acyl lipid stores (lipids containing O- or N-ester or ether linked fatty acids), and it is believed that their acyl lipids are confined to membranes. Phospholipids represent 90 to 98% of bacterial lipids (King et al., 1977; White et al., 1979) and around 50% of eukaryotic lipids (Vestal and White, 1989). Studying PLFA thus somehow "concentrates" the signal of the bacteria, and is more adapted than a total fatty acid study in the perspective of identifying the fatty acids that are produced by the symbionts from the assimilation of CH_4 and CO_2 (in the presence of sulfide).

In the present study, we thus investigated the incorporation of ^{13}C-labeled HCO_3^- (in the presence of H_2S) or CH_4 into gill-extracted PLFA from *B. azoricus* in order to assess the metabolic activity of the endosymbionts. This enabled us to establish specific fatty acid (FA) patterns which provide insights into the symbiont phenotypes. On the contrary, a technique with a high yield of tissue total fatty acid (tFA) recovery was preferred to retain most of the information relative to both the symbionts and the host's fatty acids when analysing the assimilation of ^{13}C-labeled amino acids (dissolved into seawater) into *B. azoricus* gill fatty acids. This last experiment was indeed designed to observe (i) the potential for heterotrophic growth by the symbionts, and/or (ii) the occurrence of lipogenesis from the assimilation of amino acids by the Mytilid cell machinery (which could also use

free amino acids as osmotic regulators of the cells, metabolic fuel, protein synthesis or in the glucogenesis). Close examination of the labeled FA patterns helped interpreting physiological processes occurring in *B. azoricus*.

2 Material and methods

2.1 Sampling and aquarium experiments

During the MOMARETO cruise (*R/V Pourquoi Pas?*) in August 2006, a cage was deployed at the Menez Gwen site ($37°51'$ N–$32°31'$ W, 817 m) and loaded with around 400 mussels. The cage was retrieved in May 2007 by the Portuguese vessel *R/V Arquipélago*. *Bathymodiolus azoricus* specimens were transferred to cooled seawater (9°C) for a 14 h transit to Horta, Faial Island (Azores). Their valves were scrubbed clean of visible material, rinsed in chilled seawater and transferred to the Azorean land-based hydrothermal vent laboratory, LabHorta (Colaço and Santos, 2003). Before the start of the experiments, mussels were kept for 38 days in aerated seawater amended with methane and hydrogen sulfide, as described in Riou et al. (2008).

2.2 Mussels incubation with ^{13}C -enriched substrates

All stable isotope enriched chemicals were purchased from Campro Scientific (The Netherlands). For the experiments, 42 adult mussels (55.6 to 72.4 mm Shell Length) were dispatched into 4 separate refrigerated 4-L aquaria with aerated 0.2 μm-filtered natural seawater replaced every second day and monitored daily for temperature (7.8–9.8°C), pH (7.1–8.6) and O_2 saturation (median: 45%). The available quantity of labelled methane (25% ^{13}C) allowed running the tracer experiment with ^{13}CH$_4$ for a period of 15 days with a final dissolved concentration ranging between 14–200 μmol L^{-1} (Riou et al., 2008). The control experiment (with filtered seawater only) and the experiments with NaH^{13}CO$_3$ (99% added to the filtered seawater to obtain a concentration of 2.85 mmol L^{-1}, 16.3% ^{13}C) + H_2S (0–32 μmol L^{-1}, Riou et al., 2008) or with 9 mg L^{-1} of a ^{13}C-labeled amino acid mixture (98% ^{13}C, Riou et al., 2010) were continued over a period of 20 days. The amino acid mixture consisted of Gly 20–25%, Ala 15–20%, Tyr 10–15%, Leu 5–10%, Lys 5–10%, Ser 5–10%, Thr 2–5%, Phe 1–5%, Pro 1–5%, Val 1–5%, Met <3%, Trp <1%, Ile <1%, His <1%.

Three mussels from each experiment were selected for fatty acid analysis, and dissected into gill, mantle, muscle, and remaining tissues. Mussel tissues were immediately stored at −20°C till they were freeze-dried, a few days after dissection.

2.3 PLFA and tFA preparation

Prior to use, all glassware was precombusted 4 h at 450°C. Lipids were extracted from approximately 100 mg dry tissue

(ground to a fine powder right before extraction, using a mortar and pestle) by a modified Bligh and Dyer protocol (Boschker et al., 1998), whereby water in the first extraction step was replaced by Sörens phosphate buffer pH 7.4 (White et al., 1979). Lipid extracts were fractionated on silicic acid columns into different polarity classes by sequential elution with chloroform, acetone and finally methanol. The phospholipids collected in the methanol fraction were derivatized using mild-alkaline methanolysis (using a methanolic KOH solution) to yield fatty acid methyl esters (hereafter refered to as PLFA since they were derived from the phospholipid fraction).

Total fatty acid methyl esters (tFA) were obtained from 25 mg tissue powder by direct acid methanolysis catalysed for 2 h at 90 °C (modified from Lewis et al., 2000) in 3 mL of a fresh solution of methanol/hydrochloric acid 37%/chloroform (10:1:1 volume). After cooling down to room temperature chloroform (1 mL) and water (1 mL) were added to the samples which were then well mixed before the biphasic system was allowed to separate. The chloroform fraction was transferred to a fresh tube and the upper phase was re-extracted with fresh chloroform (1 mL). Chloroform extracts were pooled, cleaned with 2 mL water, dried under a mild nitrogen flow and the tFA were re-suspended in 300 µL hexane.

2.4 FAME fractionation on Ag$^+$-SPE cartridges

PLFA and tFA fractions were resolved further as a function of degree of unsaturation (saturated, *trans*-mono-unsaturated, *cis*-mono-unsaturated, di-unsaturated and poly-unsaturated), using silver ion solid phase extraction columns (Ag$^+$-SPE cartridges, Supelco Inc., Bellafonte, PA). The protocol used by Kramer et al. (2008) was modified to recover poly-unsaturated fatty acids (PUFA) using 5 mL acetone/acetonitrile (60:40 volume), directly after eluting the di-unsaturated fraction.

2.5 FAME ^{13}C content analyses and identification

The isotopic composition of individual FAME was analysed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) using an HP6890 coupled to a Thermo Finnigan delta+XL via a GC/C III interface. The FAME mix and Ag$^+$-SPE fractions were resolved on a fused-silica capillary column (100 m × 0.25 mm) coated with 0.20 µm CP-*Sil* 88 (100% cyanopropyl polysiloxane, Varian BV, The Netherlands) after injection in splitless mode at 270 °C. Helium was used as a carrier gas at a flow rate of 1 mL min^{-1} and the following temperature program was applied: 4 min at 45 °C; an increase at 10 °C min^{-1} to 135 °C and a plateau of 90 min; an increase at 5 °C min^{-1} to 170 °C and a plateau of 25 min; an increase at 10 °C min^{-1} to 195 °C and a plateau of 15 min; an increase at 10 °C min^{-1} to 235 °C and a plateau of 5 min. A careful selection of the type of chromatographic column and the optimization of the
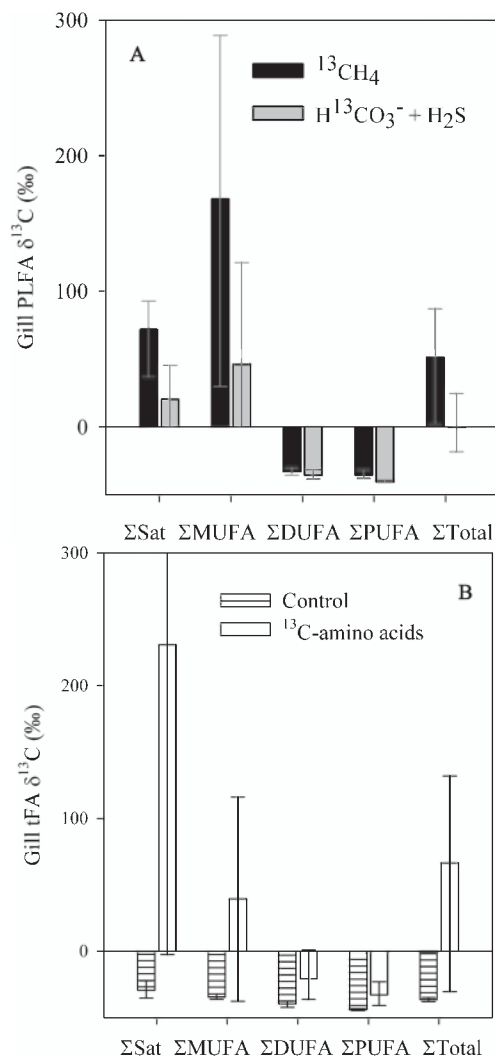
Fig. 1. *Bathymodiolus azoricus* ($n = 3$ specimens, mean ± min-max) gill FAME carbon isotopic signatures. The δ^{13}C signatures were corrected for the methyle group added during the transesterification using formula (1), and weighed averages were obtained using formula (2) **(A)** Gill PLFA after 15 days with 28 µM 25% ^{13}CH$_4$ (black bars) or 20 days with 6 µM H$_2$S + 16% H^{13}CO$_3^-$ (grey bars). **(B)** Gill total fatty acids (tFA) after 20 days in control conditions (striped bars) or 20 days with 300 µM dissolved 98% ^{13}C-amino acids (white bars). Sat: saturated, MUFA: mono-unsaturated, DUFA: di-unsaturated, PUFA: poly-unsaturated chains.

temperature program (enabling the detection of FAME 10:0 to 24:0) ensured baseline resolution of the IRMS m/z 44 trace for the majority of the components (including 16:1 and 18:1 positional isomers) and enabled an accurate identification of labeled PLFA. The δ^{13}C ratios of each FAME was corrected for the addition of one methanol carbon per molecule to obtain the isotopic signature of the fatty acid as in Abrajano et al. (1994) using the mass balance equation taking a measured δ^{13}C$_{CH_3OH}$ value of −40.3‰:

$$\delta^{13}C_{FAME} = [x]\delta^{13}C_{FA} + [1-x]\delta^{13}C_{CH_3OH} \qquad (1)$$

Where x is the fractional carbon contribution of the free fatty acid to the methyl ester (e.g.: 18/19 for FA $18:1(n-7)$).

The weighed $\delta^{13}C$ signature of fatty acid methyl esters, grouped according to their degree of unsaturation (Fig. 1), was obtained using the following formula (with mono-unsaturated fatty acids -MUFA- taken as an example of fatty acid class):

$$\delta^{13}CMUFA = (\%MUFA_1 * \delta^{13}CMUFA_1 + ... + \qquad (2)$$
$$\%MUFA_z * \delta^{13}CMUFA_z)/(\Sigma\%MUFA_{1\ to\ z})$$

where $\%MUFA_x$ is the area obtained from $MUFA_x$ on the chromatogram relative to the sum of the areas obtained from all fatty acids, and $\delta^{13}C\ MUFA_x$ is the carbon isotopic signature of $MUFA_x$.

For each PLFA, the incorporation of ^{13}C (I, expressed as micrograms of ^{13}C per gram of total PLFA) was calculated as in Knief et al. (2003):

$$I = (F_l - F_u) \cdot (A_x) \qquad (3)$$

where A_x is the peak area of $PLFA_x$ divided by the sum of the peak areas of all of the PLFA. F is the fraction of ^{13}C in $PLFA_x$ of samples incubated with ^{13}C (F_l) or in tFA_x of control unlabelled samples (F_u): $F = {}^{13}C/({}^{13}C + {}^{12}C) = R/(R+1)$. The carbon isotope ratio (R) was derived from the measured $\delta^{13}C$ values as follows: $R = (\delta^{13}C/1000 - 1) \cdot R_{VPDB}$, with $R_{VPDB} = 0.0112372$.

FAME identification was achieved by GC-mass spectrometry (GC-MS) using a Thermo Finnigan TRACE GC-MS system, applying the same GC conditions as described for the GC-IRMS analyses. The mass spectrometer was operated under mild conditions of electron impact ionization (EI$^+$; 40 eV) and recorded the mass spectra in the scan mode (m/z = 50–400). Aliquots of the PLFA or tFA mixture and of the MUFA, DUFA and PUFA fractions were derivatised further by a one step reaction into dimethyloxazolines (DMOX) to locate the unsaturations by GC-MS (Fay and Richly, 1991). We used a temperature gradient similar to the one used for FAME analyses, but since DMOX derivatives are less volatile (see also Precht and Molkentin, 2000) we increased the temperature of the isotherm plateaus at 135 °C, 170 °C and 195 °C for the temperature program described above, by 10 °C to 145 °C, 180 °C and 205 °C, respectively. The International Union of Pure and Applied Chemistry PLFA nomenclature used here is described by Guckert et al. (1985): the $(n-)$ notation indicates the position of the carbon-carbon double bond in the FA aliphatic chain starting from the methyl end carbon. The Δ notation, giving the location of FA unsaturation from the carbon at the carboxyl end, is only used in the next paragraphs to discuss fatty acid synthesis.

3 Results and discussion

3.1 Methane- and sulfide-oxidizing bacteria PLFA fingerprint

Phenotypic characterization is important for accurate taxonomic description of the endosymbionts (Bowman et al., 1993) and interpretation of the compound specific natural stable isotopic signatures. Pond et al. (1998) observed that the 14:0, 18:0, $18:1(n-7)$, $20:1(n-7)$, $18:3(n-7)$, and $20:3(n-7)$ compounds in the total lipid extract from *Bathymodiolus azoricus* gills were isotopically light and concluded these compounds had been produced by the sulfide-oxidising endosymbiotic bacteria (SOX). Our experimental results contrast with these conclusions and indicate that (i) 14:0, 18:0, $18:1(n-7)$ and $20:1(n-7)$ compounds also incorporate C from $^{13}CH_4$ and (ii) 18:0, $20:1(n-7)$, $18:3(n-7)$ and $20:3(n-7)$ compounds do not incorporate significant amounts of ^{13}C from $H^{13}CO_3^-$ in the presence of H_2S.

Our study shows that mussels kept in the presence of $^{13}CH_4$ or $H^{13}CO_3^- + H_2S$ incorporate ^{13}C mainly in saturated and mono-unsaturated PLFA (Sat and MUFA, respectively, Fig. 1a). For instance, the weighed average $\delta^{13}C$ signature of $\Sigma MUFA$ from the gill PLFA fraction of mussels incubated with $^{13}CH_4$ varied between +27 and +249‰ (Fig. 1a). From the $^{13}CH_4$ and $H^{13}CO_3^- + H_2S$ experiments, three patterns can be distinguished (Fig. 2): (i) PLFA enriched in ^{13}C from both substrates (CH$_4$ and HCO$_3^-$ +H$_2$S), i.e. 14:0; 15:0; 16:0; $16:1(n-7)c+t$; $18:1(n-13)c+t$ and $(n-7)c+t$; $20:1(n-7)$; $20:2(n-9,15)$; $18:3(n-7)$ and $(n-5,10,13)$; (ii) PLFA enriched in ^{13}C only after exposure to ^{13}C-CH$_4$, i.e., 17:0; 18:0; $16:1(n-9)$; $16:1(n-8)$ and $(n-6)$; $18:1(n-8)$; and $18:2(n-7)$ (note that fatty acid $18:1(n-9)$ was enriched in only one of the replicate specimens) and (iii) PLFA enriched in ^{13}C only after exposure to $H^{13}CO_3^-$ and H$_2$S: i.e., 12:0. Exposure to $H^{13}CO_3^-$ and H$_2$S also resulted in $17:1(n-8)$, $20:2(n-9,15)$ and $20:5(n-3)$ enrichement in two, and $20:2(n-7,11)$ and $18:3(n-6)$ in only one out of the three specimens analyzed.

Phylogenetic analyses on sequences of the genes encoding the 16S rRNA subunit and the particulate methane monooxygenase (*pmoA* gene) revealed that the *B. azoricus* methane-oxidising endosymbiont is a gamma-proteobacterium related to free living MOB and to MOB symbionts from other Bathymodiolids (Duperron et al., 2006; Nakagawa and Takai, 2008; Spiridonova et al., 2006). Although Colaço et al. (2007) did not identify any MOB PLFA biomarkers in wild *B. azoricus* specimens from the Menez Gwen site they did reveal the presence of large amounts of i19:0, $18:1(n-13)$ and $18:1(n-9)$, which were attributed to the endosymbionts. Our tracer experiment with $^{13}CH_4$ revealed that $18:1(n-13)$ was indeed slightly labeled but this was not the case for $18:1(n-9)$ (not enriched in the presence of $H^{13}CO_3^-$ +H$_2$S either) or for i19:0 which was not detected in our specimens.
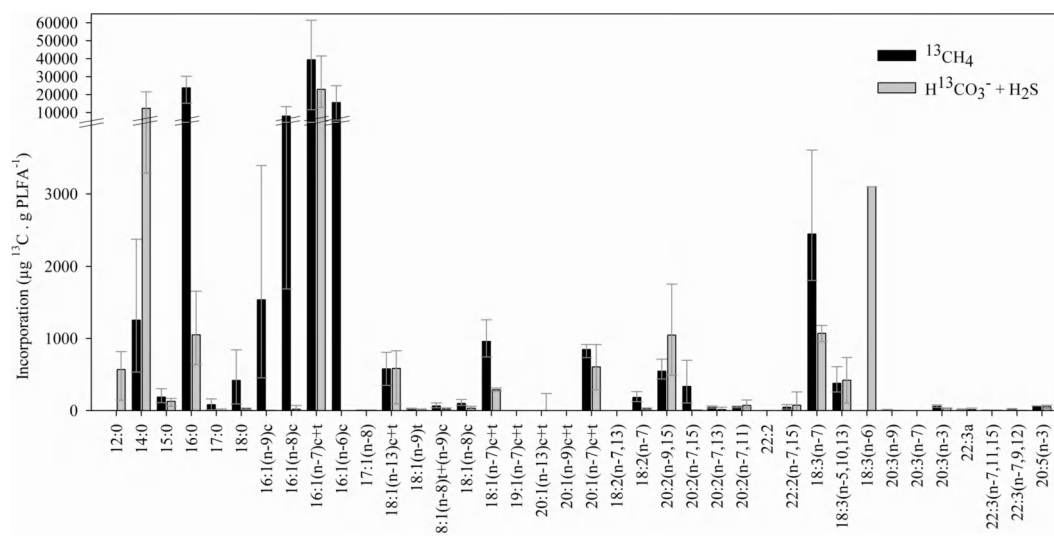
Fig. 2. *Bathymodiolus azoricus* gill tissue PLFA ($n = 3$, average $\pm$ min-max) ^{13}C incorporation per total PLFA content (as calculated using Eq. 3) after a 15 day supply with ^{13}CH$_4$ (black bars), or a 20 day supply with H^{13}CO$_3^-$ in the presence of H$_2$S (grey bars).

As mentioned above, we found that ^{13}CH$_4$ incorporated in *B. azoricus* gill tissue ended up in 14:0, 15:0, 16:0 as well as in *cis* and *trans* 16:1(n − 9,8,7 and 6) PLFA (Fig. 2). 16:1(n − 9) (one of the least abundant 16:1 isomers) also became labeled, although this compound has not been observed previously in PLFA profiles of isolated methanotrophic strains (Bodelier et al., 2009). Overall, *Methylohalobium crimeensis 1Ki* has a PLFA profile which overlaps by 98.1% with the fatty acid profile identified by stable isotope labeling with ^{13}CH$_4$ of the *B. azoricus* MOB symbiont, followed by *Methylosphaera hansonii ACAM 549* (97.0%, Table 1, Bodelier et al., 2009), *Methylocaldum sp. O–12* (95.8%) and *Methylocaldum sp. H-11* (93.0%). This result is consistent with the study of Pimenov et al. (2002) who could amplify total DNA isolated from *B. azoricus* gill tissue exclusively with 16S rRNA primers targeting the genera *Methylobacter* and *Methylosphaera*.

Phylogenetically the *B. azoricus* SOX endosymbiont stands far from any known cultured SOX bacteria. The closest strains such as the obligate chemolithoautotrophic thio-denitrifying gamma-proteobacteria *Thiohalomonas nitratireducens* (Sorokin et al., 2007) only reach around 88% 16S rRNA sequence homology (S. Duperron, personal communication, 2009). Furthermore, the PLFA labeled in our experiments represented only 69% of the fatty acid (FA) extracted from *T. nitratireducens*. It is thus difficult to relate the phenotype of *B. azoricus* SOX symbiont to any free-living bacteria.

Conway and MacDowell Capuzzo (1991) suggested that all SOX bacteria desaturate their FA via the anaerobic pathway. The anaerobic desaturation pathway produces long chain MUFA by the elongation of medium chain length *cis-3-unsaturated* intermediates, with 18:1(n − 7) as the major end-product and 16:1(n − 7) as a secondary product. The O$_2$-dependent MUFA synthesis pathway is known to produce a large variety of fatty acids (FA) with a double bond insertion occurring mainly in the Δ9 position (Conway and MacDowell Capuzzo, 1991). Note that the delta (Δ) nomenclature which imposes carbon atoms numbering to start from the carboxylic acid end of the acyl chain, is used for describing biochemical reactions. Our results show that 18:1(n − 7) and 16:1(n − 7), which are the main products of the anaerobic desaturation pathway, were significantly more labeled (−19.8 and +182.2‰, respectively) than the main product of aerobic desaturation, Δ9 MUFA 18:1(n − 9) (−29.6‰, Fig. 2). However, the latter fatty acid was less abundant, and therefore incorporation of similar levels of label in the 3 fatty acids could have resulted in a higher δ^{13}C signature in PLFA 18:1(n − 9). The fact that MUFA 16:1(n − 7), which was approximately 3 and 9 fold more abundant than 18:1(n − 7) and 18:1(n − 9), respectively (Fig. 4), was the most labeled compound together with saturated PLFA (12:0, 14:0 and 15:0; see Fig. 2), supports the contention of Conway and Mac-Dowell Capuzzo that *B. azoricus* SOX symbiont desaturates its FA via the anaerobic pathway.

3.2 Amino acid assimilation in tFA

Wright (1982) reports that *Mytilus* and *Modiolus* mussels take up free amino acids dissolved in seawater. Once inside the cell, free amino acids can be incorporated unchanged into proteins (Eccleston and Kelly, 1972) or used for the synthesis of other macromolecules. Excess amino acids (not being incorporated into proteins) cannot be stored in the cells and are generally used as metabolic fuel for the production of FA, ketone bodies or glucose (Berg et al., 2002). The ketogenic amino acids leucine, lysine, isoleucine, phenylalanine,

Table 1. PLFA displaying significant enrichement after the tracer experiments with $H_2S+^{13}CO_2$ (SOX) or with $^{13}CH_4$ (MOB). X = ^{13}C labeled. % PLFA = proportion of the PLFA content in cultured strains of *Methylosphaera hansonii* and *Thiohalomonas nitratireducens*.

|	MOB	*M. hansonii* (% PLFA) Bodelier et al., 2009	SOX	*T. nitratireducens* (% PLFA) Sorokin et al., 2007
12:0			X	3.9
14:0	X	2.5	X	0.5
15:0	X	1.5	X	0.6
16:0	X	14.5	X	25.4
18:0	X	0	X	0.5
16:1(n − 6)	X	17.5		
16:1(n − 7)c+t	X	20	X	27.3
16:1(n − 8)	X	39		
16:1(n − 9)	X	0		
17:1(n − 8)			X	0.9
18:1(n − 7)c+t	X	1.5	X	11.8
18:1(n − 8)	X	0		
18:1(n − 9)	X	0.5	X	5.1
18:1(n − 13)c+t	X	0		
20:1(n − 7)	X	0		
18:2(n − 7)	X	0		

tryptophan and tyrosine (accounting for 41 to 72% of the ^{13}C-amino acid mix used in our tracer experiment, see Riou et al., 2010) can be degraded into acetoacetyl CoenzymeA or acetyl-CoA, which constitutes the basis for *de novo* FA biosynthesis (e.g., Payne et al., 2001). Alanine, serine and cysteine can be converted to pyruvate which can further turn into acetyl CoA. Tracing the incorporation of amino acid ^{13}C into FA biomarkers may thus provide evidence for an osmotrophic activity (feeding on dissolved organics) by *B. azoricus* itself and/or by its endosymbionts. While from the present study (and Riou et al., 2008) it is now clear that *B. azoricus'* symbionts assimilate CH_4 or HCO_3^- in the presence of H_2S, their potential for osmotrophy has never been studied. The gill tissue showed the most rapid evidence of ^{13}C incorporation from labeled amino acids (Riou et al., 2010). Since the symbionts are located in the gill tissue, they might thus have increased access to such organic food source. However, mussel epithelial cells are the first to access this potential carbon and nitrogen source, since amino acids first enter the gill epithelial cells before they can reach MOB and SOX bacterial cytoplasms.

The experiment with ^{13}C labeled amino acids resulted in significant enrichment of the majority of the total fatty acids (tFA, Fig. 3). Out of the 38 gill tissue tFA only 14 did not show significant enrichment. Among these were the MUFA 16:1(n − 9) and 18:1(n − 8) (1.6% of the tFA area, Fig. 4), 19:1(n − 7) (0.5%), and 20:1(n − 13) (4.4%); the non-methylene interrupted di-unsaturated (NMID) fatty acids 18:2(n − 7,13) (0.4%), 20:2(n − 7,15) (6.2%); and the PUFA 18:3(n − 5,10,13) (5.3%), 20:3(n − 7) (2.3%) and 22:3 isomers (1.5%). The fatty acid showing most ^{13}C incorpo-

ration from the amino acids was 12:0 (representing 2.1% of tFA, Fig. 4), followed by 16:1(n − 7) (16.0%), 18:1(n − 7) (2.7%) and 16:0 (19.1% of tFA).

Mollusc lipids contain non-methylene interrupted di-unsaturated (NMID) FA having unusual unsaturation features, since they possess double bonds with more than one methylene group between ethylenic bonds (reviewed in Barnathan, 2009). We observed that the incorporation of ^{13}C from labeled amino acids in gill tissue di-unsaturated tFA ("DUFA" with these being mainly composed of NMID FA; $\delta^{13}C_{DUFA}$ = −20.9‰) was significantly higher than the ^{13}C incorporation from CH_4 or $HCO_3^-+ H_2S$ (−32.8 and −35.6‰, respectively, Fig. 1a, b). In particular, the NMID 22:2(n − 7,15) became labeled only when supplying *B. azoricus* with ^{13}C-amino acids (Fig. 3). In bivalves, NMID 22:2(n − 9,15) and 22:2(n − 7,15) (usually the most abundant in marine invertebrates, see e.g., Dunstan et al., 1993; Kawashima et al., 2003) are synthesized *de novo* by elongation and Δ5 desaturation of 18:1(n − 9) and 16:1(n − 7) (Zhukova, 1986) and subsequent elongation of 20:2(n − 9,15) and 20:2(n − 7,15) into the 22:2(n − 9,15) and 22:2(n − 7,15) acids. The incorporation of ^{13}C label in 22:2(n − 7,15) therefore indicates that the mollusc host cells have assimilated the amino acids directly in their fatty acids (as opposed to CH_4 and HCO_3^- which are being assimilated by the symbionts). Further evidence for this comes from the observation that MUFA 18:1(n − 9) as well as 20:1(n − 9) were more enriched in gills of specimens incubated with ^{13}C-amino acids (Fig. 3) than in mussels kept in the presence of $^{13}CH_4$ or $^{13}CO_2+H_2S$ (Fig. 2), even though $\sum$MUFA in the amino acid experiment was less labeled (+39‰, Fig. 1b) than $\sum$MUFA for
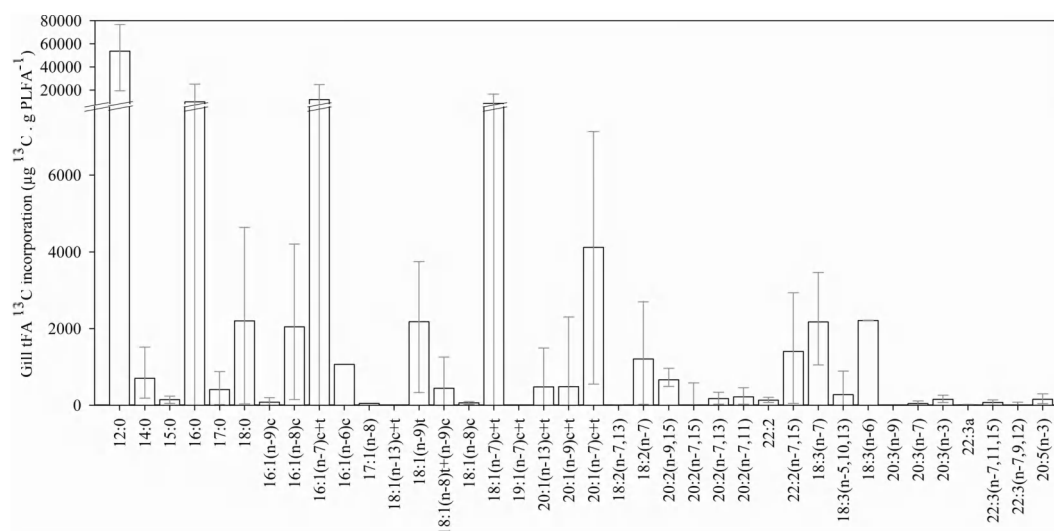
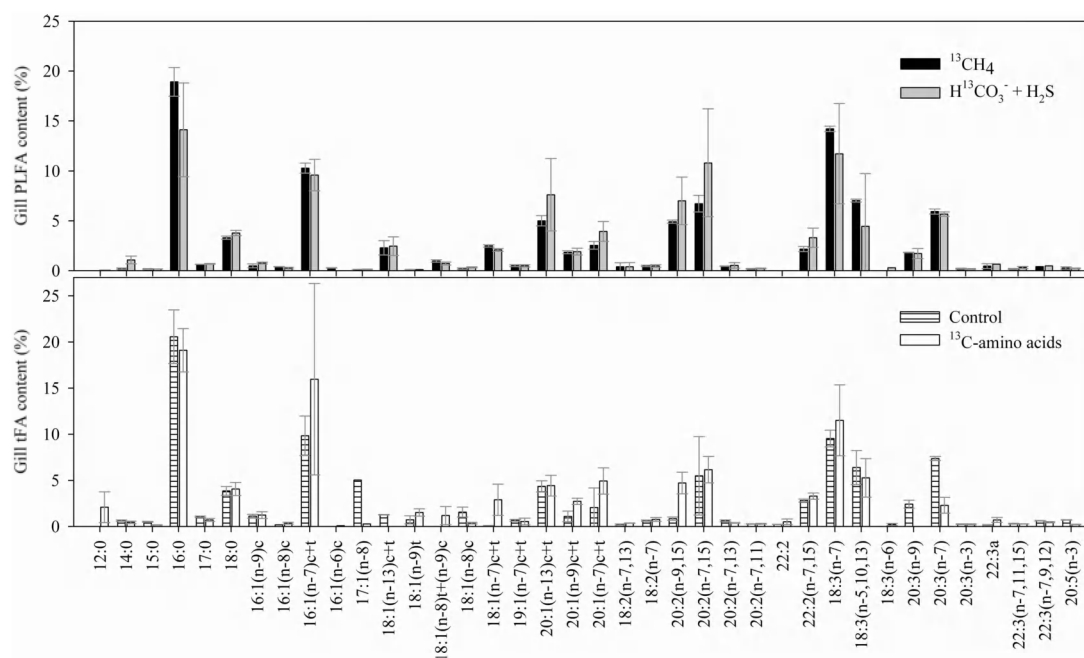**Fig. 3.** *Bathymodiolus azoricus* gill tissue tFA ($n = 3$, average ± min-max) ^{13}C incorporation per total PLFA content after a 20 day supply of dissolved ^{13}C-amino acids.

the ^{13}CH$_4$ or ^{13}CO$_2$+ H$_2$S experiments (+168 and +46‰, respectively, Fig. 1a). In animal cells, mono-unsaturation of fatty acids is catalyzed by a $\Delta9$ desaturase which has a maximum affinity for FA 18:0 and yields 18:1$\Delta9$ (18:1($n-9$); Cook, 1996). Since filter-feeding molluscs elongate *de novo* produced 18:1($n-9$) to 20:1($n-9$) (Zhukova, 1986), the observed preferential incorporation of ^{13}C from amino acids in 18:1($n-9$) as well as 20:1($n-9$), indeed points to direct assimilation of amino acids by the Mytilid cell machinery.

As can be noticed from Fig. 3, FA belonging to the ($n-7$) series were particularly enriched, with 16:1($n-7$) showing the highest ^{13}C incorporation within this series, followed by 18:1($n-7$), 20:1($n-7$), 18:3($n-7$) and 18:2($n-7$). One of the pathways of 18:1($n-7$) synthesis is the anaerobic pathway of desaturation, and Tunlid and White (1992) proposed 18:1($n-7$) to be a general indicator of the presence of bacteria. However, the high enrichment from ^{13}C-amino acids observed for 18:1($n-7$), 18:2($n-7$), 18:3($n-7$) and 20:1($n-7$) could also indicate preferrential use of the labeled acetyl coA resulting from amino acid breakdown to elongate 16:1($n-7$), which is one of the most abundant FA (around 10%, Fig. 4) in gill tissue. Indeed, had the ^{13}C-amino acids been assimilated by the SOX endosymbionts first, 16:1($n-7$) would have ended up being much more labeled than 18:1($n-7$), as was observed in the H^{13}CO$_3^-$ + H$_2$S experiment. Although it is true that 16:1($n-7$) and 18:1($n-7$) are the most abundant FA in SOX bacteria (McCaffrey et al., 1989) and that 18:1($n-7$) dominates the PLFA profiles of facultative methanotrophic alpha-proteobacteria *Methylocella* species (Dedysh et al., 2005), these particular FA are also biosynthesized by eukaryotic organisms. The elongation of 16:1($n-7$) to form 18:1($n-7$) by the incorporation of enriched acetyl coA resulting from amino acid degradation might thus be performed by the mussel itself.

The incorporation of amino acid ^{13}C into SOX and MOB FA biomarkers (12:0, and 16:1($n-8$) and ($n-6$), respectively) might indicate that the symbionts could also be assimilating the amino acids directly. Bacteria indeed possess effective metabolic mechanisms to survive long periods of low food supply and to react rapidly to available suitable nutrients (including low concentrations of dissolved organic matter; Sepers, 1977). The SOX *Beggiatoa* bacteria, for instance, can grow facultatively or mixotrophically on inorganic and soluble organic compounds (Zhang et al., 2005). However, in general, organic compounds do not stimulate the growth of specialist phototrophs, lithotrophs, or methylotrophs. Although some of the isolated bacteria strains using methane and other C$_l$ compounds also grow on sugars and acids (Patt et al., 1974), many isolates capable of growth on methane have proven incapable of growth on conventional organic media (Whittenbury et al., 1970). Several factors may explain the lack of capacity to assimilate organic compounds such as an inhibition effect by these compounds (although balanced mixtures of amino acids can cancel the inhibition by one or the other amino acid, Smith and Hoare, 1977), the impermeability of the cell (e.g. Eccleston and Kelly, 1972) or the loss of enzymes from the main assimilation pathways (Theisen et al., 2005; Smith and Hoare, 1977). In obligate methane-oxidising gamma-proteobacteria, the chemical conversion of carbohydrates, lipids and proteins into CO$_2$ and H$_2$O for energy production is blocked at the level of the tricarboxylic acid (TCA) cycle, due to the concurrent absence of one of its key enzymes, α-ketoglutarate dehydrogenase, and of isocitrate lyase and malate synthase from the glyoxylate shunt (Trotsenko, 1983). In some cases, succinyl CoA synthetase, another enzyme from the TCA cycle, is also missing, like in *Thiobacillus denitrificans* or *M. capsulatus* (Smith and Hoare, 1977). However, our finding that some

Fig. 4. *Bathymodiolus azoricus* gill tissue PLFA or tFA content ($n = 3$, average ± standard deviation) after a 15 day supply with ^{13}CH$_4$ (black bars), or a 20 day supply with H^{13}CO$_3^-$ in the presence of H$_2$S (grey bars), or with ^{13}C-amino acids (white bars); striped bars represent control mussels.

of the MOB (16:1$(n - 8)$ and $(n - 6)$) and SOX (12:0) symbionts biomarkers were labeled after incubation with ^{13}C-amino acids indicates that the symbionts had at least access to acetyl coA (or acetate) produced during the degradation of the amino acids by the host, and/or that they have the capacity to absorb and metabolise external amino acids. Further experiments are needed to ascertain these hypotheses.

4 Conclusions

PLFA and total FA ^{13}C-fingerprinting in *Bathymodiolus azoricus* gill tissues allowed us to trace the assimilation of ^{13}C-enriched HCO$_3^-$ (in the presence of H$_2$S) and CH$_4$ into the endosymbionts and host. Based on the selective labeling pattern of PLFA in each of the experimental treatments we could establish qualitative FA profiles of MOB and SOX endosymbiotic bacteria living in association with *B. azoricus*. Additionally, carbon from dissolved free amino acids was found to be incorporated into host specific FA and also into some of the symbiont biomarkers.

The tracer uptake experiments in the present study could only be performed under conditions of atmospheric pressure. It therefore needs to be verified through future experiments whether or not the activated metabolic pathways are the same under high, in situ pressure conditions and atmospheric pressure. While more experimentation is needed to better understand the physiology of the ensosymbionts, this experiment has shown its usefulness for positioning the endosymbionts

among described strains, as well as for the direct identification of the symbiont biomarkers, for which until now only assumptions had been made.

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