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Hydrogen isotopic fractionation in lipid biosynthesis by H_2-consuming *Desulfobacterium autotrophicum*

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Abstract

We report hydrogen isotopic fractionations between water and fatty acids of the sulfate-reducing bacterium *Desulfobacterium autotrophicum*. Pure cultures were grown in waters with deuterium (D) contents that were systematically varied near the level of natural abundance ($-37‰ \leqslant \delta D \leqslant 993‰$). H_2 of constant hydrogen isotope (D/H) ratio was supplied to the cultures. The D/H ratios of water, H_2, and specific fatty acids were measured by isotope-ratio mass spectrometry. The results demonstrate that *D. autotrophicum* catalyzes hydrogen isotopic exchange between water and H_2, and this reaction is conclusively shown to approach isotopic equilibrium. In addition, variation in the D/H ratio of growth water accounts for all variation in the hydrogen isotopic composition of fatty acids. The D/H ratios of fatty acids from cultures grown on H_2/CO_2 are compared with those from a separate set of cultures grown on D-enriched formate, an alternative electron donor. This comparison rules out H_2 as a significant source of fatty acid hydrogen. Grown on either H_2/CO_2 or formate, *D. autotrophicum* produces fatty acids in which all hydrogen originates from water. For specific fatty acids, biosynthetic fractionation factors are mostly in the range $0.60 \leqslant \alpha_{FA-water} \leqslant 0.70$; the 18:0 fatty acid exhibits a lower fractionation factor of 0.52. The data show that $\alpha_{FA-water}$ generally increases with length of the carbon chain from C_{14} to C_{17} among both saturated and unsaturated fatty acids. These results indicate a net fractionation associated with fatty acid biosynthesis in *D. autotrophicum* that is slightly smaller than in another H_2-consuming bacterium (*Sporomusa* sp.), but much greater than in most photoautotrophs.

1. INTRODUCTION

Sedimentary lipids potentially record useful geochemical information in their stable hydrogen isotope (D/H) ratios. Although the rate of hydrogen exchange between organic matter and water over geologic time remains unknown, lipid compounds apparently can retain their primary hydrogen isotopic composition for at least 20 million years under appropriate conditions (Yang and Huang, 2003; Sessions et al., 2004). Lipid-bound hydrogen is often studied by compound-specific isotopic analysis, a technique whose important applications include investigation of the history of sedimentary organic matter (reviewed by Schimmelmann et al., 2006) and geochemical reconstruction of the paleoenvironment (e.g., Xie et al., 2000; Andersen et al., 2001; Sauer et al., 2001; Huang et al., 2002; Dawson et al., 2004; Huang et al., 2004; Sachse et al., 2004a; Sachse et al., 2004b; Hou et al., 2006; Sachse et al., 2006; Smith and Freeman, 2006).

A robust interpretation of such natural samples depends on knowledge of the original isotopic composition of lipid compounds. Key factors in determining that isotopic composition include the extracellular origins of lipid hydrogen and the fractionations that accompany transfer of hydrogen into

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lipids. Understanding these factors requires investigation of the biochemical pathways of hydrogen in living organisms. Compound-specific D/H measurements permit identification and analysis of reaction networks involving hydrogen in the cell, including the associated isotopic fractionations.

Many studies of the hydrogen isotopic composition of lipids focus on photoautotrophic organisms. Indeed, large lipid–water fractionations ($\alpha \approx 0.85$) of hydrogen were first observed in total lipid extracts from cyanobacteria, algae, and plants (Estep and Hoering, 1980; Sternberg et al., 1986; Sternberg, 1988). Unlike other metabolic groups, (oxygenic) photoautotrophs metabolize the water in which they grow. Because growth water is the ultimate source of all hydrogen to the photoautotrophic cell, it also exerts primary control of the hydrogen isotopic composition of biomass (Sauer et al., 2001). In this case, interpretation of the isotopic fractionation between lipids and water is relatively straightforward. Even so, the isotopic composition of lipids may vary between organisms and among lipid classes because of several factors, e.g., different pathways of lipid biosynthesis, variations in the fractionations associated with the enzymes of a given pathway, and differing metabolic fluxes. Such variability is observed in lipids from an assortment of algae and plants (Sessions et al., 1999), from many native terrestrial and aquatic plants (Chikaraishi et al., 2004a,b,c; Chikaraishi, 2006; Chikaraishi and Naraoka, 2007; Hou et al., 2007), and from freshwater green algae cultivated in water of controlled D/H ratio (Zhang and Sachs, 2007).

Heterotrophic and some chemoautotrophic organisms present more complicated systems in which growth water is not the only available source of cellular hydrogen. Instead of metabolizing water, these organisms consume other hydrogen-bearing metabolites, ranging from complex organic matter to simple molecules like H_2, H_2S, or CH_4. As a result, their cellular hydrogen isotopic composition may reflect mixed contributions from growth water and hydrogen-bearing metabolites. So far, few studies have examined the hydrogen isotopic composition of lipids produced by these organisms. In one example, the aerobic, methane-oxidizing bacterium *Methylococcus capsulatus* derives up to ~20% of lipid hydrogen from CH_4 and the remainder from water, assuming lipid–water fractionations similar to those reported for plants (Sessions et al., 2002; Sessions and Hayes, 2005). In another example, the anaerobic, acetogenic bacterium *Sporomusa* sp. strain DMG 58 produces strongly D-depleted lipids during growth on H_2/CO_2 (Valentine et al., 2004b). Variation in the D/H ratio of growth water (δD_{water}) accounts for all variation in δD_{lipid}; i.e., the isotopic composition of lipids appears independent of δD_{H2}. However, during growth of *Sporomusa*, δD_{H2} decreases rapidly towards a predicted isotopic equilibrium with growth water. To explain the strongly D-depleted lipids of *Sporomusa*, the authors hypothesize a dual mechanism: (1) hydrogenase-catalyzed isotopic equilibration of H_2 with growth water followed by (2) transfer of hydrogen from D-depleted H_2 to lipids by way of an intermediate compound such as NADPH.

The experiments reported here employ *Desulfobacterium autotrophicum* to examine both parts of the above hypothesis. This marine, anaerobic, sulfate-reducing bacterium was cultivated with an electron donor (H_2 or formate) of controlled D/H ratio, in water of controlled D/H ratio. In one experiment, the initially supplied H_2 and water were out of isotopic equilibrium, and δD_{H2} and δD_{water} were observed over time to test for hydrogen exchange. In another experiment, D/H ratios of fatty acids from *D. autotrophicum* grown on H_2/CO_2 were compared to those of fatty acids from cultures grown on formate, an alternative electron donor. This study of anaerobes constitutes one part of an ongoing survey of the hydrogen isotopic composition of bacterial lipids.

2. MATERIALS AND METHODS

2.1. Preparation of D-enriched water and formate

Batches of water and formate were prepared with various concentrations of deuterium, ranging from 2 to 8 times the level of natural abundance (~155 ppm). A commercial stock of 99.9% D_2O (C/D/N Isotopes, Inc., Pointe-Claire, Que., Canada) was volumetrically diluted with deionized (Milli-Q Element A10 System, Millipore Corp., Billerica, MA), UV-disinfected (Model MP-2-SL, Aquafine Corp., Valencia, CA) water to provide a new stock of 10% D_2O. This stock in turn was volumetrically diluted to two different final δD values of approximately +500‰ and +1000‰. Volumes of 2 L or less were stored at room temperature in acid-washed glass vessels with ground–glass stoppers and a wrapping of Parafilm® M. Larger volumes were stored at room temperature in 21 L polycarbonate carboys that had been soaked overnight in 5% HCl and rinsed thoroughly to remove organic contaminants.

Similarly, D-enriched formate salts were produced by volumetric mixing of saturated solutions of 99% DCOONa (Cambridge Isotope Laboratories, Inc., Andover, MA) and nonenriched anhydrous sodium formate (Alfa Aesar, Ward Hill, MA). Formate crystals were precipitated from the combined saturated solution and then were dried at 130 °C to a constant weight. In each case, the final weight was identical within error to the known mass of anhydrous sodium formate originally added, indicating that the D-enriched salts were also anhydrous. While still warm, the dried formate salts were transferred into screw-top glass vials and stored in a vacuum desiccator.

2.2. Experimental conditions

2.2.1. Cultivation

D. autotrophicum strain HRM2 (DSM 3382) is a facultatively lithoautotrophic, sulfate-reducing bacterium originally isolated from marine sediments (Brysch et al., 1987). Inoculum was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. It was cultivated in bicarbonate-buffered, sulfide-reduced, liquid medium containing biotin, *p*-aminobenzoate, and nicotinate, as described by Brysch et al. (1987). To ensure sufficiently reduced conditions, 20 mg sodium dithionite was added per L of medium. Initial pH was approximately 6.7. Based on preliminary experiments, at least 10 mg of yeast extract per L of medium was required

for reliable, exponential growth of *D. autotrophicum* (see the electronic annex EA-1, Fig. EA-1-1). This amount of yeast extract was therefore added to all media. In each experiment, waters of differing D/H ratio were used to prepare treatments of otherwise identical media. All glass equipment used in the preparation and storage of water and media, as well as in the cultivation of *D. autotrophicum*, was cleaned with mild detergent, soaked in dilute HCl, and rinsed thoroughly with deionized water between uses. All cultures were grown at 26 °C with gentle stirring or shaking (100–120 rpm). Unless otherwise specified, gases were obtained from Accu Air, Inc. (Ventura, CA) and were purified by flow through a heated stainless-steel column filled with reduced copper filings (Hungate, 1969).

2.2.2. Isotopic exchange between water and H_2

To investigate hydrogen isotopic exchange between water and H_2 in the culture medium, triplicate cultures, alongside triplicate heat-killed controls, were grown in two different δD_{water} treatments (−34‰ and +3940‰). 40 mL liquid cultures, including 5% inoculum (v/v), were grown under a headspace of H_2/CO_2 (80/20, v/v) in 120 mL borosilicate glass serum bottles closed with butyl rubber stoppers (13 mm inner diameter, 20 mm outer) and crimped aluminum seals. After inoculation, controls were autoclaved. Then all cultures and controls were incubated with shaking at 100 rpm. Growth was monitored daily by measurement of optical density at a wavelength of 600 nm (Genesys Spectronic 10 UV/Vis, ThermoFinnigan Scientific, Waltham, MA). Sulfate concentrations were monitored using the nephelometric technique of Gieskes et al. (1991), in which sulfate is precipitated as $BaSO_4$ and quantified from absorbance at 400 nm.

When live cultures reached late exponential phase, sodium molybdate was added to a final concentration of 20 mM in all cultures and controls to inhibit further sulfate reduction. Stock molybdate solutions were prepared to have δD_{water} values identical to those of each culture. All cultures and controls were incubated with shaking for one additional hour; then the headspaces were replaced with fresh H_2/CO_2. Water and gas samples for D/H analysis were collected, and the vessels were returned to the incubator. Gas samples were taken at regular intervals for the next 73 h, after which a water sample was collected from each bottle. Final gas and water samples were taken after a period of several weeks.

Liquid samples from bottle cultures were taken via sterile syringes purged with O_2-free gas. Optical density and pH of the samples were measured immediately; syringe-filtered (0.2 μm nominal pore size) subsamples were used for sulfate quantification. Samples for D/H analysis of water were passed through disposable syringe filters (0.2 μm nominal pore size) into glass vials with lined screw-caps, and were stored in the dark at 4 °C until analysis, typically 2–4 weeks.

Gas samples were taken via sterile syringes purged with ultrahigh-purity helium (He). Samples for D/H analysis of H_2 were stored at a pressure of ~3 atm in 4-cc serum bottles sealed with butyl rubber stoppers and aluminum crimps. These vessels previously had been flushed with He and evacuated via a 60-cc syringe equipped with a stopcock. Gas samples were stored in the dark at room temperature for 4 weeks prior to analysis.

2.2.3. Determination of external hydrogen sources

2.2.3.1. Growth on H_2/CO_2. To maximize yield of biomass, *D. autotrophicum* was cultivated in a 14 L benchtop fermentor (Microferm MF-114, New Brunswick Scientific Co.), and growth media were prepared with 45 mM instead of 30 mM sodium sulfate. The fermentor was continually supplied from a single cylinder with a mixture of H_2 and CO_2 (80/20, v/v) purified of trace O_2 by flow through a heated column of copper filings. Flow rate was regulated by a mass flow controller (8100 series controller, DX-5 digital power supply, Celerity, Inc., Milpitas, CA) and was independently monitored with a digital flow meter (ADM2000, Agilent Technologies, Inc., Santa Clara, CA). To conserve gas, flow rate typically was set at 50 mL/min during lag phase and increased to 100 mL/min at the onset of exponential phase. The fermentor was stirred by impeller at 120 rpm. Because of the relatively low temperature (26 °C), evaporation of the medium was not appreciable. Therefore, coolant was not provided to the reflux condenser, and the condenser's ceramic beads were removed to prevent contamination of the medium.

Each culture consisted of 10 L, including 5% inoculum (v/v). Liquid media were prepared in three batches of different δD_{water} (−39, +498, +993‰). A duplicate batch of medium was prepared with the −39‰ water. Prior to inoculation of the duplicate (culture II), yeast extract was added directly to its salt solution, to a concentration of 200 mg/L, to test for a contribution from yeast extract to lipid hydrogen. In the other three cultures, direct addition was avoided. Instead, the 500 mL inocula were prepared with 200 mg/L yeast extract to provide the minimal requirement (10 mg/L; Fig. EA-1-1) when diluted into the fermentor. As shown in EA-1, results from this test indicate that a 20-fold increase in yeast extract in the low-δD_{water} culture affects the apparent fractionation factors by ~0.02 (Fig. EA-1-2). Because the small amounts of yeast extract employed here do not significantly affect the D/H ratios of lipids, both duplicates are included in the data analysis below.

For D/H analysis of water, initial and final samples of growth medium were taken from the liquid sample port of the fermentor. The first 15–20 mL of liquid was discarded to clear the line before sampling. Liquid samples were passed through disposable syringe filters (0.2 μm pore size) and injected into sealed glass serum bottles, which were filled completely. Samples were stored at 4 °C until D/H analysis, typically 2–4 weeks.

For D/H analysis of H_2, samples of fermentor headspace gas were taken from the exhaust line. To remove hydrogen-bearing gases other than H_2, the exhaust was diverted through a water vapor trap containing anhydrous $CaSO_4$ and a H_2S trap containing copper sulfate-coated Chromosorb® (Johns-Manville, Denver, Colorado). Glass serum bottles were first flushed with N_2, then with H_2S- and H_2O-free fermentor exhaust for more than 10 times the residence time. Serum bottles were stored under slight positive pressure, in the dark at room temperature, for 10–14 weeks prior to analysis.

Biomass was harvested in late exponential or stationary phase via the liquid sample port. Because of the large liquid volume, sampling involved both centrifugation (20 min., 15,000*g*, 4 °C, Sorvall RC-5B Plus superspeed centrifuge with Sorvall SLA-1500 fixed-angle rotor, Thermo Scientific, Waltham, MA) and filtration onto multiple 47 mm glass-microfiber filters (GF/F, 1.0 μm pore size, Whatman, Inc., Florham Park, NJ). Cell pellets and biomass-laden filters were stored in precombusted aluminum foil at −15 °C until lipid extraction.

2.2.3.2. Growth on formate. Cultures using formate were grown in bottles, at three different δD_{water} and three different $\delta D_{formate}$ conditions for a 3 × 3 matrix of nine treatments (Table 1). An uninoculated batch of medium was also prepared and sampled in parallel to control for lipid contamination during harvesting. Media were prepared in 1100 mL borosilicate glass bottles (modified for closure with butyl rubber stoppers and aluminum crimps) that had been combusted at 450 °C for at least 6 h to ensure removal of organic contaminants. Sodium formate was added from a sterile solution to a final concentration of 30 mM. Cultures consisted of 800 mL liquid, including 5% inoculum (v/v) from an active culture with the same treatment of δD_{water} and $\delta D_{formate}$, under a headspace of 80% N_2 and 20% CO_2. Headspace gas was replenished daily. Optical density was monitored at 600 nm. Growth media and headspace gas were sampled for D/H analysis, and biomass was harvested during late exponential growth.

Biomass from *D. autotrophicum* cultures was harvested via the vacuum filtration system described above. The sole difference was that different cultures were harvested in succession. Between treatments, the filtration apparatus was rinsed sequentially with milli-Q water, methanol, and DCM, which was allowed to evaporate. Biomass-laden GF/F filters were stored in precombusted aluminum foil envelopes at −15 °C.

Table 1
Isotopic ratios (δD, ‰) of available external hydrogen sources in *D. autotrophicum* cultures.

Culture	Water[a]	Electron donor[b]	
		Initial	Final
H_2			
I	−38 (5)	−168 (3)	−259 (3)
II	−39 (1)	−165 (3)	−205 (5)
III	498 (4)	−160 (4)	−184 (4)
IV	993 (3)	−165 (6)	−189 (7)
Formate			
V	−37 (3)	814 (37)	nd[c]
VI	−39 (3)	1350 (15)	nd
VII	−40 (1)	1840 (12)	nd
VIII	500 (3)	814 (37)	nd
IX	502 (2)	1350 (15)	nd
X	498 (3)	1840 (12)	nd
XI	993 (3)	814 (37)	nd
XII	966 (2)	1350 (15)	nd
XIII	986 (1)	1840 (12)	nd

[a] Mean (±1σ), based on triplicate injections of one initial sample and one final sample.
[b] H_2 values are mean (±1σ) of replicate injections ($n = 5$) from a single sample; formate values are mean (±1σ) of duplicate measurements of a single sample.
[c] Not determined.

2.3. Analytical techniques

2.3.1. D/H analysis of water

D/H ratios of water were measured by high-temperature reduction to H_2 using a ThermoElectron thermal conversion/elemental analyzer (TC/EA), ConFlo III continuous-flow interface, and Delta$^+$XP isotope-ratio mass spectrometer (IRMS) at UCSB. The TC/EA was fitted with a glassy carbon reactor tube packed with glassy carbon granules, maintained at a temperature of 1425 °C. Reaction products (CO and H_2) from injection of the water samples (1.5 μL) were separated on a packed GC column (80–100 mesh molsieve 5A, 0.6 m × ¼″ × 4 mm) at 55 °C. Multiple injections of each sample were employed to minimize memory effects between samples of very different δD_{water}. Two laboratory working standards ($\delta D_{water} = -89.4‰$ and −29.6‰) were measured with each batch of water samples and were used to normalize data to the VSMOW scale. For pooled measurements ($n = 49$) of individual standards, mean precision was 2.1‰ (1σ) and the root-mean-square (RMS) error was 2.5‰. For samples enriched in D beyond the range of standards, analytical uncertainty is presumably larger than 2.5‰ but is not quantifiable.

For the single batch of water samples with $\delta D \approx 4000‰$ (used in the water-H_2 isotopic exchange experiment), an additional standard ($\delta D = 3419‰$) was prepared by gravimetric addition of 99.9% D_2O to milli-Q water of known D/H. This standard was included in the normalization curve of water samples with δD of ∼4000‰. For replicate analyses of individual standards, mean precision was 2.3‰ (1σ). For pooled measurements of standards ($n = 36$), mean precision was 5.3‰ (1σ), and RMS error was 13‰. Absolute error was 10‰. Based on these uncertainties, the overall error associated with the most D-enriched water samples is probably less than 20‰.

2.3.2. D/H analysis of sodium formate

The D/H ratio of anhydrous sodium formate was measured using the same TC/EA system described above. Uncertainty was estimated from analyses of NIST Standard Reference Material 8540 (polyethylene film, $\delta D = -100‰$) and of a laboratory working standard (acetanilide, $\delta D \approx -140‰$). All data were corrected by −14‰ for an offset in the measured value of the NIST Reference Material, and this is regarded as the minimum absolute uncertainty in measurement of $\delta D_{formate}$. No D-enriched organic standards were available for normalization of the data. Precision (1σ) of replicate analyses was 7.8‰ for SRM 8540 ($n = 6$) and 5.0‰ for acetanilide ($n = 15$). For duplicate analyses of formate samples, mean precision was 21‰.

2.3.3. D/H analysis of H_2

Gas samples were first diluted by a factor of ∼100 by transfer of subsamples into septum-capped Exetainer® vials (Labco International, Inc., Houston, TX) previously

flushed with ultrahigh-purity helium. The δD value of H_2 was measured using a ThermoElectron GasBench device coupled to the same IRMS described above. A laboratory reference H_2 ($\delta D = -181.8‰$) was measured by triplicate injection with each unknown H_2 sample. For 91 such measurements of the reference gas, mean precision was 0.9‰ (1σ) and the RMS error was 0.5‰. For replicate injections ($5 \leqslant n \leqslant 7$) of unknown samples, mean precision was 3.4‰ (1σ). For samples D-enriched or D-depleted far beyond δD of the reference gas, analytical uncertainty is presumably greater but is not quantifiable.

Samples from the first 3 days of culture growth were analysed in a single batch, and all six killed controls exhibited constant δD values (equal to that measured for the supplied gas). Several weeks later, final samples were taken and were analysed in a separate batch. At that time, all six killed controls exhibited a consistent offset of −27‰ from the earlier value. This offset is unlikely to represent a real fractionation in all controls, and all δD values measured in this batch were therefore corrected by +27‰. Because we cannot identify the source of the offset, we conservatively estimate the uncertainty associated with δD values from this batch at 30‰.

2.3.4. Fatty acid extraction and analysis

Biomass-laden GF/F filters were first lyophilized for two days (K-Series Lyophilizer, VirTis, Gardiner, NY). Dried filters were weighed, cut into small pieces, and transferred to glass tubes with 2.5 mL hexane. Extraction and methylation of fatty acids were performed simultaneously by addition of 5 mL freshly prepared methanol/acetyl chloride (20:1, v/v) followed by heating at 100 °C for 15 min (Rodriguez-Ruiz et al., 1998). After cooling to room temperature, 5 mL water was added and fatty acid methyl esters (FAME) were extracted three times with 5 mL hexane. The collected extract was dried over anhydrous Na_2SO_4 and was concentrated under N_2 to a volume of 1 mL.

A 5% or 10% aliquot of the total lipid extract was analysed by gas chromatography/mass spectrometry (GC/MS) on a ThermoFinnigan Trace GC equipped with a DB-5 ms column (30 m × 0.25 mm × 0.25 μm) and a PTV injector operated in splitless mode. The GC effluent was split (3:1) between a ThermoFinnigan DSQ mass spectrometer for peak identification and a flame-ionization detector (FID) for quantification. Compounds were identified by comparison of their mass spectra and retention times to library data and to a 37-component FAME standard (Supelco). FAME abundances were calculated by comparison of FID peak areas to those of an internal standard (palmitic isobutyl ester, typically 10 μg per sample). Positions of unsaturation were positively identified by GC/MS analysis of the dimethyl disulfide adducts of fatty acids, prepared following the methods of Yamamoto et al. (1991).

The remaining lipid extract was used for compound-specific D/H analysis, which was performed at Caltech on a ThermoFinnigan Trace GC coupled to a Delta$^+$XP IRMS via a ThermoFinnigan GC/TC pyrolysis interface operated at 1440 °C. Three *n*-alkanes of known isotopic composition were coinjected with each sample. Two of these compounds (C_{17} and C_{22}) were used as reference peaks for the calibration of isotopic analyses, while the third (C_{23}) was treated as an unknown to assess accuracy. Data were normalized to the SMOW/SLAP isotopic scale by comparison to an external standard containing 15 *n*-alkanes with δD values from −41‰ to −256‰ (Sessions et al., 2001). FAME δD values were corrected for added methyl hydrogen by isotopic mass balance, with the δD value of methyl H derived from analyses of methylated phthalic acid of known isotopic composition (Sessions, 2006).

The mean precision for replicate analyses of external *n*-alkane standards was 1.6‰, and for the coinjected C_{23} internal standard it was 2.8‰. RMS error for the C_{23} internal standard was 2.8‰ ($n = 28$). For unknown FAMEs with δD values in the range of available standards, accuracy is probably better than 4.0‰. For FAMEs that were D-enriched outside this range, accuracy is presumably worse but cannot be quantified.

2.4. Data analysis

Experimental data were reported by instrument software as δD values relative to VSMOW. The data were processed by converting these values to absolute D/H ratios using the accepted value of R_{VSMOW}, 155.76×10^{-6} (Hagemann et al., 1970; Bohlke et al., 2005). The data were then analysed by Model I linear regression of R_{FA} on R_{water} and of R_{FA} on $R_{formate}$. Regression parameters and their standard errors were computed, and slopes were compared in an *F*-test (Rohlf and Sokal, 1995; Sokal and Rohlf, 1995). In Model I regression, standard errors of the slopes and intercepts assume no uncertainty in the *x*-variable, which is not the case in R_{water} and $R_{formate}$. However, uncertainties in those variables are random and normal, so standard errors are reported.

Estimation of uncertainties in these calculations is complicated by the fact that fatty acids were analysed on one IRMS system at Caltech, while water, H_2, and formate were analysed on a separate system at UCSB. Imperfect normalization to the VSMOW/SLAP scale in each lab, particularly for D-enriched samples outside the range of available standards, introduces additional uncertainty into comparisons of the δD values of fatty acids to those of water or formate. Currently we lack a way to quantify the magnitude of this uncertainty (e.g. measuring water and FAME samples on the same system by the same method), but a conservative estimate is ±50‰. Thus a slope based on linear regression of data generated from these two systems would be subject to an absolute uncertainty of approximately ±0.05. However, all samples would be affected in a consistent fashion by this effect, so that relative differences between fatty acids or between cultures would be subject only to the smaller uncertainties associated with each analytical method.

3. RESULTS

3.1. Isotopic exchange between water and H_2

In the headspace of sealed *D. autotrophicum* cultures grown on H_2/CO_2, δD_{H2} changed systematically over time (Fig. 1). No such change occurred in killed controls. In all

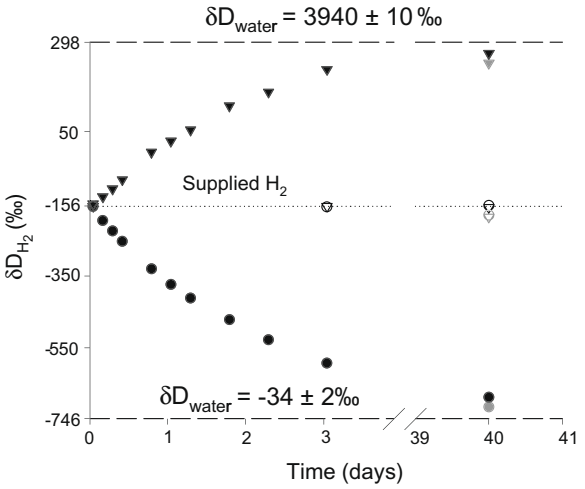

Fig. 1. Hydrogen exchange in the direction of isotopic equilibrium between water and H_2. Solid symbols represent experimental cultures; open symbols represent heat-sterilized controls. Error bars (1σ) are concealed by the symbols. The shape of the symbol indicates δD_{water} of growth media (triangles, relatively enriched; circles, relatively depleted). Dashed lines indicate δD_{H2} in theoretical equilibrium, at 26 °C, with the measured δD_{water} of growth media. The dotted line indicates δD_{H2} of the initial gas. Samples from day 40, analysed separately, were corrected for a uniform offset in the six controls (gray symbols, uncorrected; black symbols, corrected).

live cultures, δD_{H2} approached the value predicted for theoretical equilibrium with water at the growth temperature (Horibe and Craig, 1995). Further, the change in δD_{H2} occurred in both directions, positive and negative, depending on the value of δD_{water}. The latter result distinguishes this experiment from others in which δD_{H2} approached the predicted equilibrium value from only one direction (Chidthaisong et al., 2002; Romanek et al., 2003; Valentine et al., 2004a,b; Vignais, 2005). The rate of change in δD diminished with time, such that on the third day of each culture at least 73% of the predicted change in δD_{H2} had already occurred in both directions. On day 40, at least 90% of the predicted change in δD_{H2} was evident, but δD_{H2} still had not attained the equilibrium value.

After growth but prior to the sampling interval, sodium molybdate was added to all cultures to inhibit sulfate reduction. The goal was to halt the net consumption of H_2, such that the observed changes in δD would reflect only the activity of reversible hydrogenase enzymes. A similar experiment without sodium molybdate (data not shown) yielded similar results, indicating that net consumption of H_2 is not accompanied by significant fractionation.

3.2. Determination of external hydrogen sources

3.2.1. D/H of water and electron donors

Table 1 summarizes δD values of water, H_2, and formate in cultures of *D. autotrophicum* grown for lipid analysis. In cultures grown on H_2/CO_2 (I–IV), δD_{H2} of the exhaust gas decreased between initial and final sampling. In all cases, this decrease represents a change in the direction of isotopic equilibrium with growth water, consistent with the occurrence of water–H_2 isotopic exchange documented above. The decrease in δD_{H2} was largest in cultures I and II, in which growth water was farthest from isotopic equilibrium with the supplied H_2. However, final δD_{H2} values consistently failed to achieve theoretical equilibrium with growth water, which would have been −476‰ in culture IV and lower in the other cultures. This shortfall indicates that the rate of hydrogen exchange was insufficient for complete isotopic equilibration of H_2 and water to occur during the residence time of gas in the fermentor vessel (at least 40 min, based on a headspace of 4 L and a flux of 100 mL/min).

Table 2
Cellular fatty acid composition of *D. autotrophicum* strain HRM2 grown on different electron donors.

Fatty acid[a]	t_R (min)[b]	Relative abundance (%)[c]			
		H_2 (Cultures I–IV)		Formate (Cultures V–XIII)	
Individual					
13:0	14.38	0.36	(0.25)	0.42	(0.32)
14:1Δ7	15.97	0.07	(0.06)	0.39	(0.31)
14:0	16.34	6.28	(0.49)	3.28	(1.01)
15:1Δ7	17.88	0.28	(0.22)	0.47	(0.29)
15:1Δ9	18.00	9.04	(2.54)	7.35	(1.41)
15:0	18.32	14.52	(4.19)	15.92	(5.09)
me-16:0	19.22	0.91	(0.27)	0.01	(0.04)
16:1Δ7	19.77	0.21	(0.06)	0.45	(0.11)
16:1Δ9	19.87	28.13	(2.80)	23.45	(3.66)
16:0	20.25	14.18	(2.95)	25.03	(3.07)
me-17:0	21.05	6.21	(1.34)	1.35	(0.11)
17:1Δ9	21.68	2.64	(0.49)	2.05	(0.07)
17:1Δ11	21.81	9.52	(1.95)	6.90	(0.38)
17:0	22.09	4.08	(1.35)	7.18	(0.70)
me-18:0	22.89	0.31	(0.06)	trace	
18:1Δ9	23.45	0.34	(0.18)	0.56	(0.21)
18:1Δ11	23.54	2.10	(1.16)	2.07	(0.67)
18:0	23.88	0.80	(0.44)	3.10	(1.06)
Sum					
Unsaturated		52.35		43.71	
Short-chain (<16C)		30.56		27.83	
Branched		7.43		1.36	

[a] FAME identified by mass spectra. Carbon number indicates the total number of carbon atoms in the FA. Positions of unsaturation were determined by GC/MS analysis of DMDS adducts (see methods). Prefix *me-* denotes a methyl group of undetermined position.

[b] Retention time in GC. See methods for chromatographic conditions.

[c] Percentage of total fatty acids extracted from a sample, as determined by FID peak areas; expressed as mean (±1σ) of data from the indicated cultures. Unquantified trace amounts and non-detections are treated as zero in the mean.

3.2.2. Relative abundance of fatty acids

The cellular fatty acid compositions of cultures grown on H_2/CO_2 and on formate are summarized in Table 2. For overall comparison on the basis of electron donor, the table presents the mean relative abundance of each fatty acid for cultures I–IV (H_2/CO_2) and for cultures V–XIII

(formate). The detected fatty acids are tentatively identified as follows: tridecanoic acid (13:0); 7-tetradecenoic acid (14:1Δ7); tetradecanoic acid (14:0); 7-pentadecenoic acid (15:1Δ7); 9-pentadecenoic acid (15:1Δ9); pentadecanoic acid (15:0); methylpentadecanoic acid (me-16:0); 7-hexadecenoic acid (16:1Δ7); 9-hexadecenoic acid (16:1Δ9); hexadecanoic acid (16:0); methylhexadecanoic acid (me-17:0); 9-heptadecenoic acid (17:1Δ9); 11-heptadecenoic acid (17:1Δ11); heptadecanoic acid (17:0); methylheptadecanoic acid (me-18:0); 9-octadecenoic acid (18:1Δ9); 11-octadecenoic acid (18:1Δ11); and octadecanoic acid (18:0). The most abundant fatty acids are 15:0, 16:1Δ9, and 16:0. The proportions of unsaturated and branched fatty acids are larger in the cultures grown on H_2/CO_2 than in those on formate. These increases come mainly at the expense of 16:0, which is more abundant in the cultures grown on formate.

3.2.3. D/H of fatty acids

Table 3 summarizes δD values of fatty acids (δD_{FA}) from *D. autotrophicum* grown on H_2/CO_2 and on formate. In general, the fatty acids are significantly D-depleted relative to growth water. In cultures grown on H_2/CO_2, variation in δD_{water} between −39‰ and +993‰ corresponds to variation in δD_{FA} between −325‰ and +519‰; individual fatty acids exhibit slightly different ranges of δD. Similarly, in cultures grown on formate, variation in δD_{water} between −40‰ and +993‰ corresponds to variation in δD_{FA} between −370‰ and +494‰. To quantify the fractionation of D among fatty acids, growth water, and the electron donors, the data were analysed by linear regression. These calculations are summarized in the electronic annex EA-2 (Tables EA-2-1 and EA-2-2) and Table 4. The results are discussed in terms of the mass-balance equation describing isotopic fractionation and mixing.

Linear regression of compound-specific R_{FA} on R_{water} yields a unique slope and intercept for each fatty acid from *D. autotrophicum* grown on H_2/CO_2. Slopes are in the range from 0.61 to 0.74 (Fig. 2). The value of δD_{H2} was not varied systematically in this experiment.

In contrast, for *D. autotrophicum* grown on formate, both δD_{water} and $\delta D_{formate}$ were varied systematically and independently. The results are examined in terms of each variable. First, δD_{FA} is compared to $\delta D_{formate}$ (Fig. 3a). For each δD_{water} treatment, δD_{FA} is not at all correlated with $\delta D_{formate}$, which varies over a range of approximately 1000‰. For individual fatty acids within each δD_{water} treatment, linear regression of R_{FA} on $R_{formate}$ yields negligible slope in the range -0.04 ± 0.03 to 0.03 ± 0.04, where uncertainties are one standard error (Table EA-2-1). Because there is no significant difference among slopes of the different δD_{water} treatments, all data are pooled for each fatty acid. Linear regression of pooled R_{FA} on $R_{formate}$ yields a slope of 0.0 for every fatty acid.

In these same cultures grown on formate, variation in δD_{water} correlates with a variation in δD_{FA} that is constant among the $\delta D_{formate}$ treatments (Fig. 3b). Intercept magnitude is not correlated to $\delta D_{formate}$. For every fatty acid except 15:1Δ9, separate linear regression (R_{FA} on R_{water}) of the three $\delta D_{formate}$ treatments yields slopes that are indistinguishable at one standard error. An *F*-test confirms homo-

Table 3
Isotopic ratios (δD, ‰)[a] of fatty acids from *D. autotrophicum* grown on electron donors of various δD.

Fatty acid	Cultures Grown on H_2				Cultures Grown on Formate								
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
13:0	−273	−254	nd	407	nd	nd	nd	51 (2)	55 (3)	nd	389 (2)	nd	396 (7)
14:1Δ7	nd[b]	nd	nd	nd	nd	nd	nd	nd	nd	nd	411 (3)	nd	415 (5)
14:0	−322 (2)	−325 (1)	−21 (10)	314 (19)	−309 (9)	−310 (3)	−304 (1)	−3 (2)	−6 (1)	−13 (1)	304 (3)	282 (2)	307 (3)
15:1Δ7	nd	nd	nd	nd	nd	nd	nd	145 (4)	140 (3)	nd	494	nd	494 (2)
15:1Δ9	−317 (3)	−318 (5)	29 (10)	371 (4)	−306 (5)	−305 (1)	−350 (2)	36 (4)	36 (4)	−7 (0.04)	362 (3)	265 (5)	367 (9)
15:0	−317 (2)	−304 (3)	43 (3)	377 (1)	−288 (1)	−286	−302 (2)	43 (3)	44 (2)	46 (1)	386 (4)	394 (4)	382 (3)
me-16:0	−276 (4)	−275 (6)	nd	412	nd	nd	nd	nd	nd	nd	nd	nd	nd
16:1Δ9	−308 (5)	−289 (3)	60 (8)	402 (3)	−298 (5)	−293 (1)	−291 (1)	62 (3)	56 (5)	58 (2)	392 (6)	403 (3)	395 (8)
16:0	−323 (8)	−313 (3)	17 (9)	347 (1)	−294 (3)	−288 (1)	−292 (1)	42 (3)	40 (5)	43 (4)	371 (6)	378 (4)	359 (6)
me-17:0	−297 (2)	−284 (1)	65 (12)	418 (3)	−284	nd	−266	68	59	58 (6)	394 (2)	405 (2)	399 (3)
17:1Δ9	−252 (1)	−238 (2)	133 (4)	519 (16)	−248 (6)	−239 (2)	−248 (3)	115 (3)	103 (5)	114 (1)	456 (8)	460 (3)	447 (2)
17:1Δ11	−301 (5)	−282 (2)	70 (12)	418 (8)	−292 (6)	−288 (1)	−292 (1)	62 (5)	54 (6)	62 (2)	386 (5)	399 (3)	385 (3)
17:0	−322 (4)	−296 (2)	48 (22)	370 (2)	−269 (6)	−267 (3)	−275 (0.3)	91 (3)	89 (1)	93 (3)	444 (5)	452 (8)	431 (11)
me-18:0	−257	nd	nd	503	nd	nd	nd	nd	nd	nd	nd	nd	nd
18:1Δ9	−221 (11)	nd	nd	479	nd	nd	nd	nd	nd	nd	nd	nd	nd
18:1Δ11	−310 (6)	−312 (10)	7	370 (17)	−370 (6)	−358 (3)	−358 (6)	−43 (9)	−43 (3)	−11 (4)	301 (8)	348 (5)	328 (4)
18:0	−283 (5)	nd	nd	296	−247 (2)	−234 (3)	−240 (0.01)	29 (0.02)	41 (4)	52 (2)	292 (3)	284 (7)	267 (2)

[a] Values are mean (±1σ) of replicate measurements ($n = 2$ or 3) of a single sample, except where no uncertainty is given ($n = 1$).
[b] Not determined.

Table 4
Comparison of *D. autotrophicum* cultures grown on different electron donors by linear regression of R_{FA} on R_{water}.

Fatty Acid	H_2 (Cultures I–IV)[a]			Formate (Cultures V–XIII)[a]			Comparison of slopes[b]				Cultures I–XIII pooled[a]		
	Slope		Intercept × 10^4	Slope		Intercept × 10^4	F	v_1	v_2	$F_{0.05}$	Slope		Intercept × 10^4
14:0	0.61	(0.02)	0.13 (0.04)	0.592	(0.009)	0.18 (0.02)	1.23	1	9	5.12	0.600	(0.008)	0.16 (0.02)
15:1Δ9	0.665	(0.009)	0.06 (0.02)	0.64	(0.03)	0.10 (0.06)	0.43	1	9	5.12	0.65	(0.02)	0.09 (0.04)
15:0	0.666	(0.008)	0.08 (0.02)	0.66	(0.01)	0.10 (0.03)	0.01	1	9	5.12	0.667	(0.009)	0.09 (0.02)
16:1Δ9	0.68	(0.01)	0.07 (0.03)	0.676	(0.009)	0.08 (0.02)	0.03	1	9	5.12	0.677	(0.006)	0.08 (0.01)
16:0	0.64	(0.01)	0.10 (0.02)	0.65	(0.01)	0.13 (0.02)	0.04	1	9	5.12	0.65	(0.01)	0.11 (0.03)
me-17:0	0.68	(0.01)	0.08 (0.03)	0.66	(0.01)	0.13 (0.03)	1.21	1	8	5.32	0.670	(0.009)	0.11 (0.02)
17:1Δ9	0.74	(0.02)	0.07 (0.04)	0.685	(0.009)	0.14 (0.02)	9.28	1	9	5.12[d]	0.70	(0.01)	0.12 (0.03)
17:1Δ11	0.69	(0.01)	0.07 (0.03)	0.667	(0.009)	0.10 (0.02)	1.73	1	9	5.12	0.672	(0.008)	0.09 (0.02)
17:0	0.66	(0.02)	0.09 (0.03)	0.70	(0.01)	0.08 (0.02)	4.44	1	9	5.12	0.69	(0.02)	0.07 (0.04)
18:1Δ11	0.65	(0.03)	0.08 (0.06)	0.67	(0.02)	−0.03 (0.05)	0.24	1	9	5.12	0.66	(0.02)	0.02 (0.05)
18:0	0.6[c]		0.3	0.511	(0.009)	0.42 (0.02)	8.22	1	7	5.59[e]	0.52	(0.01)	0.39 (0.03)

[a] All uncertainties (in parentheses) are one standard error; for every set of regressed data, R^2 is 0.99 or greater.
[b] *F*-test performed according to Sokal and Rohlf (1995). Critical values of the *F*-distribution, calculated for degrees of freedom between groups (v_1) and within groups (v_2), are found in Rohlf and Sokal (1995).
[c] Cultures II and III did not yield sufficient 18:0 fatty acid for measurement of R_{FA}; $n = 2$.
[d] $F_{0.01} = 10.6$.
[e] $F_{0.01} = 12.2$.

geneity of the slopes for every fatty acid, including 15:1Δ9 (Table EA-2-2). Accordingly, data from cultures V-XIII are pooled for each fatty acid. Linear regression (R_{FA} on R_{water}) of the pooled data yields a common slope for each fatty acid (Table 4). These slopes range from 0.59 to 0.70, except for that of the 18:0 fatty acid (0.51). Intercepts range from -0.03×10^{-4} to 0.18×10^{-4}, again with the exception of 18:0 (0.42×10^{-4}).

Table 4 compares the slopes and intercepts calculated for cultures V–XIII (formate) to those calculated for cultures I–IV (H_2/CO_2). For most fatty acids, slopes and intercepts associated with the different electron donors are identical within one standard error. In the other cases (17:1Δ9, 17:0, and 18:0), a statistical comparison of the slopes shows that F does not exceed the critical value $F_{0.01}$, indicating no significant difference in slope between cultures on H_2/CO_2 and cultures on formate. Therefore, the data from all cultures I–XIII are pooled for linear regression of R_{FA} on $R_{formate}$. The resulting slopes range from 0.60 to 0.70, with the 18:0 fatty acid being an outlier at 0.52. The intercepts range from 0.02×10^{-4} to 0.16×10^{-4}, except for 18:0 at 0.39×10^{-4}.

4. DISCUSSION

4.1. Origins of fatty acid hydrogen

4.1.1. Mass balance

Because there is more than one possible external source of hydrogen to the fatty acids of *D. autotrophicum*, fatty acid–water fractionation factors ($\alpha_{FA-water}$) cannot be calculated directly from R_{FA} and R_{water}. Instead, the isotopic composition of fatty acids must be considered in terms of the available external sources of hydrogen: water and the electron donor (H_2 or formate). Isotopic fractionation and mixing of these two components is described by the mass balance

$$R_{FA} = X_{water}\alpha_{FA\text{-}water}R_{water} + (1 - X_{water})\alpha_{FA\text{-}donor}R_{donor} \quad (1)$$

where X_{water} represents the molar fraction of fatty acid hydrogen derived ultimately from water, α is the net fractionation associated with each hydrogen source, and R is the absolute D/H ratio (Sessions and Hayes, 2005). The solution to Eq. (1) cannot be uniquely determined from measured values of R_{FA}, R_{water}, and R_{donor} alone. However, for an experiment in which R_{water} is varied systematically, linear regression of R_{FA} on R_{water} yields a slope equal to $X_{water}\alpha_{FA-water}$ and an intercept equivalent to the entire R_{donor} term. Similarly, when R_{donor} is varied systematically, linear regression of R_{FA} on R_{donor} yields a slope equal to $(1\text{-}X_{water})\alpha_{FA\text{-}donor}$ and an intercept equal to the entire R_{water} term.

4.1.2. Cultures grown on formate

For the experiment in which *D. autotrophicum* grew on formate, R_{water} and $R_{formate}$ were varied independently. The results provide a unique solution to the mass balance (Eq. 1). For every fatty acid, linear regression of R_{FA} on $R_{formate}$ yields a slope of 0.0, indicating that the two variables are entirely uncorrelated. Because $\alpha_{FA\text{-}donor}$ cannot

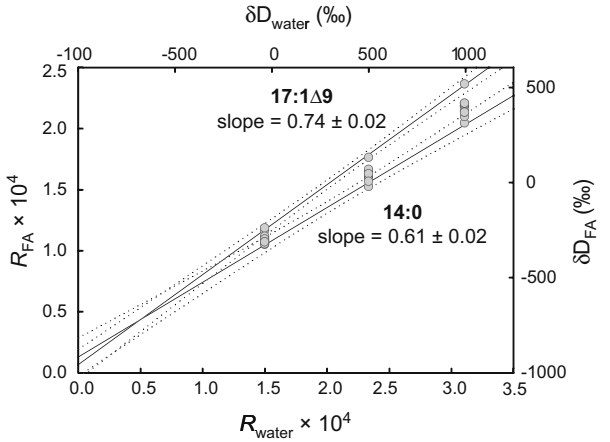

Fig. 2. Compound-specific δD of fatty acids from *D. autotrophicum* grown on H_2/CO_2. (δD of the 18:0 fatty acid, not shown, was not measurable in two cultures.) Error bars (1σ) are concealed by the symbols. Regression lines (solid) and 95% confidence intervals (dotted) are shown for the two fatty acids with the greatest and least slopes, as indicated (with uncertainties of one standard error).

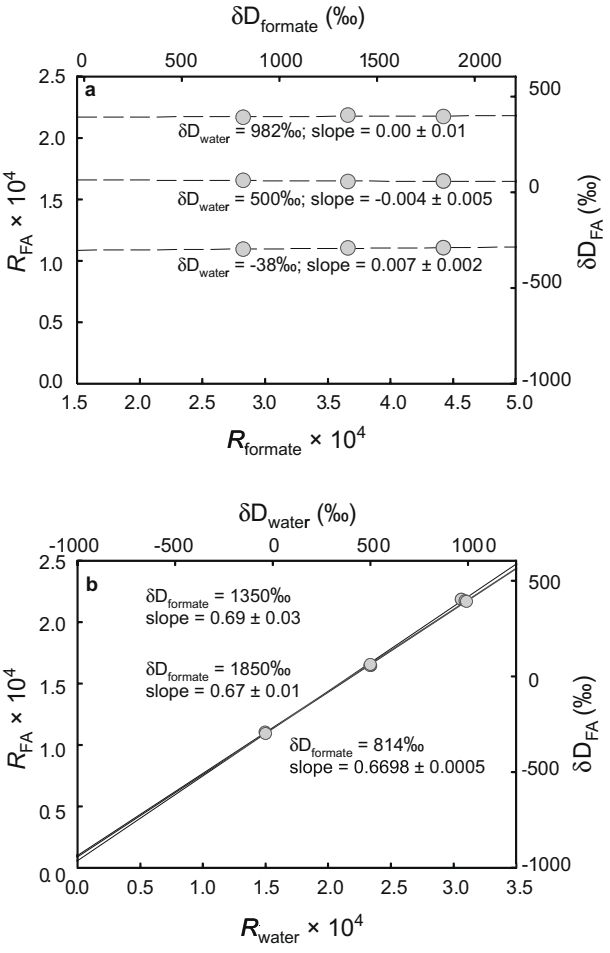

Fig. 3. δD of the 16:1Δ9 fatty acid from *D. autotrophicum* grown at various δD_{water} and various $\delta D_{formate}$. Abundant in every culture, 16:1Δ9 is representative of the other fatty acids (not shown). Error bars (1σ) are concealed by the symbols. (a) Linear regression (dashed lines) of R_{FA} on $R_{formate}$; slopes are indicated. (b) Linear regression (solid lines) of R_{FA} vs. R_{water} for the same set of cultures; slopes are indicated.

have a value of zero, this result implies that formate does not contribute hydrogen to fatty acids. Therefore, all fatty acid hydrogen originates from water ($X_{water} = 1$).

By Eq. (1), if $X_{water} = 1$, then regression of R_{FA} on R_{water} should yield an intercept of zero. In fact, the computed intercepts are small but nonzero (Table EA-2-2, Table 4); almost uniformly, they are positive and differ from zero by more than two times the standard error. The small, nonzero intercepts are interpreted as an artifact of analysis, probably due to subtle mismatches in calibration between the two labs, extrapolated over a 2000‰ range. Alternatively, we cannot rule out trace levels of contamination from yeast extract, which could also lead to a positive intercept; this possibility seems most likely for the 18:0 fatty acid, which is common in eukaryotes.

When intercepts are negligible, the mass balance simplifies to the following form:

$$R_{FA} = \alpha_{FA\text{-}water} R_{water} \quad (2)$$

In this case, linear regression of R_{FA} on R_{water} yields a slope equal to $\alpha_{FA\text{–}water}$. Therefore, the slopes calculated for pooled $\delta D_{formate}$ treatments (Table 4) are equivalent to the net fractionations between fatty acids and growth water.

Hydrogen from the extracellular formate pool is not transmitted into *D. autotrophicum* fatty acids, even though formate is an intermediate in the reduction of CO_2 to acetate (a precursor to lipids) by the reductive acetyl-CoA/carbon monoxide dehydrogenase pathway (Schauder et al., 1989). The data presented here do not identify any particular mechanism preventing transmission of formate hydrogen to fatty acids, but one possibility is hydrogen exchange between formate and water. Given the large ratio of water to formate in the cell, hydrogen exchange could render formate a negligible source of hydrogen. Such hydrogen exchange is possible in at least two processes within *D. autotrophicum*. One is the biochemical reduction of formate to acetate; the proposed pathway includes several reactions involving addition of hydrogen, and it is unknown whether carbon-bound hydrogen remains intact. Another potential exchange occurs with the catabolic oxidation of formate, catalyzed by formate dehydrogenase in *D. autotrophicum*. Formate–water hydrogen exchange observed in methanogenic sediments (deGraaf and Cappenberg, 1996) is attributed to the activity of formate dehydrogenase (Ferry, 1990).

A related possibility is that *D. autotrophicum* carries out the reversible interconversion of formate and H_2 via activity of an unidentified formate–hydrogen lyase (FHL) enzyme complex. Found in *Escherichia coli* and other organisms, the FHL complex consists of a formate dehydrogenase coupled with a hydrogenase to perform the reversible decomposition of formate to H_2 and CO_2 (Peck and Gest, 1957). However, if this reaction were to occur in *D. autotrophicum*, the interconversion between formate and H_2 would be unlikely to involve a direct exchange of protons

because the two enzyme subunits are coupled by electron transfer. Therefore, although such a reaction could alter the isotopic composition of formate, the alteration would occur by hydrogen exchange between formate and water rather than between formate and H_2.

4.1.3. Cultures grown on H_2/CO_2

For the experiment in which *D. autotrophicum* grew on H_2/CO_2, only R_{water} was varied experimentally. Without independent variation in R_{H2}, the results do not provide a unique solution to the mass balance (Eq. 1) and the experiment itself does not rule out a contribution to fatty acids from H_2. However, the results from *D. autotrophicum* grown on H_2/CO_2 are nearly identical to the results from cultures grown on formate (Fig. 4). A statistical comparison (Table 4) shows that for every fatty acid, varying the electron donor produced no significant difference in slope. Similarly, for every fatty acid except 18:0, varying the electron donor produced no significant difference in intercept (i.e., values overlap at two times the standard error). These closely matching results strongly suggest that, for *D. autotrophicum* grown on H_2/CO_2 as well as on formate, all fatty acid hydrogen originates from water. As explained above, the mass balance takes the form of Eq. (2), and the slopes in Table 4 are interpreted as $\alpha_{FA-water}$.

As in the case of formate, hydrogen from H_2 is clearly not transmitted into *D. autotrophicum* fatty acids. This result is in agreement with the expected metabolic fate of H_2: unlike formate, H_2 is not an intermediate compound in the biosynthesis of fatty acids. Some hydrogenase enzymes couple H_2 to an electron donor that is also an anabolic intermediate, such as NADPH, and there is evidence

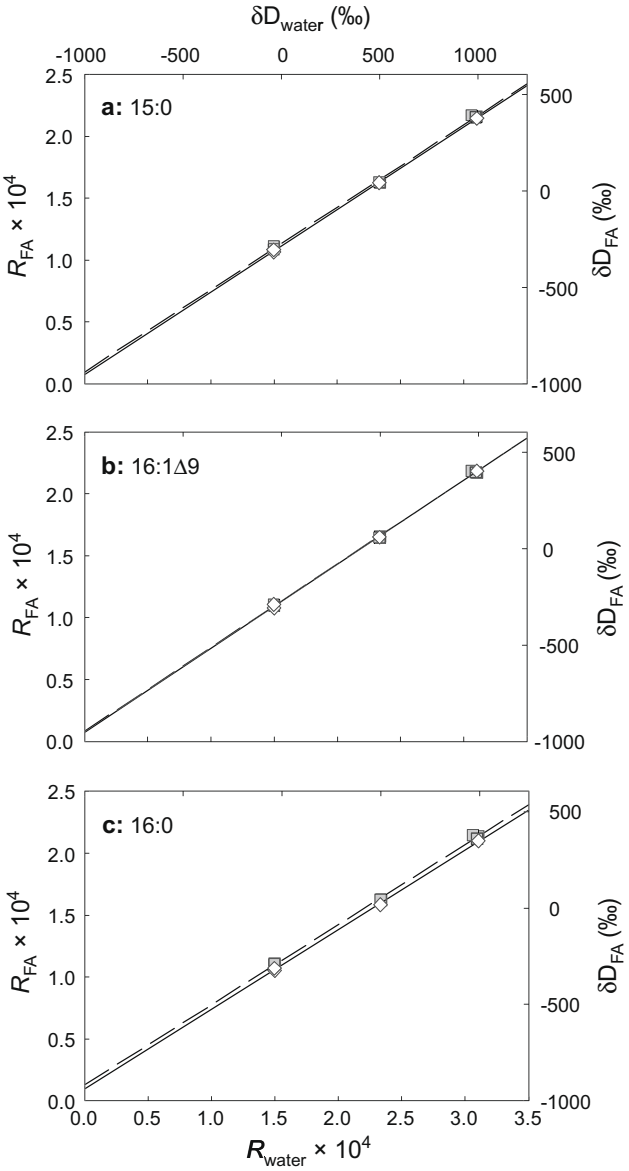

Fig. 4. Comparison of δD_{FA} in *D. autotrophicum* grown on formate (gray squares, dashed lines) and in *D. autotrophicum* grown on H_2/CO_2 (white diamonds, solid lines). Selected fatty acids are shown: (a) 15:0, (b) 16:1Δ9, (c) 16:0. Error bars (1σ) are concealed by the symbols. Lines represent linear regression of R_{FA} vs. R_{water}. Data from all cultures grown on formate were pooled for regression.

for NADPH-coupled hydrogenase (Malki et al., 1997). However, for transmission of hydrogen to fatty acids, the reaction would have to involve not just electrons but also protons, perhaps in the form of a hydride ion transferred intact from H_2 to NADPH. Studies of the hydrogenase enzyme find no such mechanism; on the contrary, they indicate that electron flow is decoupled from the flow of protons, which are released into solution (de Lacey et al., 2005). Therefore, the intact transfer of hydrogen from H_2 to a biosynthetic intermediate compound is unlikely.

A second possibility worth considering is that hydrogenase-catalyzed isotopic exchange and/or the oxidation of H_2 to H_2O might alter the δD value of intracellular water, thereby affecting the δD of fatty acids that derive their hydrogen from water. This mechanism (in combination with the above) was hypothesized to explain the unusually D-depleted lipids of *Sporomusa* sp. (Valentine et al., 2004b). According to evidence from oxygen isotopes, intracellular water in log-phase *E. coli* can become isotopically distinct from extracellular water, with up to 70% of intracellular water generated in metabolic reactions (Kreuzer-Martin et al., 2005). However, this phenomenon is unlikely in *D. autotrophicum*, whose metabolic rates are orders of magnitude lower than those of *E. coli*. Hydrogenase-catalyzed isotopic exchange between H_2 and water clearly occurs (Fig. 1). If it were altering the δD of intracellular water, one would predict a difference in δD_{FA} between cultures grown on H_2/CO_2 and those grown on formate. No such difference is observed.

Based on the evidence, the above mechanisms do not occur in *D. autotrophicum*. However, they illustrate a possible role for hydrogenase enzymes in establishing the isotopic composition of lipid hydrogen. The influence of hydrogenase on the isotopic composition of fatty acids is examined further in a companion study of *Cupriavidus necator* strain H16 and its hydrogenase mutants (Campbell, 2007).

4.2. Biosynthetic fractionations

For *D. autotrophicum* grown on either H_2/CO_2 or formate, all hydrogen in fatty acids is derived from water. Because there is no statistical difference between the slopes associated with the different electron donors, they are pooled to give a common slope (Table 4). The common slope provides a robust value of $\alpha_{FA-water}$ for lithoautotrophic *D. autotrophicum*. Individual fatty acids exhibit net biosynthetic fractionation factors between 0.6 and 0.7, except for the 18:0 fatty acid ($\alpha_{FA-water} = 0.52$). Grouping the fatty acids by carbon chain length (Fig. 5) reveals a general trend of increasing $\alpha_{FA-water}$ (i.e., relative D-enrichment of fatty acids) as chain length increases from C_{14} to C_{17}. The pattern is consistent among both saturated and unsaturated fatty acids, as is the deviation of the C_{18} fatty acids from the trend (with the 18:0 fatty acid being particularly D-depleted, as noted above). By contrast, within the groupings by chain length, there are no consistent trends in $\alpha_{FA-water}$ with respect to fatty acid abundance or degree of saturation. These observations suggest that variability in hydrogen isotopic composition is related to the elongation reactions of fatty acid biosynthesis, as opposed to the desaturation reactions. In this respect, *D. autotrophicum* differs from several species of marine macroalgae that produce fatty acids exhibiting an overall pattern of D-enrichment with carbon chain length, but in which the observed variations are explained by the hydrogen isotopic fractionation during desaturation, which "should be much larger than that during elongation in the complex network of polyunsaturated fatty acid biosynthesis" (Chikaraishi et al., 2004c). Finally, a slight trend of D-enrichment with increasing fatty acid chain length has been observed in several other algae and in the leaves of the carrot plant (Sessions et al., 1999).

The fractionation factors calculated above represent unusually strong biosynthetic fractionations of the stable hydrogen isotopes. The fatty acids of *D. autotrophicum* are D-depleted to a range similar to that of fatty acids in the acetogenic bacterium *Sporomusa* sp., strain DMG 58 (Valentine et al., 2004b). Linear regression of R_{FA} on R_{water} for pooled data from the three most abundant *Sporomusa* fatty acids (β-OH 12:0, 16:1, and 16:0) yields a slope of 0.59. If, as in *D. autotrophicum*, the slope is equivalent to the net fatty acid–water fractionation, then it can be com-

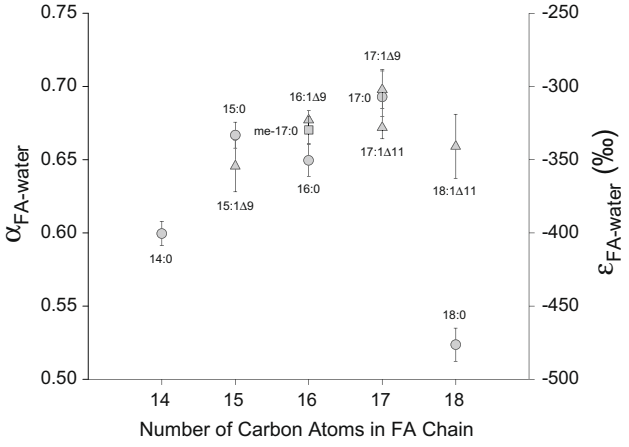

Fig. 5. Fractionation factors associated with biosynthesis of individual fatty acids (FA) in *D. autotrophicum*, based on pooled regression of D/H values from cultures grown on H_2/CO_2 and formate. Circles represent saturated FA; triangles, monounsaturated FA; and squares, methylated FA. Error bars represent one standard error.

pared directly to the biosynthetic fractionation factors observed in *D. autotrophicum*. For the most abundant *D. autotrophicum* fatty acids (15:0, 16:1, and 16:0), the range is 0.65–0.68. In general, fractionation in *Sporomusa* would appear slightly stronger than *D. autotrophicum* (although the 18:0 fatty acid of *D. autotrophicum* exhibits an anomalously low fractionation factor).

Photoautotrophic organisms also produce lipids in which all hydrogen originates from water, so their lipids can be compared directly to those of *D. autotrophicum*. The direct comparison here is restricted to fatty acids because they are the predominant lipid compounds produced by *D. autotrophicum*; according to the studies cited below, fatty acids and other acetogenic lipids are generally D-enriched relative to lipids produced by other biosynthetic pathways, including sterols and phytols. The comparison shows that fatty acid biosynthesis in *D. autotrophicum* entails a hydrogen isotopic fractionation significantly greater than in plants. For example, a variety of algae and higher plants produce fatty acids with fractionation factors in the range $0.738 \leqslant \alpha_{FA-water} \leqslant 0.887$ (Sessions et al., 1999). Chikaraishi et al. (2004b) observed different ranges associated with different plant classes (C3, C4, CAM, etc.), with all $\alpha_{FA-water}$ greater than 0.8. Freshwater green algae, cultivated under controlled D/H conditions and subjected to regression analysis as in this study, produce fatty acids with slopes ranging from 0.768 (16:0 fatty acid) to 0.984 (Zhang and Sachs, 2007). Finally, compound-specific D/H ratios of cyanobacterial fatty acids have not been reported, but total lipid extracts from cyanobacteria exhibit a range of δD values very similar to that of algae (Estep and Hoering, 1980). Further, Sachse and Sachs (2008) showed that alkanes extracted from sediments of saline and hypersaline ponds, and presumably derived from the cyanobacteria living in those ponds, exhibit lipid–water fractionations similar to those of algae and higher plants.

In summary, the available data suggest that the D/H ratio of water is the primary control on the D/H ratio of lipids, not only in photoautotrophs but also in non-phototrophic bacteria that consume hydrogen-bearing substrates (such as H_2) in addition to water. In *D. autotrophicum*, significantly D-depleted fatty acids express a strong biosynthetic isotope effect, not a contribution from the hydrogen-bearing substrate. If true for other non-phototrophic bacteria, this conclusion could prove useful, for example, in distinguishing their contribution to sedimentary organic matter from that of primary producers (photoautotrophs). To establish the ubiquity and metabolic specificity of these isotopic signals, a much broader survey of bacterial lipids is necessary.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.gca.2009.02.034.

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