

Hydrogen isotope fractionation during H₂/CO₂ acetogenesis: hydrogen utilization efficiency and the origin of lipid-bound hydrogen

D. L. VALENTINE,¹ A. L. SESSIONS,^{2,*} S. C. TYLER³ AND A. CHIDTHAISONG⁴

¹Department of Geological Sciences and Marine Science Institute, University of California, Santa Barbara, CA, USA

²Department of Geology and Geophysics, Woods Hole Oceanographic Institution, Woods Hole, MA, USA

³Department of Earth System Science, University of California, Irvine, CA, USA

⁴The Joint Graduate School of Energy & Environment, King Mongkut's University of Technology Thonburi, Bangkok, Thailand

*Present address: Division of Geological and Planetary Sciences, California Institute of Technology, Pasadena, CA, USA

ABSTRACT

Hydrogen metabolism was studied in the anaerobic bacterium, *Sporomusa sp.* strain DMG 58, by measuring natural abundance levels of deuterium in H₂, H₂O, and individual fatty acids during acetogenic growth on H₂/CO₂. Four cultures were grown, each in medium with a distinct hydrogen-isotopic composition ($\delta\text{D-H}_2\text{O}$). The δD value of H₂ was quantified in the residual gas exiting the growth chambers and found to decrease concurrently with net H₂ consumption, indicating rapid isotope exchange between H₂ and H₂O. An isotopic mass balance was used to constrain the efficiency with which H₂ was activated by the cell and the reducing equivalents catabolized, which we term the H₂ utilization efficiency. Results indicate that H₂ utilization efficiency in these cultures is less than 20% during the growth phase, and less than 2% after the growth phase. The gross rate of cellular H₂ activation was similar in the growth phase and afterward. Biomass harvested at the end of each experiment was used to analyse the D/H of individual membrane lipids. Values of δD were highly correlated between lipids and water ($\delta\text{D-lipids} = 0.59 \times \delta\text{D-water} - 381\text{‰}$; $R^2 = 0.995$), indicating the source of lipid hydrogen is in isotopic equilibrium with water. Results are consistent with two possibilities: (i) water is the sole source of hydrogen to lipids, and the fractionation during biosynthesis is significantly larger than previously observed ($\alpha = 0.59$), or (ii) hydrogen from H₂ is incorporated into lipids, but only after reaching isotopic equilibrium with H₂O. Fatty acids were strongly depleted in deuterium relative to all other organisms studied thus far, and such large depletions may prove useful as biomarkers for studying H₂ cycling in anoxic environments as well as in the geological record.

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Corresponding author: D. L. Valentine. Tel.: (805) 893-2973; fax: (805) 893-2314; e-mail: valentine@geol.ucsb.edu

INTRODUCTION

Molecular hydrogen, H₂, is important in anoxic environments as an interspecies electron carrier and as a thermodynamic master variable (Wolin, 1982; Hoehler *et al.*, 1998). To use H₂, either as a source or as a sink for electrons, organisms must carry out the reversible reaction:



Electrons are passed to (or from) electron carriers such as nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NADH). H⁺ is presumed to be in isotopic equilibrium with cellular water. The forward

direction of reaction 1 represents consumption of H₂, such as during chemoautotrophic acetogenesis, while the reverse direction represents production of H₂, such as during fermentation. The reaction is catalysed by hydrogenase enzymes, of which three general families have been identified (see Vignais *et al.*, 2001; Vignais & Colbeau, 2004).

Hydrogenase enzymes are known to facilitate the exchange of isotopes between H₂ and H₂O:



Thus, organisms fed only on DH or D₂ in H₂O medium will rapidly release H₂, even though there is a net consumption of DH/D₂. In the absence of a catalyst, isotopic exchange

between H₂ and H₂O occurs very slowly (Koehler *et al.*, 2000). The enzymatic H-D exchange reaction has been known for decades (Farkas *et al.*, 1934) and used widely in studies of hydrogenase activity and mechanism (Arp & Burris, 1982; Schworer *et al.*, 1993; Klein *et al.*, 1995; Hartmann *et al.*, 1996; Vignais *et al.*, 2000). However, most studies of H-D exchange have utilized heavily deuterated reactants (e.g., D₂ or D₂O), and such high levels of deuterium can alter the biochemical functions of organisms. The mechanism of exchange varies between different hydrogenase enzymes (e.g., Fauque *et al.*, 1988; McTavish *et al.*, 1996).

As part of our continuing work to understand the cycling of H₂ in anoxic environments (Bilek *et al.*, 1999; Valentine *et al.*, 2000a; Valentine *et al.*, 2000b; Valentine, 2001; Chidthaisong *et al.*, 2002; Chong *et al.*, 2002; Valentine *et al.*, 2004) and the D/H composition of lipid biomarkers (Sessions *et al.*, 1999; Sessions *et al.*, 2002), we performed a series of studies designed to assess the efficiency of hydrogen utilization in active cultures of the homoacetogen, *Sporomusa* strain DMG 58. These studies were further designed to determine if hydrogen atoms from H₂ are incorporated into cellular lipids – potential biomarkers of H metabolism in nature. Unlike most previous work, this study examines natural abundance levels of D, using compound-specific isotopic analysis, to minimize the potential for experimental artifacts.

MATERIALS AND METHODS

Isotope nomenclature

Previous studies of the H-D exchange reaction have utilized elevated levels of deuterated substrate (e.g. in the percentage range). Natural abundance levels of D are several orders of magnitude lower, and so different techniques for measurement and analysis of isotopic data are required. These methods are common in the geochemical literature but not widespread in microbiology, so we provide a brief summary here. More detailed treatment is available in several references (Hayes, 1983; Criss, 1999; Meier-Augenstein, 1999).

The natural abundance of deuterium is measured and reported in terms of the isotope ratio:

$$R = [D]/[H] \quad (3)$$

The D/H ratio of most Earth materials is around 150 ppm. To avoid discussing ratios with 6 or more significant decimal places, and because absolute abundance is difficult to quantify experimentally, the delta notation is commonly used to express the relative difference between the sample's isotope ratio and that of a standard:

$$\delta D = [(R_{\text{sample}}/R_{\text{VSMOW}}) - 1] \times 1000\% \quad (4)$$

The units of δD are parts per thousand (or permil, ‰), and are reported relative to the international standard VSMOW

(Vienna Standard Mean Ocean Water, as established by the International Atomic Energy Agency (IAEA)).

The introduction of a heavy isotope into a chemical reaction will affect the rate of that reaction, a phenomenon known as an isotope effect (Bigeleisen, 1965). In unidirectional reactions, the effect is referred to as a kinetic isotope effect and defined mathematically as the ratio of rate constants for the light and heavy isotopes:

$$\text{KIE} = k_{\text{H}}/k_{\text{D}} \quad (5)$$

Because molecules with a natural abundance of D will statistically contain either one or zero deuterium atoms, it is impossible to directly measure the rate constant for the heavy isotope without also having protium atoms participating in the reaction. Instead, we can measure the isotope fractionation induced in the reactant and product by the isotope effect. The fractionation factor (α) is defined as the ratio of isotope ratios in the reactant and product, and is numerically equal to the ratio of rate constants.

$$\alpha = R_{\text{react}}/R_{\text{prod}} (= \text{KIE}) \quad (6)$$

In reversible reactions, the form of Equation 5 must be modified to account for the rates of both isotopes in both the forward and reverse directions. Conveniently, though, calculation of the fractionation factor using Equation 6 remains the same, regardless of whether the product and reactant reach isotopic equilibrium. This is particularly relevant to isotopic studies of hydrogen utilization, for which both kinetic (i.e., Equation 5) and equilibrium (Equation 2) isotope effects are confounded in measurements of isotopic fractionation. For these reasons, fractionation factors are calculated and reported here using Equation 6, without specifying whether they result from equilibrium or kinetic isotope effects.

Bacterial strains and culture conditions

Sporomusa sp. strain DMG 58 (DSMZ 3301; Möller *et al.*, 1984) is an anaerobic, gram negative, spore-forming acetogenic bacterium capable of chemoautotrophic growth on H₂/CO₂, according to Equation 7:



This strain was originally isolated from sediments of the Leine River, Germany and was used in these studies due to high 16S rDNA sequence similarity with an enrichment culture obtained from Northern California (USA) rice paddies that was studied previously in our laboratory. Cultures were grown at 32 °C in basal medium as described by Möller *et al.* (1984), except trace element and vitamin solutions were based on the recipe of Boone *et al.* (1989). Four batches of media were prepared, all identical except for varying δD -H₂O values of -67‰,

+235‰, +571‰, and +1228‰. Concentrated D₂O was serially diluted in deionized water to achieve the different deuterium concentrations. Studies were performed in a 300 mL fermentor with constant CO₂/H₂ supply (Valentine *et al.*, 2000a). The mixing ratios of CO₂ and H₂ were set by mass flow controllers and confirmed by measurements of both gas entering the reactor vessel and of that in the exhaust stream. The flow rate of H₂ was set to 16 mL min⁻¹ (normalized to standard temperature and pressure) and the flow of CO₂ was set to 4 mL min⁻¹. Calculations based on the rate of acetate production indicate net H₂ consumption never exceeded 5% of H₂ supply. The average δD of H₂ supplied in all cases was $-210 \pm 2.5\%$.

Analytical techniques

Cells were counted using standard DAPI staining techniques (Kepner & Pratt, 1994). Acetate was quantified in liquid samples by HPLC as previously described (Valentine *et al.*, 2000a). Isotopic analyses of H₂ and H₂O were performed at the University of California Irvine by dual-inlet mass spectrometry, as described by Chidthaisong *et al.* (2002). At the end of each experiment, cell pellets were collected by centrifugation (15 000 × g, 60 min) and kept frozen until they were analysed.

Biomass for lipid analysis was transferred to glass vessels then lyophilized. Blanks accompanying the samples in the freeze dryer were free of both fatty acids and hydrocarbons. Because of the relatively small amount of biomass available for lipid analysis, samples were first hydrolysed in KOH/methanol (70 °C, 6 h) to provide the maximum yield of free fatty acids. The pH of the solution was adjusted to <2 by adding HCl and fatty acids were extracted into methyl *t*-butyl ether (MTBE). The extract was then purified by elution from Supelclean LC-NH₂ solid phase extraction tubes (Supelco, Bellefonte, PA) as described by Sessions *et al.* (1999). A 10% aliquot of each sample was derivatized as trimethylsilyl (TMS) ethers by reaction with bis(trimethylsilyl)trifluoroacetamide and analysed by gas chromatography/mass spectrometry (GC/MS) on a 60 m DB-5MS analytical column. The effluent from the column was split between a quadrupole mass spectrometer and a flame ionization detector (FID). Fatty acids were identified from their mass spectra, and the identity of β-hydroxy lauric acid was confirmed by comparison to authentic standards. Hydroxy fatty acids with differing chain lengths were identified by comparison of their mass spectra and retention time to lauric acid, but without authentic standards. Fatty acid abundances were quantified by comparison of FID peak areas to that of an internal standard (10 ng behenic acid methyl ester).

Methyl esters of the remaining 90% of fatty acids were synthesized using anhydrous HCl/MeOH at 70 °C for 6 h. The hydroxyl groups of hydroxy fatty acids were then derivatized as acetyl esters by reaction with acetic anhydride/pyridine at 70 °C for 1 h. Compound-specific D/H analyses of the fatty acid methyl esters (FAME's) were conducted on a Finnigan Delta + XL isotope ratio mass spectrometer coupled to an

Agilent 6890 GC via a 1440 °C pyrolysis reactor (Finnigan GC/TC), using coinjected *n*-alkane standards as reference peaks (Sessions *et al.*, 1999). Data were normalized to the SMOW/SLAP scale by comparison to an external standard containing 15 *n*-alkanes with δD values ranging from -41 to -256‰ (Sessions *et al.*, 2001). The *n*-alkane standards are identical to those being distributed by Dr Arndt Schimmelmann at the Biogeochemical Laboratories, Indiana University. Correction for H added by both methylation and acetylation reagents was performed by analysing dimethyl phthalate and acetic anhydride as described by Sessions *et al.* (2002). Typical uncertainties for isotopic analyses obtained by these techniques, quantified by replicate measurements of standards, are: δD-H₂ ($\pm 7.3\%$), δD-H₂O ($\pm 1\%$), and δD-lipids ($\pm 6\%$).

RESULTS AND DISCUSSION

Data from each of the four experiments are shown in Fig. 1. Changes in cell density are given in panels a–d and indicate that each culture went through a rapid growth phase, lasting about 75 h, followed by stationary/death phases. The time resolution of the cellular abundance data was not sufficient to differentiate the stationary and death phases, thus they were considered together. Maximum observed densities were $\sim 2 \times 10^8$ cells/mL. The cultures grown in water with δD values of -67‰ and +235‰ grew to nearly twice the cell density of the two more deuterium-enriched cultures. However, the levels of enrichment (δD = +1227‰ corresponds to a deuterium concentration of only ~340 ppm) were so small that it seemed unlikely they could be solely responsible for the decreased growth.

The accumulation of acetate in the cultures is illustrated in Fig. 1, panels e–h. Acetate accumulated to over 50 mM in each experiment, driving a concurrent drop in pH. Because acetate is the primary end-product of H₂ catabolism, catabolic rates can be estimated from the rate of acetate accumulation. The rates of acetate accumulation during the growth phase were calculated from the first derivatives of the best fit curves shown in Fig. 1, panels e–h; results are listed in Table 1 for eight relevant time points, two from each experiment. Catabolic rates at times later in the experiment (i.e., times shown by the open symbols in Fig. 1e–h) determined from the slope of the line connecting adjacent points. Results are listed in Table 1 for four time points in the stationary/death phase, one from each experiment. Total rates of catabolism ranged from 0.44 to 2.10 mM acetate per hour during the growth phase, and from 0.22 to 0.62 mM h⁻¹ during stationary/death phase. Using cell densities for the same time points interpolated from the curves shown in Fig. 1 (panels a–d), we also calculated cell-specific catabolic rates (Table 1), which range from 7 to 19 amol acetate cell⁻¹ s⁻¹ during the growth phase down to 0.35 amol acetate cell⁻¹ s⁻¹ during stationary/death phase.

The δD of residual H₂ (gas exiting the vessel) is shown in Fig. 1, panels i–l. In each case, the δD value of H₂ dropped dramatically relative to the source gas ($-210 \pm 2.5\%$) throughout

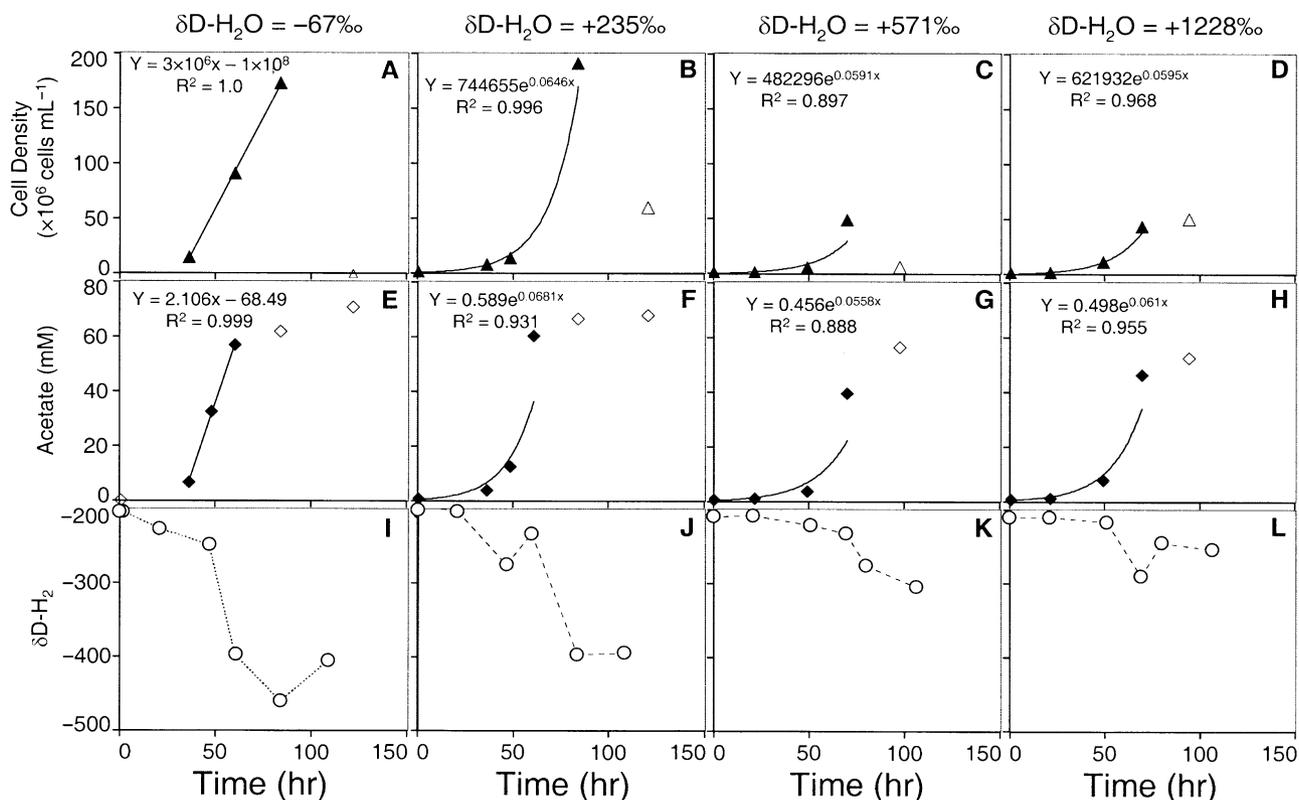


Fig. 1 Cell abundance (panels A–D), acetate accumulation (panels E–H), and $\delta\text{D-H}_2$ of residual gas (panels I–L) during growth of *Sporomusa* strain DMG 58 in media of different initial deuterium contents: -67‰ (panels A, E, and I), $+235\text{‰}$ (panels B, F and J), $+571\text{‰}$ (panels C, G and K), $+1228\text{‰}$ (panels D, H and L). The pH of the culture medium dropped from pH 7, at the beginning of each experiment, to pH 5 at the end of each experiment. The regression analyses given in panels A–H were used to interpolate cell abundance (A–D) and acetate concentration (E–H). The solid symbols were used for the regression analyses in panels A–H. The regression analyses are not valid for times corresponding the open symbols, which correspond to the stationary/death phase. An uninoculated control experiment showed no difference between the δD of H_2 supplied to the vessel and the δD of H_2 exiting the vessel. The δD of H_2 supplied to the vessel was $-210 \pm 2.5\text{‰}$ for each experiment. Slight variations ($<12\text{‰}$) were observed in the $\delta\text{D-H}_2\text{O}$ of the culture media at different sampling times, likely caused by evaporation and/or the observed isotope exchange.

Table 1 Hydrogenase efficiency and catabolic rates in *Sporomusa* strain DMG 58

Time (h)*	$\delta\text{D-H}_2\text{O}$ (‰)†	$\delta\text{D-H}_2$ (‰)‡	Catabolic Rate (mM h ⁻¹)§	Cell Density ($\times 10^6$ cells mL ⁻¹)	Efficiency¶	Cellular Rate** (amol-H ₂ cell ⁻¹ s ⁻¹)
Growth Phase						
46.5	-67	-250	2.10	40	0.23	250
59.8	-63	-398	2.10	79	0.05	630
46.5	237	-276	0.95	15	0.07	960
59.8	237	-234	2.34	35	0.31	230
51.0	573	-222	0.44	10	0.09	560
69.1	575	-233	1.18	29	0.14	330
51.0	1227	-217	0.67	13	0.18	330
69.1	1227	-291	2.03	38	0.03	1800
Stationary / Death Phase						
83.5	-56	-461	0.22	172	0.003	490
83.4	239	-398	0.26	191	0.004	340
79.8	570	-277	0.62	35	0.025	790
79.8	1226	-245	0.25	46	0.013	460

*Refers to the time at which the samples were taken as in Fig. 1.

†Slight variations were observed in $\delta\text{D-H}_2\text{O}$ of the culture media for each of the four experiments.

‡Refers to the $\delta\text{D-H}_2$ exiting the vessel.

§Catabolic rates are given in mM-acetate h⁻¹.

¶Efficiency (Equation 14) calculated assuming α_p and $\alpha_c = 0.8$ using data and equations presented in Fig. 1(E)–(H).

**Refers to the cell-specific rate of H_2 activation.

the course of the experiment as cell densities increased, whereas no change in the $\delta\text{D-H}_2$ was observed prior to inoculation or during an uninoculated control experiment (data not shown). There are two possible causes for the observed changes in isotopic composition. First, kinetic isotope effects associated with consumption of H_2 by strain DMG 58 will change the δD value of residual H_2 . Such changes should be proportional to rates of catabolism but independent of water δD values, which does not agree well with experimental data (compare panels i through l in Fig. 1). Also, a typical isotope effect should result in the deuterium enrichment (increased δD value) of residual H_2 , contrary to our observations.

The second possibility is that exchange between H_2 and H_2O has shifted δD values towards isotopic equilibrium. In this case, δD values for H_2 should depend on those of H_2O . The direction and magnitude of the observed fractionations were consistent with the residual H_2 approaching, but never reaching, isotopic equilibrium with H_2O ($\alpha = 0.272$ at 32°C ; Horibe & Craig, 1995). The exchange is presumably catalysed by hydrogenase enzymes, and so should be roughly proportionate to the amount of hydrogenase (and so total cell mass) rather than rates of catabolism, in agreement with experimental data (Fig. 1). Several other studies of anaerobes (Chidthaisong *et al.*, 2002; Romanek *et al.*, 2003; Valentine *et al.*, 2004) have also observed the δD value of H_2 approaching isotopic equilibrium with H_2O . Thus, we hypothesize that hydrogen exchange, catalysed by hydrogenase enzymes, is the dominant process modifying the δD values of H_2 . We can use these observations to understand the processing of H_2 through the hydrogenase enzyme systems of strain DMG 58 in greater detail, as described below.

H_2 utilization efficiency

In order to estimate the efficiency of H_2 utilization in strain DMG 58, a mass-balance box model was constructed for the deuterium content of dissolved H_2 in the culture (Equation 8). This approach is similar to one presented by Valentine *et al.* (2004) to calculate hydrogenase efficiency in an H_2 -utilizing methanogen. A schematic diagram highlighting this approach is available as Fig. 1 in Valentine *et al.* (2004).

$$f_{\text{out}}\alpha_{\text{aq/g}}R_{\text{out}} = f_{\text{in}}\alpha_{\text{aq/g}}R_{\text{in}} + f_{\text{p}}R_{\text{p}} - f_{\text{c}}R_{\text{c}} \quad (8)$$

R 's in Equation 8 represent D/H ratios, f 's represent molar fluxes of molecular hydrogen (H_2 or HD), and $\alpha_{\text{aq/g}}$ is the equilibrium fractionation between dissolved and gaseous H_2 . The subscripts *in* and *out* indicate gas-phase H_2 entering or exiting the culture vessel, respectively, and subscripts *p* and *c* indicate dissolved H_2 , produced and consumed by the hydrogenase enzyme system. The equilibrium fractionation factor $\alpha_{\text{aq/g}}$ is necessary to accommodate the fact that H_2 is measured in the gas phase, while the hydrogenase enzyme interacts with dissolved H_2 . The model thus assumes that H_2

gas entering and exiting the culture apparatus is in equilibrium with dissolved H_2 , and that *Sporomusa*'s hydrogenase enzymes are the only significant source and/or sink for H_2 .

The gross H_2 consumption term in Equation 8 can be expressed as the sum of three individual terms:

$$f_{\text{c}} = f_{\text{p}} + f_{\text{a}} + f_{\text{b}} \quad (9)$$

where, f_{p} is the flux of H_2 that will be subsequently released back to the medium, f_{a} , the flux of H_2 converted to acetate for catabolism, and f_{b} , the flux of H_2 diverted to biomass for growth. In strict anaerobes grown chemoautotrophically, such as homoacetogens, biomass yields tend to be quite low. Assuming the average cell contains 1×10^{-13} g C, biomass yield can be estimated from data in Fig. 1 to be less than 2%. Inclusion of f_{b} in Equation 13 would have only a minor impact on the final efficiency calculation and so f_{b} is excluded from our calculations. The isotope ratio of the H_2 consumed by hydrogenase can be related to that of the dissolved H_2 by some fractionation factor:

$$R_{\text{c}} = \alpha_{\text{c}}R_{\text{diss}} = \alpha_{\text{c}}\alpha_{\text{aq/g}}R_{\text{out}} \quad (10)$$

Because H_2 is not produced as a catabolic product under the experimental conditions, the H_2 production term (f_{p}) represents only H_2 that was originally activated by hydrogenase then subsequently released. We can then write the D/H ratio of released H_2 in terms of that of the enzyme-bound H_2 :

$$R_{\text{p}} = \alpha_{\text{p}}R_{\text{bound}} \quad (11)$$

where α_{p} represents the fractionation associated with H_2 production. If we then assume that H_2 activated by the hydrogenase enzyme can rapidly equilibrate with water – an assumption supported by isotope labelling experiments – we can write the D/H ratio of produced H_2 as:

$$R_{\text{p}} = \alpha_{\text{p}}\alpha_{\text{h/w}}R_{\text{H}_2\text{O}} \quad (12)$$

where $\alpha_{\text{h/w}}$ refers to fractionation between dissolved H_2 and liquid H_2O . Substituting Equations 9–12 into Equation 8 gives:

$$f_{\text{out}}\alpha_{\text{aq/g}}R_{\text{out}} = f_{\text{in}}\alpha_{\text{aq/g}}R_{\text{in}} + f_{\text{p}}\alpha_{\text{p}}\alpha_{\text{h/w}}R_{\text{H}_2\text{O}} - (f_{\text{p}} + f_{\text{a}})\alpha_{\text{c}}\alpha_{\text{aq/g}}R_{\text{out}} \quad (13)$$

Values for f_{out} and f_{in} , R_{out} and R_{in} , and $R_{\text{H}_2\text{O}}$ can be determined directly from the experimental data. Values for f_{a} can be estimated from the measured acetate accumulation rates using the stoichiometry of Equation 7. Knox *et al.* (1992) report a value for $\alpha_{\text{aq/g}}$ of 1.038 (at 21°C). Horibe & Craig (1995) report a value for $\alpha_{\text{H}_2(\text{g})/\text{H}_2\text{O}(\text{l})}$ of 0.272 at 32°C . Combining these values we estimate $\alpha_{\text{h/w}} = 0.282$. Values for α_{c} and α_{p} have not been reported, so we must assume a range of values

for α_c and α_p to allow calculation of f_p at any given time. Because f_p is the total flux of hydrogen exchanged by hydrogenase and depends on the size of the culture, we define the intrinsic efficiency (E) of H_2 consumption as the ratio of H_2 used for catabolism divided by the gross H_2 consumption:

$$E = \frac{f_a}{f_c} = 1 - \frac{f_p}{f_p + f_a} \quad (14)$$

Calculations for the experimental data using Equation 13 and 14 are shown in Fig. 2, applying various values for α_c and α_p . Twelve time points were considered in total, eight in the growth phase and four in the later stages (stationary/death phases). Pertinent data from these time points are given in Table 1. Efficiency was calculated to be $14 \pm 10\%$ in the growth

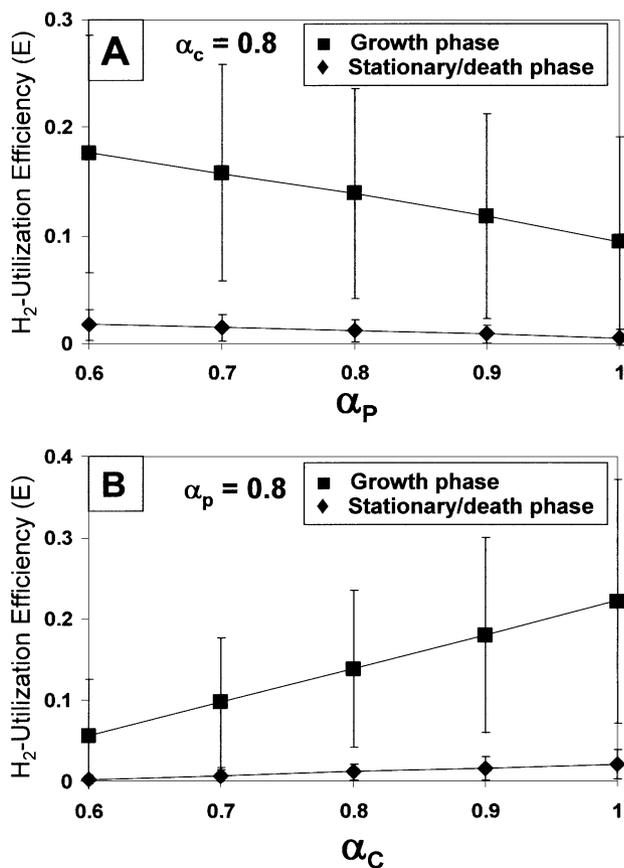


Fig. 2 (A) Calculated efficiency (E) of H_2 utilization as a function of α_p , the D/H fractionation associated with H_2 production. (B) Calculated efficiency of H_2 utilization as a function of α_c , the D/H fractionation associated with H_2 consumption. For both panels the efficiency is shown separately for the growth phase (■) and for the stationary/death phase (◆). Data for the eight time points (given in Table 1) was used to determine the mean efficiency for the growth phase, and data from four time points (given in Table 1) were used to determine the mean efficiency for the stationary/death phase. Error bars represent \pm one standard deviation from the mean. Values of 0.8 were chosen for α_c (panel A) and α_p (panel B). Arp and Burris (Arp & Burris, 1982) compared the kinetics of uptake and release of D_2 and H_2 using hydrogenase purified from soybean root nodules, and measured the discrimination associated with H_2 uptake as 0.77–0.83.

phase, but only $1.1 \pm 1\%$ in the stationary/death phase, assuming α_c and α_p both equal 0.8.

Cell-specific rates of H_2 activation can also be calculated from the experimental data, assuming each DAPI-stained cell was active, and assuming values for α_c and α_p (Table 1). The average cellular rate of H_2 activation was calculated to be 630 ± 540 amol- H_2 cell $^{-1}$ s $^{-1}$ for samples from the growth phase, and 520 ± 190 amol- H_2 cell $^{-1}$ s $^{-1}$ for samples from the stationary/death phase. These calculations indicate the rate of cellular H_2 activation is roughly independent of cellular catabolic rate as the average cellular catabolic rate is an order of magnitude higher in the growth phase compared with the stationary/death phase.

Evaluation of model assumptions

Several assumptions in this model deserve closer scrutiny. First, the H_2 -utilization efficiency model assumes that the D/H ratio of H_2 exiting the vessel (R_{out}) is in isotopic equilibrium with dissolved H_2 but *not* with H_2O . However, dissolved and gaseous H_2 may not fully achieve isotopic equilibrium during the rapid transit through the system. Furthermore, the only value available for $\alpha_{aq/g}$ (Knox *et al.*, 1992) applies to 21 °C, not the experimental temperature of 32 °C. Because the magnitude of $\alpha_{aq/g}$ is small relative to other factors in Equation 13, small variations in $\alpha_{aq/g}$ due to temperature or incomplete gas-liquid equilibration will cause only small variations in the calculated efficiencies.

The H_2 -utilization efficiency model treats the entire hydrogenase system of strain DMG 58 as one enzyme, as is common for studies of isotope fractionation in biological systems (Hayes, 2001). Strain DMG 58 may harbor multiple hydrogenases with different expression patterns, activities and fractionation factors. In this context, our model provides a view of the weighted-average activity of all active hydrogenase enzymes as they were operating at the culture conditions.

The H_2 -utilization efficiency model does not differentiate H_2 activation and immediate release (i.e., H-D exchange reaction) from enzymatic reversibility of the catabolic pathway. While this model is useful for determining the proportion of H_2 that is activated and subsequently catabolized, it is not as useful for understanding the reversible flow of electrons from metabolic intermediates in H_2 -consuming microbes.

Despite various caveats accompanying the estimates of H_2 utilization efficiency, our results clearly indicate *Sporomusa* strain DMG 58 is not efficient with regard to H_2 consumption at the experimental conditions. Efficiencies determined here (<20%) are lower than efficiencies calculated for *Methanothermobacter marburgensis* (<55%), a moderately thermophilic H_2 -utilizing methanogen (Valentine *et al.*, 2004). These efficiencies are also low compared to the high efficiencies (90–95%) reported by Arp & Burris (1982) for hydrogenase purified from soybean root nodules. One possible explanation for the discrepancy is the use of high H_2 levels (80%) in our

experiments. This idea is consistent with reducing equivalents making their way into the catabolic pathway, but readily reversing their flow – presumably due to a rate-limiting step downstream. The lowered efficiency during stationary/death phase indicates that this sort of mechanism may be applicable. In this case, lower levels of H₂ may lead to greater efficiency of H₂ utilization in strain DMG 58. However, much of the H-D exchange activity may arise in the initial bonding of H₂ to hydrogenase (Hartmann *et al.*, 1996). If the majority of exchange occurs at this step, H₂ levels may not have a major impact on efficiency.

Fatty acids

Lipids from all four cultures of *Sporomusa* were dominated by β-hydroxy lauric acid (3-OH C12 : 0), which comprised up to 66% of the total recovered fatty acids (Table 2). This fatty acid is likely a component of the lipopolysaccharide cell covering (Möller *et al.*, 1984). Palmitic (C16 : 0) and palmitoleic (*cis*-9-C16 : 1) acids were the other two most abundant lipids, with concentrations varying between cultures. Trace fatty acids included 2-hydroxy lauric, 2- and 3-hydroxy tridecylic (C13 : 0), 3-hydroxy capric (C10 : 0) and 3-hydroxy undecylic (C11 : 0) acids, which have not to our knowledge been previously reported in this organism. There were no consistent changes in lipid distribution with increasing deuterium content of the water, although one of the deuterium-enriched cultures did have a significantly lower abundance of β-hydroxy lauric acid.

Hydrogen-isotopic compositions of the three most abundant fatty acids are given in Table 3, along with δD values for the supplied H₂O and for H₂ exiting the culture vessel at three

Table 2 Identification and relative abundances of fatty acids in *Sporomusa* strain DMG 58

Fatty Acid	<i>r</i> _t (min)†	Relative Abundance*			
		–67‰‡	235‰‡	571‰‡	1228‰‡
10:0 3OH§	23.05	2.6	1.6	1.4	1.8
11:0 3OH§	24.92	0.5	0.4	0.4	0.4
14:0	25.9	1.0	1.6	2.4	0.7
12:0 2OH¶	26.03	2.4	0.8	0.4	1.1
12:0 3OH¶	26.74	62.0	66.1	38.3	60.4
15:1	27.34	ND	0.9	2.2	0.7
13:0 2OH§	27.89	1.2	1.2	0.2	0.3
13:0 3OH§	28.56	0.9	0.7	0.9	0.7
16:1	29.26	0.9	19.9	39.5	21.3
16:0	29.57	26.1	5.2	9.7	11.2
17:1	31.42	2.3	0.7	1.7	ND
18:1	32.47	ND	1.0	3.0	1.3

*Listed abundances are given as the percentage of total fatty acids calculated from FID peak areas.

†Retention time in GC. See methods for chromatographic conditions.

‡Refers to initial δD of the culture media.

§Tentative identification based on mass spectrum. No authentic standard was available for comparison.

¶Identification confirmed by comparison to authentic standards.

Table 3 Isotopic compositions of water, cellular fatty acids, and H₂

Water*	Culture			
	–67	+235	+571	+1228
Fatty Acid				
12:0 2OH	–451	–236	–31	+337
16:1	–446	–234	–30	+329
16:0	NA	–205	–22	+328
H ₂				
H ₂ (initial)	–210	–210	–210	–210
H ₂ (log phase)	–398	–234	–233	–290
H ₂ (final)	–461	–397	–277	–245

*Listed values refer to the initial δD of the culture medium, relative to VSMOW.

times during the incubation. Values of δD for the lipids range from –450‰, in the unlabelled culture, to +340‰ in the culture with the most D-enriched water. Differences of up to 31‰ exist between different fatty acids from the same culture, but these differences are not consistent between cultures.

Sources of lipid-bound hydrogen

Water and H₂ represented the only two external sources of hydrogen for biosynthesis. Thus, the isotopic mass balance requires that:

$$R_l = X_w \alpha_{l/w} R_w + (1 - X_w) \alpha_{l/h} R_h \quad (15)$$

where R_l , R_h , and R_w are the D/H ratios of lipids, H₂, and water, respectively, $\alpha_{l/h}$ and $\alpha_{l/w}$ are fractionation factors describing the overall fractionation between lipids and H₂ or water, and X_w is the fraction of lipid hydrogen that is ultimately derived from water. Note that Equation 15 refers to the isotopic compositions of the ‘ultimate’ hydrogen sources present in the cultures (H₂ and H₂O), not the ‘immediate’ biochemical precursors of lipid hydrogen (acetate, NADPH, etc.; see discussion by Sessions *et al.*, 2002). For example, H₂ is not directly involved in lipid biosynthesis, but may be indirectly involved through the reduction of NADP⁺.

Equation 15 does not have a unique solution, even given multiple values for R_h , R_w , and R_l (Sessions & Hayes, in press). However, two key observations provide some insight. The first is that a graph of R_l vs. R_w for all analysed lipids produces a single straight line (Fig. 3; $R^2 = 0.995$ for the regression). This implies that the second term in Equation 15 $[(1 - X_w) \alpha_{l/h} R_h]$ is constant, even though we know that R_h changed dramatically between experiments with different δD-H₂O (i.e., Figure 1, panels i–l). The fractionation factor ($\alpha_{l/h}$) cannot be zero, so X_w must equal one [and thus $(1 - X_w) \alpha_{l/h} R_h = 0$].

The second observation is that the regression in Fig. 3 has a slope of 0.59, and is numerically equal to $X_w \alpha_{l/w}$, the product of the mole fraction of water hydrogen and the isotopic fractionation factor. Assuming that $X_w = 1$ (from above), we can then estimate that $\alpha_{l/w} = 0.59$, a much smaller value than has

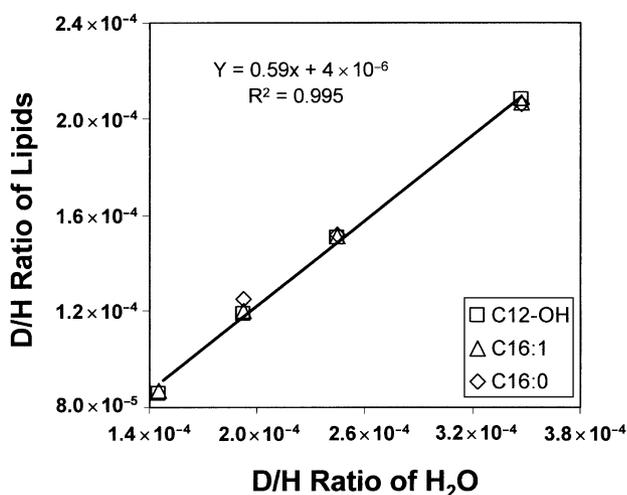


Fig. 3 Relationship between D/H ratios of the culture medium and of the three most abundant cellular lipids produced by *Sporomusa* strain DMG 58 during growth. Key to lipid identity: β -OH 12:0 (3-hydroxy dodecanoic acid), C16:0 (palmitic or hexadecanoic acid), and C16:1 (palmitoleic or *cis*-9-hexadecenoic acid). The D/H ratio is used in place of δ D because D/H more accurately fits the form of Equation 15.

been determined for fatty acids from other organisms (mainly photoautotrophs) that are typically ~ 0.90 – 0.80 (Sessions *et al.*, 1999; Sauer *et al.*, 2001; Sessions *et al.*, 2002; Chikaraishi & Naraoka, 2003; Yang & Huang, 2003). Why should the isotopic fractionation between fatty acids and water be so much larger in this bacterium than in other organisms studied to date?

One possible explanation is that the fatty acid biosynthetic pathway in *Sporomusa* is dramatically different than in eukaryotes. A second possibility is that the hydrogen carried on methyl groups of acetate (the molecular building block of fatty acids) is strongly depleted in D as a result of *Sporomusa*'s use of H₂ for carbon fixation. However, only one-quarter of fatty acid hydrogen comes directly from acetate (Sessions *et al.*, 2002), so the required depletion of acetate is unrealistically large.

Our preferred hypothesis to explain the experimental data is that H₂ is used by *Sporomusa* to directly reduce NADP⁺ (or NAD⁺), and that NADPH transfers hydrogen intact to fatty acids. The dissolved H₂ is in isotopic equilibrium with water as a result of hydrogenase activity, leading to the lack of correlation between R_l and the supplied R_h (used in Equation 15). The large equilibrium fractionation between water and H₂ also leads to the strong deuterium depletion in H₂, and hence NADPH and fatty acids. This hypothesis is supported by several previous investigations that have shown, (1) NADPH supplies \sim half of the reducing power used to synthesize fatty acids from acetate (summarized by Wakil *et al.*, 1983; White, 2000); (2) reduction by NADPH involves the direct transfer of a hydride (H⁻) ion, thus preserving isotopic fidelity (Abeles *et al.*, 1992); and (3) a hydrogenase enzyme has been identified that can directly reduce NADP⁺ to NADPH *in vitro* (Malki *et al.*, 1997).

Regardless of whether this hypothesis is correct, our experimental data show that *Sporomusa* fatty acids are consistently depleted in D relative to their growth water by $\sim 400\%$, and are essentially independent of the isotopic composition of supplied H₂. This strong deuterium depletion should distinguish them from most or all eukaryotes, and potentially from most other prokaryotes, and thus serves as a useful isotopic marker. Further studies to investigate the specificity of such a marker among other acetogens, H₂ consumers, and lithoautotrophic bacteria and archaea in general seem well-justified.

CONCLUSIONS

- 1) Active cultures of *Sporomusa* strain DMG 58 catalyse rapid isotope exchange between water and H₂. This is presumably due to the presence of hydrogenase enzymes. Hydrogenases are widespread in anaerobic bacteria and archaea, suggesting that δ D values for H₂ in anoxic environments are likely to be highly dynamic.
- 2) The efficiency of H₂ utilization is very low ($<20\%$) in these cultures, perhaps due in part to the high H₂ partial pressure supplied to the culture. Our approach offers a means for quantifying the efficiency of H₂ utilization which may be complementary to other types of biochemical studies.
- 3) Lipid D/H ratios appear to be unrelated to those of supplied H₂, indicating the isotopic composition of supplied H₂ is not efficiently transmitted to lipids. However, isotopic fractionation between water and fatty acids is extremely large ($\alpha_{l/w} = 0.6$) compared to those known for eukaryotes. This may result from unusually large fractionations during lipid biosynthesis. Alternatively, hydrogen from H₂ may be incorporated into lipids via the reduction of NADP⁺ by H₂, after the isotopic equilibration of H₂ and H₂O. In this model, the very negative δ D values (-450%) of fatty acids reflect the large fractionation between H₂ and H₂O.
- 4) Certain bacterial fatty acids may be suitable biomarkers for anoxia in natural settings based on strongly depleted δ D values. The long-term preservation of D/H ratios in such molecules (Sessions *et al.*, 2004) may enable the investigation of hydrogen cycling and anoxia in the geological record.

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