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Carbon and hydrogen isotope fractionations associated with dissimilatory iron-reducing bacteria

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Abstract

Shewanella putrefaciens strain CN-32 and *Shewanella algae* strain BrY were grown in laboratory cultures at 30 °C to characterize carbon and hydrogen isotope fractionation patterns related to the growth of iron-reducing bacteria. Ferric citrate or hydrous ferric oxide (HFO) was provided as the electron acceptor and lactate or H_2 (balanced with CO_2) was used as the electron donor. Because these bacteria are not known to grow chemoautotrophically, yeast extract was provided as a carbon source when cultures were grown on H_2/CO_2.

Siderite formed only when HFO was used as the electron acceptor, possibly because of chelation of ferrous iron with dissolved citrate when ferric citrate was used as the electron acceptor. Carbon isotope enrichment factors for the siderite–CO_2 system ($\varepsilon_{sid\text{-}CO_2}$) ranged from 13.3 ‰ to 14.5 ‰ when lactate was used as the carbon and energy source, which were consistent with theoretical calculations of equilibrium isotope fractionation ($\alpha_{sid\text{-}CO_2}$) for the siderite–CO_2 system [Geochim. Int. 18 (1981) 85]. In experiments using H_2/CO_2 as the energy source and yeast extract as the carbon source, carbon isotope enrichment factors were relatively low (0.5 ‰ to 7.4 ‰). The potential exists that a kinetic effect related to siderite precipitation rate influenced isotope partitioning or a dynamic balance was established between carbon sinks (i.e. biomass and solid carbonate) of diverging carbon isotope composition. A more quantitative estimate of $\varepsilon_{sid\text{-}CO_2}$ for biological systems that contain ambient dissolved inorganic carbon (DIC) requires a deeper understanding of carbon flow dynamics in these compartmentalized closed systems.

Finally, in experiments using H_2/CO_2 as an energy source, the hydrogen isotope composition of head gas H_2 and water were analyzed for D/H ratio. The results indicate that bacterial metabolism potentially facilitates isotope exchange between water and H_2.

Keywords: Iron-reducing bacteria; Biogenic siderite; Carbon isotopes; Hydrogen isotopes

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1. Introduction

The importance of microbial iron reduction is well known in many sedimentary environments (Suess, 1979; Lovley, 1991, 1993; Nealson and Saffarini, 1994; Coates et al., 1996; Lonergan et al., 1996). The reduction of Fe^{3+} couples to the oxidation of organic matter or hydrogen gas (Lovley and Phillips, 1988; Lovley et al., 1989, 1993; Caccavo et al., 1992) and this can result in the formation of siderite and/or magnetite (Lovley, 1991; Fredrickson et al., 1998; Zhang et al., 1997, 1998, 2001). Siderite is the most common authigenic carbonate in Fe-rich environments (James, 1966; Maynard, 1983) and many researchers have postulated that biologically mediated reactions are responsible for its occurrence (Walker, 1984; Curtis, 1987; Ellwood et al., 1988; Mozley and Carothers, 1992; Mozley and Wersin, 1992). Persuasive arguments have been based on a variety of textural and physicochemical evidence, including stable isotope compositions. The carbon and oxygen stable isotope content of siderite provides information about the sources and sinks of carbon and oxygen as well as the temperature regime(s) in which the carbonate precipitated (Curtis et al., 1986; Carothers et al., 1988; Mortimer and Coleman, 1997; Zhang et al., 2001).

The hydrogen isotope composition of biomarkers and formation waters may also reveal information about microbial processes that occur in subsurface environments (Sessions et al., 2000; Sauer et al., 2001). In addition, the history of sedimentary rocks has been deciphered from organic carbon isotope fingerprints and geochemical markers of biological activity (Freeman et al., 1990; Hayes, 1993, 2001; Macko et al., 1994). The isotopic composition of specific classes of organic compounds provides not only a biomarker of microbial activity but also an indicator of the biochemical pathway by which the compound(s) originated (Blair et al., 1985; Hayes, 1993, 2001; Summons et al., 1994; Jahnke et al., 1999; Teece et al., 1999; Pancost and Sinninghe Damste, 2003; Zhang et al., 2002a,b). This information is useful to researchers unraveling the complex histories of organic matter and sediment diagenesis in the geologic record.

The goal of this study was to characterize the carbon isotope composition of siderite produced by dissimilatory iron-reducing bacteria, and the hydrogen isotope fractionations associated with microbial metabolism of hydrogen gas. The stable isotope compositions of these solids and gases may provide insights into the nature of biogeochemical activity in modern and ancient geological settings.

2. Material and methods

2.1. Organisms and experimental conditions

Most of the experiments in this study were conducted using two strains of mesophilic iron-reducing bacteria *Shewanella putreficiens* strain CN-32 and *Shewanella algae* strain BrY. Strain CN-32 was isolated from the subsurface in New Mexico (Fredrickson et al., 1998) and BrY was isolated from the Great Bay Estuary, New Hampshire (Caccavo et al., 1992). Both strains are known to reduce iron in the presence of H_2 or short-chain fatty acids. For this study, strains CN-32 and BrY were grown in a basal medium containing the following reagents (in grams per liter of deionized water): NaCl (10), $MgCl_2{\cdot}6H_2O$ (0.8), $CaCl_2{\cdot}2H_2O$ (0.1), NH_4Cl (1.0), PIPES (piperazine-*N*,*N*′-bis [2ethanesulfonic acid]) (10), yeast extract (0.08%), and trace minerals and vitamins (Phelps et al., 1989). The pH of the medium was held at 7.0–7.5 using PIPES buffer. Experiments were performed using either 160-ml culture bottles containing 60 ml of medium or 1500-ml bottles containing 300 ml of medium.

Table 1 summarizes run conditions for iron-reduction experiments. Two types of electron donors were used, Na-lactate (25 mM) or H_2 (balanced with 20% CO_2 vol/vol, total pressure about 12.3 psi). When

Table 1
Experimental run conditions for iron reduction using hydrous ferric oxide (HFO) and ferric citrate as electron acceptors

e^- donor	e^- acceptor	Duration (h)	Purpose
Lactate	HFO ($Fe[OH]_3$)	653	C isotopes of siderite and CO_2
H_2 (balanced with CO_2)	HFO ($Fe[OH]_3$)	653	C isotopes of siderite and CO_2
H_2 (balanced with CO_2)	Ferric citrate	45	H isotopes of biomass, water and H_2

lactate was used as the electron donor, N_2 was used as the headspace gas to avoid initial dissolved inorganic carbon (DIC) in solution. When H_2 was used as the electron donor, yeast extract was provided as a carbon source because previous studies have shown that *S. putreficiens* and *S. algae* do not grow on H_2 with CO_2 as the sole carbon source (Lovley et al., 1989; Caccavo et al., 1992). External CO_2 gas was added in the headspace of some culture experiments to examine isotopic fractionation during siderite precipitation under conditions likely to be found in most subsurface environments.

The electron acceptor used in all experiments was ferric citrate (30 mM) or amorphous hydrous ferric oxide (HFO: 70 mM). Citrate could potentially serve as a carbon source for iron reduction by *S. algae* as demonstrated in a co-culture with a fermentative bacterium *Aeromonas veronii* (Knight et al., 1996), however, in pure culture *S. algae* is not known to grow with citrate as the sole carbon source. A 10% (vol/vol) inoculum from pre-grown cultures was used for all experiments and all incubation temperatures were maintained at 30 °C in the dark.

In addition to strains CN-32 and BrY, three strains of thermophilic bacteria were grown to examine hydrogen isotope fractionation between H_2 and water when H_2 was used as the energy source for bacterial growth. These bacteria include *Desulfotomaculum luciae*, *Thermoterrabacterium ferrireducens*, and *Persephonella marina*. *D. luciae* is a thermophilic sulfate-reducing bacterium (Liu et al., 1997) and *T. ferrireducens* is a thermophilic iron-reducing bacterium (Slobodkin et al., 1997), both isolated from continental hot springs. *P. marina* is a thermophilic hydrogen-oxidizing bacterium isolated from a deep sea hydrothermal vent (Reysenbach et al., 2000). *D. luciae* was grown in a medium described in Liu et al. (1997), *T. ferrireducens* was grown in a medium described in Slobodkin et al. (1997) and *P. marina* was grown in a medium described in Zhang et al. (2002a). Diatomic hydrogen gas (H_2) and medium water were collected before and after the culture experiments for D/H analysis (described below).

2.2. Bacterial cell counts

Bacterial cell numbers were determined by the acridine-orange direct-count method (Zhang et al., 1996). Approximately 1.0 ml of a culture was diluted with sterile phosphate buffer (pH 7), filtered onto a black Nuclepore membrane (0.2 μm pore diameter), stained with 1.0 ml of a particle-free acridine-orange solution, and observed using an epifluorescence microscope.

2.3. Measurements of Eh, pH, and Fe^{2+}

Measurements of Eh and pH were performed using the methods outlined in Zhang et al. (1997). Eh was measured by placing a calibrated combination platinum micro-electrode (Microelectrodes, Londonderry, N.H.) directly into a culture bottle; emf response was recorded after equilibration for 5 min. The pH of the medium was then measured by transferring 5 ml of liquid into a 10-ml beaker into which a calibrated pH probe was placed.

Ferrous iron was determined by the ferrozine method (Lovley and Phillips, 1986; Zhang et al., 1997). In this method, 0.1 ml of sample was added to 5 ml of anaerobic 0.5 M HCl solution. After 15 min, 0.1 ml of the mixture was added to 3 ml of ferrozine (1 g/l) in 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer at pH 7 (Lovley and Phillips, 1986). The sample was mixed, filtered (0.2 μm), and measured for maximum absorbance at 562 nm using a standard UV–VIS spectrophotometer. Standards for the ferrozine assay were prepared with ferrous ethylenediammonium sulfate dissolved in 0.5 M HCl.

2.4. X-ray diffraction and transmission electron microscopy

X-ray diffraction (XRD) patterns of the solid run products were obtained on a Scintag automated diffractometer using cobalt Kα radiation. Samples were filtered on glass-fiber filters (0.45 μm), dried in an anaerobic chamber, and then measured on the diffractometer. Samples were scanned over a range from 2° to 70° (2θ) at a rate of 2°/min. The mineralogical composition was determined by comparing sample diffraction patterns to known mineral standards (International Center for Diffraction Data).

The morphological characteristics of inorganic solids were examined with transmission electron microscopy (TEM). Culture medium containing bacterial cells, organic matter, and inorganic solids was fixed

with 2.5% glutaraldehyde in 0.1 M cacodelylate. After washing with buffer and an alcohol/water solution, samples were dehydrated with propylene oxide and embedded in a low-viscosity, thermally curing epoxy resin. Ultra thin sections (70–80 nm) were cut from resin blocks with a diamond knife and transferred to 300 mesh, formvar-coated Cu TEM grids for image analysis on a JEOL FX 2000.

2.5. Carbon and hydrogen stable isotope analysis

Stable isotope ratios for carbon ($^{13}C/^{12}C$) were determined from headspace CO_2 and the solid run product siderite in all the experiments (Table 1) according to methods described in Zhang et al. (2001). Three milliliters of headspace gas was transferred to a vacuum extraction line for the separation and purification of CO_2 for stable isotope analysis by isotope ratio mass spectrometry (IRMS-dual inlet technique). The solid material, which included some biomass, was washed three times with anaerobic, distilled–deionized water and dried in an anaerobic chamber. A subsample of 40–50 mg of the dry solid was reacted with "100%" phosphoric acid in an isolated reaction vessel at 60 °C for 24 h. The evolved CO_2 was then separated and purified for carbon isotope analysis. Previous experiments showed that when reagent grade glucose, spent medium (containing organic acids), or microbial biomass was added to "100%" phosphoric acid in an amount similar to the experimental protocols, insufficient CO_2 was produced to significantly alter the $\delta^{13}C$ of CO_2 liberated from a pure siderite separate (Zhang et al., 2001). From these experiments, it may be concluded that, within the error of the measurement, the $\delta^{13}C$ of CO_2 from the phosphoric acid reaction is representative of siderite in the solid phase.

Lactate, ferric citrate and yeast extract were analyzed for carbon isotope composition by loading approximately 5 mg of the dried pure material into a quartz tube containing 1.0 g CuO and 0.5 g elemental Cu. Tubes were then evacuated and sealed for combustion of the solid at 800 °C in a furnace. The resulting CO_2 was then extracted and purified for carbon stable isotope analysis.

All carbon isotope analyses were performed using a Finnigan MAT 252 isotope ratio mass spectrometer. The results were reported relative to the international V-PDB standard in conventional delta notation (Craig, 1957) with a precision of ± 0.2‰ (1σ).

Stable isotope ratios for hydrogen (D/H) were reported for headspace H_2 and the medium water from ferric citrate experiments (Table 1). Diatomic hydrogen gas was separated from CO_2 and water vapor in headspace and purified cryogenically using a vacuum extraction line. Pure H_2 was then compressed in a collection reservoir using a Toepler pump for stable isotopic analysis. Two to three microliters of the medium water was analyzed by reduction of water to H_2 gas through a uranium furnace at 725 °C before introduction to the inlet of the IRMS. All hydrogen isotope analyses were reported in standard delta notation relative to the international V-SMOW standard using the SMOW-SLAP scale correction of Coplen (1988, 1996) with a precision of ± 2‰ (1σ).

3. Results and discussion

3.1. Bacterial growth and iron reduction

Growth of iron-reducing bacteria often requires yeast extract as an essential nutrient or potential carbon source (Lovley et al., 1989; Caccavo et al., 1992). To test for the effect of yeast extract on cell growth, strains CN-32 and BrY were provided various concentrations of yeast extract under lactate and H_2/CO_2 conditions (Fig. 1a). Under H_2/CO_2 conditions, cell numbers increased 1.6-fold at 0.08% yeast extract and 5.0-fold at 0.16% yeast extract compared to cell numbers without yeast extract. Cells did not grow on yeast extract if H_2 was replaced with N_2, suggesting that bacterial growth required H_2 as the energy source and yeast extract as the carbon source. In a separate set of experiments, CO_2 in the H_2/CO_2 mixture was replaced by N_2 to qualitatively determine the relative roles of CO_2 and yeast extract as potential carbon sources (Table 2). In these experiments, more biomass was produced under H_2/CO_2 than H_2/N_2, suggesting that CO_2 may have stimulated the growth of cells when H_2 was used as the electron donor. It is possible that CO_2 and H_2 were utilized by cells to form formate, which was subsequently used in C1 metabolism (Scott and Nealson, 1994).

When lactate was used as the carbon source, the increase in cell numbers was 3.7-fold at 0.08% yeast

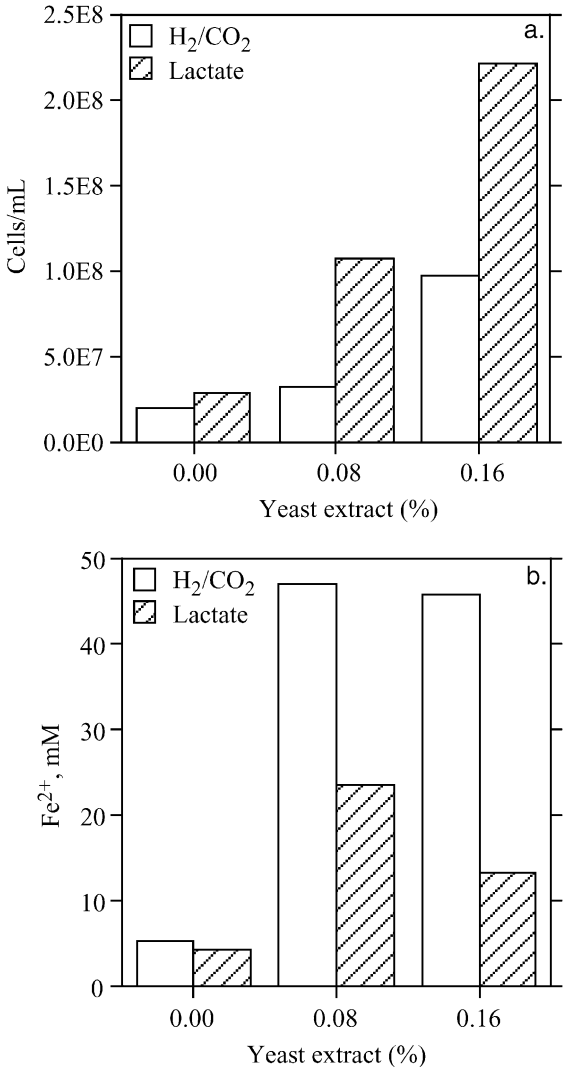

Fig. 1. Comparison of cell densities (a) and Fe^{2+} concentrations (b) at various concentrations of yeast extract for lactate and H_2/CO_2 experiments. Hydrous ferric oxide (70 mM) was used as the electron acceptor.

extract and 7.7-fold at 0.16% yeast extract (Fig. 1a). Cell numbers were always higher when bacteria were grown on lactate than on H_2/CO_2 and yeast extract, suggesting that lactate was preferentially used as a carbon source. The enhanced biomass production under lactate conditions may be a consequence of the additional energy generated through lactate oxidation by lactate dehydrogenase, which can feed electrons directly to the electron transport chain (James Scott, personal communication).

Addition of yeast extract also enhanced iron reduction. Using H_2/CO_2 and yeast extract, Fe^{2+} increased about 9-fold at 0.08% and 0.16% yeast extract compared to experiments without yeast extract (Fig. 1b). When lactate was used as the electron donor, the increase was about 6- and 3-fold at 0.08% and 0.16% yeast extract, respectively. The reason for lower Fe^{2+} at 0.16% yeast extract is unknown. Nevertheless, the results indicate that more Fe^{2+} was produced using H_2 as the electron donor than lactate. This may be related to the more highly reduced environment in culture bottles containing H_2/CO_2. Measurement of the redox potential showed Eh to be in the range of -470 to -650 mV for the H_2/CO_2 experiments compared with >-450 mV for the lactate experiments. On the other hand, the greater production of Fe^{2+} with less biomass under H_2/CO_2 conditions may simply indicate that cells were not reproducing because they were forced to utilize a more complex carbon source (yeast extract) than under the lactate conditions.

For the isotope determinations, a 0.08% yeast extract was used to stimulate bacterial growth under both lactate and H_2/CO_2 conditions. It was expected that the yeast extract functioned mainly as a nutrient source for bacterial growth on lactate, and as nutrient and carbon sources for bacterial growth on H_2.

3.2. Mineralogy of the crystalline solid produced

Results from the XRD analysis showed that siderite precipitated in experiments using HFO as the electron acceptor; however, low peak intensities and

Table 2
Comparison of biomass (cells/ml) between initial (t_i) and final (t_f) incubation times of CN-32 and BrY under H_2/CO_2 and H_2/N_2 conditions

Culture	H_2/CO_2			H_2/N_2		
	t_i	t_f	t_f/t_i	t_i	t_f	t_f/t_i
CN-32	3.4e+06	9.2e+07	27	3.8e+06	2.7e+07	7
CN-32	3.5e+06	7.0e+07	20	5.2e+06	3.5e+07	7
BrY	7.4e+06	9.1e+07	12	8.7e+06	3.0e+07	3
BrY	2.5e+06	2.1e+08	84	5.7e+06	1.5e+08	26

Ferric citrate (30 mM) was used as the electron acceptor. All experiments were terminated after 24 h.

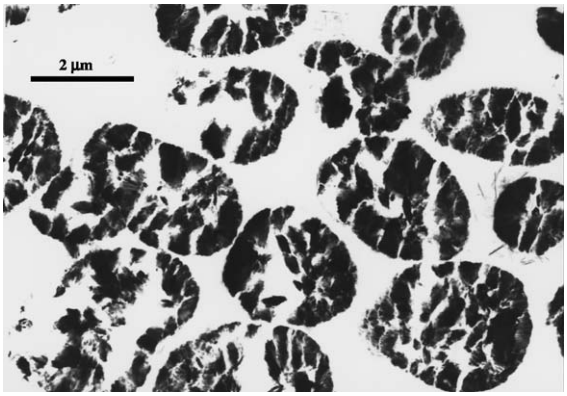

Fig. 2. TEM ultra thin section of final run product produced by strain CN-32. Individual grains are composed of siderite (as determined by XRD of bulk solid). Fracturing is artifact of thin sectioning process. Scale bar is 2 μm.

slight peak broadenings suggested the crystallinity of siderite was poor; no other minerals were identified. Siderite did not precipitate when ferric citrate was used as the electron acceptor and this is probably due to chelation of ferrous iron with dissolved citrate in solution (Martell and Smith, 1989).

Photomicrographs taken by transmission electron microscopy (TEM) showed that siderite particles were rod- or plate-shaped and approximately 2 to 3 μm in length (Fig. 2). The morphology of the crystals was uniform and distinct from the rhombohedral and saddle-shaped grains of other microbial siderites reported in the literature (Mortimer and Coleman, 1997; Fredrickson et al., 1998; Dong et al., 2000). These shapes occurred under both lactate and H_2/CO_2 conditions. The cause for the unusual morphology is unknown, and it raises the intriguing possibility that grain morphology may be influenced to some extent by dissimilatory iron-reducing bacteria under particular environmental conditions.

3.3. Carbon isotope fractionation between siderite and CO_2 using HFO as the terminal electron acceptor

Siderite precipitated in experiments using both strains CN-32 and BrY and HFO as the terminal electron acceptor. When CN-32 and BrY were grown on lactate, dissolved inorganic carbon (DIC) was produced by the dissolution of CO_2 generated through lactate respiration. In the CN-32 experiments, the $\delta^{13}C$ of headspace (respiratory) CO_2 averaged -31.5‰ while that of siderite averaged -18.5‰, and the resulting enrichment factor for the siderite–CO_2 system ($\varepsilon_{sid\text{-}CO_2}$) was about 13.4‰ (Table 3). In the BrY experiment, the $\delta^{13}C$ of respiratory CO_2 was -28.4‰, siderite was -14.3‰, and $\varepsilon_{sid\text{-}CO_2}$ was 14.5‰ (Table 3). Collectively, $\varepsilon_{sid\text{-}CO_2}$ averaged 13.7 ± 0.6‰ (Table 3).

When CN-32 and BrY were grown on H_2/CO_2 and yeast extract, siderite precipitation was accomplished by the combination of free ferrous iron with CO_3^{2-} originating from the head space CO_2, and perhaps respiratory CO_2 from yeast extract metabolism. Over the course of the CN-32 experiment, the $\delta^{13}C$ of residual CO_2 decreased from -14.4‰ to -21.2‰, while in the BrY experiment, residual CO_2 decreased to only -16.0 ‰ (Table 3). Enrichment factors for siderite–CO_2 ($\varepsilon_{sid\text{-}CO_2}$) calculated from the initial CO_2

Table 3
Carbon isotope compositions of siderite and head-gas CO_2 in CN-32 and BrY cultures grown on lactate or H_2/CO_2 (80:20, vol/vol) using 70 mM hydrous ferric oxide as the electron acceptor

Source of CO_2	$\delta^{13}C_{CO_2}$	$\delta^{13}C_{siderite}$	ε
Lactate-CN-32	-31.5[a]	-18.4	13.5
Lactate-CN-32	-31.5[a]	-18.6	13.3
Lactate-BrY	-28.4[a]	-14.3	14.5
Average			13.7 ± 0.6
H_2/CO_2-CN-32	-14.4[b]	-13.95	0.5
	-21.2[c]	-13.95	7.4
Average			4.0 ± 4.9
H_2/CO_2-BrY	-14.4[b]	-10.39	4.1
	-16.0[c]	-10.39	5.7
Average			4.9 ± 1.1

All values are in per mil (‰).
The enrichment factor ε was determined as: ε(‰)= $[(1000+\delta^{13}C_{siderite})/(1000+\delta^{13}C_{CO_2})-1]\times 1000$. $\delta^{13}C$ of lactate = -25.4‰, $\delta^{13}C$ of yeast extract = -24.6‰, $\delta^{13}C$ of ferric citrate = -25.6‰.

[a] Because head-gas CO_2 was from oxidation of organic substrate, measurement of initial head-gas CO_2 was impossible due to low concentration. We also assumed the $\delta^{13}C$ of head-gas CO_2 did not change over time. This was based on observation of constant $\delta^{13}C$ values of biogenic head-gas CO_2 during lactate oxidation when using ferric citrate as the electron acceptor (Zhang, unpublished data).

[b] Initial head-gas CO_2. The accompanying $\delta^{13}C_{of\ siderite}$ was assumed to be the same as the final value.

[c] Finial head-gas CO_2.

(−14.4‰) were 0.5‰ and 4.1‰ for CN-32 and BrY, respectively, while they were 7.4‰ and 5.7‰ if $\delta^{13}C$ values for residual CO_2 were used (Table 3). Integrating over the course of experiments, averages of $\varepsilon_{sid\text{-}CO_2}$ values were 4.0‰ and 4.9‰ for CN-32 and BrY, respectively (Table 3). More accurate estimates of ε require additional knowledge concerning the mass of headspace CO_2 consumed, the percentage of CO_2 incorporated in siderite derived from lactate respiration, and the mass of biomass and/or solid carbonate that was generated.

To determine if any of the $\varepsilon_{sid\text{-}CO_2}$ values from this study approached equilibrium isotope exchange, the magnitude of the fractionation (as $10^3 \ln\alpha_{sid\text{-}CO_2}$) was compared to data existing in the literature for the inorganic growth of siderite and theoretical calculations (Fig. 3). Carothers et al. (1988) precipitated siderite at temperatures ranging from 33 to 197 °C from solutions equilibrated with pure CO_2 gas at various gas pressures. The values reported for $\varepsilon_{sid\text{-}CO_2}$ at 33 and 103 °C were 11.6‰ and 7.0‰, respectively

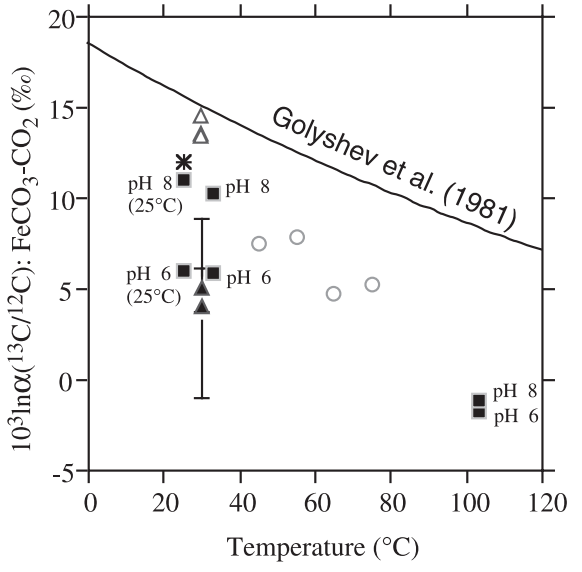

Fig. 3. Carbon isotope fractionation between siderite and CO_2. Open triangles are lactate experiments and solid triangles are averages from H_2/CO_2 experiments (error bars indicate one standard deviation from average values, see Table 3). Open circles = Zhang et al. (2001). Solid square = Carothers et al. (1988) re-calculated for pH 6 and 8 (see text for discussion); the 25 °C values were calculated based on extrapolation of their original curve. Star = Jimenez-Lopez and Romanek (2000). Also shown is a theoretical curve from Golyshev et al. (1981) for the siderite–CO_2 system.

(Table 2 of Carothers et al., 1988). These values were recently revised to 10.6‰ and −1.2‰ to reflect the equilibrium fractionation between CO_2 and HCO_3^- at the relevant temperatures of their experiment (Zhang et al., 2001). In this correction, Zhang et al. (2001) assumed the $\delta^{13}C$ of dissolved inorganic carbon (DIC) was equivalent to dissolved bicarbonate (HCO_3^-). While this assumption is valid ($\varepsilon_{HCO_3\text{-}DIC} \sim 0.2$‰) in dilute solutions at neutral to slightly basic pH (e.g. 7 to 8; Romanek et al., 1992; Jimenez-Lopez and Romanek, in review), the experiments of Carothers et al. (1988) may have precipitated siderite from more acidic and concentrated solutions. Although Carothers et al. (1988) did not report in situ pH measurements, these may be calculated using their reported data (e.g. P_{CO_2}, temperature, solution composition) and a modified version of the EQPITZ speciation model (Morse, pers. comm.) developed by He and Morse (1993) for intermediate ionic strength solutions. The results of these calculations suggest that the pH of Carothers solutions may be sufficiently low (pH < 7) to preclude the use of the $\delta^{13}C$ of dissolved inorganic carbon (DIC) as a proxy for dissolved bicarbonate (HCO_3^-). This is because aqueous CO_2 becomes an increasingly important component of DIC as pH decreases and the equilibrium carbon isotope fractionation between aqueous CO_2 and HCO_3^- (as a proxy for DIC) is substantial (Mook et al., 1974). Calculated values of $\varepsilon_{sid\text{-}CO_2}$ from Carothers et al. (1988) are plotted at the appropriate temperature for values of pH between 6 and 8 in Fig. 3. We expect the true fractionations of Carothers et al. (1988) to lie somewhere between the calculated values plotted in Fig. 3, perhaps toward the values at pH 6. The results indicate that pH makes a significant difference for calculating fractionations at lower temperatures.

Enrichment factors for the siderite–CO_2 system ($\varepsilon_{sid\text{-}CO_2}$) are also plotted from Jimenez-Lopez and Romanek (2000, in review) for synthetic siderite grown at 25 °C from solutions held at a constant state of super saturation. The value of $\varepsilon_{sid\text{-}CO_2}$ for the final solid phase of these experiments, a well-crystallized siderite, was ~12.0‰ (Fig. 3). Finally, $\varepsilon_{sid\text{-}CO_2}$ are plotted from Zhang et al. (2001) for biogenic siderite grown in culture experiments at temperatures ranging from 45 to 75 °C and pH above 7 (Fig. 3).

Collectively, all experimental values for $\varepsilon_{sid\text{-}CO_2}$, whether abiotic or biogenic, plot below a theoretical

calculation of equilibrium fractionation proposed by Golyshev et al. (1981). The empirical and theoretical estimates converge near 25 °C but depart significantly as temperature increases (Fig. 3) and the reason for this deviation is unclear at the present time.

In this study, the values of $\varepsilon_{sid\text{-}CO_2}$ for the lactate experiments are close to the theoretical curve of Golyshev et al. (1981), while the values for the H_2/CO_2 experiments fall significantly below the theoretical curve. Isotopic fractionations may be driven by different mechanisms for the lactate and H_2/CO_2 experiments. In the lactate experiments, CO_2 was probably released slowly during lactate oxidation, and siderite precipitation rate may have been sufficiently slow to facilitate isotope exchange between respiratory CO_2, dissolved inorganic carbon (DIC), and siderite. In the H_2/CO_2 experiments, gaseous CO_2 was introduced in the headspace prior to the initiation of an experiment and culture medium may have become saturated with respect to siderite as quickly as ferric iron was reduced (Zhang et al., 2001). Siderite precipitation rate may have been sufficiently high to preclude time for isotope exchange between dissolved inorganic carbon and siderite, producing the low $\varepsilon_{sid\text{-}CO_2}$ values observed in the H_2/CO_2 experiments. An alternative explanation is that a dynamic equilibrium was established between carbon sinks (i.e. biomass and siderite) of distinct and opposing carbon isotope composition. But in this case, DIC would have to function as a carbon source for bacterial metabolism. This may indeed have happened as shown by an increase in biomass when CO_2 was used (Table 1). However, the extent of CO_2 utilization was uncertain.

In the most general terms, the difference in carbon isotope enrichment factors can be explained by the presence of two isotopically distinct pools of CO_2 in the H_2/CO_2 experiments, respiratory CO_2 with a $\delta^{13}C$ value near −25‰ and head space CO_2 with a $\delta^{13}C$ value near −14.5‰. These pools probably mixed to some extent as solid carbonate formed leading to carbon isotope compositions for siderite that were somewhat higher than that formed in the lactate experiments where only a single pool of CO_2 ($\delta^{13}C$ near −25‰) was present. A Rayleigh fractionation effect potentially fractionated the carbon isotopes in these pools further as ^{13}C was preferentially sequestered in the solid phase. Further evaluation of carbon isotope partitioning cannot be made without additional information concerning the extent of reaction and contribution of respiratory CO_2 in the H_2/CO_2 experiments.

3.4. Hydrogen isotopic fractionations between H_2 and water using ferric citrate as the electron donor

Fractionation of hydrogen isotopes during microbial metabolism has been extensively studied for the CO_2 reduction pathway (e.g. Whiticar et al., 1986; Balabane et al., 1987; Burke et al., 1988; Waldron et al., 1988; Grossman et al., 1989; Burke, 1993; Sugimoto and Wada, 1995; Hornibrook et al., 1997; Whiticar, 1999). When formation water is the sole source of hydrogen for CO_2 reduction, there is a strong kinetic isotope effect (160‰ to 180‰), with the metabolic end product CH_4 being preferentially enriched in hydrogen relative to deuterium. Landmeyer et al. (2000) showed that when H_2 was an available electron donor, methanogens preferentially utilized H_2 enriched in ^1H for CO_2 reduction. Hydrogen isotope fractionation during lipid biosynthesis has recently been documented by Sessions et al. (2000). Various enzymatic pathways were identified that preferentially utilized ^1H with resulting differences in bulk lipids and cellular water of approximately −150‰. Differences were ascribed to isotopically distinct precursors (e.g. acetyl-CoA), isotope effects and exchange reactions during biosynthesis (e.g. through the formation of mevalonic acid), and hydrogen additions (e.g. from isotopically distinct, intracellular pools of NADPH). These processes would all tend to deplete deuterium in microbial biomass but the transition state(s) and/or exchange mechanism(s) are unclear or only poorly defined with respect to the contribution of hydrogen isotopes from H_2 or water.

The hydrogen isotope compositions of headspace H_2 and medium water were determined from CN-32 culture experiments (Table 4) to determine if isotope fractions were expressed in either system component. If strain CN-32 incorporated ^1H from hydrogen gas into biomass, residual H_2 would be enriched in deuterium, but apparently this did not occur because of the observed depletion of D (^2H) in residual H_2 (Table 4). This would also apply if iron reduction was coupled to H_2 oxidation. The δD of biomass would help to constrain the isotope systematics but unfortunately biomass was treated with HCl solution prior to anal-

ysis. Isotope exchange between HCl solution and biomass may have occurred making the measurement of δD ambiguous. Nevertheless, the effect of bacterial metabolism on partitioning between H_2 and water can be evaluated without significant complications. This was demonstrated by fractionations associated with the mesophilic methanogen *M. formicicum*, and the thermophilic sulfate-reducing bacterium *D. luciae* and iron-reducing bacterium *T. ferrireducens* (Fig. 4). The open symbols are fractionations between water and initial H_2 before bacterial growth. The solid symbols are fractionations between water and residual H_2 after bacterial growth. In each case over time, final fractionation factors approached the line representing the theoretical equilibrium fractionation between water and H_2 from both directions (Fig. 4). This suggests that bacteria may function as catalysts, perhaps through hydrogenase activities, and facilitate isotope exchange between H_2 and water. Many other factors, however, can affect isotopic exchange mediated by hydrogenase activities (Yagi et al., 1973; Adams et al., 1981; McTavish et al., 1996), which appear to impose significant challenges for quantitative characterization of equilibrium fractionation facilitated by hydrogenases.

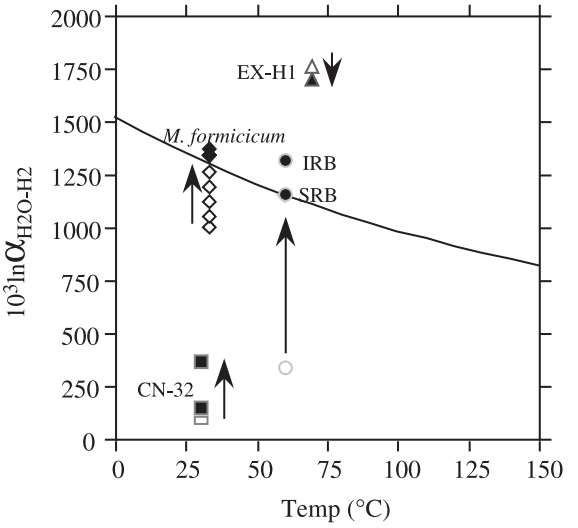

Fig. 4. Hydrogen isotope fractionation between water and H_2 in bacterial cultures that use H_2 as the electron donor for growth. The open symbols are fractionations between water and initial H_2 before bacterial growth; the solid symbols are fractionations between water and residual H_2 after bacterial growth (see Table 4). All error bars are smaller than the symbols. Arrows represent direction of change in $10^3 \ln\alpha_{H_2O-H_2}$ over time. The curve is the theoretical equilibrium fractionation between water and H_2 calculated by Horibe and Craig (1995) according to Horita and Wesolowski (1994) and Bardo and Wolfsberg (1976).

Table 4
Hydrogen isotope compositions of medium water and hydrogen gas at the beginning (H_2-intl) and end of experiments of different cultures

Sample	Temperature (°C)	δD_{H_2O}	δD_{H_2}	$10^3 \ln\alpha_{H_2O-H_2}$ [a]
H_2-intl	30	−47	−157	123
CN-32	30	−47 ± 2 [b]	−179 ± 3	149 ± 1
CN-32	30	155	−201	369
H_2-intl	70	−72.5	−840	1758
EX-H1	70	−72.5	−830 ± 5	1696 ± 31
H_2-intl	60	−50	−322	337
T. ferrireducens	60	−50	−745 ± 2	1316 ± 8
D. luciae	60	−50	−700 ± 11	1154 ± 37
H_2-intl	34	−43	−647	997
M. formicicum [c]	34	−42 ± 2	−749 ± 3	1338 ± 10
H_2-intl	34	−9	−653	1049
M. formicicum	34	−21 ± 4	−743 ± 6	1336 ± 28
H_2-intl	34	55	−655	1118
M. formicicum	34	43 ± 5	−727 ± 2	1338 ± 3
H_2-intl	34	132	−654	1185
M. formicicum	34	115 ± 5	−708 ± 6	1338 ± 23
H_2-intl	34	208	−656	1256
M. formicicum	34	188 ± 1	−696 ± 3	1363 ± 8

All values are in per mil (‰).

[a] Determined according to $10^3 \ln\alpha_{H_2O-H_2} = \ln[(1000 + \delta D_{H_2O})/(1000 + \delta D_{H_2})] \times 1000$.

[b] Average ± 1σ standard deviation (n = 2 to 4).

[c] Reproduced from Balabane et al. (1987).

4. Conclusions

Isotopic fractionations associated with bacterial iron reduction were evaluated for two *Shewanella* species, *putreficiens* CN-32 and *algae* BrY. Examination of bacterial growth with and without yeast extract suggested that lactate could be used as the sole electron donor and carbon source during lactate oxidation. During hydrogen oxidation, however, yeast extract was used as a necessary carbon source. Carbon isotope fractionation between siderite and CO_2 was

significantly different for lactate oxidation versus H_2 oxidation. During lactate oxidation, siderite was formed by combining ferrous iron with CO_3^{2-} derived from respiratory CO_2. This process apparently was slow and permitted significant isotope exchange between siderite and CO_2. During H_2 oxidation, siderite did not form in equilibrium with co-existing CO_2. The disequilibrium may result from either a kinetic effect related to siderite precipitation rate or isotope partitioning influenced by a Rayleigh fractionation effect. Comparison of the δD of head space H_2 and water before and after microbial growth indicated that microorganisms potentially facilitated the partitioning of hydrogen isotopes in biological systems.

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