

Carbon isotope signatures of fatty acids in *Geobacter metallireducens* and *Shewanella algae*

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Abstract

The goal of this study was to examine carbon isotope fractionations associated with lipid biomarkers of different iron-reducing bacteria. Experiments were conducted using a *Geobacter metallireducens* strain GS-15 (grown on acetate) and a *Shewanella algae* strain BrY (grown on lactate). Both organisms were grown anaerobically using ferric citrate as the electron acceptor. The $\delta^{13}\text{C}$ of total biomass of GS-15 was -34.3‰ , which was about 7.2‰ lower than the substrate acetate ($\delta^{13}\text{C} = -27.1\text{‰}$). The $\delta^{13}\text{C}$ of total biomass of BrY was -30.0‰ , which was about 7.1‰ lower than the substrate lactate ($\delta^{13}\text{C} = -22.9\text{‰}$). Isotopic fractionations (ϵ) between fatty acid and biomass within the same strain were consistently greater for BrY (-10.9‰ to -15.5‰) than for GS-15 (-4.5‰ to -8.6‰). The lipid biomarker 10Me16:0, which was only found in GS-15 cultures, had an ϵ value of $-5.39 \pm 0.73\text{‰}$ ($n=2$). The differences in fractionation may reflect the carbon assimilation pathways by which the fatty acids were synthesized: the smaller fractionations in GS-15 may be associated with the tricarboxylic cycle, whereas the greater fractionations in BrY may be associated with the serine pathway. The difference in isotope fractionations between lipid biomarkers and total biomass of different iron-reducing bacteria may be used to enhance our understanding of the microbial community structure in an iron-reducing environment.

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Keywords: Iron-reducing bacteria; *Geobacter metallireducens*; *Shewanella algae*; Lipid biomarkers; Carbon isotopes

1. Introduction

Microbial Fe(III) reduction is a major respiratory process in low temperature sedimentary environments. It plays an important role in cycling of organic carbon and inorganic nutrients such as phosphorus and trace metals (Lovley, 1991; Nealson and Saffarini,

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1994). The most commonly identified Fe(III)-reducing bacteria belong to the genera *Geobacter* and *Shewanella*, which have been isolated from diverse sedimentary environments including fresh water and marine sediments, deep pristine aquifers, and contaminated shallow ground waters (Lovley and Phillips, 1988; Myers and Nealson, 1988; Lovley et al., 1993; Caccavo et al., 1994; Nealson and Saffarini, 1994; Coates et al., 1996; Lonergan et al., 1996). Certain species of *Geobacter* such as *G. metallireducens* can oxidize multi-carbon compounds including acetate completely to CO₂ when coupled to Fe(III) reduction, whereas species of *Shewanella* such as *Shewanella putrefaciens* can only partially oxidize multi-carbon compounds to acetate (Coates et al., 1996). Species of *Geobacter* are strict anaerobes, whereas those of *Shewanella* are facultative anaerobes and can use oxygen as a terminal electron acceptor (Lovley, 1991; Nealson and Saffarini, 1994; Lonergan et al., 1996).

Geobacter and *Shewanella* can also use a variety of oxidized metals as electron acceptors and thus have the potential for bioremediation of metal- or radionuclide-contaminated environments (Lovley, 1995; Fredrickson and Gorby, 1996). In particular, *G. metallireducens* and *S. putrefaciens* can enzymatically reduce soluble U(VI) to insoluble U(IV) by coupling the oxidation of acetate or hydrogen (Lovley et al., 1991; Gorby and Lovley, 1992; Truex et al., 1997).

Because of their differences in physiology and metabolic capabilities, *Geobacter* and *Shewanella* may have distinct activities and occur in different abundances in natural environments. *Geobacter* species were found to be predominant in a variety of anaerobic, freshwater environments (Coates et al., 1996; Snoeyenbos-West et al., 2000), whereas *Shewanella* species were found to be prevalent at the oxic/anoxic interfaces in oceanic environments (Nealson et al., 1991; Brettar and Hoefle, 1993; Nealson and Saffarini, 1994). Zhou et al. (unpublished data) found that *Shewanella* species dominated the microbial community for iron reduction in samples from permafrost environments.

Microbial lipids provide quantitative information about the community structure without the necessity of culturing and isolation (White, 1988). Because bacteria have ester-linked lipids and Archaea have ether-linked lipids, analysis of lipid structures pro-

vides unequivocal distinctions between these two domains. Ester-linked polar lipid fatty acids (PLFA) have been validated as a measure of bacterial biomass in complex ecosystems, such as fresh water or marine sediments (Baird et al., 1985; Guckert et al., 1985). Furthermore, some PLFA are specific to a single genus or species and when corroborated with environmental conditions, can be used to distinguish between discrete microbial populations in natural environments. For example, among the Fe(III)-reducing bacteria, *G. metallireducens* contains 10Me16:0 (Lovley et al., 1993), which is absent in *Shewanella* species (Moule and Wilkinson, 1987; Rossello-Mora et al., 1994). Thus, 10Me16:0 may be used to distinguish between *Geobacter* and *Shewanella* species in iron-reducing environments. This compound is also present in *Desulfobacter* but not in other sulfate-reducing bacteria (Dowling et al., 1986), and may be used as a biomarker for *Desulfobacter* when sulfate reduction is the dominant process.

Recent advances in compound-specific isotope-ratio mass spectrometry have enhanced our capability to trace the flow of organic compounds at the molecular level along autotrophic and heterotrophic pathways (Hayes et al., 1990; Macko et al., 1994; Hayes, 2001). Microorganisms fractionate ¹³C and ¹²C differently depending on the substrates available and the metabolic pathways used (Monson and Hayes, 1982; Blair et al., 1985; Preuß et al., 1989; Hayes, 1993; Summons et al., 1994; Jahnke et al., 1999; Teece et al., 1999). Microorganisms are known to have diverse metabolic pathways for carbon assimilation and energy generation (Madigan et al., 2000) and as a result, major isotopic differences may exist in lipid biomarkers from different microorganisms (Hayes, 1993, 2001; Jahnke et al., 1999; van der Meer et al., 1998, 2000, 2001; Zhang et al., 2002a).

So far, carbon isotope fractionation associated with lipid biomarkers of Fe(III)-reducing bacteria has only been reported for a *S. putrefaciens* species (Teece et al., 1999). In this study, our goal was to determine the isotopic compositions of fatty acids in *G. metallireducens*, strain GS-15, and *Shewanella algae*, strain BrY. *S. algae* was recently renamed from *S. alga* (Trüper and de' Clari, 1997; Venkateswaran et al., 1999).

2. Material and methods

2.1. Sources of organisms and culturing procedures

GS-15 was first isolated from bottom sediments in the Potomac River, Maryland (Lovley and Phillips, 1988), and BrY was first isolated from bottom sediments of the Great Bay estuary, New Hampshire (Caccavo et al., 1992). In this study, axenic cultures of GS-15 and BrY were grown in an anaerobic medium containing the following reagents (g l^{-1}): Ferric citrate (13.7), NaHCO_3 (2.5), NH_4Cl (0.25), $\text{NaH}_2\text{PO}_4 \cdot 4\text{H}_2\text{O}$ (0.6), KCl (0.1) as well as trace minerals and vitamins (Lovley and Phillips, 1988). Sodium acetate (20 mM) was used as the electron donor for GS-15 and sodium lactate (20 mM) was used for BrY. The medium was prepared using a strictly anaerobic technique (Lovley and Phillips, 1988) and transferred into multiple 160-ml serum bottles with each containing 80 ml of the medium. The gas phase overlying the medium was 20% (v/v) CO_2 balanced with N_2 . Final pH was about 7.0.

Six identical bottles were used to grow each culture. Incubation was at 30 °C in the dark without shaking. Experiments were terminated when bacterial growth reached the stationary phase (about 24 h). Cells were harvested from each bottle and combined as a single sample. Cell pellets were obtained by centrifugation at $18,000 \times g$ for 10 min. About 20% of the biomass were treated with 1.0 N HCl for 2 h to remove inorganic carbon and oven dried (60 °C) before isotopic measurement. The remaining 80% of the biomass were freeze-dried and used for lipid extraction and carbon isotope analysis of the derivatized fatty acids.

2.2. Extraction of cellular lipids

Freeze-dried cell pellets were extracted by a single-phase organic solvent system comprised of chloroform, methanol, and aqueous 50 mM phosphate buffer (pH 7.4) in the ratio of 1:2:0.8 (v/v/v) (White et al., 1979). A blank sample was processed in the same manner to serve as a control. After extraction overnight, equal volumes of chloroform and nanopure water were added to the extractant, resulting in a two-phase system. The lower organic (lipid-containing) phase was collected and fractionated on a silicic acid column into neutral lipids, glycolipids, and polar

lipids (Guckert et al., 1985). The polar lipids were treated using a mild alkaline methanolysis to produce fatty acid methyl esters (FAME).

Definitive identification of FAME was done on an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 mass selective detector using a 20-m non-polar column (0.1 mm I.D., 0.1 μm film thickness). The injector and detector were maintained at 230 and 300 °C, respectively. The column temperature was programmed from 60 °C for 1 min, ramping at 20 °C/min to 150 °C and holding for 4 min. This was followed by ramping at 7 °C/min to 230 °C and holding for 2 min, and finally ramping at 10 °C/min to 300 °C and holding for 3 min. Mass spectra were determined by electron impact at 70 eV. Methyl nonadecanone was used as the internal standard, and the PLFA expressed as equivalent peak response to the internal standard. Double-bond positions of monounsaturated FAME were determined by GC-MS analysis of the dimethyl disulfide adducts (Nichols et al., 1986).

Fatty acids are designated as $A:B\omega C$, where A is the total number of carbon atoms, B the number of double bonds, and C the position of the double bond from the methylene of the fatty acid. The suffixes c for *cis* and t for *trans* refer to geometric isomers. The prefixes a and i refer to anteiso and iso methyl-branching, respectively, 10Me indicates a methyl group on the 10th carbon atom from the carboxyl end of the fatty acid, cy refers to a cyclopropane fatty acid, and br refers to an unknown methyl branching position.

2.3. Isotope analysis of lipid biomarkers and total biomass

Carbon isotope compositions of methylated fatty acids were determined on a Finnigan MAT 252 Mass Spectrometer (Finnigan MAT, Bremen, Germany), which was connected to a combustion interface with a HP 5890A gas chromatograph (Hewlett Packard, Avondale, PA) (Hayes et al., 1990; Ricci et al., 1994; Merritt et al., 1994). The fatty acid sample was dissolved in hexane and injected into the GC column (Chrompack CP-SIL5CB-MS, 50 m \times 0.32 mm (id) \times 0.4 μm , 100% dimethylpolysiloxane) along with perdeuterio n -alkanes (C_{16} , C_{20} , C_{24} , C_{36}) (MSD Isotopes of St. Louis, MO; now defunct) of known carbon isotope compositions. Temperature program was 60–320 °C at a rate of 4 °C/min, isothermal for 25 min. The

column effluent was combusted online at 850 °C in a reactor containing CuO. Water generated from the combustion was removed by a Nafion membrane as the effluent passed through it. Each sample was run at least twice at different injection sizes (0.2–0.6 µl). Greater injection sizes allowed detection and analysis of smaller peaks but resulted in over flow of larger peaks (ion current > 8 V), which gave erroneous $\delta^{13}\text{C}$ values. These larger peaks were reduced to less than 8 V using smaller injection sizes to have the valid $\delta^{13}\text{C}$ values. Another potential problem is the co-elution of some fatty acid isomers (e.g., 16:1 or 18:1), which could not be baseline resolved on the GC column (Fig. 1). For this reason, a composite $\delta^{13}\text{C}$ value was reported for the sum of the isomers.

Measured isotope ratios of the methylated fatty acids were corrected for the methyl moiety according to [Abrajano et al. \(1994\)](#) using the following equation:

$$\delta^{13}\text{C}_{\text{FA}} = [(C_n + 1)\delta^{13}\text{C}_{\text{FAME}} - \delta^{13}\text{C}_{\text{MeOH}}]/C_n$$

where $\delta^{13}\text{C}_{\text{FA}}$ is the $\delta^{13}\text{C}$ of the fatty acid, C_n is the number of carbons in the fatty acid, $\delta^{13}\text{C}_{\text{FAME}}$ is the $\delta^{13}\text{C}$ of the methylated fatty acid, and $\delta^{13}\text{C}_{\text{MeOH}}$ is the $\delta^{13}\text{C}$ of the methanol used for the methylation reaction.

The oven-dried biomass was combusted using a Carlo Erba Model 1500 elemental analyzer. The resultant CO_2 was cryogenically trapped and analyzed for $^{13}\text{C}/^{12}\text{C}$ ratios using the dual-inlet system of the isotope mass spectrometer. Repeated analyses of an

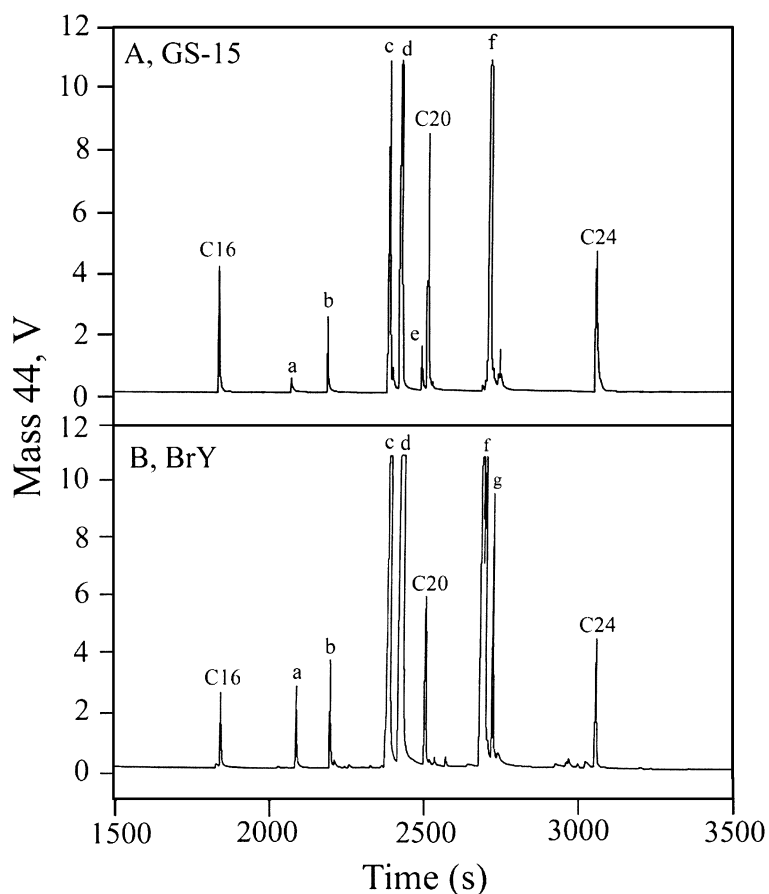


Fig. 1. GC-IRMS mass-44 current of the phospholipid fatty acids (as methyl esters) extracted from GS-15 (A) and BrY (B). Peak numbers are: a, 14:0; b, 15:0; c, 16:1 ω 7c/t; d, 16:0; e, 10Me16:0; f, 18:1 ω 9c/t or 18:1 ω 7c; g, 18:0. Peaks C16, C20, and C24 are internal standards of perdeuterio *n*-alkanes.

organic standard (acetanilide) gave an averaged $\delta^{13}\text{C}$ value of $-27.98 \pm 0.09\text{‰}$ ($n=3$).

3. Results and discussion

3.1. Lipids

The profiles of phospholipid fatty acids in GS-15 and BrY were dominated by even-numbered fatty acids (Table 1). The three most abundant fatty acids in GS-15 were 16:1 ω 7c (23%), 16:0 (26%), and 18:1 ω 7c (29%). The four most abundant fatty acids in BrY were 16:1 ω 7c (24%), 16:0 (33%), 18:1 ω 9c (18%) and 18:1 ω 7c (13%). The abundance of 14:0 was much less in both species (6% in GS-15 and 3% in BrY). The detected odd-numbered fatty acids included i15:0, a15:0, 15:0, i17:0, a17:0, cy17:0, 17:0, and cy19:0, but not all were present in the same species. The abundance of i15:0 was 8% in GS-15 and 3% in BrY. The abundance of i17:0 was about 1% in GS-15 and less than 0.5% in BrY (Table 1). The rest odd-num-

bered fatty acids were present as trace concentrations (data not shown).

The distribution of fatty acids in GS-15 has been reported by Lovley et al. (1993) and the distribution of fatty acids in BrY has been reported by Rossello-Mora et al. (1994). The fatty acids of *S. putrefaciens* strains have been reported by Moule and Wilkinson (1987), Nichols et al. (1992), and Teece et al. (1999). Fatty acids in other *Shewanella* species have been reported by Venkateswaran et al. (1999). For GS-15, our results agreed with those of Lovley et al. (1993), who also grew GS-15 on acetate. In both studies, 16:1 ω 7c (>20%) and 16:0 (>25%) were the dominant fatty acids. However, in this study, GS-15 also produced significant amount of 18:1 ω 7c (29%), which was less than 5% in Lovley et al. (1993). For BrY, our results agreed with those of Nichols et al. (1992) and Teece et al. (1999) who grew the *S. putrefaciens* strains using lactate as the substrate.

The distribution of fatty acids in BrY and MR-4 was significantly different from the distribution of fatty acids in BrY (Rossello-Mora et al., 1994) and other *S. putrefaciens* species (Moule and Wilkinson, 1987; Venkateswaran et al., 1999) grown on multiple carbon sources such as the nutrient agar. In studies by Moule and Wilkinson (1987) and Rossello-Mora et al. (1994), the fatty acids were dominated by odd-number components (i15:0, 15:0, and 17:1 ω 9c), which were low in abundance in Teece et al. (1999) and this study (Table 1). This suggests that different substrates can affect the composition of fatty acids in the same bacterial species.

Despite the variability in fatty acid compositions of *Shewanella*, this group of iron-reducing bacteria does not produce 10Me16:0, an unusual fatty acid that is characteristic of GS-15 (Table 1, this study; Lovley et al., 1993) and other acetate-metabolizing bacteria (Taylor and Parkes, 1983; Dowling et al., 1986; Vainshtein et al., 1992). Thus, 10Me16:0 may be used as a reliable lipid biomarker for distinguishing between *Geobacter* and *Shewanella* species in iron-reducing and low-sulfate environments.

3.2. Carbon isotope compositions of fatty acids and total biomass

For both GS-15 and BrY, carbon isotope compositions of fatty acids were depleted in ^{13}C relative to the corresponding total biomass, which was, in turn,

Table 1
Phospholipid fatty acids in GS-15 grown on 20 mM acetate and in BrY grown on 20 mM lactate

Fatty acid (methyl ester)	Fatty acids (%)			
	This study		Teece et al. (1999)	
	GS-15	BrY	MR-4 (O ₂)	MR-4 (NO ₃)
14:0	6	3	4	3
i15:0	8	3	4	3
16:1 ω 9c	0	1	tr ^a	tr
16:1 ω 7c	23	24	41	45
16:1 ω 7t	1	0	tr	tr
16:1 ω 5c	2	0	tr	tr
16:0	26	33	35	34
10Me16:0	2	0	tr	tr
i17:0	1	0	tr	tr
18:1 ω 9c	0	18	tr	tr
18:1 ω 7c	29	13	13	14
18:1 ω 7t	1	0	tr	tr
18:0	0	5	3	3
br18:1	1	0	tr	tr

Ferric citrate was used as the electron acceptor. Also shown are fatty acids in a *S. putrefaciens* strain MR-4 grown aerobically (O₂) and anaerobically (NO₃) using lactate as the electron donor (Teece et al., 1999).

^a Trace concentration or not detected (personal communication with Teece).

depleted in ^{13}C relative to the organic carbon the bacteria were grown on (Table 2). The $\delta^{13}\text{C}$ of individual fatty acids ranged from -38.0 to -43.4 for GS-15 and ranged from -40.1 to -44.3 for BrY (Table 2). In GS-15, the highest value (-38.0) occurred in 14:0 and the lowest value (-43.4) occurred in a composite of 16:1 ω 7c (dominant in abundance) and 16:1 ω 7t. In BrY, the higher value occurred in i15:0 (-40.1) and the lower values occurred in 16:0 (-44.2) and 18:0 (-44.3) (Table 2). We expected that less than 10% of substrates (acetate or lactate) were depleted during bacterial growth (data not shown), these isotopic compositions may represent the maximum depletion in ^{13}C relative to substrate or total biomass (Mariotti et al., 1981). It must be noted that the total organic carbon was treated with 1.0 N HCl, which may cause the isotopic composition to be slightly heavier or lighter than untreated samples (House, personal communication). Thus, the fractionation between fatty acids and total biomass should be explained with caution.

The difference in $\delta^{13}\text{C}$ values of individual fatty acids between GS-15 and BrY can be better expressed using the fractionation factor (ϵ) between fatty acids and the biomass (Table 2), an approach advocated by Hayes (1993) and was followed by others (Summons et al., 1994; Abraham et al., 1998). For GS-15, ϵ varied

between -3.8 (14:0) and -9.4 (16:1 ω 7c/t) with an average value of -6.5 ± 2.2 . The fatty acid, 10Me16:0, which was characteristic of GS-15, had an ϵ value of -5.5 (Table 2). For BrY, ϵ varied between -10.4‰ (i15:0) and -14.7‰ (16:0 and 18:0) with an average value of $-13.0 \pm 1.8\text{‰}$ (Table 2).

In a similar study, Teece et al. (1999) compared the fractionation by a *S. putrefaciens* strain MR-4, which was grown under aerobic and anaerobic (nitrate reduction) conditions. Their study showed that under the aerobic condition, the fractionation between fatty acids and biomass ranged from -1.3‰ to -3.1‰ . Under the anaerobic condition, the fractionation was about two- to six-fold greater, ranging from -5.4‰ to -10.3‰ (Teece et al., 1999) (Table 2). It is interesting to note that the smallest fractionation (-5.6‰) in MR-4 under the anaerobic condition coincided with the smallest fractionation (-10.4‰) of the same fatty acid (i15:0) in BrY, and the greatest fractionation (-10.7‰) in MR-4 (NO_3) coincided with one of the greatest fractionation (-14.7‰) in BrY (Table 2).

Variation in carbon isotopic compositions of lipids from a single species or between different species may be caused by variation in biosynthetic pathways, growth phase, and environmental conditions (Summons et al., 1994; Sakata et al., 1997; Schouten et al.,

Table 2

Carbon isotope compositions of phospholipid fatty acids in GS-15 grown on acetate ($\delta^{13}\text{C} = -27.1\text{‰}$) and in BrY grown on lactate ($\delta^{13}\text{C} = -22.9\text{‰}$)

Fatty acids	GS-15					Peak ^a ID	BrY				MR-4		
	$\delta^{13}\text{C}$ (‰)				ϵ (‰)		$\delta^{13}\text{C}$ (‰)			ϵ (‰)	Peak ^a ϵ (‰)		
	Run-1	Run-2	Run-3	Mean			Run-1	Run-2	Mean		NO ₃	O ₂	
14:0	-38.0			-38.0	-3.8	a	-43.3		-43.3	-13.7	a	-7.3	-2.4
i15:0	-42.7	-41.8		-42.3	-8.2	b	-40.1		-40.1	-10.4	b	-5.6	-3.1
16:1 ω 7c/t	-45.7	-39.8	-44.6	-43.4	-9.4 ^b	c		-40.9	-40.9	-11.3 ^c	c	-8.0	-1.3
16:0		-41.3	-38.2	-39.8	-5.6 ^b	d		-44.2	-44.2	-14.7	d	-10.7	-2.1
10 Me16:0	-40.1	-39.1		-39.6	-5.5	e					e		
18:1 ω 9c/t						f	-43.7	-42.5	-43.1	-13.5 ^d	f	-9.6	-2.4
18:1 ω 7c		-39.2	-37.3	-38.3	-4.1 ^b	f					f		
18:0						g	-44.5	-44.0	-44.3	-14.7	g	-8.3	-2.8
Biomass				-34.3					-30.0				

The fractionation was determined as: ϵ (‰) = $[(1000 + \delta^{13}\text{C}_{\text{fatty acid}})/(1000 + \delta^{13}\text{C}_{\text{biomass}}) - 1] \times 1000$. Also shown are fractionations for strain MR-4 grown on lactate under anaerobic (NO_3) and aerobic (O_2) conditions (recalculated from Teece et al., 1999).

^a Peaks in Fig. 1a (GS-15) and b (BrY), respectively.

^b Standard deviation greater than 1.0‰.

^c Also contain trace amount of 16:1 ω 9c (see Table 1).

^d Also contain 18:1 ω 7c (see Table 1).

1998; van der Meer et al., 1998; Jahnke et al., 1999). Isotope fractionation associated with lipid biosynthesis most commonly occurs during the formation of acetyl-CoA, which is the precursor for fatty acid biosynthesis (Monson and Hayes, 1980, 1982; Hayes, 1993; Jahnke et al., 1999). Acetyl-CoA can be produced by many enzyme-mediated reactions. In organisms using the tricarboxylic acid (TCA) cycle, the key enzyme for isotopic fractionation is pyruvate dehydrogenase, which oxidizes pyruvate to acetyl-CoA (DeNiro and Epstein, 1977; Monson and Hayes, 1982; Melzer and Schmidt, 1987). According to DeNiro and Epstein (1977), the ^{13}C depletion of the lipid fraction resulted from kinetic isotope effects during this oxidation step, which produced the ^{13}C -depleted acetyl-CoA. Subsequent synthesis of lipids from the ^{13}C -depleted acetyl groups resulted in the ^{13}C depletion of the lipids. Monson and Hayes (1982) confirmed and substantiated this hypothesis by using indigenous techniques of intramolecular analysis. A latter study by Blair et al. (1985) suggested that a kinetic isotope effect was

associated with the conversion of acetyl phosphate to acetyl-CoA by the enzyme phosphotransacetylase. This enzyme, similar to the pyruvate dehydrogenase, caused ^{13}C -depletion in fatty acids. The fractionation between biomass and fatty acids in MR-4 grown under aerobic conditions was similar to the fractionation for *Escherichia coli* grown aerobically on glucose (Teece et al., 1999). Since pyruvate dehydrogenase has been shown to be active when MR-4 was grown aerobically (Scott and Nealson, 1994), it was suggested that fractionation associated with lipid synthesis in MR-4 under aerobic conditions probably resulted from the same mechanisms that operate in *E. coli* (Teece et al., 1999).

In methanotrophs using the ribulose monophosphate pathway, the activity of pyruvate dehydrogenase was also detected (Summons et al., 1994; Jahnke et al., 1999), which caused fractionations in the same range as in *E. coli* and MR-4 grown on O_2 (Fig. 2).

Organisms using the serine pathway have very low or no measurable PDH, and malyl-CoA-lyase is the

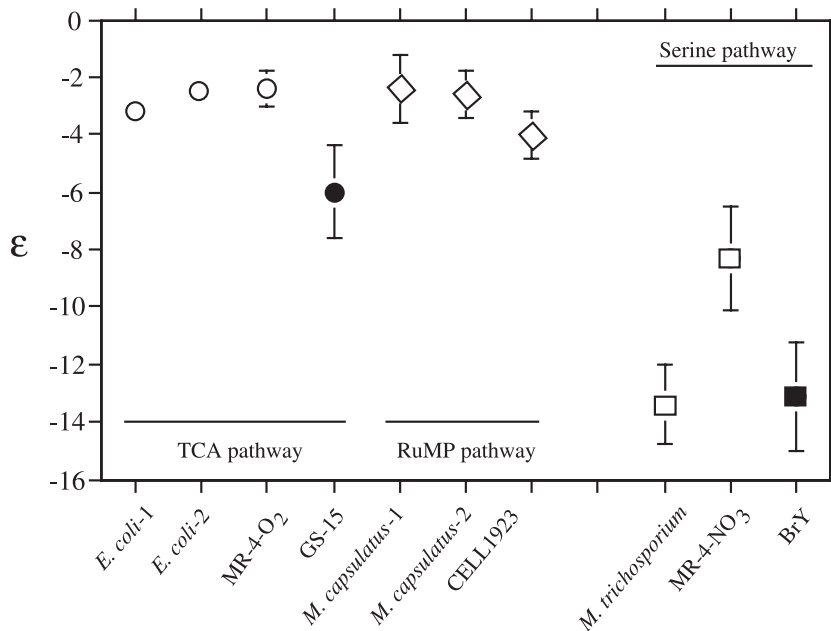


Fig. 2. Carbon isotope fractionation (ϵ) between fatty acids and total biomass for different carbon assimilation pathways. *E. coli*-1 is from Monson and Hayes (1982), *E. coli*-2 from Blair et al. (1985), aerobic (O_2) and anaerobic (NO_3) MR-4 from Teece et al. (1999), *M. capsulatus*-1 from Summons et al. (1994), *M. capsulatus*-2, CELL1923 and *M. trichosporium* from Jahnke et al. (1999), and GS-15 and BrY from this study. Error bars represent one standard deviation of the mean, which is the average fractionation of individual fatty acids. Organisms using the TCA (tricarboxylic acid) and RuMP (ribulose monophosphate) pathways contain sufficient pyruvate dehydrogenase (PDH) activity, whereas organisms using the serine pathway lack the PDH activity.

presumed source of acetyl-CoA for lipid biosynthesis (Anthony, 1982; Jahnke et al., 1999). Mechanisms of isotope fractionation associated with this reaction network are not well understood. However, it was clearly demonstrated that methanotrophs utilizing the serine pathway produced lipids that were about 12‰ depleted in ^{13}C relative to the biomass (Jahnke et al., 1999).

The *S. putrefaciens* species MR-4, when grown anaerobically, had decreased pyruvate dehydrogenase activity but enhanced activity of hydroxypyruvate reductase, which is indicative of the serine pathway (Scott and Nealson, 1994). It is unknown, however, whether the malyl-CoA-lyase is present in MR-4. On the other hand, the dominant pathway of acetyl-CoA production appeared to be from the cleavage of pyruvate by the enzyme pyruvate formate lyase (Teece et al., 1999). Thus, it is possible that the pyruvate formate lyase might have contributed to the depletion in ^{13}C in fatty acids of MR-4 grown anaerobically in addition to the possible contribution of malyl-CoA-lyase. Because the average fractionation (-8.3%) in MR-4 was lower than the fractionation of the pure serine pathway in, for example, the methanotrophs (-13.4%) (Fig. 2), the isotope effect associated with the pyruvate formate lyase may be smaller than the isotope effect associated with the malyl-CoA-lyase.

Pathways of carbon utilization in BrY have not been determined. Although BrY and MR-4 both belong to the *Shewanella* genus (Nealson and Saffarini, 1994) and may use similar biosynthetic pathways for carbon utilization under anaerobic conditions (Fig. 3A), the much larger ϵ values in BrY than in MR-4 (Table 2) suggest that the exact mechanisms of fractionation during fatty acid biosynthesis may be quite different between the two species. Interestingly, the fractionation in BrY was very close to that in the methanotroph using the serine pathway (Fig. 2). Thus, it is tempting to suggest that the malyl-CoA lyase might be mainly responsible for the acetyl-CoA production in BrY. Other enzymes during biosynthesis may potentially cause isotopic fractionation between intermediates (Jahnke et al., 1999). Furthermore, the formation of acetyl-CoA by the serine pathway requires the incorporation of carbon from CO_2 (Fig. 3A), which may come from the oxidation of formate or serine (Scott and Nealson, 1994; Ringo et al.,

1984), or from CO_2 added to the headspace. The uptake of CO_2 proceeds by equilibration with HCO_3^- , which was then assimilated via the enzyme phosphoenolpyruvate carboxylase (Jahnke et al., 1999). In this study, the isotopic composition of CO_2 was unknown, making it difficult to estimate its contribution to isotopic variation in the lipids.

In GS-15, the oxidation of acetate was performed by the TCA cycle (Champine and Goodwin, 1991) (Fig. 3B). The two important enzymes in GS-15 are acetate kinase, which activates acetate to form acetyl phosphate, and phosphate acetyltransferase, which transforms acetyl phosphate to acetyl-CoA (Champine and Goodwin, 1991). Acetyl-CoA was then utilized for fatty acid synthesis and for catabolism by the TCA cycle (Fig. 3B). The isotope fractionation between fatty acids and acetate for GS-15 may be due to the isotope effects associated with either acetate kinase or phosphate acetyltransferase, or both. Because the fractionation in GS-15 was different from those reported for *E. coli* and the methanotroph using the RuMP pathway (Fig. 2), acetate kinase or phosphate acetyltransferase may have different isotope effect than the pyruvate dehydrogenase that is dominant in *E. coli* and the RuMP pathway-utilizing methanotrophs. These results suggest that a variety of enzymes can cause isotope fractionation during heterotrophic growth.

Understanding the fractionation mechanisms of Fe(III)-reducing bacteria is important for determining the role of these organisms play in carbon cycling coupled to iron reduction. For example, the large difference in fractionation between GS-15 and BrY may help determine the community structure of an iron-reducing environment. In addition, the abundance of 10Me16:0 and its isotopic composition may be used in certain environments to indicate the population and activity of *G. metallireducens* or other members within the *Geobacteraceae* group existing in the iron-reducing environment. Molecular technique showed that *G. metallireducens* were dominated in several metal-contaminated environments; however, the presence of other iron-reducing bacteria cannot be ruled out (Lovley, 2000). Identification and isotopic analysis of the 10Me16:0 biomarker may help elucidate the presence and activity of the *G. metallireducens* species. Fatty acid biomarkers have also been used to understand the community structure in

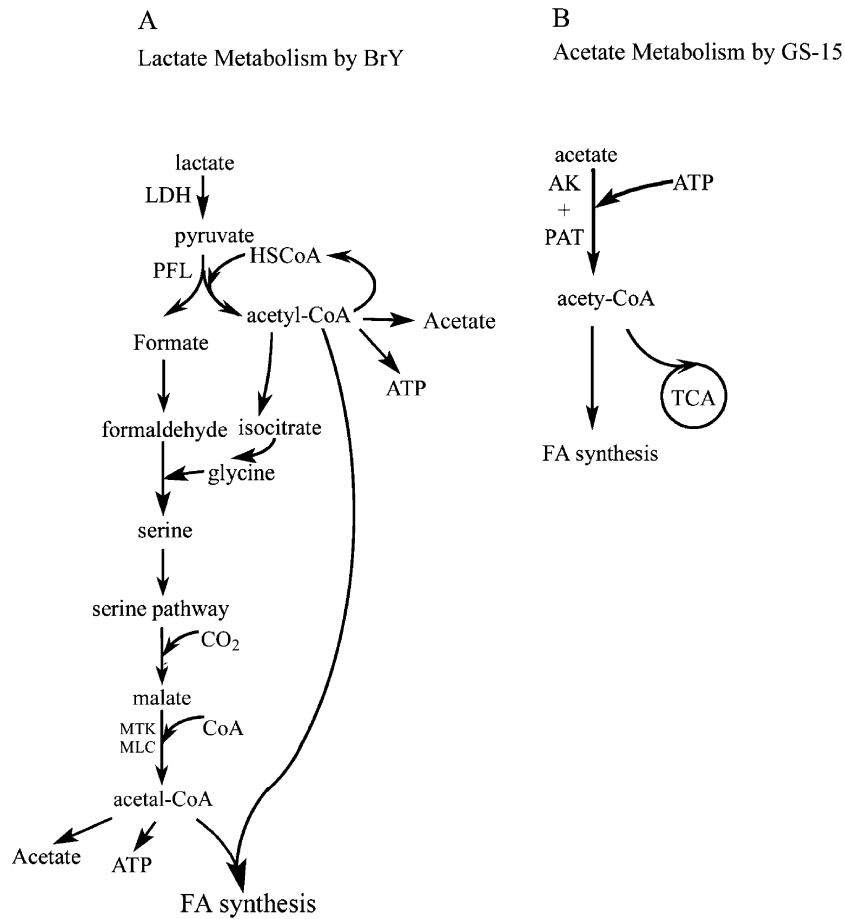


Fig. 3. Proposed pathways for carbon assimilation by (A) *S. algae*, strain BrBY and (B) *G. metallireducens*, strain GS-15. (A) is modified from Scott and Neilson (1994), Teece et al. (1999), and Jahnke et al. (1999). (B) is based on an enzymatic study of GS-15 by Champine and Goodwin (1991). The key enzymes are lactate dehydrogenase (LDH), pyruvate formate lyase (PFL), malate thiokinase (MTK) and malyl-CoA-lyase (MLC) for BrY, and acetate kinase (AK) and phosphate acetyltransferase (PAT) for GS-15. TCA stands for tricarboxylic acid (cycle).

other geological environments. For example, based on the $\delta^{13}\text{C}$ of fatty acids, Fang et al. (1993) distinguished two different chemosynthetic processes of invertebrates in the Gulf of Mexico hydrocarbon seep communities. Hinrichs et al. (1999, 2000) and Pancost et al. (2000, 2001) used the ^{13}C -depleted lipid biomarkers characteristic of Archaea to demonstrate that methane was consumed by Archaea in marine sediments. The lipid- and isotope-biomarkers will become increasingly useful as we need to better understand the microbial complexity in the natural environments (Zhang, 2002; Zhang et al., 2002b; Pancost and Sinninghe Damsté, in press).

4. Summary and conclusions

Geobacter and *Shewanella* represent two major groups of iron-reducing bacteria in the natural environments. While acetate plays a major role in the anaerobic metabolism of certain *Geobacter* species such as *G. metallireducens*, formate appears to be the key substrate for the anaerobic metabolism of certain *Shewanella* species such as *S. putrefaciens*. As a result, two distinct pathways may be utilized by iron-reducing bacteria under anaerobic conditions: *Geobacter* species use the TCA cycle and *Shewanella* species use the serine pathway. The difference in carbon assimilation

pathways may result in distinct carbon isotope fractionation patterns between fatty acids and total biomass, which can serve as biogeochemical criteria for distinguishing between these two populations in an iron-reducing environment. Two specific conclusions can be drawn for GS-15 and BrY when grown anaerobically using ferric iron as an electron acceptor:

1. Both GS-15 and BrY made abundant even-carbon numbered fatty acids including 16:1 ω 7c, 16:0, and 18:1 ω 7c or 18:1 ω 9c. However, GS-15 contained the fatty acids 10Me16:0 and br18:1, which were absent in BrY. The isotopic composition of 10Me16:0 may serve a lipid and isotope biomarker for iron reduction performed by *Geobacter* species.
2. Carbon isotope fractionations between fatty acids and biomass were smaller for GS-15 ($\epsilon = -3.8$ to -9.4) and greater for BrY ($\epsilon = -10.4$ to -14.7); the former may reflect the TCA cycle, whereas the latter may reflect the serine pathway for carbon assimilation.

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