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Compound-Specific Isotopic Fractionation Patterns Suggest Different Carbon Metabolisms among *Chloroflexus*-Like Bacteria in Hot-Spring Microbial Mats†

Marcel T. J. van der Meer,[1]* Stefan Schouten,[1] Jaap S. Sinninghe Damsté,[1] Jan W. de Leeuw,[1] and David M. Ward[2]

Department of Marine Biogeochemistry and Toxicology, Royal Netherlands Institute for Sea Research, 1790 AB Den Burg, Texel, The Netherlands,[1] and Department of Land Resources and Environmental Sciences, Montana State University, Bozeman, Montana 59717[2]

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Stable carbon isotope fractionations between dissolved inorganic carbon and lipid biomarkers suggest photoautotrophy by *Chloroflexus*-like organisms in sulfidic and nonsulfidic Yellowstone hot springs. Where co-occurring, cyanobacteria appear to cross-feed *Chloroflexus*-like organisms supporting photoheterotrophy as well, although the relatively small ^{13}C fractionation associated with cyanobacterial sugar biosynthesis may sometimes obscure this process.

Chloroflexus aurantiacus and its phylogenetic relatives, which comprise a deeply branching kingdom-level lineage in the domain *Bacteria* (24), are major components of photosynthetic microbial mats in both sulfidic and nonsulfidic hot springs in Yellowstone National Park, Wyo. (3, 4, 5, 12, 23, 47). *C. aurantiacus*, the most studied representative of the green nonsulfur bacteria available in pure cultures, can grow heterotrophically by aerobic respiration, photoheterotrophically (using light to incorporate prereduced organic compounds), and photoautotrophically (using light to fix inorganic carbon) (26). Photoautotrophic metabolism by an obligately phototrophic relative of *C. aurantiacus*, the predominant phototroph in sulfidic hot-spring microbial mats, has been reported (12). However, based on culture (25, 26) and radiolabeling studies (2, 32), it has been hypothesized (44) that *Chloroflexus*-like organisms are mainly photoheterotrophic in mats where they live together with cyanobacteria. In such mats cyanobacteria are thought to be the main primary producers, which cross-feed reduced organic compounds to *Chloroflexus*-like organisms, thus supporting their photoheterotrophic metabolism. We exploited the distinctive lipid biomarkers of cyanobacteria and *Chloroflexus*-like organisms to test this hypothesis (39). If *Chloroflexus*-like organisms were purely photoheterotrophic in cyanobacterial mats, their biomarkers should have a $\delta^{13}C$ signature, similar to biomarkers of cyanobacteria. Lipids derived from cyanobacteria in hot-spring mats were found to have $\delta^{13}C$ values typical of the -20 to $-25‰$ fractionations relative to the inorganic carbon source expected of the Calvin cycle (27, 31). However, long-chain polyunsaturated alkenes (e.g., the $C_{31:3}$ alkene hentriacontatriene) and $C_{30\text{-}37}$ wax esters that are typical of *Chloroflexus* (17, 35, 40) and mats containing *Chloroflexus* relatives (9, 35, 37, 39) were approximately 10 to 15‰ enriched in ^{13}C relative to cyanobacterial lipids (37, 39). Such values are expected based on autotrophic metabolism by *Chloroflexus*, which is known to use an inorganic carbon fixation pathway, the 3-hydroxypropionate pathway (15), that imparts an unusually heavy $\delta^{13}C$ signature to both biomass and lipids (16, 40). Thus, the enrichment in ^{13}C of *Chloroflexus* lipids relative to cyanobacterial lipids in microbial mats pointed towards autotrophic growth of *Chloroflexus*-like organisms in hot-spring microbial mats (39).

Here, we examine an alternative explanation—*Chloroflexus*-like biomarkers could have heavy $\delta^{13}C$ signatures because they are cross-fed isotopically heavy fixed organic matter from cyanobacteria. Radiolabeling studies have shown that light-driven CO_2 fixation in hot-spring cyanobacterial mats leads mainly to production of polysaccharides (18, 22), presumably by cyanobacteria, and the polysaccharides are fermented during darkness to short-chain fatty acids known to be photoassimilated by *Chloroflexus*-like organisms (2, 23, 32). It has been shown that sugars may be significantly enriched in ^{13}C relative to lipids in cyanobacteria and other Calvin cycle photoautotrophs (7, 43). Hence, we now have significantly expanded our previous study by investigating not only the $\delta^{13}C$ signatures of lipid biomarkers and biomass but also sugars and dissolved inorganic carbon (DIC) species to enable determination of isotopic fractionations. Furthermore, we included more representatives of mats where cyanobacteria and *Chloroflexus*-like organisms live together in different environments, thereby enabling us to better observe differences in isotopic fractionation patterns.

MATERIALS AND METHODS

Samples were taken from five different hot-spring microbial mats containing *Chloroflexus*-like bacteria with and without cyanobacteria located in Yellowstone National Park, Wyo. (Table 1). Samples for lipid analysis were frozen in the field and were kept frozen until lyophilization and lipid extraction. Samples for microscopy were stored on ice and directly analyzed after returning from the field. The presence of cyanobacteria and *Chloroflexus*-like bacteria was determined by phase contrast and autofluorescence microscopy. Water samples for sulfide anal-

* Corresponding author. Mailing address: Royal Netherlands Institute for Sea Research, Department of Marine Biogeochemistry and Toxicology, P.O. Box 59, 1790 AB Den Burg, Texel, The Netherlands. Phone: (31) 222-369565. Fax: (31) 222-319674. E-mail: mmeer@nioz.nl.

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TABLE 1. General description of mat samples

Mat samples	Geothermal area	Temp (°C)	pH	[S^{2-}] (μM)	*Chloroflexus*-like	Cyanobacteria	Sampling date[d]
NMA source[a]	Mammoth Terraces	58.9	6.1	133	+	−	05-12-1995
NMA downstream[a]	Mammoth Terraces	56.3	6.4	19	+	+	05-12-1995
Tangerine Spring[b]	Mammoth Terraces	60	6.4	40	+	+	26-08-1999
Mushroom Spring[c]	Lower Geyser Basin	64	8.3	BD[e]	+	+	25-08-1997
Octopus Spring[c]	Lower Geyser Basin	58–64	8.3	BD	+	+	27-08-1997

[a] Previously reported by van der Meer et al. (39).
[b] On the shoulder of Orange Mound Spring along the Mammoth Upper Terrace loop road.
[c] See Ward et al. (45) and Ramsing et al. (28) for location.
[d] Day-month-year.
[e] BD, below detection.

ysis were collected from above each mat, preserved in zinc acetate, and analyzed by the method of Cline (6). Inorganic carbon in water overflowing mats was trapped as $BaCO_3$ by increasing the pH of 200 ml of spring water to approximately pH 11 by adding a saturated NaOH solution (pH 13) and solid $BaCl_2$ (36). From the stable carbon isotopic composition of the $BaCO_3$, the isotopic composition of the CO_2 in the spring water was calculated using the temperature-dependent isotopic equilibrium equation of Mook et al. (21).

Lipids were extracted, derivatized, and analyzed by using gas chromatography, gas chromatography-mass spectrometry, and isotope-ratio-monitoring gas chromatography-mass spectrometry (33). Cell-associated sugars were analyzed by using approximately 20 to 160 mg of microbial mat residue after lipid extraction. This material was hydrolyzed, and sugar monomers were derivatized and analyzed by gas chromatography, gas chromatography-mass spectrometry, and isotope-ratio-monitoring gas chromatography-mass spectrometry (42). Stable carbon isotopic compositions of the bulk cell material and $BaCO_3$ were determined by automated on-line combustion (Carlo Erba CN analyser 1502 series) followed by conventional isotope ratio-mass spectrometry (Fisons optima [11]). The stable carbon isotope compositions are reported in the delta notation relative to the Vienna PeeDee Belemnite ^{13}C standard.

RESULTS AND DISCUSSION

The mat samples studied are compared in Table 1. The New Mound Annex (NMA) mat, found in a sulfidic, high-carbonate hot spring at Mammoth Terraces, is comprised of *Chloroflexus* without cyanobacteria (39). The sulfide is thought to poison cyanobacteria but is utilized by *Chloroflexus* spp. in a photoautotrophic metabolism (12). All other mats are comprised of both cyanobacteria (*Synechococcus*) and *Chloroflexus*-like filamentous bacteria. The Tangerine Spring mat, like the downstream NMA mat (39), occurs in a slightly acidic, high-carbonate spring in the Mammoth Terraces group, where sulfide levels have been reduced due to its use by *Chloroflexus* spp. and other upstream sulfide-utilizing organisms. The Octopus Spring and Mushroom Spring mats occur in low-sulfide, alkaline silica-rich springs of the Lower Geyser Basin.

Lipid and sugar composition. The lipid compositions of the five mat samples are compared in Fig. 1 and Table 2, which also includes information for cultures of *C. aurantiacus* and its phylogenetic relative, *Roseiflexus castenholzii* (13), for reference. Lipids characteristic of *Chloroflexus*-like organisms were abundant in all mats. For instance, wax esters ranging from C_{31} to C_{37} were among the predominant lipids in all mats, with small-scale variation in distribution pattern among mat samples. Differences in the carbon skeletons of the wax esters between the environmental samples and the cultures may reflect physiological differences between cultivated and natural populations. For instance, the different mat systems do not contain monounsaturated wax esters as does *C. aurantiacus* but rather contain *iso*-branched wax esters (35, 39). Also, the mats contain C_{31} to C_{37} wax esters, whereas *R. castenholzii* produces C_{37} to C_{40} wax esters (41). Long-chain ($C_{29\text{-}32}$) alkenes, predominantly hentriacontatriene ($C_{31:3}$) (38), were abundant in mats in Mammoth Terraces springs but were present at only trace levels in mats from the Lower Geyser Basin. This may reflect the predominance of *Chloroflexus* spp. in Mammoth Terraces (46) and more distantly related *Roseiflexus*-like organisms in springs of the Lower Geyser Basin (23, 30), since the latter organism lacks hentriacontatriene (41). C_{17} *n*-alkane, a biomarker for cyanobacteria (34), was found in all mats containing cyanobacteria but was absent from the NMA source mat. More common lipids, such as $C_{15\text{-}18}$ fatty acids and $C_{17\text{-}18}$ alcohols, were also detected in all mats.

The sugar fractions of all mats contained arabinose, xylose, rhamnose, and glucose, the latter being dominant (approximately 30 to 80% of the sugar fraction). The sugar distribution was similar to that reported for *C. aurantiacus* (40).

Stable carbon isotopic compositions. The isotopic compositions of DIC, bulk biomass, lipids, and sugars for all mats are reported in Table 3. The values for DIC species determined for Tangerine Spring was within the range previously reported for other Mammoth Terraces hot springs (19). The values for Mushroom Spring were somewhat lower. Isotopic composition of bulk biomass ranged from −13 to −17‰, except for the NMA downstream and Tangarine Spring mats, which were isotopically lighter (−24 and −25‰, respectively). Isotopic compositions of specific lipids ranged from −8.9 to −36.3‰. Compounds known from or possibly contributed by cyanobacteria (e.g., C_{17} *n*-alkane and C_{16} and C_{18} fatty acids, respectively) were isotopically lighter (−21.3 to −36.3‰) than biomarkers of *Chloroflexus*-like organisms ($C_{31:3}$ and wax esters; −8.9 to −27.0‰). The isotopic composition of glucose ranged from −5.1 to −21.8‰. Isotopic compositions in cyanobacterial mats were generally more depleted in ^{13}C in springs from Mammoth Terraces (Tangerine and NMA downstream) than in springs from the Lower Geyser Basin (Octopus Spring and Mushroom Spring).

Comparison of ^{13}C fractionation in mats and cultures. Isotopic fractionations between bicarbonate, the principle DIC species, and bulk biomass, lipids, and glucose for all mat samples and *C. aurantiacus* are compared in Fig. 2. The ^{13}C fractionations between biomass, lipids, and glucose relative to DIC are similar for the photoautotrophically grown *C. aurantiacus* culture and the *Chloroflexus* spp.-dominated NMA source pool microbial mat (Fig. 2). The smallest ^{13}C fractionation relative to DIC ($\Delta\delta^{13}C_{DIC}$) was observed for glucose, followed by hen-

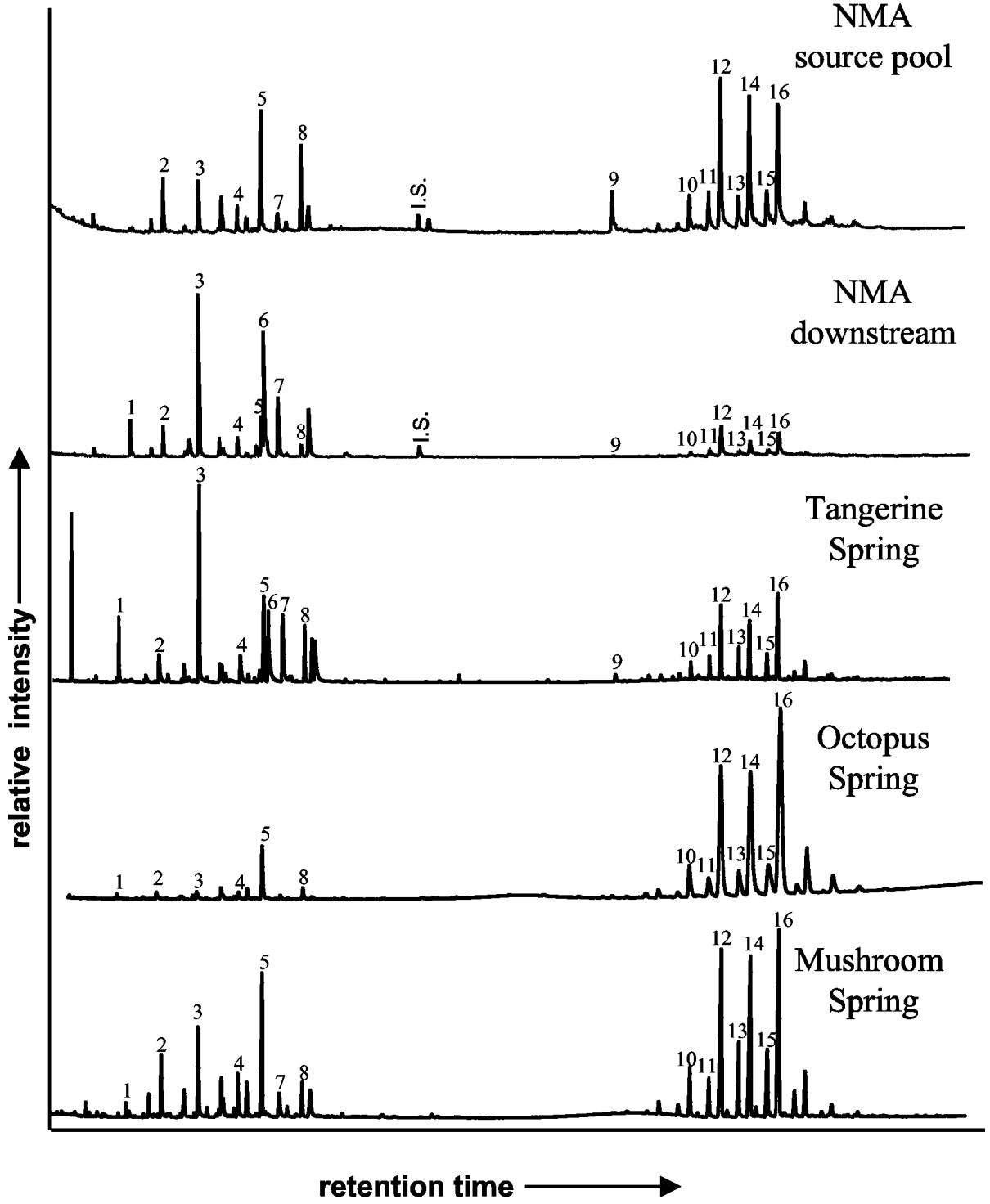

FIG. 1. Partial gas chromatograms of the total lipid extracts from the investigated microbial mats. 1, C_{17} *n*-alkane; 2, C_{15} fatty acid; 3, C_{16} fatty acid; 4, C_{17} fatty acid; 5, C_{17} alkanol; 6, $C_{18:1}$ fatty acid; 7, C_{18} fatty acid; 8, C_{18} alkanol; 9, $C_{31:3}$ alkene; 10, C_{31} wax ester; 11, C_{32} *iso*-wax ester; 12, C_{32} wax ester; 13, C_{33} *iso*-wax ester; 14, C_{33} wax ester; 15, C_{34} *iso*-wax ester; 16, C_{34} wax ester; I.S., internal standard.

triacontatriene, the bulk cell material, the fatty acids, and wax esters. This confirms earlier observations of *Chloroflexus* photoautotrophy in these high-sulfide, *Chloroflexus*-dominated microbial mats (12, 39) and shows that carbon isotopic observations made in culture experiments can be extended to environmental settings.

Chloroflexus biomarkers showed much larger fractionation relative to DIC in the two high-carbonate mats (NMA down-

TABLE 2. Relative abundance of different lipids within mat samples

Category or compound	Peak no.[a]	Relative abundance[b]						
		NMA source[c]	NMA downstream[d]	Tangerine Spring	Octopus Spring	Mushroom Spring	*C. aurantiacus*[d]	*R. castenholzii*[e]
Chloroflexus biomarkers								
$C_{31:3}$ alkene	9	++	+	+	Trace	Trace	++	−
n-C_{31} wax ester	10	++	+	+	++	++	−	
iso-C_{31} wax ester		+	+	+	+	+		
n-C_{32} wax ester	12	+++	++	++	+++	+++	+	
iso-C_{32} wax ester	11	+	+	+	+	+		
$C_{32:1}$ wax ester							+	
n-C_{33} wax ester	14	+++	++	++	+++	+++	+	
iso-C_{33} wax ester	13	+	+	+	+	+		
n-C_{34} wax ester	16	+++	++	++	+++	+++	++	
iso-C_{34} wax ester	15	+	+	+	+	+		
$C_{34:1}$ wax ester							+	
n-C_{35} wax ester		+	+	+	++	++	++	
n-C_{36} wax ester		+	−	+	+	+	++	
$C_{36:1}$ wax ester							+	
n-C_{37} wax ester		+	−	+	+	+	+	+
n-C_{38} wax ester								++
n-C_{39} wax ester								+
n-C_{40} wax ester								++
Cyanobacterial biomarker								
C_{17} *n*-alkane	1	−	++	++	+	+	−	−
Nondiagnostic lipids								
C_{15} fatty acid	2	++	++	+	+	++	+	−
C_{16} fatty acid	3	++	+++	+++	+	++	+++	−
C_{17} fatty acid	4	+	+	+	+	++	+++	−
C_{17} alkanol	5	+++	++	++	++	+++	+	−
$C_{18:1}$ fatty acid	6	−	+++	++	−	−	+++	−
C_{18} fatty acid	7	+	++	++	+	+	+++	−
C_{18} alkanol	8	+++	+	++	+	++	++	−

[a] See Fig. 1.
[b] +, detected; ++, ~50% of base peak; +++, base peak; trace; detected in very low abundance; −, not detected.
[c] Previously reported by van der Meer et al. (39).
[d] Knudsen et al. (17), Shiea et al. (35), and van der Meer et al. (40).
[e] van der Meer et al. (41).

stream and Tangerine Spring) where they live together with cyanobacteria (Fig. 2). This indicates that *Chloroflexus*-like organisms are not purely autotrophic in these cyanobacterial mats. Some isotopically lighter carbon must be obtained from an alternative source. The large $\Delta\delta^{13}C_{DIC}$ for the cyanobacterial biomarker C_{17} *n*-alkane suggests cyanobacteria as a possible source for this light carbon. We considered whether cyanobacterial glucose synthesis and fermentation coupled with cross-feeding could explain the observed isotopic fractionation patterns. The $\Delta\delta^{13}C_{DIC}$ of glucose in these two high-carbonate mats is smaller than that observed for the cyanobacterial biomarker, C_{17} *n*-alkane, consistent with the possibility that cyanobacterial glucose biosynthesis imparts a heavier isotopic signature than lipid biosynthesis (7, 43). However, even in the unlikely event that all of the glucose detected was from cyanobacteria and *Chloroflexus* derived all of its carbon from cyanobacterial sugar fermentation, the $\delta^{13}C$ value for all *Chloroflexus* biomarkers should be lower than the $\delta^{13}C$ of glucose, due to isotopic effects of subsequent lipid biosynthesis pathways (1, 14, 20). The fact that the $\delta^{13}C$ value of the $C_{31:3}$ alkane is higher than (NMA downstream) or similar to (Tangarine Spring) that of glucose and that the $\delta^{13}C$ value of wax esters (NMA downstream) is similar to that of glucose therefore suggests that cyanobacterial cross-feeding alone is unlikely to explain the observed results. Apparently, photoautotrophy is also occurring in *Chloroflexus* spp. in these mats. This could reflect either the inputs of separate heterotrophic and autotrophic *Chloroflexus* populations or mixotrophic carbon metabolism in a single *Chloroflexus* population. Since both cyanobacteria and *Chloroflexus*-like organisms contribute organic compounds to the mats, the $\delta^{13}C$ values for the bulk biomass and glucose must be intermediate to cyanobacterial and autotrophic *Chloroflexus* isotope signatures.

Relative to the Mammoth Terraces cyanobacterial mats, the two mats in alkaline siliceous springs show smaller $\Delta\delta^{13}C_{DIC}$ values (Fig. 2). The fractionation pattern of the bulk biomass, glucose, *Chloroflexus* biomarkers, and more general lipids resembles that of the autotrophically grown *C. aurantiacus* culture and the NMA source pool mat, suggesting the possibility of photoautotrophic metabolism by *Chloroflexus* relatives. However, in these mats the $\delta^{13}C$ values of glucose are sufficiently heavy to support the hypothesis that cyanobacterial sugar biosynthesis imparts a heavier isotopic signature to sugars than to lipids; cross-feeding of sugar fermentation products could then impart the heavier signatures of *Chloroflexus* biomarkers. A complicating factor in Octopus Spring and Mush-

TABLE 3. Stable carbon isotopic compositions of bicarbonate, CO_2 (calculated from bicarbonate), bulk biomass, *Chloroflexus* and cyanobacterial biomarkers, nondiagnostic lipids, and glucose in mat samples expressed in per mille relative to the PeeDee Belemnite standard

Category or compound	Peak no.[a]	$\delta^{13}C$ values of samples					
		NMA source[b]	NMA downstream[b]	Tangerine Spring	Octopus Spring	Mushroom Spring	*C. aurantiacus*[c]
Bicarbonate		−2 to −4[d]	−2 to −4[d]	−3.3	~6.8[e]	−6.8	0
CO_2[f]		~9	~9	~9	~12	~11	
Bulk biomass		−14.9	−23.5	−24.8	−16.9	−13.2	−13.0
Chloroflexus biomarkers							
$C_{31:3}$ alkene	9	−8.9	−15.1	−20.0	−18.9[g]	NM[h]	−12.5
C_{32} wax ester	12	−17.7	−22.7	−26.0	−18.3	−16.5	NM
C_{33} wax ester	14	−17.9	−24.1	−27.0	−18.5	−16.4	−15.0
C_{34} wax ester	16	−17.4	−23.4	−26.7	−18.5	−16.5	−14.4
Cyanobacterial biomarker							
C_{17} n-alkane	1		−36.3	−36.3	−34.1	−29.6	
General lipids							
C_{15} fatty acid	2	−19.4	−23.4	−27.1	−20.2	−17.5	NM
C_{16} fatty acid	3	−19.3	−34.5	−35.3	−21.3	−21.3	−13.8
C_{17} fatty acid	4	−18.9	−22.1	−27.6	−18.7	−16.8	−14.8
C_{17} alkanol	5	−18.0	−22.0	−27.9	−19.4	−15.5	NM
$C_{18:1}$ fatty acid	6		−32.7	−35.5			−12.5
C_{18} fatty acid	7	−16.0	−33.9	−35.1	NM	−24.4	−13.6
C_{18} alkanol	8	−16.1	−24.3	−29.1	−22.2	−21.5	−12.5
Sugar							
Glucose		−5.1	−21.8	−20.0	−11.0	−11.5	−5.7

[a] See Figure 1.
[b] Previously reported by van der Meer et al. (39), except for the glucose isotope values.
[c] From van der Meer et al. (40). Isotope values for organic compounds are reported relative to bicarbonate.
[d] Madigan et al. (19).
[e] Assuming similar $\delta^{13}C$ value as for Mushroom Spring.
[f] Calculated based on temperature-dependent isotope equilibrium equation (21).
[g] Partial coelution.
[h] NM, not measured.

room Spring could be the effect of CO_2 limitation on stable carbon isotope fractionation by cyanobacteria in these much more alkaline and lower-DIC settings (8). The Mammoth Terraces mats occur in carbonate-depositing springs that are high in DIC and, at pH 6.4, are poised near the pK_a of H_2CO_3/HCO_3^- (i.e., CO_2 is readily available). In contrast, the mats in alkaline silicious springs of the Lower Geyser Basin have midday pHs as high as 9.4, approaching the pK_a of HCO_3^-/CO_3^{2-} (29). CO_2 limitation in these alkaline silicious hot springs may decrease the degree of isotopic fractionation by cyanobacteria, resulting in higher $\delta^{13}C$ values for all organic compounds produced by cyanobacteria, including the C_{17} *n*-alkane and glucose. However, the very large differentials in $\delta^{13}C$ values of C_{17} *n*-alkane and glucose (18 to 22.4‰) exceed those observed so far in Calvin cycle organisms (1 to 16‰ [43]). This large isotopic difference between the cyanobacterial biomarker and glucose, especially in combination with very small or no isotopic fractionation between CO_2 and glucose, makes it unlikely that all of the glucose is derived from cyanobacteria even when the possible effect of CO_2 limitation on the stable carbon isotope ratios of cyanobacterial products is considered. The high abundance of *Chloroflexus* biomarkers (i.e., wax esters) relative to cyanobacterial biomarkers (i.e., C_{17} *n*-alkane) (Fig. 1) and the heavier isotopic signatures of C_{16} and C_{18} fatty acids (Table 3), which could be contributed by either type of phototroph (10, 17, 35, 40), indeed suggest that a large fraction of

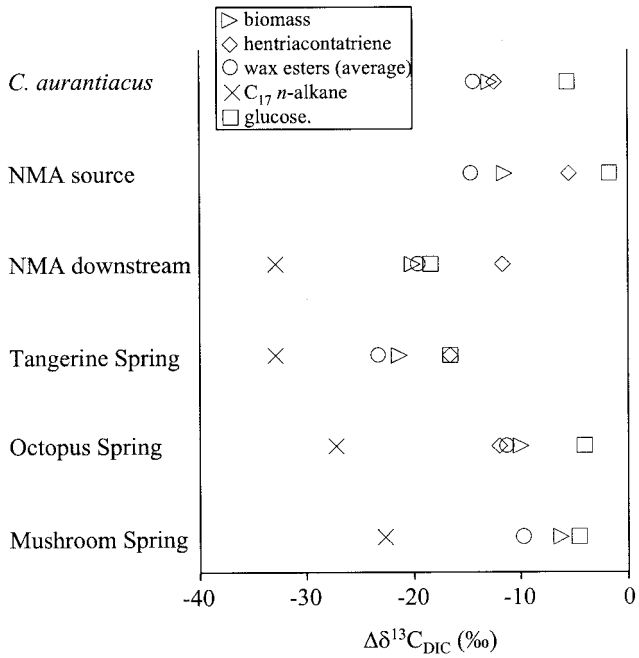

FIG. 2. Depletions in ^{13}C of biomass, key biomarkers, and glucose relative to DIC. ▷, biomass; ◇, hentriacontatriene; ○, wax esters (average); ×, C_{17} *n*-alkane; □, glucose.

the total biomass might be *Chloroflexus* derived. This might be due to our analysis of thicker mat samples (i.e., >1 cm for Lower Geyser Basin mats versus 1 to 2 mm for Mammoth Terraces mats) and the persistence of *Chloroflexus* carbohydrates in deeper layers of the mats in alkaline siliceous springs (39).

By comparing isotopic compositions of compound classes from replicate cyanobacterial mats from different hot-spring settings, we were able to observe that both autotrophic and heterotrophic carbon metabolisms are employed by *Chloroflexus*-like bacteria. Heavier isotopic signatures of *Chloroflexus*-like bacteria may be due in part to their unique autotrophic biochemistry and in part to the differences in isotopic fractionation in sugar and lipid biosynthetic pathways of cyanobacteria. Further work will be necessary, however, in order to observe the degree to which cyanobacterial sugar biosynthesis affects isotopic compositions and, thus, the relative importance of heterotrophy (via cross-feeding from cyanobacteria) and autotrophy in the carbon metabolism of *Chloroflexus*-like bacteria in these mats. This is especially true of mats in alkaline siliceous springs, where CO_2 limitation effects make resolution of the two types of carbon metabolism more difficult.

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