Carbon Isotope Fractionation by Autotrophic Bacteria with Three Different CO_2 Fixation Pathways

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Carbon isotope fractionation during autotrophic growth of different bacteria which possess different autotrophic CO_2 fixation pathways has been studied. $^{13}C/^{12}C$-Ratios in the cell carbon of the following bacteria were determined (CO_2 fixation pathway suggested or proven in parentheses): *Alkaligenes eutrophus* (reductive pentose phosphate cycle), *Desulfobacterium autotrophicum* and *Acetobacterium woodii* (reductive acetyl-CoA pathway), *Desulfobacter hydrogenophilus* and *Thermoproteus neutrophilus* (reductive citric acid cycle). The $\Delta\delta^{13}C$ values, which indicate the *per mille* deviation of the ^{13}C content of cell carbon from that of the CO_2 used as the sole carbon source, range from −10‰ (reductive citric acid cycle) over −26‰ (reductive pentose phosphate cycle) to −36‰ (reductive acetyl-CoA pathway). Acetate formed *via* the acetyl-CoA pathway by the acetogenic *Acetobacterium woodii* showed a $\Delta\delta^{13}C = -40‰$. These data are discussed in view of the different CO_2 fixation reactions used by the bacteria and especially with regard to the isotopic composition of sedimentary carbon through time.

Introduction

Enzyme-catalyzed carboxylation reactions and subsequent reactions entail carbon isotope effects as a result of both thermodynamic and kinetic fractionations [1]. An outstanding example is the bias in favor of ^{12}C exercised by the ribulose 1,5-bisphosphate carboxylase reaction of Calvin cycle (C_3) autotrophy, that discriminates against "heavy" carbon (^{13}C) by about −20 to −35‰ (the negative sign indicates the ^{13}C content in cell carbon is 20 to 35‰ less than in the substrate CO_2). Chemical reactions show much less pronounced carbon isotope discrimination. The isotopic composition of sedimentary carbon, which in the average deviates from inorganic carbonates by approximately −28‰ (or from CO_2 in the air by −21‰), is being taken as indication for biological CO_2 fixation by autotrophs. According to these assumptions and data autotrophic ecosystems became a dominant component of the terrestrial carbon cycle as early as 3.5×10^9, if not 3.8×10^9 years ago.

In the past decade novel autotrophic CO_2 fixation pathways have been unravelled occurring mostly in strictly anaerobic prokaryotes; so far, the reductive pentose phosphate cycle (Calvin cycle) could not be found in this group ([2], for possible exception see [3]). Since the first autotrophic organisms on earth most likely were strict anaerobes, the knowledge of the magnitude of carbon isotope fractionation during autotrophic cell material synthesis from CO_2 in these bacteria might be of some value for the interpretation of geological isotopic composition data. On the other hand, if different CO_2 fixation pathways result in different carbon isotope fractionation, isotopic composition data may be taken as indication for the kind of CO_2 fixation reactions used.

Here we report on the carbon isotope fractionation during autotrophic growth by different bacteria which possess three different CO_2 fixation pathways. Special emphasis was put on comparable growth conditions and excess supply of CO_2, such that the data reflect differences in the main CO_2 fixation reac-

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tions, rather than differences in temperature, residual CO_2 concentration, pH, or salinity of the medium.

Experimental

Organisms

Desulfobacter hydrogenophilus strain AcRS 1 (DSM 3380) *(Dbr. hydrogenophilus)* [4], *Desulfobacterium autotrophicum* strain HRM2 (DSM 3382) *(Dbm. autotrophicum)* [5], and *Acetobacterium woodii* (DSM 1030) *(Ac. woodii)* [6] were a kind gift of Dr. F. Widdel, University of Marburg (F.R.G.). *Alkaligenes eutrophus* strain H16 (DSM 428) [7] was a kind gift of Prof. B. Friedrich, Free University of Berlin (F.R.G.). *Thermoproteus neutrophilus* (DSM 2338) [8] was a kind gift of Prof. K. O. Stetter, University of Regensburg (F.R.G.).

Growth of organisms

In order to compare $^{13}C/^{12}C$ carbon isotope fractionation by different autotrophic microorganisms, the bacterial strains were grown under comparable conditions, with a large excess of CO_2. The conditions were: Growth temperature 24 °C, except for *T. neutrophilus* (85 °C); culture volume 1 l; pH of medium between 6.8–7.2; mineral salt medium with CO_2 as the sole carbon source except for *Ac. woodii* which was grown with additional 0.02% yeast extract; continuous gassing with a 80% H_2/20% CO_2/0.1% H_2S gas mixture at a minimal rate of 50 ml · min^{-1} · l^{-1} culture, except for *A. eutrophus* which was grown with 50% air/40% H_2/10% CO_2; stirring rate (magnetic stirring bar) was 100–150 rpm; the same type of all-glass 1 l fermenter with a filter candle, porosity 3 (Schott, Mainz, F.R.G.), for gassing was used. Except for *A. eutrophus* all organisms were grown under strictly anaerobic conditions. The cell yields were between 0.15–0.3 g dry cell matter · l culture^{-1}, except for *A. eutrophus* which reached 2 g dry cell matter · l culture^{-1}. The medium used for growth of *A. eutrophus* was described by [7], for *T. neutrophilus* by [9]; *Ac. woodii, Dbr. hydrogenophilus,* and *Dbm. autotrophicum* were grown in a salt water medium [10] as modified by [11]. Growth was determined by measuring the optical density at 578 nm (d = 1 cm), except for *T. neutrophilus* where the cell number was determined. Cells were harvested by centrifugation and lyophilized. Parallel to each experiment an aliquot of the gas mixture (Linde AG, Höllriegelskreuth) was continuously passed through two consecutive alkaline CO_2 traps containing 4 N KOH. Carbonate was precipitated as $BaCO_3$ [12]. From the culture supernatant of *Ac. woodii* acetate was obtained by diethylether extraction in a Kutscher-Steudel apparatus of the concentrated medium supernatant followed by vacuum distillation. Acetate from vinegar prepared from wine (Frings, Bonn, F.R.G) was obtained after vapor distillation, diethylether extraction and crystallization.

Determination of $\delta^{13}C$ values

Cell material and Na-acetate were oxidized quantitatively to CO_2 with O_2 [13, 14] and precipitated as $BaCO_3$. The ^{13}C analyses of $BaCO_3$ were performed in CO_2 gas extracted from the samples after acidification with 95% *ortho*-phosphoric acid. The $\delta^{13}C$ value of CO_2 was determined on a specially equipped mass spectrometer MAT 230 (Varian). Corrections for the small isotope contribution of ^{17}O to the mass 45 peak were made. The $\delta^{13}C$ values are presented as *per mille* deviation from the PDB standard (CO_2 obtained from Peedee Belemite from the Peedee Formation, Upper-Cretaceous, South Carolina), as defined by [15]:

$$\delta^{13}C = \left[\frac{^{13}C/^{12}C \text{ sample}}{^{13}C/^{12}C \text{ standard}} - 1 \right] \times 10^3 \text{ [‰]}.$$

Errors in determination of $\delta^{13}C$ were about 0.3‰. The $\delta^{13}C$ values of CO_2 in the gas mixture used in the different series of experiments were −49.8‰, −49.5‰, and −47.6‰, respectively. The $\Delta\delta^{13}C$ cell value refers to the deviation of ^{13}C content of cell carbon from that of the CO_2 used as carbon source.

Results and Discussion

The discrimination between the stable carbon isotopes ^{12}C and ^{13}C by five different facultatively autotrophic bacteria was studied under autotrophic growth conditions, *i.e.* CO_2 was supplied as the only source for cell carbon. All strains but one were strict anaerobes *(Desulfobacterium autotrophicum, Desulfobacter hydrogenophilus, Acetobacterium woodii, Thermoproteus neutrophilus);* as a reference strain the aerobic *Alkaligenes eutrophus* was grown under similar but aerobic conditions. The ^{13}C content in cell matter (after combustion to CO_2) and in CO_2 gas used as carbon source was determined by mass spectroscopy. The values are related to a carbonate standard of defined ^{13}C content and are referred to as $\delta^{13}C$

values. The difference in ^{13}C content between CO_2 and cell carbon is referred to as $\Delta\delta^{13}C$ value. The cultures were grown in 1 l fermenters under continuous gassing with gas mixtures containing CO_2. Fig. 1 summarizes the growth conditions and shows representative growth curves of the 5 strains. The generation time in the exponential phase varied between 10 h and 20 h and the maximal cell yield was 0.3 g dry weight $\cdot l^{-1}$. Since 50% of cell dry weight is carbon the maximal CO_2 assimilation rate of the 1 l culture under those conditions (t_d = 10 h, $\mu = 1.15 \times 10^{-3} \cdot min^{-1}$; cell mass X = 0.3 g = 150 mg C = 12.5 mmol C) follows from the growth equation $\frac{dx}{dt} = \mu \cdot X$ as 15 μmol CO_2 assimilated $\cdot min^{-1} \cdot l$ culture^{-1}. The CO_2 supply varied in the experiments from 330 μmol CO_2 supplied $\cdot min^{-1} \cdot l$ culture^{-1} *(A. eutrophus)* to 1.34 mmol CO_2 supplied $\cdot min^{-1} \cdot l$ culture^{-1} *(Ac. woodii);* usually 440 μmol CO_2 was supplied $\cdot min^{-1} \cdot l$ culture^{-1}. Since CO_2 is well soluble it was supplied in large excess. This is important in order to prevent that due to preferential $^{12}CO_2$ fixation $^{13}CO_2$ selectively accumulates and therefore the $\delta^{13}C$ value of cell carbon becomes artificially low. The results of several independent growth experiments are given in Table I; the values represent means of at least two determinations. The table also includes the CO_2 fixation pathways, which have been proven or suggested, as well as the most important CO_2 fixation reactions and their estimated contribution to total fixed cell carbon. The most important CO_2 fixing enzymes and reactions are summarized in Table II.

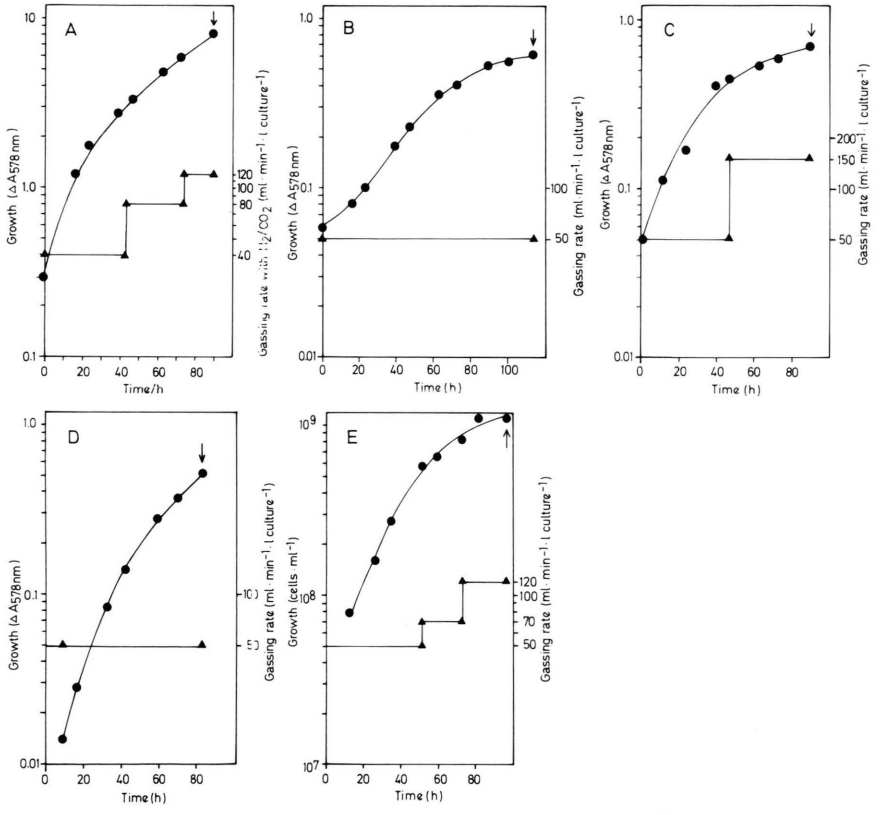

Fig. 1. Autotrophic growth of different bacteria on mineral salt medium at neutral pH and under excess gassing with CO_2. CO_2 was the only carbon source added.
A. *Alkaligenes eutrophus* grown aerobically with H_2 + O_2 as energy source (24 °C).
B. *Desulfobacterium autotrophicum* grown anaerobically with H_2 + sulfate as energy source (24 °C).
C. *Acetobacterium woodii* grown anaerobically with H_2 + CO_2 as energy source (24 °C).
D. *Desulfobacter hydrogenophilus* grown anaerobically with H_2 + sulfate as energy source (24 °C).
E. *Thermoproteus neutrophilus* grown anaerobically with H_2 + elementary sulfur as energy source (85 °C).
The arrow indicates the time of harvest.

Table I. Fractionation of stable carbon isotopes $^{13}C/^{12}C$ during autotrophic growth of bacterial cultures excessively gassed with 20% CO_2/80% H_2 and 10% CO_2/40% H_2/50% air *(Alkaligenes)*, respectively. The main CO_2 fixation reactions are presented in Table II. The estimation of the contribution of the individual CO_2 fixation reaction to total carbon in the sample is based on the assumption that in the Calvin cycle 90% of cell carbon is derived from ribulose 1,5-bisphosphate carboxylase, and 10% from anaplerotic and other minor CO_2 fixation reactions. In anaerobes in which acetyl CoA is central carbon fixation intermediate, approximately 2/3 of cell carbon is derived from acetyl CoA, the rest from additional carboxylations.

Organism or sample	Culture conditions	Autotrophic CO_2 Fixation pathway	$\delta^{13}C_{cell}$ [‰]	$\delta^{13}C_{CO_2}$ [‰]	$\Delta\delta^{13}C_{cell}$ [‰]	Main CO_2 fixation reactions (see Table II)	Estimated contribution of individual CO_2 fixation step to total cell carbon [%]
Alkaligenes eutrophus	28 °C pH 6.7	Reductive pentose phosphate cycle	−75.6	−49.5	−26.1	1 7	90 5
Desulfobacterium autotrophicum	28 °C pH 6.8–7.2	Reductive acetyl CoA pathway	−85.4 −84.8	−49.5 −49.8	−35.9 −35.0	2 3 4 7	33 33 20 10
Acetobacterium woodii	28 °C pH 6.8–7.0	Reductive acetyl CoA pathway	−70.4 −76.3 −62.4	−49.8 −49.5 −47.6	−20.6 −26.8 −14.8	2 3 4 8	33 33 20 10
Acetate from *Ac. woodii* culture	28 °C pH 6.8–7.0	Reductive acetyl CoA pathway	−89.5 −87.2	−49.8 −47.6	−39.7 −39.6	2 3	50 50
Desulfobacter hydrogenophilus	28 °C pH 6.8–7.2	Reductive citric acid cycle	−59.2 −57.9 −60.9	−49.8 −49.5 −47.6	− 9.4 − 8.4 −13.3	5 6 4 7	33 33 20 10
Thermoproteus neutrophilus	85 °C pH 6.8–6.9	Modified reductive citric acid cycle (?)	−55.8	−47.6	− 8.2	5 6 4 7	33 33 20 10

Table II. Main CO_2 fixing enzymes and CO_2 fixation reactions catalyzed by these enzymes in bacteria. [H] refers to reducing equivalents which cannot be specified in many cases. [CO] refers to carbonyl bound to CO dehydrogenase. For literature see [2, 18]; for active species of "CO_2" utilized by these enzymes see [24] and literature quoted herein.

No.	CO_2 fixing enzyme	(E.C.)	CO_2 fixation reaction
1	Ribulose 1,5-bisphosphate carboxylase	4.1.1.39	CO_2 + D-Ribulose 1,5-bisphosphate + H_2O → 2 × 3 phospho-D-glycerate
2	Formate dehydrogenase	1.2.–.–	CO_2 + 2[H] → formate
3	CO dehydrogenase	1.2.99.2	CO_2 + 2[H] → [CO] + H_2O
4	Pyruvate synthase	1.2.7.1 (?)	CO_2 + acetyl CoA + 2[H] → pyruvate + coenzyme A
5	2-Oxoglutarate synthase	1.2.7.3 (?)	CO_2 + succinyl CoA + 2[H] → 2-oxoglutarate + coenzyme A
6	Isocitrate dehydrogenase ($NADP^+$)	1.1.1.42	CO_2 + 2-oxoglutarate + 2[H] → isocitrate
7	PEP carboxylase	4.1.1.31	HCO_3^- + phosphoenolpyruvate → oxaloacetate + phosphate
8	PEP carboxykinase (pyrophosphate)	4.1.1.38	CO_2 + phosphoenolpyruvate + P_i → oxaloacetate + pyrophosphate

A. eutrophus, which assimilates CO_2 *via* the Calvin cycle [16], discriminated ^{13}C by approximately −26‰. *Dbm. autotrophicum*, which assimilates CO_2 probably *via* a reductive acetyl CoA pathway [17–20], discriminated the heavy isotope even stronger (−36‰). *Ac. woodii* formes acetate *via* acetyl CoA from 2 CO_2 and 4H2 by a similar reductive acetyl CoA pathway [6, 21, 22]; this is reflected by a similar strong isotope discrimination (−40‰). In contrast cell carbon of this acetate forming bacterium which mostly derives from acetyl CoA had a $\Delta\delta^{13}C$ value of −20 to −27‰. This relatively low value is unexpected because *Dbm. autotrophicum* and *Ac. woodii* use a similar CO_2 fixation mechanism. The interpretation of the data has to take into account that in the acetogenic bacterium two pathways are competing for acetyl CoA, *i.e.* conversion to acetyl phosphate and acetate for ATP synthesis [6], and assimilation into cell carbon *via* pyruvate, citrate, malonyl CoA and other acetyl CoA metabolizing reactions of intermediary metabolism [22]. Since approximately 12 times more acetyl CoA is converted to acetate than assimilated this eventually will lead to an accumulation of [^{13}C]acetyl CoA due to the isotope effects exerted by phosphotransacetylase and acetate kinase. Consequently cell carbon will be enriched in ^{13}C relative to the acetate excreted. As a control acetate formed from ethanol by aerobic acetic acid bacteria in vinegar had a $\Delta\delta^{13}C$ value of −25.3‰ (data not shown), if the $\delta^{13}C$ value of atmospheric CO_2 was used as reference. This value corresponds to the $\Delta\delta^{13}C$ value of C_3 plant material [reviewed in 1]. *Dbr. hydrogenophilus*, which assimilates CO_2 *via* acetyl CoA formed in a reductive citric acid cycle [23], exhibited a rather low ^{13}C discrimination (−10‰); a similar value was observed with *T. neutrophilus* which seems to assimilate CO_2 *via* a similar mechanism [9, unpublished results].

In conclusion the autotrophic organisms showed remarkable differences in their ability to discriminate the stable carbon isotopes. Three groups with similar fractionation were recognized each probably characterized by a distinct autotrophic CO_2 fixation mechanism. This is an additional argument – although indirect – for the existence of alternatives to the Calvin cycle in prokaryotes. Table III summarizes representative carbon isotope fractionation data in bacteria. Table II summarizes the main CO_2 fixation reactions in autotrophic organisms. We would like to emphasize three points. First, biological carbon isotope fractionation data should be interpreted with caution. One has to be aware of several CO_2 fixation mechanisms; even not all CO_2 fixation pathways in bacteria and their isotope effects may be known. Second, the Calvin cycle most likely was not the predominant autotrophic pathway in the early anaerobic biosphere, and attempts to relate isotope fractionation in geological specimens to this CO_2 fixation mechanism must be rejected on the basis of this study. A combination of the alternative CO_2 fixation pathways would lead to a similar discrimination as exerted by ribulose-1,5-bisphosphate carboxylase.

Table III. Stable carbon isotope $^{13}C/^{12}C$ fractionation reported for autotrophic bacteria.

Organism	$\Delta\delta^{13}C_{cell}$ [‰]	References
Alkaligenes eutrophus	−26.1	this paper
Desulfobacterium autotrophicum	−35.5	this paper
Desulfobacter hydrogenophilus	−10.4	this paper
Thermoproteus neutrophilus	− 8.2	this paper
Acetobacterium woodii	−15 to −26.8	this paper
Chromatium, strain D	−22.5	[25]
Rhodospirillum rubrum	−20.5	[25]
Chlorobium limicola thiosulfatophilum	−12.2	[25]
Methanobacterium thermoautotrophicum	+5 to −34	[26]
Chlorobium phaeovibrioides	− 3.5	[27]
Chlorobium vibrioforme	− 3.9	[27]
	− 3.8	[27]
Chromatium vinosum	−19.6	[27]
Rhodospirillum rubrum	−12.4	[27]
Rhodopseudomonas capsulata	−10.6	[27]
Thiomicrospira sp.	−24.6	[28]
Thiobacillus neapolitanus	−25.1	[28]

Third, physical parameters such as temperature, pH of solution, salinity, limiting concentrations of CO_2, and especially reactions of energy metabolism effectively competing for CO_2 (CO_2 reduction to methane or acetate) greatly influence the size of isotope discrimination by autotrophs. These parameters are completely unknown for the early biosphere. Their effect on biological isotope discrimination should be studied in bacteria. No rationale for the magnitude of isotope fractionation can be given at present, since the isotope effects of the individual CO_2 metabolizing reactions are mostly unknown.

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