Research Paper

An Examination of the Carbon Isotope Effects Associated with Amino Acid Biosynthesis

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ABSTRACT

Stable carbon isotope ratios (δ^{13}C) were determined for alanine, proline, phenylalanine, valine, leucine, isoleucine, aspartate (aspartic acid and asparagine), glutamate (glutamic acid and glutamine), lysine, serine, glycine, and threonine from metabolically diverse microorganisms. The microorganisms examined included fermenting bacteria, organotrophic, chemolithotrophic, phototrophic, methylotrophic, methanogenic, acetogenic, acetotrophic, and naturally occurring endocryptolithic communities from the Dry Valleys of Antarctica. Here we demonstrated that reactions involved in amino acid biosynthesis can be used to distinguish amino acids formed by life from those formed by nonbiological processes. The unique patterns of δ^{13}C imprinted by life on amino acids produced a biological bias. We also showed that, by applying discriminant function analysis to the δ^{13}C value of a pool of amino acids formed by biological activity, it was possible to identify key aspects of intermediary carbon metabolism in the microbial world. In fact, microorganisms examined in this study could be placed within one of three metabolic groups: (1) heterotrophs that grow by oxidizing compounds containing three or more carbon-to-carbon bonds (fermenters and organotrophs), (2) autotrophs that grow by taking up carbon dioxide (chemolitotrophs and phototrophs), and (3) acetoclastic microbes that grow by assimilation of formaldehyde or acetate (methylotrophs, methanogens, acetogens, and acetotrophs). Furthermore, we demonstrated that endocryptolithic communities from Antarctica grouped most closely with the autotrophs, which indicates that the dominant metabolic pathways in these communities are likely those utilized for CO_2 fixation. We propose that this technique can be used to determine the dominant metabolic types in a community and reveal the overall flow of carbon in a complex ecosystem. Key Words: Amino acids—Stable carbon isotope ratio—Isotopic-ratio gas chromatography mass spectrometry—CO_2 fixation. Astrobiology 6, xxx–xxx.

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INTRODUCTION

ONE OF THE EFFECTS of biological activity is to alter and rearrange the stable carbon isotope composition (δ^{13}C) of an environment. How biology alters an environment has become a way for scientists to identify and quantify the impact of life on the planet (Schidlowski *et al.*, 1983; Schidlowski, 2001). Insight into many elemental cycles on the planet has been garnered by examining the bulk changes in carbon, nitrogen, sulfur, hydrogen, and oxygen isotopes in response to biology. Advancements in the 1960s (Abelson and Hoering, 1961) increased the efficiency of examining the stable carbon isotope composition of individual amino acids. Abelson and Hoering (1961) showed that this chromatographic approach provided a more rapid and powerful way to examine the biochemistry of life and the different ways in which life impacts the environment. Specifically, they demonstrated that individual amino acids formed by biology had significant variations in their δ^{13}C. These variations or isotope anomalies were shown to be associated with metabolism being carried out by life. Thus, it is easy to understand the analytical power of amino acid specific stable isotope ratio analysis. Since amino acid biosynthesis is a ubiquitous process for biology, specific stable isotope ratio analysis of amino acids holds enormous promise as an approach for delineating life from abiotic processes and examining the subtle differences between the biosynthetic pathways exploited by life.

Life has evolved a great deal of flexibility with regard to what can be used as a source of food. This flexibility is due in large part to the myriad of ways life has devised with which to assimilate carbon for growth. The metabolic flexibility we observe is due in large part to the adaptability of the citric acid cycle (CAC), also known as the tricarboxylic acid or Krebs cycle (Gest, 1987; Huynen *et al.*, 1999).

To date, every cell characterized utilizes some portion of the CAC. Despite its ubiquity, however, few microorganisms utilize a complete battery of CAC enzymes (Huynen *et al.*, 1999). The reactions of the CAC that have been demonstrated to be well conserved are those that are essential for the maintenance of oxaloacetate, pyruvate, and α-ketoglutarate pools (Kornberg and Quayle, 1958; Kornberg, 1970). The maintenance of cellular pools of oxaloacetate, pyruvate, and α-ketoglutarate is essential for the biosynthesis of all amino acids. Therefore, the defining feature of metabolic diversity is how a range of carbon assimilation pathways can be utilized while still satisfying this fundamental biochemical requirement for growth by adapting the CAC. Compound-specific stable isotope analysis of amino acids provides a straightforward analytical approach to examine life's metabolic diversity in the world by tracking the isotopic change in the pools of oxaloacetate, pyruvate, and α-ketoglutarate. Therefore, such an approach makes it possible to tie variations in intermediary carbon metabolism in biological communities back to biogeochemical phenomena. The examination of carbon isotope anomalies in individual amino acids has been utilized as a tracer of physiological, environmental, ecological, and archaeological phenomena (Sarbu *et al.*, 1996; Abraham *et al.*, 1998; Pelz *et al.*, 1998; Boschker *et al.*, 1999; Pancost *et al.*, 2000, 2001; Zhang *et al.*, 2005). It has allowed for the examination and comparison of the dietary practices of modern and ancient humans (Fogel *et al.*, 1997; Fogel and Tuross, 2003). It has also been utilized to examine the allocation of nutritional resources in insect reproduction (O'Brien *et al.*, 2002) and document climatic changes over time in various regions of the world (Johnson *et al.*, 1997, 1998).

While amino acids are ubiquitous in extant life, it has been shown that certain amino acids commonly formed by life (*i.e.*, glycine, alanine, aspartic acid, and glutamic acid) are also formed by nonbiological processes (Miller, 1953; Yuen *et al.*, 1984; Haberstroh and Karl, 1989; Hennet *et al.*, 1992; Marshall, 1994; Amend and Shock, 1998; Bada and Lazcano, 2002; Bernstein *et al.*, 2002; Muñoz Caro *et al.*, 2002; Shock, 2002). An important illustration of this phenomenon has been observed in meteoritic samples examined to date (Kvenvolden *et al.*, 1970, 1971; Epstein *et al.*, 1987; Engel *et al.*, 1990; Bada *et al.*, 1998; Glavin *et al.*, 1999). It is clear that amino acids can be formed abiologically under the conditions meteorites encounter before they enter the planets influence (Yuen *et al.*, 1984; Bernstein *et al.*, 2002; Muñoz Caro *et al.*, 2002). We propose that biochemical pathways imprint a carbon isotopic pattern that allows amino acids formed by life to be readily distinguished from those formed by nonbiological processes.

MATERIALS AND METHODS

Description of microbial samples

Microorganisms examined in this study were obtained from the American Type Culture Collection (Table 1). The strains utilized were cultivated as previously described (http://www. ATCC.org/) unless otherwise noted. The complex microbial communities studied were isolated from the Dry Valley of Antarctica. The mineralogy, sampling, and storage of the Antarctic sandstone communities and lichens examined in this study have been previously described (Friedmann *et al.*, 1993; Sun and Friedmann, 2003).

Preparation of samples

Dried cellular material (1–3 mg) was hydrolyzed in 1 ml of 6.0 *N* hydrochloric acid at 110°C for 20 h. After the sample was carefully dried under a stream of dinitrogen gas, the hydrolyzate was derivatized with acidified isopropanol followed by esterification with trifluoroacetic acid anhydride, following a previously described method (Silfer *et al.*, 1991). The resulting mixture of derivatized amino acids was diluted in 200 μl of dichloromethane for subsequent analysis. All Antarctic rock extracts were initially extracted from the rock in nanopure water at 100°C for 24 h. After extraction, the amino acids extracted from enriched samples were derivatized and prepared as described above.

Gas chromatography-isotope ratio mass spectrometry analysis

Stable carbon isotope data are presented using the standard convention $\delta^{13}C = [R_{sample}/R_{standard} - 1] \times 10^3$, where R is the $^{13}C/^{12}C$ ratio of the sample and standard, respectively. Stable carbon isotope values are reported relative to the Pee Dee Belemnite standard. Derivatized amino acids were injected on a split-splitless injector (1:10 split) at 220°C and separated on a 50-m HP Ultra-1 column in a Varian model 3400 gas chromatograph (Hewlett Packard, Palo Alto, CA). The resolved amino acids were then combusted individually in a Finnigan GC continuous flow interface (Thermo Electron Corp., Waltham, MA) at 940°C and then measured as CO_2 on a Delta XL Plus isotope ratio mass spectrometer (Thermo Electron Corp.). Samples were run in triplicate, unless noted (Table 1), along with standards of

known isotopic composition. The $\delta^{13}C$ (‰) values of samples were corrected and calculated by using the measurements of known standards as previously described (Fogel and Tuross, 2003). The analytical error in measuring the derivatized amino acids was typically ±0.4‰. Errors in the standards used to correct for the carbon added during derivatization were approximately ±0.3‰. Standard deviations of corrected $\delta^{13}C$ values for all variance in sample and standard preparation and the correction for the addition of C from the derivatization step were 1.5 ± 0.9‰. For interpretation, amino acids with $\delta^{13}C$ values within 1.5‰ of each other have statistically similar values.

Data analysis

To determine whether there were any strong statistical relationships in the $\delta^{13}C$ measured for amino acids that share common intermediates and carbon backbones (Fig. 1) and are interconnected by intermediary carbon metabolism (Fig. 1), the $\delta^{13}C$ values of amino acids (Table 2) were characterized by least squares linear regression. In each case and for consistency, the $\delta^{13}C$ of the precursor amino acid was designated as the independent variable (*X*), and the $\delta^{13}C$ of the downstream amino acid was designated the dependent variable (*Y*). We used the 95% confidence estimates of the slopes to determine whether they differed significantly from 1:1. Residuals were tested for normality using Shapiro-Wilks algorithm; in all cases they satisfied the assumptions of parametric analysis.

The $\delta^{13}C$ values of amino acids from various cultured microorganisms were compared and contrasted utilizing discriminant function analysis (DFA). DFA allowed us to determine whether a set of individual $\delta^{13}C$ values of amino acids from representative microorganisms could be used to place various microorganisms into groups defined using *a priori* metabolic characteristics. Three groups were identified, and variations between groups were compared to evaluate whether the data significantly discern similarities or differences among these predefined groups. DFA analysis allowed us to define a statistical plane, where the characteristics and differences between various sets of $\delta^{13}C$ values of amino acids could be visualized statistically and graphically in two dimensions utilizing JMP version 5.0 (SAS Institute, Cary, NC).

TABLE 1. STRAINS UTILIZED IN THIS STUDY AND SOME KEY METABOLIC CHARACTERISTICS

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| Organism	Kingdom	Metabolic pathway		ATCC designation
Desulfotomaculum acetoxidans	Bacteria	Oxidative acetyl coenzyme A pathway	Acetotroph	ATCC49208
Methanobacterium formicicum	Archaea	Reductive acetyl coenzyme A pathway	Methanogen	ATCC33274
Methanosarcina acetivorans	Archaea	Reductive acetyl coenzyme A pathway	Methanogen	ATCC35395
Moorella thermacetica	Bacteria	Reductive acetyl coenzyme A pathway	Acetogen	ATCC35608
Aquifex pyrophilus	Bacteria	Reductive TCA pathway	Chemolithotroph	ATCC51433
Chlorobium tepidum	Bacteria	Reductive TCA pathway	Phototroph	ATCC49652
Spirulina platensis	Bacteria	Ribulose bisphosphate pathway	Phototroph	ATCC53843
Synechocystis sp.	Bacteria	Ribulose bisphosphate pathway	Phototroph	ATCC27184
Nitrosomonas europaea Winogradsky	Bacteria	Ribulose bisphosphate pathway	Chemolithotroph	ATCC19718
Methylophilus methyltrophus	Bacteria	Serine pathway	Methylotroph	ATCC53528
Methylobacterium exotorquens	Bacteria	Serine pathway	Methylotroph	ATCC14718
Thermococcus celer	Archaea	TCA cycle	Organotroph	ATCC35543
Pyrococcus furiosus	Archaea	TCA cycle	Organotroph	ATCC43587
Thermotoga maritime	Bacteria	TCA cycle	Organotroph	ATCC43589
Desulfovibrio africanus	Bacteria	TCA cycle	Organotroph	ATCC19996
Clostridium beijeerinckii	Bacteria	TCA cycle	Fermenter	ATCC35702
Clostridium acetobutylicum	Bacteria	TCA cycle	Fermenter	ATCC39236
Escherichia coli K12	Bacteria	TCA cycle/glyoxylate shunt	Organotroph/acetotroph	ATCC700926
Sulfolobus solfataricus	Archaea	TCA cycle/glyoxylate shunt/ reductive TCA cycle	Organotroph	ATCC35091
Shewanella oneidensis MR1	Bacteria	TCA cycle/glyoxylate shunt/ serine pathway	Organotroph/acetotroph/ methylotroph	ATCC700550
Sandstone community	Polyphylogenetic	Unknown	Metabolic consortia	Not applicable
Isolated lichen community	Polyphylogenetic	Unknown	Metabolic consortia	Not applicable

TCA, tricarboxylic acid.
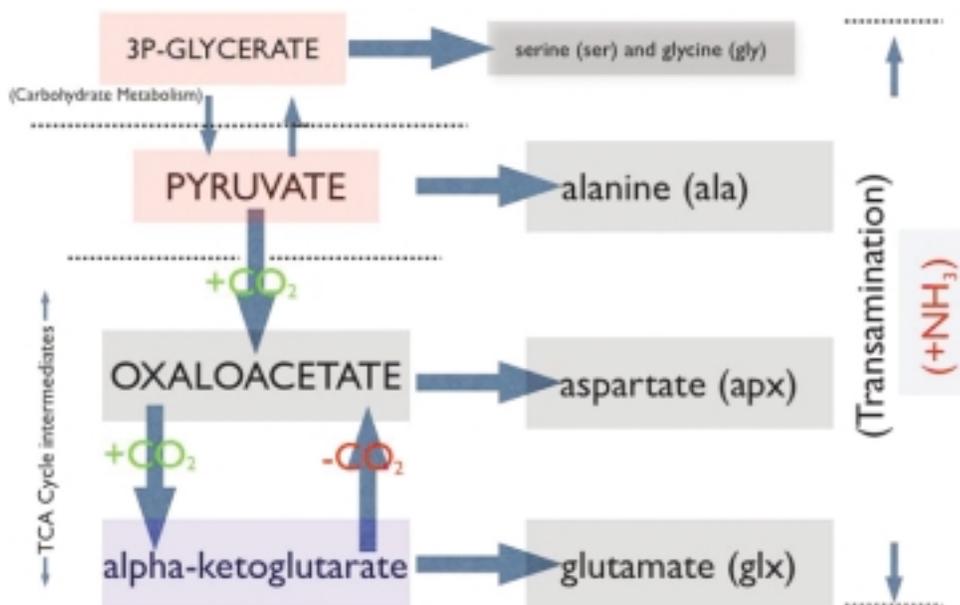
FIG. 1. Schematic showing the connection of amino acids through CAC-intermediate pools. TCA, tricarboxylic acid.

RESULTS

Amino acids connected through intermediary metabolism and CAC reactions

Here, it should be noted that, because of the process utilized for hydrolysis, it is impossible to distinguish between glutamic acid and glutamine, as well as aspartic acid and asparagine. In both instances, therefore, we refer to the total pools of glutamic acid and glutamine as glutamate, and of aspartic acid and asparagine as aspartate.

Biology forms aspartate and alanine by adding an amine group to oxaloacetate and pyruvate, respectively (Fig. 1). This process of nitrogen assimilation is referred to as transamination. It has been shown that, during transamination, a major shift in the $\delta^{15}N$ occurs (Macko *et al.*, 1987). During this process, however, no significant shift in $\delta^{13}C$ is observed. This is because the significant carbon isotope effects during amino acid biosynthesis occur during reaction steps where carbon-carbon bonds are formed (carboxylation) and broken (decarboxylation) (Hayes, 2001). Therefore, it is valid to treat aspartate and alanine as carbon isotope proxies for oxaloacetate and pyruvate. When the $\delta^{13}C$ values of aspartate and alanine, formed by a representative group of microorganisms, were compared and contrasted utilizing least square linear regression analysis, we found a strong linear correlation: $Y = (0.90)X + (-2.6)$, with r^2 of 0.95. The strong linear correlation is a consequence of well-conserved anapleurotic pathways that link the replenishment of oxaloacetate pools to the replenishment of pyruvate pools.

Glutamate is formed by the transamination of α-ketoglutarate. Utilizing the same rationale as previously discussed, we were able to consider glutamate an isotopic proxy of α-ketoglutarate (Fig. 1). When the $\delta^{13}C$ values of aspartate and glutamate from individual microorganisms were examined, a strong linear correlation was observed: $Y = (1.01)X + (-1.8)$, with r^2 of 0.88. The strong linear relationship between glutamate and aspartate is a consequence of α-ketoglutarate and oxaloacetate being linked in all cells through well-conserved reactions of the CAC.

Pyruvate and 3-phosphoglycerate are two of the most important carbon intermediates in the carbon metabolism of every cell. Also, pyruvate and 3-phosphoglycerate are connected through reactions that link carbohydrate metabolism and the CAC (Fig. 1). Serine is formed from the transamination of 3-phosphoglycerate. Therefore, serine can be viewed as a carbon isotopic proxy for 3-phosphoglycerate. When the $\delta^{13}C$ values of biologically formed serine and alanine were compared and contrasted, we observed a linear correlation defined as a line represented by the equation $Y = (1.17)X + (4.8)$, with r^2 of 0.74. The linear

TABLE 2. STABLE CARBON ISOTOPE RATIO OF AMINO ACIDS ASSOCIATED WITH THE MICROORGANISMS IN THIS STUDY

Organism	$\delta^{13}C$											
	Ala	Gly	Thr	Ser	Val	Leu	Ileu	Pro	Asp	Glu	Phe	Lys
E. coli												
Aerobic (pyruvate)	−4.08	5.67	−5.38	7.09	−12.41	−11.94	−11.58	−6.76	−3.52	−0.30	−10.65	−8.13
Anaerobic (pyruvate)	−4.81	11.25	−13.62	0.76	−11.99	−16.59	−15.23	−10.46	−7.38	−7.80	−19.42	−10.65
Acetate	−29.80	−16.42	−30.57	−18.02	−34.61	−32.34	−32.39	−25.19	−27.35	−21.25	−34.45	−24.11
S. oneidensis	—											
Aerobe (fumarate)	−21.96	−11.73	−24.50	−11.10	−30.89	−30.69	−28.07	−26.12	−22.75	−21.59	−30.48	−24.42
Aerobe (nitrate)	−24.13	−17.39	−25.64	−10.73	−34.95	−30.25	−28.68	−24.01	−25.98	−23.18	−30.75	−26.65
Anaerobe (fumarate)	−23.36	−15.21	−27.33	−10.36	−32.85	−34.94	−28.96	−33.07	−25.80	−31.78	−30.02	−28.73
Anaerobe (nitrate)	−24.55	−19.15	−27.85	−4.50	−33.52	−35.88	−28.34	−28.15	−26.72	−29.89	−31.65	−27.47
Desulfovibrio	−20.00	−6.28	−15.33	−21.82	−26.53	−29.86	−25.52	−27.94	−17.06	−25.28	−24.77	−20.68
Methylophilus	−53.18	−41.66	−53.70	−57.64	−58.63	−59.12	−60.08	−55.73	−48.08	−54.84	−59.02	−51.30
Methylobacterium	—											
Methylamine	−42.38	−30.25	−44.40	−51.51	−47.96	−48.07	−48.02	−42.02	−40.63	−39.95	−51.15	−37.64
Methanol	−43.37	−35.69	−39.77	−55.11	−51.74	−45.86	−43.38	−41.36	−39.57	−40.37	−47.16	−38.39
Methanosarcinia	−29.04	−23.39	−21.77	−30.00	−37.43	−33.59	−34.92	−28.02	−22.40	−29.78	−29.84	−29.56
Methanobacterium	−12.82	−2.06	−9.60	−4.57	−27.57	−29.55	−27.85	−8.11	−7.79	−4.34	−20.59	−12.36
Aquifex	−23.27	−12.92	−36.42	−26.74	−38.01	−30.83	−32.25	−29.03	−25.89	−27.98	−30.31	−28.81
Pyrococcus	−8.34	−2.41	−7.76	−19.67	−24.79	−25.87	−21.46	−13.62	−11.85	−9.69	−23.44	−18.41
Thermococcus	−1.09	−2.46	−12.3	−23.23	−26.88	−26.37	−28.53	−16.18	−15.38	−11.04	−24.54	−21.36
Sulfolobus	−15.69	−9.95	−15.34	−26.26	−30.09	−31.7	−25.54	−21.35	−22.64	−21.52	−27.79	−23.53
C. acetobutylicum	−13.33	−11.16	−17.75	−9.16	−30.48	−32.37	−29.92	−21.37	−16.76	−19.51	−29.56	−23.83
Thermotoga	−14.31	−6.04	−13.38	−19.51	−22.54	−26.89	−23.73	−18.71	−16.82	−21.67	−27.57	−20.16
Desulfomaculum	−25.25	−17.27	−24.11	−18.88	−29.19	−29.42	−28.22	−23.91	−23.50	−25.70	−27.64	−23.25
C. beijerinckii	−18.23	−7.28	−15.99	−11.21	−29.77	−30.78	−28.29	−20.47	−18.49	−20.03	−26.76	ND
Nitrosomonas	−52.72	−25.75	−63.19	−61.58	−69.72	−70.47	−64.44	−64.31	−54.18	−60.93	−73.53	ND
Moorella	−28.36	−32.78	−25.7	−33.24	−29.43	−27.32	−22.71	−24.41	−28.1	−28.68	−28.93	ND
Sandstone (Antarctic) community	—											
Exterior	−22.27	−3.87	−30.14	−38.61	−36.22	−37.92	−26.43	−26.24	−23.25	−23.25	−32.49	ND
Interior	−22.68	−6.72	−22.05	−19.73	−46.45	−33.04	−22.63	−24.36	−25.49	−24.8	ND	ND
Lichens (Antarctic) community	−18.12	0.32	−16.31	−8.56	−30.47	−35.87	−23.36	−24.27	−18.49	−22.29	29.5	−27.23
Chlorobium	−4.48	−1.06	−1.16	−2.52	−17.15	−13.4	11.61	−10.71	−5.96	−13.31	−17.04	ND
Synchocystis	−13.06	−4.61	−11.04	ND	−25.68	−28.25	19.44	−14.5	−11.84	−19.51	−23.02	ND
Spirulina	−5.88	4.57	−6.15	−3.88	−18.76	−24.18	−17.06	−12.84	−7.82	−21.43	−20.76	ND

Ala, alanine; Gly, glycine; Thr, threonine; Ser, serine; Val, valine; Leu, leucine; Ileu, isoleucine; Pro, proline; Asp, aspartate; Glu, glutamic acid; Phe, phenylalanine; Lys, lysine; ND, not detected.

correlation between serine and alanine is not as strong as previous examples. This, however, is consistent with what is known about the reactions that link carbohydrate metabolism and the CAC. Significant variation exists in the enzyme reactions and pathways that connect carbohydrate metabolism to the entrance to the CAC. For example, depending on the carbon substrate being used, either the cell will need to breakdown complex carbohydrates (or sugars) to pyruvate, or it will take pyruvate and form sugar through gluconeogenesis (Kornberg and Quayle, 1958; Kornberg, 1970; Gest, 1987). Because of this, there is more variation in what carbon bonds are broken and formed than in the previous linkage we have observed. So while there is a linear correlation between the δ^{13}C of serine and alanine, it is not surprising in retrospect that it is not as strong as for alanine and aspartic acid or glutamic acid or aspartic acid. This is an indication that the enzymatic and chemical linkages between carbohydrate metabolism and the CAC have more diversity and variation than they do between the CAC and anapleurotic pathways. Despite this fact, however, biology still places a stable carbon isotope imprint on alanine and serine it forms.

Can the analysis of amino acids with DFA provide insight into the patterns of intermediary metabolism?

Three distinct *a priori* groups defined on the basis of their ability to assimilate and utilize carbon nutrients were selected to determine whether there were any patterns of intermediary carbon metabolism that could be elucidated from the δ^{13}C values of the amino acids characterized in this study. Typically, DFA is exploited as an *a priori* analytical approach to ascertain whether groups of data sets can be related by a set of predictors. The mathematical and statistical foundation of DFA consists of finding a functional transformation that gives the maximum ratio of difference between a set of group multivariate means and the multivariate variance within two groups (Davis, 1986). Accordingly, an attempt is made to delineate based upon maximization between varying groups, while simultaneously maximizing within group variance. The predictors characteristics, in this case the δ^{13}C values of nine amino acids, are related to form groups based upon metabolic similarities of nine-dimensional space, which are then compared with groups that are input by the user as *a priori* knowledge of carbon utilization by microorganisms. DFA enabled us to test the validity of our metabolic groups based on the δ^{13}C values of the amino acids noted above.

DFA can be viewed as the opposite of multiple analysis of covariance (MANCOVA). MANCOVA is used to determine the effect on multiple dependents of a single categorical independent, while DFA analysis is utilized to see the effect on a categorical dependent of multiple interval independents. DFA differs from cluster analysis in that, in DFA, the groups are determined beforehand and the object is to determine the linear combination of independent variables that best discriminates among groups. In cluster analysis, the groups are not predetermined, and, in fact, the object is to determine in which cases groups may be clustered (Davis, 1986; Puroit and Rocke, 2003; Dworzanski *et al.*, 2004; Tsai *et al.*, 2005). Conversely, DFA has two steps: (1) an *F* test or Wilks-lambda is used to test whether the discriminant model as a whole is significant (Tables 3 and 4), and (2) whether the *F* test shows significance. Then the individual independent variables are assessed to see which ones differ significantly in mean by group, and these are used to classify the dependent variable. The independent variables or predictors in this case are the individual δ^{13}C for alanine, proline, glutamate, aspartate, valine, leucine, isoleucine, threonine, and glycine from heterotrophs, including fermentative anaerobes, methylotrophs, methanogens, acetogens, chemolithoautotrophs, and phototrophs. The Wilks-lambda is used in an analysis of variance (*F*) test of mean differences in discriminant analysis, such that the smaller the lambda for an independent variable, the more that variable contributes to the DFA. The *F* test of Wilks-lambda shows which variable's contributions are significant. Wilks-lambda is sometimes called the U statistic. More relevant to this study is the finding that the Wilks-lambda can also be used in a second context to test the statistical significance of the DFA model as a whole.

Our analysis indicates that the DFA model that relied on (1) heterotrophs, which included the fermenters and organotrophs, (2) autotrophs, which included the chemolitotrophs and phototrophs, and (3) acetoclastic microbes, which included the methylotrophs, methanogens, acetogens, and acetotrophs as *a priori* descriptions of metabolic groups was well described by the δ^{13}C values of the nine amino acids in our study (Fig. 2).

TABLE 3.   RESULTS OF LINEAR REGRESSION ANALYSIS BETWEEN AMINO ACID PRECURSORS AND PRODUCTS

Y (dependent)	X (independent)	Number	r^2	Intercept	Slope	Slope 95% confidence interval[a]	P
Aspartate (Asx)[b]	Alanine (Ala)[b]	29	0.95	−2.64	0.90	0.084	<0.0001
Threonine (Thr)	Aspartate (Asx)	29	0.93	−2.73	1.15	0.123	<0.0001
Lysine (Lys)	Aspartate (Asx)	22	0.89	−7.71	0.80	0.129	<0.0001
Proline (Pro)	Glutamate (Glx)	29	0.94	−1.65	0.96	0.099	<0.0001
Serine (Ser)	Glycine (Gly)	28	0.59	−0.49	0.55	0.362	<0.0001
Serine (Ser)	Alanine (Ala)	28	0.74	4.81	1.17	0.274	<0.0001
Glutamate (Glx)[c]	Aspartate (Asx)[c]	29	0.88	−1.75	1.01	0.283	<0.0001
Valine (Val)	Alanine (Ala)	29	0.86	−13.05	0.90	0.142	<0.0001
Leucine (Leu)	Alanine (Ala)	29	0.82	−14.72	0.82	0.151	<0.0001

[a]All slopes except threonine versus aspartate and serine versus glycine were within one confidence interval of 1.0.
[b]Although aspartate is not synthesized from alanine, the metabolic intermediate from which alanine is synthesized (pyruvate) supplies carbon skeletons for the intermediate from which aspartate is synthesized (oxaloacetate), via anapleurotic pathways (phosphoenolpyruvate carboxylase and pyruvate carboxylase).
[c]Although glutamate does not share a common biosynthetic pathway with aspartate, the intermediates that both are directly formed from (α-ketoglutarate and oxaloacetate) are connected by common portions of the tricarboxylic acid cycle in all cells.

Isotopic relationship between amino acids that share common carbon intermediates

The δ^{13}C of amino acids formed from α-ketoglutarate have a strong statistical correlation due to a shared carbon backbone (Table 3). Proline shares a common five-carbon backbone with glutamic acid and glutamine. We were able to determine the δ^{13}C for both proline and glutamate in 29 microbial samples (Table 2). The δ^{13}C of the proline examined in our study ranged from −6.8‰ to −64.3‰ with a mean of −24.9 ± 13.0‰, while the δ^{13}C of the glutamate pool ranged from −0.3‰ to −60.9‰ with a mean of −24.2 ± 13.1‰. When the δ^{13}C values of proline and glutamate from individual microorganisms were treated as individual points and analyzed utilizing least square linear regression analysis, a strong linear relationship was observed as a line represented by the equation $Y = (0.96)X + (−1.65)$ with a correlation of confidence (r^2) of 0.94.

The δ^{13}C values of amino acids formed from oxaloacetate have a strong statistical correlation due to a shared carbon backbone (Table 3). Threonine shares a common four-carbon backbone with aspartic acid and asparagine. We were able to determine the δ^{13}C for both threonine and aspartate in 29 samples (Table 2). The δ^{13}C of threonine examined in our study ranged from −1.2‰ to −63.2‰ with a mean of −22.7 ± 14.4‰, while the aspartate pool ranged from −3.5‰ to −54.2‰ with a mean of −22.1 ± 12.1‰. The δ^{13}C of threonine in comparison with that of aspartate from individual microorganisms showed a strong linear relationship represented by the equation $Y = (0.93)X + (−2.73)$ with an r^2 of 0.93.

While both lysine and isoleucine have more than four carbons in their backbone, they still share oxaloacetate as their common metabolic intermediate with aspartate. The δ^{13}C for lysine was determined in 22 microbial samples, and the δ^{13}C for isoleucine was determined in 29 samples. The δ^{13}C values of lysine ranged from −8.1‰ to −51.3‰ with a mean of −24.9‰ ± 9.4‰. The δ^{13}C of isoleucine examined in our study ranged from −11.6‰ to −64.4‰ with a mean of −28.9‰ ± 12.1‰. When the δ^{13}C values of lysine and aspartate were plotted relative to each other, a linear relationship represented by the equation $Y = (0.80)X + (−7.71)$ with an r^2 of 0.89 was determined. When we compared the δ^{13}C of isoleucine and aspartate by a similar approach,

TABLE 4.   RESULTS OF WILKS TEST FOR ANALYSIS IN TABLE 3

Test	Value	Approx F	NumberDF	DenDF	Prob > F
Wilks' lambda	0.0122	6.5644	27	50.291	0.0001
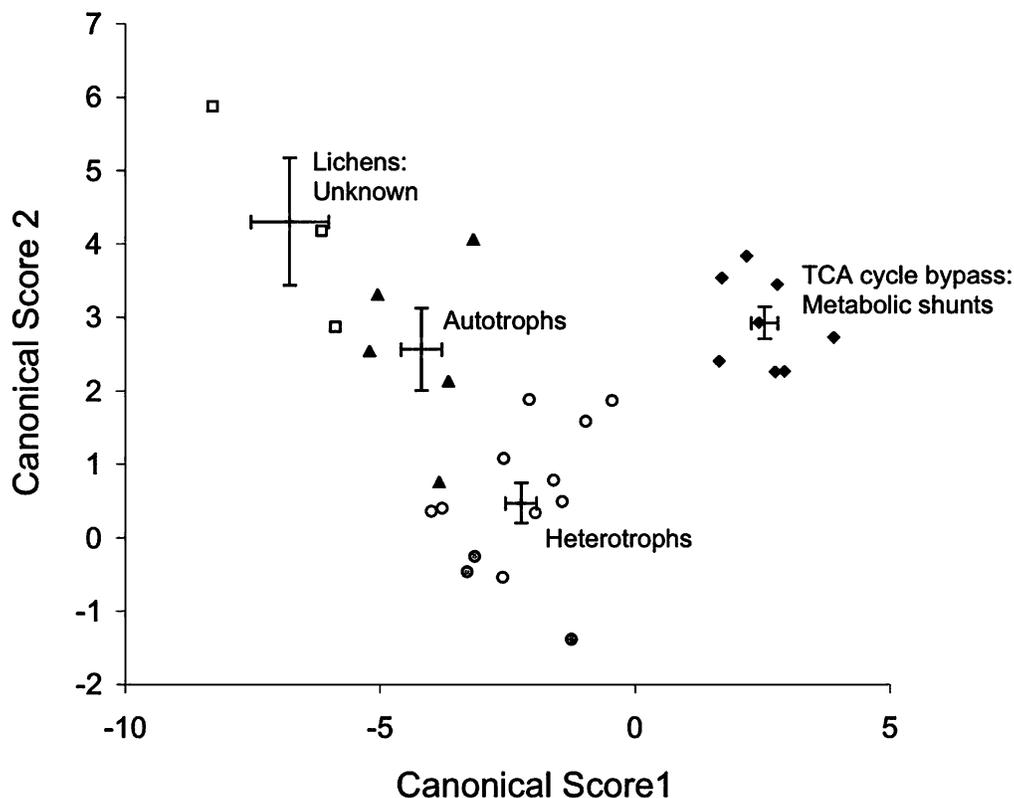
FIG. 2. Schematic of metabolic classification revealed by DFA. Results of the DFA are shown, with categories selected by mode of carbon acquistion. Heterotrophs (circles), autotrophs (triangles), organisms with tricarboxylic acid (TCA) shunts (diamonds), and lichens (squares) formed distinct clusters based on amino acid $\delta^{13}C$, with no statistical mismatches. Archaea are indicated by gray-filled symbols. Crosshairs indicate means ± standard error for each group.

the data scatter was correlated along a line represented by the equation $Y = (0.91)X + (-8.66)$ with an $r^2 = 0.83$.

The $\delta^{13}C$ of amino acids formed from pyruvate have a strong statistical correlation due to a shared carbon backbone (Table 3). Valine and leucine contain five and six carbon backbones, respectively; however, they both still share pyruvate with alanine as a common metabolic intermediate. The $\delta^{13}C$ for valine, alanine, and leucine were determined in 29 microbial samples (Table 2). The $\delta^{13}C$ values for valine ranged from $-12.0‰$ to $-69.7‰$ with a mean of $-32.6‰ ± 12.7‰$ The $\delta^{13}C$ values for leucine were nearly identical to those observed for valine, $-11.9‰$ to $-70.5‰$ with a mean value of $-32.5‰ ± 11.9‰$. The $\delta^{13}C$ of both valine and leucine were compared and contrasted to the $\delta^{13}C$ of alanine from individual microorganisms utilizing least square linear regression analysis. Lines with best fits of $Y = (0.90)X + (-13.0)$ with an r^2

of 0.86 and $Y = (0.82)X + (-14.7)$ with an r^2 of 0.82, respectively, were calculated.

The $\delta^{13}C$ of amino acids formed from 3-phosphoglycerate have a strong statistical correlation due to a shared carbon backbone (Table 3). The $\delta^{13}C$ for both serine and glycine was determined in 28 samples (Table 2). In our study, the weakest linear correlation observed between amino acids that are connected through biosynthetic pathways in microorganisms was between serine and glycine. We found that scatter between the $\delta^{13}C$ of serine and glycine yielded a line represented by the equation $Y = (0.55)X + (-0.49)$ with an r^2 of 0.59.

Complex microbial communities: cryptoendolithic eommunities from the Antarctic Dry Valleys

The Antarctic Dry Valleys are some of the coldest and driest environments on the planet. This extreme environment is seen by some as a possible analog for Mars environments, past and pres-

ent. While the Dry Valleys may be barren of multicellular life, such as plants and animals, complex communities of microorganisms that are able to survive despite the harsh conditions exist there. In the Dry Valleys of Antarctica, microorganisms colonize the pore spaces of exposed rocks and are, thereby, protected from the desiccation. Culture-based physiological studies and microscopy have been used to characterize the cryptoendolithic communities (Friedmann *et al.*, 1993; Sun and Friedmann, 2003). Recently, a 16S rRNA phylogenetic study allowed the community structure of lichen-dominated and cyanobacterial-dominated endolithic communities from Antarctica to be compared and contrasted (de la Torre *et al.*, 2003). Both communities are dominated by a relatively small number of phylotypes that, due to their relative abundance, presumably represent the main primary producers. In lichen-dominated communities, de la Torre *et al.* (2003) found that three rRNA sequences, from a fungus, a green alga, and a chloroplast, which had been previously identified by other approaches, accounted for over 70% of the clones analyzed. In contrast, cyanobacterium-dominated communities were found to contain, in addition to cyanobacteria, a member of the α-subdivision of the Proteobacteria (potentially capable of aerobic anoxygenic photosynthesis) and an apparent member of a new 16S rRNA defined clade within the *Thermus-Deinococcus* bacterial phylogenetic division. Despite the phylogenetic differences observed in both communities, however, one would anticipate that CO_2-fixing microorganisms would dominate the carbon isotope characteristics of the organic material formed in the ecosystems.

To test this hypothesis, we analyzed the isotopic signature of the amino acids extracted from sandstone rocks from the Antarctic Dry Valleys. The sandstone had previously been examined by a range of microscopic and organic techniques, and found to have a complex microbial community dominated by cyanobacteria (Sun and Friedmann, 2003). As shown (Fig. 3), we found that the amino acids examined from similar communities were clearly formed by biological activity. We were able to see the signal despite the physical and chemical barriers anticipated due to interactions between the sandstone-matrix and organic material. We found that the $\delta^{13}C$ pattern of the amino acid pools isolated from the sandstone samples shared many of the isotopic characteristics previously observed in pure cultures cultivated in the laboratory for this study. Further-

more, DFA analysis of the $\delta^{13}C$ of the alanine, proline, glutamate, aspartate, valine, leucine, isoleucine, threonine, and glycine indicates that they were formed in communities where CO_2 fixation was the dominant metabolism (Fig. 3).

The results of our analysis of the $\delta^{13}C$ of amino acids from cryptoendolithic communities from the McMurdo Dry Valley were consistent with the interpretations of the physiology of such communities made previously by microscopy, culture-based investigations, and molecular studies. Therefore, DFA analysis of these Antarctic communities made it possible to reveal the autotrophic signal produced by the primary-producing bacteria that dominate the carbon cycle in the ecosystem. This indicates that amino acid-specific stable carbon isotope analysis provides a powerful method for determining what metabolic pathways are involved in the cycling of carbon in the environment.

DISCUSSION

Amino acids are defined as molecules that contain both amino and carboxylic acid functional groups. Over 500 molecules have been discovered and synthesized that can fit this ubiquitous chemical description. Extant life uses a subset of α-amino acids to form macromolecules. In particular, life synthesizes α-amino acids in which the amino and carboxylate functional group are attached to the α-carbon. Furthermore, 20 "common" amino acids have been found in extant life. These amino acids have been well characterized, and their linkage to the universal genetic code has been well documented (Crick, 1968; Sjostrom and Wold, 1985; Wetzel, 1995). However, it should be noted that our knowledge of the code is constantly evolving. For example, two novel amino acids were recently isolated and characterized (Stadtman, 1987; Hao *et al.*, 2002). Seleno-cysteine is a selenium-containing amino acid with the structure of cysteine, in which a selenium atom replaces the sulfur atom. The amino acid is selectively incorporated into many redox enzymes at a UGA codon, which typically indicates a stop codon (Zinoni *et al.*, 1986; Stadtman, 1987). The novel amino acid pyrrolysine in methanogens is incorporated into several enzymes involved in the synthesis of methane (Zinoni *et al.*, 1986; Stadtman, 1987; Hao *et al.*, 2002). The physiological importance of these two amino acids is still not entirely understood.
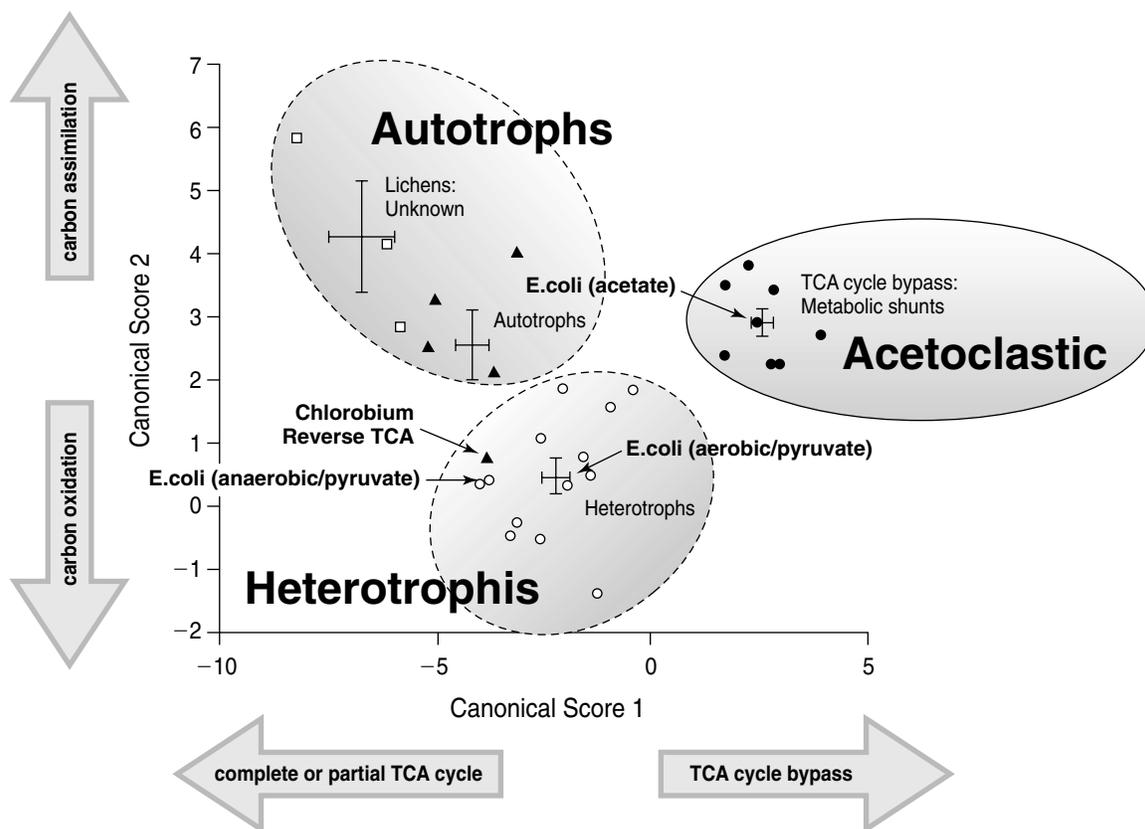
FIG. 3. Graphical representation of metabolic groups identified utilizing the stable carbon isotope fractionation associated with the synthesis of amino acids. The schematic shows the connections between large amino acid intermedicate pools. TCA, tricarboxylic acid.

Finally, life demonstrates a bias toward certain enantiomers in the formation of proteins. For example, amino acids used to form peptides are in the L-configuration, while those found associated with peptidoglycan, which occurs in cell walls in bacteria, are biased toward the D-configuration (Madigan *et al.*, 2002). Recent research has focused on chirality as an important feature of life origins and as a means of distinguishing biologically formed amino acids from those formed by nonbiological processes (Engel and Nagy, 1982; Bada, 1997; Cronin and Pizarello, 1997; Engel and Macko, 1997; Bonner and Bean, 2002; Brinton *et al.*, 2002; Sparks *et al.*, 2005; Vandenabeele-Trambouze *et al.*, 2005).

Amino acids can be formed by processes that require no biology. In the interstellar medium, it has been proposed that amino acids can be formed using a Fischer-Tropsch chemical mechanism. This mechanism allows amino acid formation to occur in cooled nebular gases (CO, H_2, and NH_3) on the surfaces of catalytic icy-dust grains (Hayatsu and Anders, 1981). On planetary bodies (*e.g.*, Earth and Mars), it has been shown

that amino acids can be formed utilizing spark-discharge gas-phase chemistry. Miller (1953) and Miller and Urey (1959) demonstrated that amino acids could be formed when a mixture of reduced gases (CH_4, NH_3, H_2, and H_2O) is subjected to electrical discharge in a closed system. Interestingly, the resulting product of this experiment is a mixture of amino acids and dicarboxylic acids, which closely mimic the distribution of organics observed in the Murchison meteorite (Wolman *et al.*, 1972).

Amino acid synthesis by extant life proceeds by a set of mechanisms that are very different from those proposed for the formation of meteoritic amino acids (Hayes, 2001; Savidge and Blair, 2004; McDonald and Storrie-Lombardi, 2006). For example, amino acid synthesis includes the fixation of nitrogen in the form of ammonia by assimilation into keto-acids. The keto-acids are provided by intermediates formed by carbohydrate metabolism (*i.e.*, glycolysis or gluconeogenesis) in the case of serine, and CAC intermediates for the formation of alanine, aspartate, and glutamate. Formation of serine, alanine, aspar-

tate, and glutamate serves as an initial step in the biosynthetic pathways that lead to the formation of every other amino acid. The reactions that make up these branching and interconnected pathways consist of rearrangements of the initial carbon skeleton and the assimilation of CO_2 and methyl groups onto the basic carbon framework supplied by the CAC and glycolysis (Kornberg, 1970).

We are comparing the mechanistic differences between amino acids formed by a Fischer-Tropsch or Miller-Urey type reaction and the one-carbon additions and carbon-backbone rearrangements used by extant biology. How carbon bonds are formed and broken in nonbiological versus biological synthesis of amino acids can be differentiated by measuring the stable carbon isotope patterns in the amino acids. In fact, when the isotopic composition of meteoritic amino acids and the data set from this study are compared and contrasted, clear differences emerge. The isotopic fractionation in amino acids formed by nonbiological processes looks very different from a pool of amino acids formed by biology (Engel and Nagy, 1982; Cronin and Pizarello, 1997; Engel and Macko, 1997; Fox, 2002). Furthermore, it has been reported that there is almost unity in the $\delta^{13}C$ of the alanine and aspartate formed by a Miller-Urey synthesis (Engel *et al.*, 1995). This is also consistent with the mechanism proposed for the formation of aspartate and alanine in the Murchison meteorite (Yuen *et al.*, 1984). The $\delta^{13}C$ values of alanine between aspartate pools formed by biology differ significantly. The fractionation pattern in the pools of aspartate and alanine formed by biology differ in their $\delta^{13}C$ values by $\Delta^{13}C = 2–6$ parts per million (Tables 2 and 3). Also, there is a distinctive range of isotope carbon fraction between the pools of aspartate and glutamate formed by biology (Tables 2 and 3). The observed isotope fractionation anomalies are associated with biology. For example, they are caused by carboxylation and decarboxylation of oxaloacetate and pyruvate. These key carboxylation and decarboxylation reactions are catalyzed by pyruvate and phosphoenolpyruvate carboxylases. The other enzymes that link the pools of oxaloacetate, pyruvate, and α-ketoglutarate are found within the CAC pathway (Kornberg, 1970). The isotopic anomalies reported for these classes of enzymes (Schmidt and Winkler, 1979; O'Leary *et al.*, 1981; Hayes, 2001) are consistent with the $\Delta^{13}C = 2–6$ parts per million (Tables 2 and 3) carbon isotope effect measured in our study.

In this study, we looked at complex communities that formed inside sandstone rocks found in the Dry Valleys of Antarctica to determine whether a biochemical signature could be measured. We admit work with more communities is required before the power of this method as a means of analyzing environmental samples can be definitively determined. It is clear that the next key stage of this work will be to apply DFA to natural samples characterized by a greater number of phylotypes and metabolic diversity (*e.g.*, it was not known to what degree the large portion of organics synthesized in these communities by primary producing autotrophic microorganisms influenced the ability to obtain the CO_2 fixation signature). Despite these concerns, however, it is clear that unique carbon isotopic signatures can be teased from a complex microbial community using our method. Biology imprints, on a pool of amino acids, a biosignature that indicates how and what a microbial community metabolizes. Our method can also be used as a means by which to distinguish amino acids formed by biology and nonbiological processes.

ABBREVIATIONS

CAC, citric acid cycle; DFA, discriminant function analysis; MANCOVA, multiple analysis of covariance.

REFERENCES

Abelson, P.H. and Hoering, T.C. (1961) Carbon isotope fractionation in formation of amino acids by photosynthetic organisms. *Proc. Natl. Acad. Sci. USA* 47(5), 623–632.

Abraham, W.R., Hesse, C., and Pelz, O. (1998) Ratios of carbon isotopes in microbial lipids as an indicator of substrate usage. *Appl. Environ. Microbiol.* 64(11), 4202–4209.

Amend, J.P. and Shock, E.L. (1998) Energetics of amino acid synthesis in hydrothermal ecosystems. *Science* 281(5383), 1659–1662.

Bada, J.L. (1997) Extraterrestrial handedness? *Science* 275(5302), 942–943.

Bada, J.L. and Lazcano, A. (2002) Origin of life. Some like it hot, but not the first biomolecules. *Science* 296(5575), 1982–1983.

Bada, J.L., Glavin, D.P., McDonald, G.D., and Becker, L. (1998) A search for endogenous amino acids in Martian meteorite ALH84001. *Science* 279(5349), 362–365.

Bernstein, M.P., Dworkin, J.P., Sandford, S.A., Cooper, G.W., and Allamandola, L.J. (2002) Racemic amino acids from the ultraviolet photolysis of interstellar ice analogues. *Nature* 416(6879), 401–403.

Bonner, W.A. and Bean, B.D. (2002) Asymmetric photo-lysis with elliptically polarized light. *Orig. Life Evol. Biosph.* 30(6), 513–517.

Boschker, H.T.S., de Brouwer, J.F.C., and Cappenberg, T.E. (1999) The contribution of macrophyte-derived organic matter to microbial biomass in salt-marsh sediments: stable carbon isotope analysis of microbial biomarkers. *Limnol. Oceanogr.* 44(2), 309–319.

Brinton, K.L.F., Tsapin, A.I., Gilichinsky, D., and McDonald, G.D. (2002) Aspartic acid racemization and age-depth relationships for organic carbon in Siberian permafrost. *Astrobiology* 2(1), 77–82.

Crick, F.H.C. (1968) The origin of the genetic code. *J. Mol. Biol.* 38(3), 367–379.

Cronin, J.R. and Pizarello, S. (1997) Enatiometric excesses in meteoritic amino acids. *Science* 275(5302), 951–955.

Davis, J. (1986) *Statistics and Data Analysis in Geology*, John Wiley and Sons, Toronto.

de la Torre, J.R., Goebel, B.M., Friedmann, E.I., and Pace, N.R. (2003) Microbial diversity of cryptoendolithic communities from the McMurdo Dry Valleys, Antarctica. *Appl. Environ. Microbiol.* 69(7), 3858–3867.

Dworzanski, J.P., Synder, A.P., Chen, R., Zhang, H., Wishart, D., and Li, L. (2004) Identification of bacteria using tandem mass spectrometry combined with a proteome database and statistical scoring. *Anal. Chem.* 76(8), 2355–2366.

Engel, M.H. and Macko, S.A. (1997) Isotopic evidence for extraterrestrial non-racemic amino acids in the Murchison meteorite. *Nature* 389(6648), 265–268.

Engel, M.H. and Nagy, B. (1982) Distribution and enantiomeric composition of amino acids in the Murchison meteorite. *Nature* 296(29 April), 837–840.

Engel, M.H., Macko, S.A., and Silfer, J.A. (1990) Carbon isotope composition of individual amino acids in the Murchison meteorite. *Nature* 348(6296), 47–49.

Engel, M.H., Macko, S.A., Qian, Y., and Silfer, J.A. (1995) Stable isotope analysis at the molecular level: a new approach for determining the origins of amino acids in the Murchison meteorite. *Adv. Space Res.* 15(3), 99–106.

Epstein, S., Krishnamurthy, R.V., Cronin, J.R., Pizzarello, S., and Yuen, G.U. (1987) Unusual stable isotope ratios in amino acid and carboxylic acid extracts from the Murchison meteorite. *Nature* 326(6112), 477–479.

Fogel, M.L. and Tuross, N. (2003) Extending the limits of paleodietary studies of humans with compound specific carbon isotope analysis of amino acids. *J. Archaeol. Sci.* 30(5), 535–545.

Fogel, M.L., Tuross, N., Johnson, B.J., and Miller, G.H. (1997) Biogeochemical record of ancient humans. *Org. Geochem.* 27(5/6), 275–285.

Fox, A. (2002) Chemical markers for bacteria in extraterrestrial samples. *Anat. Rec.* 268(3), 180–185.

Friedmann, E.I., Kappen, L., Meyer, M.A., and Nienow, J.A. (1993) Long-term productivity in the cryptoendolithic microbial community of the Ross Desert, Antarctica. *Microb. Ecol.* 25(1), 51–69.

Gest, H. (1987) Evolutionary roots of the citric acid cycle in prokaryotes. *Biochem. Soc. Symp.* 54, 3–16.

Glavin, D.P., Bada, J.L., Brinton, K.L.F., and McDonald, G.D. (1999) Amino acids in the Martian meteorite Nakhla. *Proc. Natl. Acad. Sci. USA* 96(16), 8835–8838.

Haberstroh, P.R. and Karl, D.M. (1989) Dissolved free amino acids in hydrothermal vent habitats of the Guaymas Basin. *Geochim. Cosmochim Acta* 53(11), 2937–2945.

Hao, B., Gong, W., Ferguson, T.K., James, C.M., Krzycki, J.A., and Chan, M.K. (2002) A new UAG-encoded residue in the structure of a methanogen methyltransferase. *Science* 296(5572), 1462–1466.

Hayatsu, R. and Anders, E. (1981) Organic compounds in meteorites and their origins. *Top. Curr. Chem.* 99, 1–37.

Hayes, J.M. (2001) Fractionation of isotopes of carbon and hydrogen in biosynthetic processes. In *Stable Isotope Geochemistry*, edited by J.W. Valley and D.R. Cole, Mineralogical Society of America and Geochemical Society, Washington, DC, pp. 225–278.

Hennet, R.J.-C., Holm, N.G., and Engel, M.H. (1992) Abiotic synthesis of amino acids under hydrothermal conditions and the origin of life: a perpetual phenomenon? *Naturwissenschaften* 79(8), 361–365.

Huynen, M.A., Dandekar, T., and Bork, P. (1999) Variation and evolution of the citric-acid cycle: a genomic perspective. *Trends Microbiol.* 7(7), 281–291.

Johnson, B.J., Fogel, M.L., Miller, G.H., and Beaumont, P.B. (1997) The determination of late Quaternary paleoenvironments at Equus Cave, South Africa, using stable isotopes and amino acid racemization in ostrich eggshell. *Paleogeogr. Palaeclimatol. Palaeoecol.* 136(1), 121–137.

Johnson, B.J., Fogel, M.L., and Miller, G.H. (1998) Stable isotopes in modern ostrich eggshell: a calibration for paleoenvironmental applications in semi-arid regions of southern Africa. *Geochim. Cosmochim. Acta* 62(14), 2451–2462.

Kornberg, H.L. (1970) The role and maintenance of the tricarboxylic acid cycle in *Escherichia coli. Biochem. Soc. Symp.* 30, 155–171.

Kornberg, H.L. and Quayle, J.R. (1958) The metabolism of C2 compounds in microorganisms. 2. The effect of carbon dioxide on the incorporation of ^{14}C acetate by acetate-grown *Pseudomonas* KB1. *Biochem. J.* 68(3), 542–549.

Kvenvolden, K.A., Lawless, J.G., Pering, K., Peterson, E., Flores, J., Ponnamperuma, C., Kaplan, I.R., and Moore, C. (1970) Evidence from extraterrestrial amino-acids and hydrocarbon in Murchison meteorite. *Nature* 228(5 December), 923–926.

Kvenvolden, K.A., Lawless, J.G., and Ponnamperuma, C. (1971) Nonprotein amino acids in the Murchison Meteorite. *Proc. Natl. Acad. Sci. USA* 68(2), 486–490.

Macko, S.A., Fogel (Estep), M.L., Hare, P.E., and Hoering, T.C. (1987) Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms. *Chem. Geol.* 65(1), 79–92.

Madigan, M.T., Martinko, J., and Parker, J. (2002) *Brock Biology of Microorganisms*, 10th ed., Prentice-Hall, Upper Saddle River, NJ.

Marshall, W.H. (1994) Hydrothermal synthesis of amino acids. *Geochim. Cosmochim. Acta* 58(9), 2099–2106.

McDonald, G.D. and Storrie-Lombardi, M.C. (2006) Amino acid distribution in meteorites: diagenesis, extraction methods, and standard metrics in the search

for extraterrestrial biosignatures. *Astrobiology* 6(1), 17–33.

Miller, S.L. (1953) A production of amino acids under possible primitive Earth conditions. *Science* 117(3046), 528–529.

Miller, S.L. and Urey, H. (1959) Organic compound synthesis on the primitive Earth. *Science* 130(3370), 245–251.

Muñoz Caro, G.M., Meierhenrich, U.J., Schutte, W.A., Barbier, B., Arcones Segovia, A., Rosenbauer, H., Thiemann, W.H.-P., Brack, A., and Greenberg, J.M. (2002) Amino acids from ultraviolet irradiation of interstellar ice analogues. *Nature* 416(?), 403–406.

O'Brien, D.M., Fogel, M.L., and Boggs, C.L. (2002) Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. *Proc. Natl. Acad. Sci. USA* 99(March), 4413–4418.

O'Leary, M.H., Rife, J.E., and Slater, J.D. (1981) Kinetic and isotope effect studies of maize phosphoenolpyruvate carboxylase. *Biochemistry* 20(1), 7308–7314.

Pancost, R.D., Damsté, J.S.S., de Lint, S., van der Maarel, M.J.E.C., Gottschal, J.C., and the Medinaut Shipboard Scientific Party (2000) Biomarker evidence for widespread anaerobic methane oxidation in Mediterranean sediments by a consortium of methanogenic Archaea and Bacteria. *Appl. Environ. Microbiol.* 66(3), 1126–1132.

Pancost, R.D., Hopmans, E.C., Damsté, J.S.S., and the Medinaut Shipboard Scientific Party (2001) Archaeal lipids in Mediterranean cold seeps: molecular proxies for anaerobic methane oxidation. *Geochim. Cosmochim. Acta* 65(10),1611–1627.

Pelz, O., Cifuentes, L.A., Hammer, B.T., Kelley, C.A., and Coffin, R.B. (1998) Tracing the Assimilation of organic compounds using $\delta^{13}C$ analysis of unique amino acids in the bacterial peptidoglycan cell walls. *FEMS Microbiol. Ecol.* 25(3), 229–240.

Purohit, P.V. and Rocke, D.M. (2003) Discriminant models for high-throughput proteomics mass spectrometer data. *Proteomics* 3(9), 1699–1703.

Sarbu, S.M., Kane, T.C., and Kinkle, B.K. (1996) A chemoautotrophically based cave ecosystem. *Science* 272(5270), 1953–1955.

Savidge, W.B. and Blair, N.E. (2004) Patterns of intramolecular carbon isotopic heterogeneity within amino acids of autotrophs and heterotrophs. *Oecologia* 139(2), 178–189.

Schidlowski, M. (2001) Carbon isotopes as biogeochemical recorders of life over 3.8 Ga of Earth history: evolution of a concept. *Precambrian Res.* 106(1-2), 117–134.

Schidlowski, M., Hayes, J.M., and Kaplan, I.R. (1983) Isotopic inference of ancient biochemistries: carbon, sulfur, hydrogen and nitrogen. In *Earth's Earliest Biosphere: Its Origin and Evolution*, edited by J.W. Schopf, Princeton University Press, Princeton, NJ, pp. 149–186.

Schmidt, H.-L. and Winkler, F.J. (1979) Studies of ^{13}C isotope effects on enzyme catalyzed carboxylations and decarboxylations. In *Stable Isotopes: Proceedings of the 3rd International Conference*, edited by E.R. Klein and P.D. Klein, Academic Press, New York, pp. 295–298.

Shock, E.L. (2002) Seeds of life? *Nature* 416(6879), 380–381.

Silfer, J.A., Engel, M.H., Macko, S.A., and Jumeau, E.J. (1991) Stable carbon isotope analysis of amino acid enantiomers by conventional isotope ratio mass spectrometry and combined gas chromatography/isotope ratio mass spectrometry. *Anal. Chem.* 63(4), 370–374.

Sjostrom, M, and Wold, S. (1985) A multivariate study of the relationship between the genetic code and the physiochemical properties of the amino acid. *J. Mol. Evol.* 22(3), 272–277.

Sparks, W., Hough, J.H., and Bergeron, L.E. (2005) A search for chiral signatures on Mars. *Astrobiology* 5(6), 737–748.

Stadtman, T.C. (1987) Specific occurrence of selenium in enzymes and amino acid tRNAs. *FASEB J.* 1(5), 375–379.

Sun, H. and Friedmann, E.I. (2003) Growth on geological time scales in the Antarctic cryptoendolithic microbial community. *Geomicrobiology* 16(2), 193–202.

Tsai, C.A., Lee, T.C., Ho, I.C., Yang, U.C., Chen, C.H., and Chen, J.J. (2005) Multi-class clustering and prediction in the analysis of microarray data. *Math. Biosci.* 193(1), 79–100.

Vandenabeele-Tramouze, O., Claeys-Bruno, M., Dobrijevic, M., Rodier, C., Borruat, G., Commeyras, A., and Garrelly, L. (2005) Comparison of methods for measurement of organic compounds at ultra-trace level: analytical criteria and applications to analysis of amino acids in extraterrestrial samples. *Astrobiology* 5(1), 48–65.

Wetzel, R. (1995) Evolution of the aminoacyl-tRNA synthases and the origin of the genetic code. *J. Mol. Evol.* 40(5), 545–550.

Wolman, Y., Haverland, W.J., and Miller, S.L. (1972) Nonprotein amino acids from spark discharges and their comparison with the Murchison meteorite amino acids. *Proc. Natl. Acad. Sci. USA* 69(4), 809–811.

Yuen, G., Blair, N., Des Marais, D.J., and Chang, S. (1984) Carbon isotopic composition of low molecular weight hydrocarbons and monocarboxylic acids from the Murchison meteorite. *Nature* 307(January), 252–242.

Zhang, C.L., Huang, Z., Cantu, J., Pancost, R.D., Brigmon, R.L., Lyons, T.W., and Sassen, R. (2005) Lipid biomarkers and carbon isotope signatures of a microbial (*Beggiatoa*) mat associated with gas hydrates in the Gulf of Mexico. *Appl. Environ. Microbiol.* 71(4), 2106–2112.

Zinoni, F., Birkmann, A., Stadtman, T.C. and Bock, A. (1986) Nucleotide sequence and expression of selenocysteine-containing polypeptide of formate dehydrogenase (formate-hydrogen-lyase-linked) from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* 83(13), 4650–4654.

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AU1
Shortened to ≤45 spaces.

AU2
Provide heading

QU1
Should [4] be changed to [5]?