J Forensic Sci, Sept. 2004, Vol. 49, No. 5
Paper ID JFS2003226
Available online at: www.astm.org

Helen W. Kreuzer-Martin,[1] *Ph.D.; Lesley A. Chesson,*[1] *B.S.; Michael J. Lott,*[1,2] *B.S.; Janet V. Dorigan,*[3] *Ph.D.; and James R. Ehleringer,*[1,2] *Ph.D.*

Stable Isotope Ratios as a Tool in Microbial Forensics—Part 1. Microbial Isotopic Composition as a Function of Growth Medium*

ABSTRACT: The stable isotope ratios of a seized pathogen culture could potentially reveal information about the environment in which the agent was produced. In this paper we describe general relationships between stable isotopes of carbon, nitrogen, and hydrogen in bacteriological culture media and spores of *Bacillus subtilis*, an endospore-forming soil bacterium. In numerous media that varied both in nutrient composition and water stable isotope ratios, medium to spore enrichment in carbon isotopes was 0.3 ± 2.0‰ (parts per thousand), and in nitrogen, 4.5 ± 0.7‰. We achieved mass balance for the contribution of hydrogen isotopes from nutrients (70%) and water (30%) to spores in independent experiments by varying the isotope ratios of nutrients or water. A model was derived for predicting the isotope ratio values of spores from those in nutrients and water.

KEYWORDS: forensic science, stable isotopes, isotope ratio mass spectrometry (IRMS), *Bacillus subtilis*, spores, bacteria, biological agents, bioweapons, bioterrorism

Organisms record aspects of their growth environments in the stable isotope ratios of their organic compounds. Studies of the relationship between stable isotopes of carbon and nitrogen in the diets of various animals and in their tissues have established that isotope ratios in a consumer are a function of diet (1–3). The physiological differences between the photosynthetic pathways of C_3 and C_4 plants, which result in large differences in carbon isotope ratios, allow one to trace the flow of organic carbon as differential dietary inputs (4), to reconstruct paleodiets (5,6) and to follow the transport of carbon across ecosystems (7). The characteristic enrichment of nitrogen isotope ratios from one trophic level to the next has been used to trace food webs (8,9).

Factors such as continentality, storm-track trajectories, moisture origins, and average temperatures impart strong gradients to the oxygen and hydrogen stable isotope ratios of precipitation and, therefore, of local waters (10), causing these isotope ratios to carry a strong geographic signature. Because the stable isotope composition of local waters is recorded in the oxygen and hydrogen isotope ratios of plant cellulose (11,12) and in the bones, blood and tissues of animals (13–15), stable isotope ratio analysis has been used to trace the origins of migratory populations of birds (16,17), butterflies (18), and elephants (19,20). The sourcing application of stable isotope ratio analysis has also been used to address forensic questions such as determining the point-of-origin of illicit drugs (21,22) and adulteration of food and beverages (23–25).

Since the anthrax attacks of 2001, the need for methods to trace the origins of microbial agents has become even more urgent. Stable isotope ratio analysis of pathogens could presumably reveal information about the conditions in which the agent was produced, complementing information gained from genotype analysis. In the event that a sample of a suspect agent, such as bacterial spores, was seized, it would be highly desirable to extract all possible information that could link the agent to its producers. We recently showed that stable isotope ratios of oxygen and hydrogen in bacterial spores can potentially provide geolocation information by linking spores to the water in which they were grown (26).

The nutrient medium in which bacteria are produced provides another essential component of their growth environment. To fully exploit stable isotope information that may be present in a sample such as spores, it is necessary to understand (1) general rules governing the relationship between stable isotopes in growth media and spores, and (2) stable isotopic variation in microbiological growth media. We have therefore undertaken a two-pronged investigation in which we have explored both of these issues. Here we report our initial investigations into the relationship between stable isotope ratios in nutrient media and bacterial spores, using *Bacillus subtilis* as a model system. We report our study of stable isotope variation in over 500 samples of microbiological media and components in a companion paper (27).

Methods

Our experimental organism was *Bacillus subtilis* strain 6051 (ATCC, Rockville, MD). Cultures were grown in the following media: Schaeffer's Sporulation Medium (28) made with Difco and Oxoid Nutrient Broth mixes, SSM + 0.2% glucose made with Difco and with Oxoid Nutrient Broth mixes; Luria Broth with added SSM salts, and the following media mixtures, each with a total of 8 g media components in the following ratios per liter and with added SSM salts: 25:75, 50:50, and 75:25, respectively, of (1) beef extract

[1] Stable Isotope Ratio Facility for Environmental Research, 257 S. 1400 E., Department of Biology, University of Utah, Salt Lake City, Utah 84112.
[2] IsoForensics, Inc., Salt Lake City, Utah.
[3] Central Intelligence Agency, Washington, DC 20505.

* The research described in this paper was supported by the Central Intelligence Agency of the United States of America. Portions of the results described in this paper were presented in poster form at the 2003 General Meeting of the American Society of Microbiology, Washington, DC.

Received 29 June 2003; and in revised form 28 March 2004; accepted 28 March 2004; published 4 Aug. 2004.

TABLE 1—*Hydrogen atoms of culture medium nutrients undergo no apparent exchange with medium water during autoclaving or subsequent storage. Confidence intervals of measurements are twice the average standard deviation of repeated measurement of standard water or medium samples (see Methods).*

	δD, ‰			
			Lyophilized Medium Solids, ±6‰	
Medium Water	Water, ±2.4‰	Medium Powder, ±6‰	1 Day	69 Days
Water 1	−124	−92	−95	−100
Water 2	17	−92	−95	−100
Water 3	145	−92	−95	−100
Water 4	271	−92	−95	−97

and peptone (an enzymatic digest of meat protein), (2) beef extract and tryptone (an enzymatic digest of casein), (3) yeast extract and peptone, and (4) yeast extract and tryptone. SSM contained 8 g Difco or Oxoid Nutrient Broth powder per liter plus the following volumes of solutions: 10% KCl, 10 mL; 1.2% $MgSO_4$ heptahydrate, 10 mL; 1 M NaOH, 1 mL; 1 M $Ca(NO_3)_2$, 1 mL; 0.01 M $MnCl_2$, 1 mL; 1 mM $FeSO_4$, 1 mL.

To make the media, the nutrient broth powder was dissolved in 976 mL water, the KCl, $MgSO_4$, and NaOH solutions were added, and the medium was autoclaved. The autoclaved solution was cooled to approximately 50°C and the remaining sterile solutions were added. The same salt solutions were used to make all the media, even when the water was varied. For SSM with 0.2% glucose, 8 mL of sterile 25% glucose was also added after autoclaving. To make Luria Broth with SSM salts, 10 g tryptone, 5 g yeast extract, and 10 g NaCl were dissolved in 976 mL water, and salts were added as described above for SSM. Samples of prepared culture medium were taken before inoculation of cultures and lyophilized, and the stable isotope ratios of carbon, nitrogen, oxygen, and hydrogen in the recovered powder were determined as described.

We prepared four containers of water of different oxygen and hydrogen isotope ratios by adding various amounts of D_2O and $H_2{}^{18}O$ to local deionized water. We used waters from these containers throughout our experiments. The containers had airtight lids and were kept in the cold room. Isotope ratios of the four waters are listed in Table 1.

Cultures were grown in triplicate in 100 mL media in 1 L flasks or 50 mL media in 500 mL flasks at 37°C and aerated by shaking at 200–225 rpm. Spore cultures were grown for 48 h, and sporulation was confirmed by microscopic examination of wet mounts under phase contract. Spore cultures were centrifuged for 10 min at 8000 rcf, resuspended in 1/4 volume of culture water, recentrifuged, and resuspended in 1/5 volume of culture water (20 mL for a 100 mL culture). Spores were purified by shaking in water for at least one week, centrifuging each day for 20 min at 20,000 rcf and resuspending the resulting pellet (29). Finally, the spore pellet was resuspended in 1 mL water, frozen, and lyophilized for analysis.

Stable Isotope Ratio Analysis

Stable isotope ratios are measured relative to internationally recognized standards. We calibrate laboratory standards to the international standards, and then include the laboratory standards as internal standards in every run. Stable isotope contents are expressed in "delta" notation as δ values in parts per thousand (‰), where $\delta‰ = (R_A/R_{Std} - 1) \cdot 1000‰$, and R_A and R_{Std} are the molar ratios of the rare to abundant isotope (e.g., $^{13}C/^{12}C$) in the sample and the standard. The standard used for both oxygen and hydrogen is Vienna Standard Mean Ocean Water [VSMOW (30)]. The standard for carbon is Peedee Belemnite [VPDB (30)], a fossil limestone from South Carolina, and the standard for nitrogen is air.

For C and N stable isotope analysis, 2 mg ± 10% organic samples (growth media or spores) were weighed and placed into tin capsules. Carbon and nitrogen isotope ratios of each sample were determined on a Finnigan-MAT Delta S isotope ratio mass spectrometer (IRMS, Bremen, Germany) interfaced with an Elemental Analyzer (Model 1108; Carla Erba, Milan, Italy). Growth medium samples were analyzed in duplicate, while spore samples were analyzed in triplicate and the results averaged. Our average standard deviations of repeated measurements of the same microbial growth media and spore samples, made at different times over a year or more, were 0.2‰ for carbon and nitrogen isotope ratios. Confidence intervals for measurements of individual samples are reported as ×2 these standard deviations, or 0.4‰.

For H stable isotope analysis, 150 μg ± 10% spore samples or 200 μg ± 10% medium samples were weighed and placed into silver capsules, which had been treated to remove silver oxide. Hydrogen isotopic composition of each sample was determined on a ThermoFinnigan-MAT Delta Plus XL isotope ratio mass spectrometer (IRMS, Bremen, Germany) equipped with a Thermo Chemical Elemental Analyzer (ThermoFinnigan-MAT, Bremen Germany) and a zero blank auto sampler (Costech Analytical, Valencia, CA). Growth media samples were analyzed in duplicate, while spore samples were analyzed in triplicate and the results averaged. The average standard deviations of repeated measurements of powdered growth media and spores, taken over the course of a year or more, was 3‰. Confidence intervals for measurements of individual samples are reported as ×2 this standard deviation, or 6‰.

Hydrogen isotope ratios of water samples were obtained by reducing the hydrogen in 2 μL of water to H_2 using 100 mg of Zn reagent (University of Indiana) in a Pyrex tube at 500°C (31). The resulting hydrogen gas was analyzed on a Finnigan-MAT Delta S IRMS (Bremen, Germany) equipped with a dual inlet. The standard deviation of repeated measurements of a standard water sample made over several months was 1.2‰.

Results

Carbon and Nitrogen Stable Isotopes

To investigate the relationship between stable isotope ratios of carbon and nitrogen in culture media and spores, we grew a minimum of three cultures of *B. subtilis* in 17 different nutrient media and allowed the cultures to sporulate. Stable isotope ratios of the culture media and the resulting spores were determined. Figure 1 shows a graph of $\delta^{13}C$ values of spores versus $\delta^{13}C$ values of their respective culture media, where each point represents the average of the triplicate cultures. The relationship between the carbon stable isotope ratios of spores and media is described by the equation

$$\delta^{13}C_{spores} = (0.94 \pm 0.08) \cdot \delta^{13}C_{media} - 0.9 \pm 1.6$$

The ranges of the slope and y-intercept values indicate the 95% confidence intervals of the two parameters.

A plot of the $\delta^{15}N$ values of spores versus the $\delta^{15}N$ of their respective media, again using average values from the triplicate cultures (Fig. 2), yielded the following equation describing the relationship between nitrogen stable isotopes in media and spores:

$$\delta^{15}N_{spores} = (0.92 \pm 0.15) \cdot \delta^{15}N_{media} + (4.8 \pm 0.7)$$

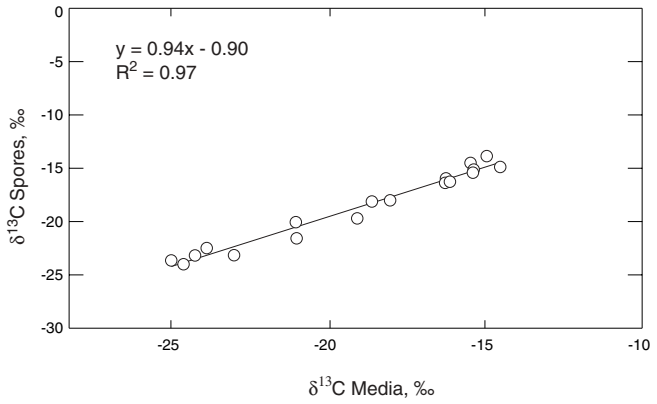

FIG. 1—*$\delta^{13}C$ values of spores vs. media. Spores were produced in 17 different liquid nutrient media. Each point is the average value of at least three cultures. Error bars representing one standard error of the mean are hidden within the data points.*

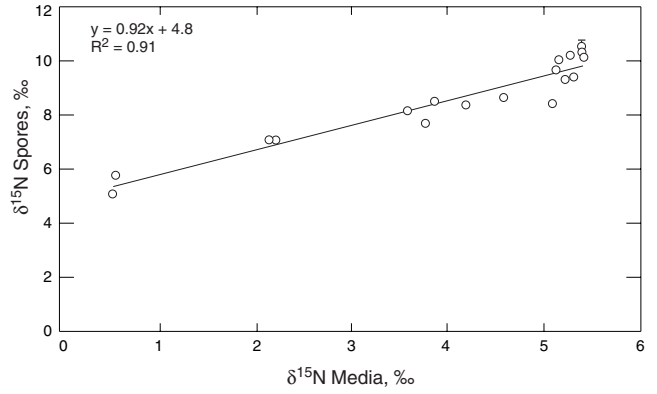

FIG. 2—*$\delta^{15}N$ values of spores vs. media. Spores were produced in 17 different liquid nutrient media. Each point is the average value of at least three cultures. Error bars representing one standard error of the mean are hidden within the data points, with one exception (shown).*

The ranges again indicate the 95% confidence intervals of the slope and y-intercept.

A common manner of discussing the incorporation of stable isotopes of carbon and nitrogen from the diet of a heterotroph into its tissues or from one trophic level to the next in a food web [for example, see (32)] is in terms of diet-organism enrichment. When discussing diets, the enrichment for a particular isotope can be defined as

$$\delta_{\text{organism}} = \delta_{\text{diet}} + \Delta_{\text{do}}$$

where δ_{organism} is the particular stable isotope ratio in the organism, δ_{diet} is the ratio of that stable isotope in the diet, and Δ is the enrichment between diet and organism. This discussion framework assumes that the slope of the line relating δ_{organism} to δ_{diet} is 1.0 and the y-intercept is the value of Δ. In the case of our data, a slope of 1.0 is within the 95% confidence intervals of the lines relating both $\delta^{13}C$ and $\delta^{15}N$ in growth medium and microbes. If the value of the slopes of the regression lines is set to 1.0, the value of the intercepts and, hence, the value of Δ becomes 0.3‰ for carbon and 4.5‰ for nitrogen.

Hydrogen Stable Isotopes

Hydrogen atoms bound to oxygen or nitrogen as well as carbonyl oxygen atoms have been reported to exchange freely with atoms from water molecules (33,34). We showed previously that no exchange between isotopes in spores and environmental water occurs (26), but the growth media we used contained amino acids and oligopeptides, which we expected would undergo such exchange. To determine the extent of isotopic exchange during media preparation and storage, we prepared SSM medium in four isotopically different waters (Table 1). The medium samples were autoclaved for 25 min at 121°C and cooled; an aliquot from each was withdrawn and lyophilized, and the resulting powder analyzed. The sterile media were stored in bottles at room temperature for over two months and then sampled again. No apparent exchange of hydrogen isotopes occurred as a result of autoclaving or storage (Table 1). The isotope ratios measured on dry media powder therefore represent the isotope ratios of the nutrient molecules after media preparation.

In our earlier study, we found that spores of *B. subtilis* grown in SSM derived 28% of their hydrogen atoms from the water used to make the liquid culture medium (26). The percentage of atoms derived from water is denoted by the slope of the line relating δ_{spore} (y) to δ_{water} (x), where δ is the hydrogen stable isotope ratio in the water (see Eq 2). In these experiments, the growth medium was always constant and so its contribution to the stable isotope content of the spores would be reflected in the y-intercept of the above-described line. We anticipated two possible results upon changing the growth medium: (1) the slope of the spore versus water isotope line could change because the proportion of atoms originating from water or nutrients could change with different nutrient media, and (2) the y-intercept of the line could change because of different stable isotope ratios in the culture medium itself (see Discussion for an explanation of the significance of the slope and intercept values).

To investigate these possibilities, we prepared five different growth media in each of four isotopically different waters (Table 1). The growth media were SSM made with Difco Nutrient Broth mix (D-SSM), SSM made with Oxoid Nutrient Broth mix (O-SSM), D-SSM + 0.2% glucose (D-SSM + G), O-SSM + 0.2% glucose (O-SSM+G), and Luria Broth with SSM salts (LB). A minimum of three cultures of *B. subtilis* were grown in each of the 20 medium types and allowed to sporulate. Spores were harvested and analyzed for hydrogen, carbon, and nitrogen stable isotope ratios. The carbon and nitrogen stable isotope data from these cultures accounted for 5 of the 17 culture media in the carbon and nitrogen studies described above.

The slopes of the lines relating the δ^2H value of culture water to spores grown in the different media ranged from 0.27 to 0.33, and the y-intercept values ranged from −73.9 to −57.3‰ (Fig. 3, Table 2). The reason that the value of the y-intercept reported here for growth on D-SSM is slightly different from the values reported in our previous study (26) is that we grew several additional cultures on D-SSM for this study, and the isotope ratios of the spores from the additional cultures changed the average value of the intercept from −57.2 to −56.2‰. The 95% confidence intervals of the slopes of the two curves for the media with added glucose did not overlap the confidence intervals for the slopes of D-SSM and LB (Table 2 presents rounded values). The 95% confidence interval of the slope of the D-SSM + G curve barely overlapped the slope for O-SSM.

The other 12 growth media in which we grew spores were made with laboratory deionized water but different combinations of medium components. We made four series of two components: either beef extract or yeast extract, and either tryptone or peptone. Three proportions of each mixture were used: 75:25, 50:50, and 25:75. After the mixtures were made, we lyophilized a small aliquot of each and measured the C, N, and H isotope ratios of

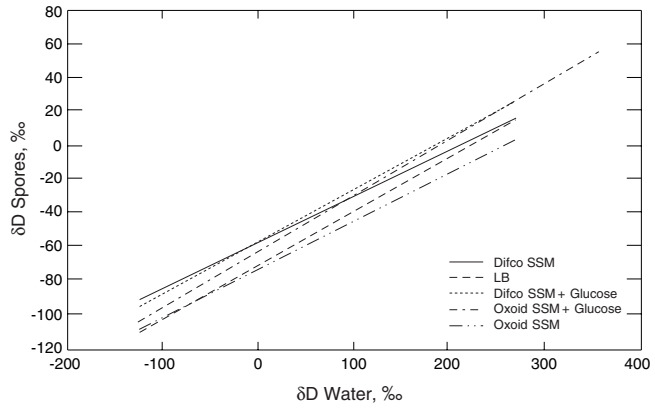

FIG. 3—*δ^2H(D) values of spores vs. culture water. Results shown are for spores produced in five different nutrient media, where each medium was made with four isotopically distinct waters (Table 1). Three cultures were grown in each nutrient-water mixture. Slopes, intercept values, and statistics of each line are presented in Table 2. The slopes of all the regression lines can be approximated as 0.3.*

TABLE 2—*Statistics of spore δ^2H vs. water δ^2H curves for spores grown in five different liquid growth media.*

Medium	Average Slope	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Difco SSM	0.28	0.25	0.30
Oxoid SSM	0.29	0.27	0.30
LB	0.32	0.29	0.35
Difco SSM + glucose	0.31	0.30	0.32
Oxoid SSM + glucose	0.33	0.32	0.35
AVERAGE y-INTERCEPT			
Difco SSM	−56.2	−59.7	−52.6
Oxoid SSM	−73.9	−76.5	−71.2
LB	−71.4	−75.5	−67.3
Difco SSM + glucose	−57.3	−58.9	−55.6
Oxoid SSM + glucose	−62.9	−65.7	−60.1

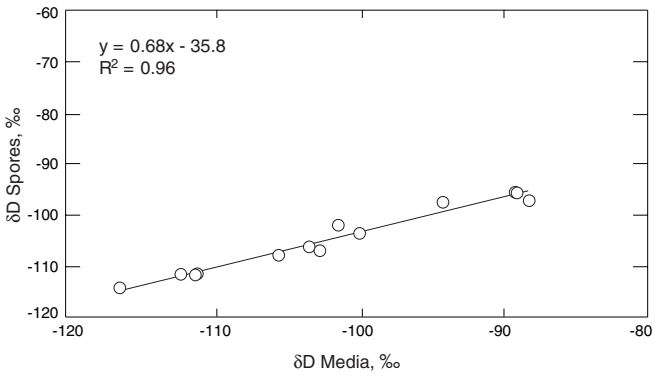

FIG. 4—*δ^2H(D) values of spores vs. medium nutrients. Each point represents the average value of three cultures. Error bars representing one standard error of the mean are hidden within the data points.*

the solids. We used each of the 12 different mixtures (4 series × 3 mixtures/series) to grow three independent cultures, harvested spores, and measured C, N, and H isotope ratios.

The results for carbon and nitrogen isotopes are presented in Figs. 1 and 2. Figure 4 shows the relationship between δ^2H of the media and δD of the resulting spores, where each point is the average of the three cultures grown on one medium mixture. The relationship between the δ^2H values for the 12 different media mixtures and the resulting spores is well described by the linear regression line on the graph.

Discussion

Carbon

The fractionation of carbon and nitrogen isotopes we observed between culture media and spores is consistent with fractionation between food source isotopes and consumer tissues in other heterotrophic systems, including mammals, birds, insects, and other invertebrates. DeNiro and Epstein (1) first showed that the δ^{13}C of a consumer is a function of the δ^{13}C of its diet, and they reported a slight enrichment from diet to consumer. Macko and Estep (35) studied enrichment of both carbon and nitrogen isotopes between diet and cells of the heterotrophic marine bacterium *Vibrio harveyi* grown on individual amino acids and amino acid-cell δ^{13}C differences ranging from < -5 to $> +9$‰. Field and laboratory studies with a variety of organisms, reviewed by Kelly (3), report either slight enrichments or depletions from diet to consumer, in the range of ±1.5‰. We observed a slight average enrichment in δ^{13}C from medium to spores. The preservation of carbon isotope ratios between trophic levels in a food chain has proven very useful in identifying the base of the chain itself, as there are distinct differences between δ^{13}C values of terrestrial C_3 plants, terrestrial C_4 plants, and marine plants (36–38).

From this study we can conclude that carbon isotope ratios measured in spores will similarly allow approximate determination of the carbon isotope ratio of the culture medium upon which they were grown. Carbon isotope ratios of commercially available bacteria culture media showed the same variation as δ^{13}C values of plants and animals (27), and so such a determination has potential utility for testing whether media seized from a suspect facility could have been used to produce seized spores.

Nitrogen

The average enrichment in δ^{15}N of about 4.5‰ observed in the spores with respect to their growth media is also generally consistent with what has been observed in other systems. DeNiro and Epstein (2) first documented an approximate 3‰ increase in δ^{15}N from diet to the whole body of consumers. Since then, field and laboratory studies support the generalization that an enrichment of about 3–4‰ occurs at each trophic level for higher animals (3).

Macko and Estep (35) observed nitrogen isotope fractionation in *V. harveyi* cultured on individual amino acid substrates and saw changes from < -12 to >22‰, depending on the amino acid substrate. Amino acids entering bacterial cells are deaminated and the ammonia fixed into glutamate to form glutamine and then passed to other amino acids by transamination (39). When an excess of nitrogen is present, ammonia is lost via deamination of glutamic acid. This deamination reaction yields a ^{15}N-depleted pool of ammonia, which is excreted (35). The glucose-free growth media we used had C/N ratios of about 3–3.5, while the C/N ratio of the spores was 3.9–4.1, indicating that the media contained an excess of nitrogen. Excretion of the excess nitrogen as isotopically depleted ammonia is a plausible explanation for the observed greater enrichment in δ^{15}N values.

The culture medium that showed the least enrichment in δ^{15}N from medium to spores was D-SSM + G, where Δ was only 3.1‰. The O-SSM + G spores had an enrichment of 4.1‰, still less than the average enrichment seen in the cultures grown on media without

glucose. Quick tests with glucose strips following growth of the organisms showed that these cultures had been completely depleted of glucose. It seems reasonable that the organisms consequently had to use less amino acid material for energy and thus had to excrete less nitrogen. Removing these cultures from the dataset used for the linear regression shown in Fig. 2 changed the slope from 0.92 ± 0.15 to 0.95 ± 0.13 and narrowed the confidence interval of the y-intercept from ± 0.7 to ± 0.6, neither being a significant change.

Hydrogen

Unlike atoms of carbon and nitrogen found in spores, which could only originate in the culture medium nutrients, hydrogen atoms could originate from either the nutrients or the water used to make the medium. Although we observed no isotopic exchange between nutrient molecules and water during media preparation and storage (Table 1), such exchange could occur when the nutrients undergo biochemical processing by the bacteria. Since the strength of bonds formed by different isotopes of the same element vary, two isotopes of the same element do not react at the same rates during exchange, and a net change in the ratio of the two isotopes is seen when ratios before and after exchange are compared. Similarly, different isotopes of the same element react at different rates in enzyme-mediated reactions even if no exchange with water is involved. The changes in isotope ratios after exchange with water or after anhydrous enzyme-mediated reactions can be described in terms of fractionation effects.

The relationship between isotopes in water, organic substrates, and synthesized organic material is described as

$$\delta_{\text{cellulose}} = n(\delta_{\text{water}} + \varepsilon) + (1 - n)\delta_{\text{NE}} \quad (1)$$

where $\delta_{\text{cellulose}}$, δ_{water} and δ_{NE} are the δ^2H values of synthesized organic matter, water, and the nonexchangeable stable isotopes of the substrate, respectively; n is the proportion of hydrogen derived from medium water during organic biosynthesis, and ε is the isotope fractionation effect for enzyme-mediated exchange or addition (34,40). Equation 1 was originally applied to studies of the synthesis of a single product (cellulose) from a single substrate via a pathway of limited, defined steps and assuming the atoms that were not exchangeable with water were not otherwise fractionated by biosynthetic enzymes. In considering the aggregate biosynthesis of all cellular products in a microbe from a complex medium, we must assume that even H atoms unavailable for exchange with water might undergo enzymatic reactions in which isotope fractionation could occur. If hydrogen or oxygen isotopes in the substrate molecules undergo enzyme-mediated rearrangement without being available for exchange with water, then we add the fractionation factor ε_{NE} to the equation to account for that fractionation:

$$\delta_{\text{microbe}} = n(\delta_{\text{water}} + \varepsilon_{\text{E}}) + (1 - n)(\delta_{\text{media}} + \varepsilon_{\text{NE}}) \quad (2)$$

Rearranging this equation gives the form

$$\delta_{\text{microbe}} = n\delta_{\text{water}} + n\varepsilon_{\text{E}} + (1 - n)\delta_{\text{media}} + (1 - n)\varepsilon_{\text{NE}} \quad (3)$$

For the data shown in Fig. 3, we varied the water isotope ratios and kept the media constant for each medium tested. Thus for each series of experiments with one medium, n, ε_{E}, ε_{NE}, and δ_{media} are constant, yielding the form $y = mx + b$, where the slope = n and the value of the intercept is given by the expression

$$b = n\varepsilon_{\text{E}} + (1 - n)\delta_{\text{media}} + (1 - n)\varepsilon_{\text{NE}} \quad (4)$$

The slopes of the lines relating spore δ^2H values to water δ^2H values (Fig. 3) show that, regardless of the nutrient medium tested, about 30% of the hydrogen atoms in the spores underwent exchange with water. The confidence intervals for the slopes of the cultures grown with glucose show a statistical difference from those of the same media without glucose (D-SSM and O-SSM), but again the numbers are based on small numbers of cultures grown with glucose. The findings that about 30% of the hydrogen atoms in the spores were derived from water is similar to the findings of Horita et al. (41), who measured isotope ratios of single cultures of *Bacillus globigii* and *Erwinia agglomerans* grown in one medium made with three isotopically different waters. They estimated that 26 and 28% of the hydrogen atoms in the two organisms, respectively, came from culture water. Similarly, Hobson et al. (42), who studied the contribution of hydrogen isotopes in food and drinking water to the hydrogen isotope ratios of various tissues in quail, found about 20–30% of the hydrogen atoms in the quail tissues were derived from drinking water. Sharp et al. (43), in a deuterium-enriched drinking study, found that approximately 31% of the hydrogen atoms in human hair were derived from ingested water.

If about 30% of the hydrogen atoms in spores are derived from water used to make the culture medium, then the remaining 70% of the hydrogen atoms must be derived from the nutrient molecules. Figure 4 shows the results of experiments in which the composition of the culture medium was varied and the water held constant. In this graph, the value of x is the hydrogen isotope ratio of the culture medium; the slope of the line represents the fraction of spore hydrogen atoms derived from nutrients $(1 - n)$, and the value of the y-intercept is given by the expression

$$b = n\varepsilon_{\text{E}} + n\delta_{\text{water}} + (1 - n)\varepsilon_{\text{NE}} \quad (5)$$

The slope of the line $(1 - n)$ in Fig. 5 is 0.68, with a 95% confidence interval of ± 0.07, accounting for the remaining 70% of the hydrogen atoms and achieving a mass balance for hydrogen in spores.

Multivariate Model for Predicting Spore Hydrogen Isotope Ratios

If we could be reasonably certain that the values of ε_{E} and ε_{NE} were the same for each medium tested, and that the overall δ_{media} measured for the media powder represented the isotope ratios of the nutrients actually used by the bacteria during growth, then we could combine data from experiments in which the water was varied (Fig. 3) with data from experiments in which the media was varied (Fig. 4) to build a model for predicting spore isotope ratios from water and media isotope ratios. The data suggest that we can make these above assumptions for hydrogen isotopes. All of the points relating the hydrogen isotope ratio of culture media powder to the hydrogen isotope ratio of spores grown on the various media fall onto a single line (Fig. 4). The most parsimonious explanation for this result is that (1) the measured δ^2H value of the media reflects what the organisms absorb, and (2) the isotope fractionation effects of *B. subtilis* growing on the different media are the same or very close to the same. If the isotope fractionation effects varied, or the measured δ^2H of the media powder did not consistently reflect what the organisms absorb, we would expect to see different y-intercept values or slopes for different media mixtures (Eqs 2–5).

The nutrient mixtures were composed of combinations of yeast extract, beef extract, peptone, and tryptone. We had previously grown spores in nutrient media made from isotopically different waters and combinations of these nutrients: D-SSM (beef extract and peptone), O-SSM (beef extract, yeast extract, and peptone), and LB (yeast, extract and tryptone). In each case, some of the cultures were grown on deionized water from our laboratory (Water 1, Table 1) and thus could be compared to cultures grown in the

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Additional information and reprint requests:
Helen W. Kreuzer-Martin, Ph.D. or James R. Ehleringer, Ph.D.
Department of Biology, University of Utah
257 South 1400 East, Rm 201
Salt Lake City, UT 84112-0840
E-mail: kreuzer@biology.utah.edu or ehleringer@biology.utah.edu