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# Stable Isotope Ratios as a Tool in Microbial Forensics—Part 2. Isotopic Variation Among Different Growth Media as a Tool for Sourcing Origins of Bacterial Cells or Spores*

**ABSTRACT:** Since the anthrax attacks of 2001 the need for methods to trace the origins of microbial agents has become urgent. The stable isotope ratios of bacteria record information from both the nutrients and the water used to make their culture media (1) and could potentially be used to provide information about their growth environment. We present a survey of carbon (C), nitrogen (N), and hydrogen (H) stable isotope ratios in 516 samples of bacteriological culture media. The observed variation was consistent with expected isotopic variation in the plant and animal products upon which the media are based. The variation is sufficient to translate into substantial isotope variation in cultures grown on different batches of media, and thus to allow investigators to determine whether seized media could have been used to produce seized bioweapons agents.

**KEYWORDS:** forensic science, isotope ratio mass spectrometry (IRMS), stable isotopes, bacterial cultures, culture media, peptones, carbohydrates, yeast extract, bioterrorism, biological agent, bioweapons

Both plants and animals record aspects of their growth environment in the stable isotope ratios of their organic compounds. Studies of the relationship between stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) in the diets of various animals and in their tissues have established that consumer isotope ratios are a function of diet (2–4). The physiological differences between $C_3$ and $C_4$ photosynthetic pathways, which result in large differences in $\delta^{13}C$ values, allow one to trace the flow of organic carbon as differential dietary inputs (5), to reconstruct paleodiets (6,7) and to follow the transport of carbon across ecosystems (8). The characteristic enrichment of nitrogen isotope ratios from one trophic level to the next has been used to delineate food webs (9,10). Similarly, the stable isotope composition of local waters is recorded in the oxygen isotope ratios ($\delta^{18}O$) of plant cellulose (11,12) and in the bones, blood, and tissues of animals (13–15). Thus the stable isotope ratios of tissues can reveal information about the growth environment of the organism.

Since the anthrax attacks of 2001 in the United States, the need for methods to trace the origins of microbial agents has become even more urgent. In the event that a sample of a bioterror agent such as the anthrax spores is seized, stable isotope ratio analyses could reveal information about the conditions under which the agent was produced. We recently showed that stable isotope ratios of oxygen and hydrogen in spores can provide geolocation information by associating spores with the water in which they were grown (1).

The nutrient medium in which bacteria are produced provides another essential component of their growth environment. We hypothesized that bacterial spores would contain an isotopic record of their growth medium in addition to that of the water used to prepare the medium. We have undertaken a two-pronged investigation into the relationship between stable isotopes of C, N, and H in bacteriological growth media and spores produced on this medium. One aspect of our investigation consisted of experiments to explore the relationship between stable isotope composition of medium and resulting spores. We describe these relationships in a companion paper. The second aspect of our investigation was a survey of the C, N, and H stable isotope composition of bacteriological growth media. In this, the second of two papers, we report the results of our investigations of isotopic variability in over 500 samples of commercially available bacteriological culture media.

## Methods

### *Samples*

We obtained our samples by direct purchase or from colleagues at the University of Utah, Brigham Young Univeristy, Los Alamos National Laboratory, Lawrence Livermore National Laboratory, Dugway Proving Ground, Wake Forest University, Elon University, the University of North Carolina at Charlotte, the University of North Carolina at Greensboro, North Carolina State University, and Duke University.

### *Stable Isotope Ratio Analysis*

Stable isotope ratios are measured relative to internationally recognized standards. We calibrate laboratory standards to the

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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 (16)]. The standard for carbon is Peedee Belemnite [VPDB (16)], a fossil limestone from South Carolina, and the standard for nitrogen is air.

For carbon and nitrogen isotope ratio analysis, 2 mg ± 10% media samples 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). 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 isotope ratio and 0.2‰ for nitrogen. Confidence intervals for measurements of individual samples are reported as ×2 these standard deviations, or 0.4‰.

For hydrogen stable isotope analysis, 200 μg ± 10% medium samples were weighed and placed into silver capsules, which had been previously baked at 500°C 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) interfaced with a Thermo Chemical Elemental Analyzer (ThermoFinnigan-MAT, Bremen, Germany) operated at 1425°C and a zero blank auto sampler (Costech Analytical, Valencia, CA). All samples were analyzed in duplicate 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‰.

## Results

No single nutrient medium will support the growth of all types of bacteria, and literally thousands of media formulations have been developed for varying purposes and microorganisms (17). In general, however, growth media contain the following elements: (i) an amino nitrogen source such as a protein hydrolysate or an infusion, (ii) a growth factor source such as blood, serum, or yeast extract, and (iii) an energy source, usually a sugar or other carbohydrate. Bacteria can use amino acids as sources of energy and carbon, and many common growth media do not contain added carbohydrates or sugars. Salts, trace metals, buffering agents, and selective agents can also be included. Since our interest was directed at stable isotopes of carbon, nitrogen and hydrogen in growth media and, in a companion report (18), their relationship to the corresponding stable isotope ratios in bacteria grown upon the media, we focused on the organic components of media.

A few types of organic medium components are combined in various proportions and combinations to make the backbones of a wide variety of growth media. These include the following components.

### *Protein Hydrolysates, Also Called Peptones*

The term peptone refers to water-soluble protein hydrolysates. The sources of protein for the hydrolysates are most commonly meat, casein (the major protein in milk), or soya. Manufacturers use various names to refer to milk, meat, and soya peptones, and the generic term peptone is sometimes used to refer to peptone from meat. Peptones are made by forming a slurry of the protein and water, digesting the protein either with enzyme (usually trypsin, pepsin, or papain) or mineral acid, then purifying and drying the resulting solution. Peptones typically contain a combination of free amino acids and oligopeptides, up to a molecular weight of about 6000 D (17).

### *Extracts and Infusions*

Extracts and infusions are made by extracting a raw material (e.g., yeast, beef) with water. No hydrolysis is involved. These medium components typically have low levels of peptides but high levels of vitamins, trace metals, and complex carbohydrates. An extract or infusion is often combined with a peptone in a given culture medium.

### *Agar*

Agar, a salt or mixture of salts of anionic polysaccharides, is used as a gelling agent. It is obtained as an aqueous extract from seaweeds of the class Rhodophyceae, typically of the genera *Gelidium*, *Gracilaria*, and *Pterocladia*. These seaweeds are found in intertidal zones to depths of more than 40 m at certain latitudes around the world. Agar is insoluble in cold water but soluble in boiling water, and forms a firm gel as it cools. Agar is typically added to culture media at concentrations of 1.2–1.5% wt/vol to solidify them. One reason agar is especially valuable as a gelling agent for culture media is that very few bacteria can metabolize it. Confusingly, culture media that have been solidified with agar are often referred to colloquially as "agars."

Because so many growth media are combinations of peptones and extracts, with optional carbohydrates, we focused on these groups of medium components in our sample collection. In addition, we made a point to collect samples of a few common, pre-mixed general growth media. These media were (1) nutrient broth, (2) Luria broth, (3) brain-heart infusion broth, and (4) tryptic soy broth. The exact formulations of these broth mixes can vary by supplier. In general, nutrient broth is composed of meat peptone and beef extract, and some suppliers also add yeast extract. Luria broth is composed of an enzymatic hydrolysate of casein (tryptone) and yeast extract. Brain-heart infusion broth contains infusion of the named organs, meat protein hydrolysate, and glucose. Tryptic soy broth contains hydrolysate of soya, hydrolysate of casein, and glucose. Altogether, we collected and analyzed 516 different samples.

In our discussion, we distinguish between manufacturers and suppliers. Many companies sell microbiological media with their own brand labels on the containers, but quite a few of those companies do not manufacture the components of those media. It is possible that medium components sold under several different labels were actually manufactured by the same producer. We use the term manufacturer to refer to whatever entity obtained the raw materials (meat, milk, etc.) and processed them. We use the term supplier to refer to the company that sold the final product. In between manufacturing and supplying is the important process of compounding, and many companies that do not manufacture peptones or infusions do compound their own media. Since sources of raw material can be viewed as confidential industrial information, it is not easy to determine where the components in a particular medium were actually produced. We necessarily refer to all companies whose media we analyzed as suppliers, though some of them also manufacture at least some of their components.

The number and types of media samples collected and analyzed are shown in Table 1. Of these, 310 bore the label of a single

TABLE 1—*Samples in medium collection.*

| Sample Type | Number of Samples |
|---|---|
| Agar | 68 |
| Carbohydrate | 55 |
| Casein peptones | 75 |
| Meat peptones | 46 |
| Yeast extracts | 37 |
| Brain-heart infusion broth | 20 |
| Luria broths | 16 |
| Nutrient broths | 25 |
| Tryptic soy broths | 28 |
| Other | 146 |

TABLE 2—*Distribution of samples by supplier.*

| Supplier | Number of Samples in Collection |
|---|---|
| Difco | 310 |
| BBL | 48 |
| Sigma | 42 |
| Fisher | 27 |
| Becton Dickinson | 21 |
| EM Science | 18 |
| Oxoid | 9 |
| Hardy Diagnostics | 8 |
| 15 other suppliers | Fewer than five samples each |

TABLE 3—*Carbon, nitrogen, and hydrogen delta value averages and ranges for various medium categories. The 95% confidence intervals for measurements of $\delta^{13}C$ and $\delta^{15}N$ in media is ±0.4‰, and for $\delta^{2}H$, ±6‰.*

| | $\delta^{13}C$ | | $\delta^{15}N$ | | $\delta^{2}H$ | |
|---|---|---|---|---|---|---|
| Category | Average | Range | Average | Range | Average | Range |
| Agar | −18.49 | −24.90 to −14.89 | n/a | n/a | −73.8 | −94.0 to −58.2 |
| Carbohydrate | −13.94 | −27.09 to −8.95 | n/a | n/a | −28.0 | −126.9 to 22.8 |
| Casein peptones | −23.49 | −28.10 to −12.44 | 6.22 | 4.80 to 8.75 | −102.4 | −116.7 to −57.2 |
| Meat peptones | −15.05 | −22.31 to −12.50 | 6.55 | 5.41 to 8.15 | −81.4 | −105.1 to −43.4 |
| Yeast extract | −24.14 | −25.37 to −20.46 | −0.15 | −1.17 to 4.05 | −114.42 | −121.1 to −96.9 |
| Brain-heart infusion broth | −17.65 | −24.58 to −15.89 | 5.57 | 4.81 to 6.00 | −94.5 | −110.6 to −81.1 |
| Luria broth | −25.29 | −26.60 to −20.64 | 3.99 | 3.12 to 4.67 | −111.1 | −118.6 to −94.5 |
| Nutrient broth | −16.74 | −23.59 to −12.08 | 5.34 | 1.9 to 6.60 | −87.3 | −103.1 to −64.1 |
| Tryptic soy broth | −22.82 | −26.55 to −13.53 | 5.19 | 3.49 to 7.83 | −101.7 | −118.4 to −88.0 |

supplier, no doubt a reflection of the purchasing habits of research scientists in the United States. Distribution of the samples by supplier is reported in Table 2. The average isotope ratio values and ranges of values for stable isotopes of carbon, nitrogen, and hydrogen of these groups of components and common growth media are presented in Table 3.

## Discussion

### *Expectations*

Microbiological media are made from materials derived from plants, animals, and fungi, and we expected to see variability in media isotope ratios as a function of variability in the source materials. A fundamental source of isotope variability in biological material is the physiological difference between $C_3$ and $C_4$ plants, which results in distinct $\delta^{13}C$ values in their organic molecules. These differences are incorporated into the tissues of animals that eat $C_3$ and $C_4$ plants, respectively.

In considering the potential isotopic variability of $^{13}C$ in culture media, we expected that plant-derived carbohydrates would show a clear $C_3$ or $C_4$ signature depending on their source (19). For example, glucose made from corn would have a $C_4\delta^{13}C$ signature while wheat starch would have a $C_3\delta^{13}C$ signature. We expected that the $\delta^{13}C$ value of animal products such as meat peptones, caseins, and lactose would depend on the proportion of $C_3$ and $C_4$ plants in the diet of the meat or dairy animal. The proportion of $C_3$ and $C_4$ in an animal's diet might well be a function of where the animal was raised, with animals from warmer regions being more likely to have eaten more $C_4$ plants.

Nitrogen isotope ratios in plants are a function of plant physiology, soil fertility, and growth environment. Factors such as whether the plant roots are nodulated by nitrogen-fixing bacteria, whether artificial fertilizer was applied to the plants, and the nature of other environmental sources of nitrogen all influence the nitrogen isotope ratio in the plant (20,21). The $\delta^{15}N$ value of animal tissues is a function of diet, not only in terms of the $\delta^{15}N$ values of the dietary materials, but also how much nitrogen an animal consumed relative to its needs, since isotope fractionation occurs during elimination of excess nitrogen (22). Given that our collection contained a large number of animal products, we expected variation in $\delta^{15}N$ values but not predictable patterns.

The hydrogen isotope ratios in plant and animal tissues are a function of the isotope ratios of the water the organism used during its growth and humidity during growth (23). In trees, for example, the isotopic composition of cellulose has been shown to correlate with that of source water (11,12). Similarly, the oxygen and hydrogen isotope composition of blood and other tissues has been correlated with that of local water (13,24). The isotope composition of precipitation and surface waters bears a strong geographic signature as a consequence of continentality, storm-track trajectories, and moisture origins, and even within the same geographic region, different plants may use deep soil water, shallow soil water, or even fog as a water source (25,26). Thus we anticipate $\delta^{2}H$ variability in plant and animal products, with a potential geographic signature from the contributing water isotopes.

Manufacturing processes should also play a part in $\delta^{2}H$ values of dehydrated culture media. Many of these products are hydrolysates, and the hydrogen atoms added during the hydrolysis products would be derived from the water in which the process was carried out. Many of these atoms would be expected to undergo exchange with water as soon as the powder was dissolved, though we have not observed such exchange in our experiments with nutrient broth powder (1). Hydrogen atoms added as a result of manufacturing could extinguish any geographic signature associated with these elements in the original plant or animal.

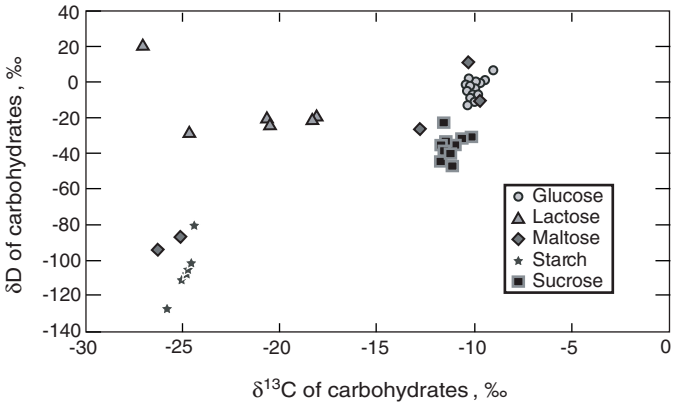


FIG. 1—*Carbohydrates, $\delta^2H$ (D) vs. $\delta^{13}C$. The $\delta^{13}C$ values of the plant-derived carbohydrates reflect whether they were derived from $C_3$ or $C_4$ plants. The $\delta^{13}C$ values of the lactose samples reflect the diets of the animals that produced the milk from which the samples were derived. The 95% confidence interval for measurements of $\delta^2H$ in media is ±6‰ and for $\delta^{13}C$, ±0.4‰.*

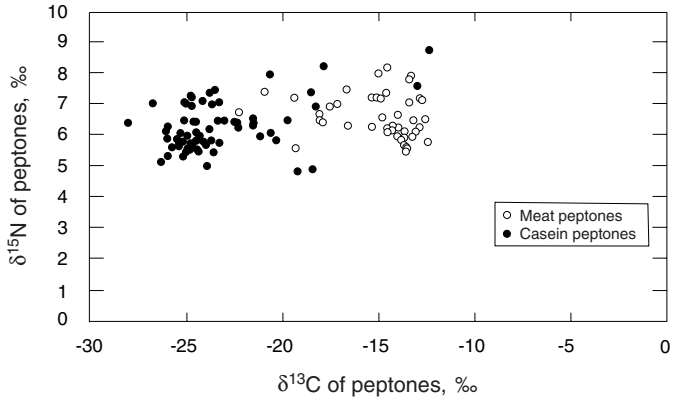


FIG. 2—*Casein and meat peptones, $\delta^{15}N$ vs. $\delta^{13}C$. The $\delta^{13}C$ values of both milk- and meat-derived peptones reflect the relative contribution of $C_3$ and $C_4$ plants to the animals' diets. The 95% confidence interval for measurements of $\delta^{13}C$ and $\delta^{15}N$ in media is ±0.4‰.*

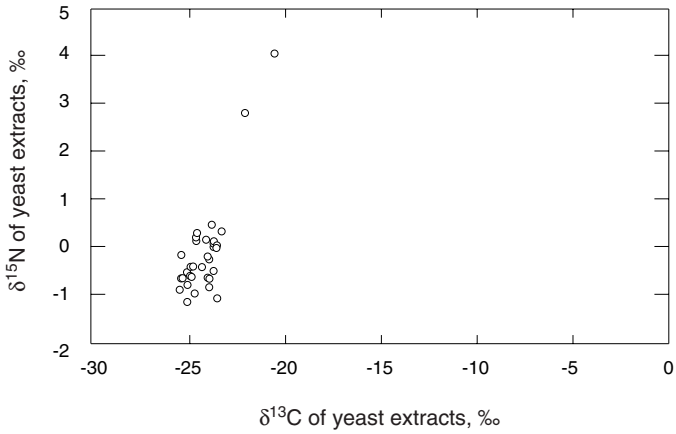


FIG. 3—*Yeast extracts, $\delta^{15}N$ vs. $\delta^{13}C$. The similarity of most samples analyzed suggest that many suppliers may obtain yeast extract from the same manufacturer. The two unique values each represent the only sample available from a particular supplier. The 95% confidence interval for measurements of $\delta^{13}C$ and $\delta^{15}N$ in media is ±0.4‰.*

### *Carbohydrates*

As expected, the $\delta^{13}C$ value of plant-derived carbohydrates both varied with and revealed $C_3$ and $C_4$ sources. Glucose is potentially derived from $C_4$ plants (usually corn) or $C_3$ plants (grapes, sugar beet), just as sucrose could have $C_4$ (sugar cane) or $C_3$ (sugar beet) origins. All 20 of the glucose samples we analyzed were clearly from $C_4$ sources, as were the 12 sucrose samples (Fig. 1). The glucose samples were supplied by five different companies, one European, as were the sucrose samples. The six samples of starch we analyzed were all derived from $C_3$ sources. One of these samples indicated its source as wheat but the others were labeled only as soluble starch. Although all the plant carbohydrates we analyzed could potentially have had $C_3$ or $C_4$ origins, only the maltose samples showed source variability. Maltose is produced by the hydrolysis of starch. Of the five maltose samples we analyzed, two were derived from $C_3$ sources and three from $C_4$ plants. Interestingly, one company supplied three of the samples, the two derived from $C_3$ plants and one of the $C_4$ samples. The $\delta^{13}C$ values of the six samples of the milk sugar lactose showed a range from –18.3‰ to –27.5‰, reflecting varying contributions of $C_3$ and $C_4$ plants to the animals' diets.

Hydrogen isotope ratios of the glucose samples showed little variability, suggesting that the glucose samples were all manufactured from corn from very similar regions. There was more variability in the hydrogen isotope ratios of the sucrose samples, suggesting that the sugar cane itself may have come from different places. The hydrogen isotope ratios of the starch samples were much lower than those of the glucose and sucrose samples, consistent with the source plants having been grown in a cooler, drier climate. The $C_3$ and $C_4$-derived carbohydrates had distinctly different $\delta^2H$ values, but the $\delta^2H$ values of the animal-derived lactose resembled those of the $C_4$-derived carbohydrates even when the $\delta^{13}C$ value of the lactose indicated that the animal's diet was almost solely composed of $C_3$ plants.

### *Peptones*

The $\delta^{13}C$ values of animal proteins varied along a continuum from a mostly $C_3$ signature to a mostly $C_4$ signature, reflecting the animals' diets [Fig. 2; (2)]. Dairy-derived medium components such as tryptone (an enzymatic hydrolysate of casein) tended to show more $C_3$ influence than meat-derived media components such as meat peptones, but there were exceptions.

### *Yeast Extracts*

The yeast extracts we analyzed were isotopically very homogeneous in their $\delta^{13}C$ and $\delta^{15}N$ values, with the exception of two samples (Fig. 3). We analyzed 37 samples bearing labels of eight different supply houses, two of them European. Of these samples, there was a range of only 2.2‰ in $\delta^{13}C$ values and 1.7‰ in $\delta^{15}N$ values in a group of 35 of the samples. The two outlier samples were each the only sample we obtained from those particular supply houses, and so we do not know if the analyzed samples are typical for those suppliers. It appears that the yeast used to make the samples from six of the suppliers had grown on very similar substrates containing $C_3$ carbon, such as might be expected from a distiller or a brewer of barley beer. It is possible that all six of those suppliers purchase yeast extract (or yeast) from the same manufacturer. The samples from the remaining samples from the remaining two suppliers were clearly produced by yeast that had grown on different substrates; these suppliers may purchase yeast from a different manufacturer or produce it differently from other companies.

*Broth Mixes*

Isotope ratios of broth mixes showed variation consistent with observed variation in isotope ratios of peptones. There were trends in the $\delta^{13}C$ values of broth mixes depending on the sources of its components (meat, dairy, yeast, and carbohydrate), but our data suggest that it would be difficult to identify a medium powder type (for example, to determine whether a powder was nutrient broth or brain-heart infusion) based on its C, N, and H stable isotope profile alone (Fig. 4). Nevertheless, given a carbon stable isotope ratio, it should be possible to say whether the medium were most likely casein-based or meat-based. Our data further suggested that it should be possible to match samples of powder by their stable isotope ratio fingerprints, particularly if stable isotope data were combined with data from other analytical methods.

In Fig. 4, most of the broth mixes in the group with $\delta^{13}C$ ratios around –25‰ were either Luria Broth (a combination of casein and yeast extract) or tryptic soya broth (a combination of casein and soya, protein from a $C_3$ plant). The more diffuse group with $\delta^{13}C$ values from about –20 to about –12‰ was primarily made up of nutrient broths (beef extract and meat peptone, some with yeast extract) and brain-heart infusion (meat infusion and meat peptone). Note that there are exceptions in each group.

Our analysis of nutrient broth powder (Fig. 5) samples illustrates two more important points. The first is that there is variation in stable isotope ratio values between different lots of a product from the same supplier. This variation could reflect differences in sources of raw materials. Even if a supplier buys from the same manufacturer, that manufacturer might use alternate sources of materials. The samples from Supplier 4 in Fig. 5 seem to fall into two groups with respect to their $\delta^{13}C$ values. Supplier 4 is European, and since the outbreak of bovine spongiform encephalopathy (BSE), media manufacturers use meat from certified BSE-free countries, particularly the U.S. (at least prior to December, 2003), Canada (at least prior to May, 2003), Australia, and New Zealand. The variation in $\delta^{13}C$ values could be a reflection of varying geographic sources of the meat. For example, Australian animals might be expected to eat more $C_4$ plant material than animals from New Zealand, based on the climates of the two countries.

The second point illustrated by Fig. 5 is that, from our analyses, it has not been possible to identify the suppliers of specific media by the stable isotope ratios of the media. We cannot rule out the possibility that in some cases it might be possible (e.g., for the isotopically unique yeast extracts in Fig. 3), but more data would be required before such a conclusion could be reached. We typically had large numbers of samples of a single type from only a single supplier, with a smattering of samples from others, and so were unable to make meaningful comparisons of isotope ratios of various sample categories by manufacturer. Finally, suppliers can and do change their sources of materials, and patterns identified today may not be repeated in the future.

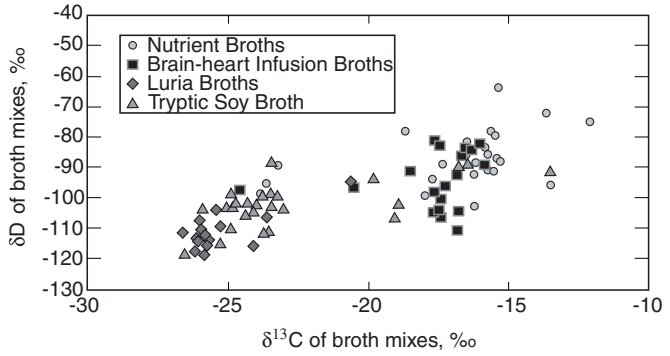


FIG. 4—*Nutrient, Luria, Tryptic Soy, and Brain-heart Infusion Broths, $\delta^{18}D$ ($^2H$) vs. $\delta^{13}C$. The values are consistent with those obtained for individual medium components. The 95% confidence interval for measurements of $\delta^2H$ in media is ±6 and for $\delta^{13}C$, ±0.4‰.*

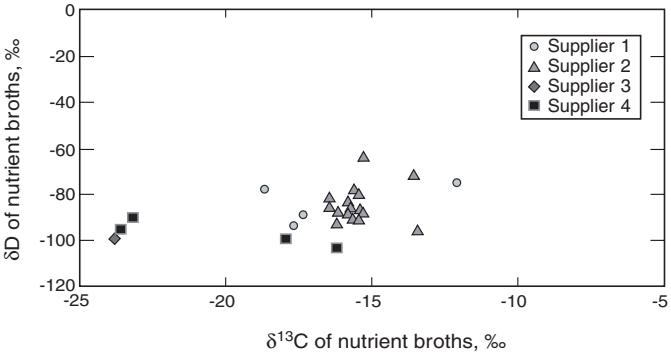


FIG. 5—*Nutrient Broth powders from various suppliers, $\delta^{18}D$ ($^2H$) vs. $\delta^{13}C$. There is considerable lot-to-lot variation among samples from the same supplier. The 95% confidence interval for measurements of $\delta^2H$ in media is ±6‰ and for $\delta^{13}C$, ±0.4‰.*

*Overall Correlations*

There is a statistically significant correlation between the $\delta^{13}C$ and $\delta^2H$ values of broth components and broth mixtures (Fig. 6). The data used in Fig. 6 include all caseins, peptones, carbohydrates, yeast extracts, and broth mixtures (except salt bases). This pattern fits predictions based on the geographical and climatic distribution of $C_3$ and $C_4$ plants, as well as geographic patterns of $\delta^2H$ content in surface water (19,26). $C_3$ plants are favored in cooler climates, where precipitation and surface water tends to be depleted in $^2H$ in comparison to warmer climates where $C_4$ plants are favored. The correlation of $\delta^{13}C$ and $\delta^2H$ values also suggests that manufacturing processes do not usually extinguish the $^2H$ signal from the original plant or animal material.

There was no correlation between $\delta^{13}C$ and $\delta^{15}N$ values of the same medium components and broth mixes. This is not surprising since $\delta^{15}N$ values are at least in part functions of agricultural practices and thus would not be expected to show geographic correlations.

*Translating Medium Variability into Spore Variability*

In a companion paper (17), we present data correlating the carbon, nitrogen, and hydrogen stable isotope ratios of culture media to those in *Bacillus subtilis* spores. Combining the relationships developed from the spore and medium data with the data presented here about isotopic variation in culture media, we can now predict potential variation in *B. subtilis* spores due to variations in the nutrient medium (Table 4). The observed variation in culture media should yield distinguishable variation in carbon, nitrogen, and hydrogen stable isotope ratios in spores.

## Conclusions

The isotope ratio values of bacterial culture media show the variation anticipated based on the biological sources of medium components, the most important of these being the $C_3$ and $C_4$ plants that are either a direct source of medium components or

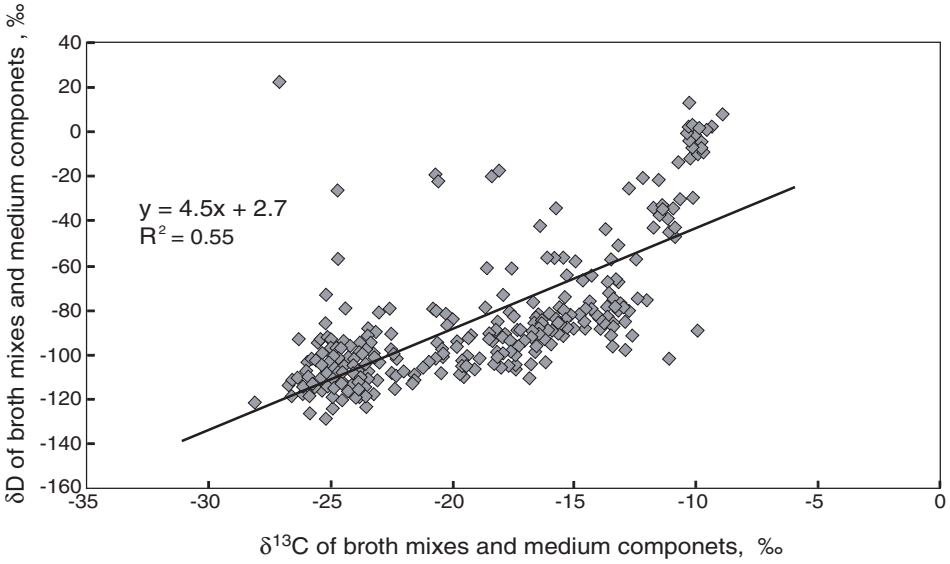


FIG. 6—*Broth mixes, yeast extracts, casein peptones, meat peptones, and carbohydrates, $\delta^2H$ (D) vs. $\delta^{13}C$. The statistically significant correlation between $\delta^2H$ and $\delta^{13}C$ values in broth mixes and medium components reflects geographic correlation of isotopically lighter precipitation and $C_3$ plant habitats. The 95% confidence interval for measurements of $\delta^2H$ in media is ±6‰ and for $\delta^{13}C$, ±0.4‰.*

TABLE 4—*Isotopic variation in bacteriological culture media and its expected translation into B. subtilis spores.*

| Isotope | Equation Relating Spores and Media (17) | Substrate Isotope Range (Broth Mixes) | Potential Spore Variation | Approximate 95% Confidence Limits of Measurement* |
|---|---|---|---|---|
| $^{13}C$ | $\delta^{13}C_{spores} = 0.94\ \delta_{media} - 0.9$ | 15‰ | 14‰ | 0.4‰ |
| $^{15}N$ | $\delta^{15}N_{spores} = 0.92\ \delta_{media} + 4.8$ | 5‰ | 4.6‰ | 0.4‰ |
| $^{2}H$ | $\delta^{2}H_{spores} = 0.29\ \delta_{water} + 0.78\ \delta_{media} + 11.1$ | 50‰ | 39‰ | 6‰ |

* The 95% confidence interval is twice the standard deviation reported in the Methods section for repeated measurements of medium components.

the base of the food chain for animal or yeast sources. The range of variation in $^{13}C$, $^{15}N$, and $^{2}H$ content of bacteriological media should yield differences in microbe isotope ratios that are readily measurable, based on our results with *Bacillus subtilis*. Analysis of stable isotope ratios of microbiological agents and seized culture media should make it possible to rule out specific batches of media (say from a laboratory seizure) as having been used to culture a specific batch of bioterror organisms.

We have demonstrated that $\delta^2H$ values of spores can potentially provide information about the geographic location at which the spores were grown (1). Approximately 70% of the hydrogen atoms in spores originate from the organic components of the culture medium; thus hydrogen isotope ratios also show promise as a means of linking media and spores. We have developed a model relating the hydrogen isotope ratios of culture media, water, and spores that works well for spores produced in media without glucose (18). Combining information from isotope analysis of spores, potential culture media, and water from a suspect location should be a powerful forensic tool, in combination with genetic evidence, for verifying that spores could have come from a particular facility.

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