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TECHNICAL NOTE

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Stable-Isotope Fingerprints of Biological Agents as Forensic Tools*

ABSTRACT: Naturally occurring stable isotopes of light elements in chemical and biological agents may possess unique "stable-isotope fingerprints" depending on their sources and manufacturing processes. To test this hypothesis, two strains of bacteria (*Bacillus globigii* and *Erwinia agglomerans*) were grown under controlled laboratory conditions. We observed that cultured bacteria cells faithfully inherited the isotopic composition (hydrogen, carbon, and nitrogen) of media waters and substrates in predictable manners in terms of bacterial metabolism and that even bacterial cells of the same strain, which grew in media water and substrates of different isotopic compositions, have readily distinguishable isotopic signatures. These "stable-isotopic fingerprints" of chemical and biological agents can be used as forensic tools in the event of biochemical terrorist attacks.

KEYWORDS: forensic science, biological agents, stable isotope ratios, fingerprints, source identification, isotope ratio-mass spectrometry

In the wake of the September 11, 2001, terrorist attacks and the subsequent incidents of "anthrax letters," there is growing public concern over large-scale attacks with hazardous biological and chemical warfare agents (anthrax, smallpox, sarin, cyanide, etc.). The prevention of such terrorist attacks and the early detection of hazardous materials are issues of high priority. It is equally important for law-enforcement agencies to characterize terrorist attacks so that the perpetrators can be apprehended and future attacks can be intercepted and prevented. For this reason, reliable forensic methods are needed to unambiguously determine the origins of biological and chemical materials used in terrorism. However, current methods for chemical and biological identifications (chemical formula, strain of bacteria) are insufficient to determine the exact source of the materials. For example, anthrax spores collected from a newspaper building in Florida in October, 2001, were identified as the Ames strain by DNA sequences (1). However, this particular strain of anthrax spores could have been produced in several different laboratories and locations within the United States or abroad. Similarly, simply identifying a chemical agent (e.g., the sarin used in the Tokyo subway attack) does not pinpoint its source. Clearly, additional methods are needed for identifying with certainty the sources of biological and chemical agents.

The abundance of natural stable isotopes of carbon, hydrogen, nitrogen, oxygen, and other light elements of biological and chemical materials could be used for identifying their sources. These elements, which are essential elemental components of biological and chemical agents, possess multiple stable isotopes ($^{13}C/^{12}C$, $D(^{2}H)/H$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, etc.). The abundance of these stable isotopes of both natural and man-made compounds varies depending on: (a) starting raw materials and substrates used, (b) manufacturing and culturing processes, and (c) geographic locations of the production sites. Variations in the abundance of stable isotopes are generally small, on the order of a few percent (a few tens of percent for hydrogen isotopes) (2). However, these small differences can be readily detected with modern mass spectrometry techniques. In fact, several fields of natural sciences are devoted to the study of variations in the abundances of these stable isotopes (isotope geochemistry, isotope hydrology, etc.). Modern techniques of stable isotope measurements are increasingly utilized in more applied fields. These include the identification of geographic regions for agricultural products (cocaine, alcohol, vanillin, etc.) and the detection of counterfeiting pharmaceutical drugs (3–5). A recent study showed that cocaine samples from South America have distinct $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios depending on their geographic locations. Such "stable-isotope fingerprints" can be used in forensic applications. It is also known that chemical reagents (PCBs, benzene, etc.) from various manufacturers have distinct isotopic ratios (6). These findings led us to hypothesize that candidate biological and chemical agents for terrorism also possess "stable-isotope fingerprints." For this purpose, we first need to understand how growth and manufacturing conditions of biological and chemical warfare agents control their isotopic composition, because cause-to-result relationships for "stable-isotope fingerprints" of these agents are crucial to their use as forensic tools. Here, we report results from our proof-of-principle study for "stable-isotope fingerprints" of biological agents, which were grown under controlled and monitored laboratory conditions.

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Materials and Methods

Bacterial Strains

Two bacterial strains were used in this experiment. *Bacillus globigii* (formerly *Bacillus subtilus*), a Gram-positive microorganism and simulant for anthrax, was obtained in lyophilized spore form from Dugway Proving Grounds, Utah. This material was originally grown in Stockholm, Sweden and sent in spore form to the United States. *Erwinia agglomerans* (formerly *Erwinia herbicola)* a Gram-negative microorganism and simulant for other Gram-negative pathogenic microorganisms such as *Vibrio cholerae* and *Yersina pestis*, was purchased from the American Type Culture Collection, Rockville, MD (ATCC No. 33243). The two bacterial strains were grown on Bacto-Tryptic Soy Agar, DIFCO Laboratories, Detroit, IL (40 g/L) to establish stock cultures and to verify viability and purity.

Media and Growth Conditions

The medium used for all experiments was BBL Trypticase Soy Broth—TSB (Soybean-Casein Digest Medium), Becton Dickinson Microbiology Systems, Cockeysville, MD. Three types of deionized water were used to grow bacterial strains. They included water from Edmonton, Alberta, Canada (CANADA), water from Oak Ridge National Laboratory (ORNL), and water from Oak Ridge National Laboratory that was spiked with a small quantity of D_2O (49.3 mg of 99.26% D_2O in 1973 g of ORNL deionized water) (D-ORNL). The three deionized waters with different isotopic compositions were prepared to examine how the isotopic composition of growth media affects that of cultured bacteria. Three grams of Trypticase Soy Broth were added to 100 mL of each type of water in 250-mL shaker flasks and autoclaved for 15 min at 15 psi (121°C). Once cool, each flask made from the same medium base (TSB), but containing different types of water, was seeded with approximately 10^3 bacteria harvested from the stock culture plates, resulting in a total of six flasks (each bacterial strain in TSB made with the three types of water). Once the media was seeded with the bacterial cultures, they were placed on a Thermolyne RotoMix 50800 shaker platform (Sybron) and incubated at 30°C in ambient air (aerobic) without CO_2 enrichment (Forma Scientific, Inc., Marietta, OH) for four days to a late log stage. Both cultures attained a concentration of approximately 10^{10}/mL.

Bacterial Harvest

After incubation, the bacterial cells were concentrated by centrifugation. All cultures were centrifuged at 10,000 × g for 30 min in a Sorvall RC-B refrigerated supercentrifuge. The supernate was then removed and the cell pellets were washed using their respective source water. After washing, the cells were again centrifuged, the supernate was removed, and the cell pellets were collected and dried overnight in 25-mm sterile petri dishes. The weight of the recovered cells was approximately 19 to 36 and 67 to 125 mg for *Bacillus globigii* and *Erwinia agglomerans*, respectively. Thus, only small fractions (0.6 to 4.2%) of the substrate were consumed. These cells were stored in a vacuum desiccator until analysis.

Isotopic Measurements

The isotopic ratios of hydrogen, carbon, and nitrogen (D/H, $^{13}C/^{12}C$, and $^{15}N/^{14}N$) were determined for both the culture media (waters and substrate) and bacterial samples. Samples of Trypticase Soy Broth and cultured bacteria were prepared for isotopic analyses using a sealed silica-tube method (7–9). Approximately 3 to 5 mg of dried samples stored in a desiccator were loaded into a 1/4 in. OD silica-glass tube with 0.5 g of CuO, which was roasted beforehand for several hours at 1000°C. Then the samples were further dried in vacuum heating to 70 to 80°C for 2 h to remove adsorbed water, and the silica tubes were sealed without exposing the samples to ambient air. The samples were then combusted to CO_2, water, and N_2 (and possibly oxides of nitrogen) within a furnace heated to 850°C for 2 h. The furnace was then slowly cooled to 500°C over 6 to 7 h and held at this temperature overnight. This slow cooling process facilitated the conversion of SO_2 to $CuSO_4$ and of any oxides of nitrogen to N_2, and the sorption of O_2 back to CuO (9). The CO_2, N_2, and water produced were cryogenically separated, and their amounts were determined with manometers in a vacuum preparation line. The water recovered, and the three waters used as culture media were reduced to H_2 with uranium metal heated at 700°C (10).

The isotopic ratios (D/H, $^{13}C/^{12}C$, and $^{15}N/^{14}N$) of the H_2, CO_2, and N_2 gases prepared were determined with a gas-source stable isotope ratio mass spectrometer (Finnigan MAT252). The isotopic ratios are presented in the δ-notation expressed as per mil (‰) deviations from international standards:

$$\delta(‰) = \left(\frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right) \cdot 1000 \qquad (1)$$

where *R* represents the ratios *D/H*, $^{13}C/^{12}C$, or $^{15}N/^{14}N$. The international standards are Vienna-Standard Mean Ocean Water (V-SMOW) for *D/H* (*D/H* of V-SMOW = $156 \cdot 10^{-6}$), Vienna-PDB for $^{13}C/^{12}C$ defined as $\delta^{13}C$ of NBS-19 standard = +1.95‰ ($^{13}C/^{12}C$ of V-PDB = 0.0112), and atmospheric N_2 for $^{15}N/^{14}N$ ($^{15}N/^{14}N$ of air = 0.00367). Overall precision (1σ) of the isotopic analysis is generally about ±0.5 to 7‰ for δ*D* values, ±0.05‰ for $\delta^{13}C$ values, and ±0.05 to 0.3‰ for $\delta^{15}N$ values, depending on the nature of samples (Table 1). We did not measure the isotopic composition of the waters and the substrate (soy broth) after culturing, but they most likely remained nearly constant, since only small fractions (0.6 to 4.2%) of the substrate were consumed.

Results and Discussion

Elemental Ratios

The C/H ratio (0.781 ± 0.006, $n = 2$) of the *Bacillus globigii* spores used as inoculum is somewhat greater than those of cultured bacteria of the same strain (0.688 to 0.706), which in turn are relatively constant (Table 1). On the other hand, the *C/N* ratios of the three cultured *Bacillus globigii* vary from 4.49 to 5.07. Both *C/H* and *C/N* ratios of three cultured *Erwinia agglomerans* are relatively constant, 0.666 to 0.689 and 4.02 to 4.14, respectively. The smaller *C/N* ratio of *Erwinia agglomerans* than that of *Bacillus globigii* is due to a higher protein content of the former bacterium (≈60%) compared to that of the latter (≈40%). Note that the *C/H* ratios determined in this study are probably larger than a true value. This is because the sealed-tube combustion methods yield low water content (≈90%), presumably due to the hydration of the inner wall of silica glass, but without isotopic fractionation (11).

$^{13}C/^{12}C$ Ratios

The *Bacillus globigii* spore originally grown in Stockholm, Sweden, has a distinctive $\delta^{13}C$ value (−7.80 ± 0.04‰, $n = 6$), com-

TABLE 1—*Results of elemental and isotopic compositions of growth media (water and substrate) and bacteria (Bacillus globigii and Erwinia agglomerans).*

Sample	Elemental Ratio		Isotopic Composition (‰)*		
	C/H	*C/N*	δ^{13}C(V-PDB)	δD(V-SMOW)	δ^{15}N(air)
Water					
CANADA				−155.3 ± 0.5 (n = 2)	
ORNL				−38.6 ± 0.1 (n = 2)	
D-ORNL				+99.6 ± 1.2 (n = 2)	
Substrate					
Soy Broth	0.621 ± 0.009 (n = 2)	4.45 ± 0.02 (n = 2)	−23.42 ± 0.07 (n = 2)	−68.4 ± 1.0 (n = 2)	+5.32 ± 0.02 (n = 2)
Bacillus globigii					
Spore (inoculum)	0.781 ± 0.006 (n = 2)	4.46 ± 0.04 (n = 2)	−7.80 ± 0.04 (n = 6)	−49.6 ± 5.1 (n = 2)	+4.89 ± 0.01 (n = 2)
CANADA	0.688	4.49	−20.60	−97.7	+9.21
ORNL	0.688 ± 0.001 (n = 2)	5.07 ± 0.05 (n = 2)	−20.72 ± 0.01 (n = 2)	−65.7 ± 1.3 (n = 2)	+8.29 ± 0.38 (n = 2)
D-ORNL	0.706 ± 0.008 (n = 2)	4.96 ± 0.01 (n = 2)	−20.86 ± 0.01 (n = 2)	−32.2 ± 0.2 (n = 2)	+7.15 ± 0.03 (n = 2)
Erwinia agglomerans					
CANADA	0.674	4.14 ± 0.01 (n = 2)	−21.58 ± 0.03 (n=2)	−77.5	+7.43 ± 0.34 (n = 2)
ORNL	0.666 ± 0.000 (n = 2)	4.02 ± 0.05 (n = 2)	−21.63 ± 0.00 (n = 2)	−36.4 ± 7.6 (n = 2)	+7.74 ± 0.23 (n = 2)
D-ORNL	0.689	4.12	−21.89	−5.2	+7.75

* $\delta(‰) = \left(\frac{R_{Sample}}{R_{Standard}} - 1\right) \cdot 1000$, where R stands for D/H, $^{13}C/^{12}C$, or $^{15}N/^{14}N$.

$R_{standard}$: D/H(V-SMOW) = $156 \cdot 10^{-6}$, $^{13}C/^{12}C$(V-PDB) = 0.0112, and $^{15}N/^{14}N$(air) = 0.00367.

pared to the three bacteria cells cultured from this spore (−20.86 to −20.60‰), because they grew on the soy broth, which has a δ^{13}C value of −23.42 ± 0.07‰ (n = 2) (Table 1 and Figs.1*a, b*). The type and isotopic compositions of a substrate are not known for the *Bacillus globigii* spore. The three samples of bacteria cells of *Erwinia agglomerans* have δ^{13}C values (−21.58 to −21.89‰) similar to, but slightly lower than, those of the *Bacillus globigii* bacteria, despite the fact that the two strains of bacteria grew on the same substrate. There seem to be very slight variations in δ^{13}C values among three cultured bacteria of each strain (0.26‰ for *Bacillus globigii* and 0.31‰ for *Erwinia agglomerans*).

Several investigators (12 to 15) reported $^{13}C/^{12}C$ isotope fractionation during the growth of several aerobic heterotrophic bacteria (*Escherichia coli, Pseudomonas aeruginasa, Shewanella putrefaciens*) from various organic substrates (glucose, lactate, glutamate). An isotopic enrichment of biomass relative to a substrate is defined:

$$\varepsilon(‰) = (\alpha_{Bacteria-substrate} - 1) \cdot 1000 = \left(\frac{R_{Bacteria}}{R_{Substrate}} - 1\right) \cdot 1000 = \left(\frac{1 + 10^{-3}\,\delta_{Bacteria}}{1 + 10^{-3}\,\delta_{Substrate}} - 1\right) \cdot 1000 \quad (2)$$

where α is the isotopic fractionation factor. The measured values of ε for $^{13}C/^{12}C$ for the above heterotrophic bacteria ranged from −0.6 to +3.0‰ at 25 to 37°C. Recently, Zhang et al. (16) reported $\varepsilon(^{13}C/^{12}C)$ values of −8.4 to −7.4‰ for thermophilic (80°C) heterotroph *Thermotoga maritima*. In this study, $\varepsilon(^{13}C/^{12}C)$ values were +2.56 to +2.82‰ for *Bacillus globigii* and +1.53 to +1.84‰ for *Erwinia agglomerans.* It is known that heterotrophs generally inherit δ^{13}C values of substrates (foods) with slightly positive offsets (ca. +1‰—"you are what you eat plus a few per mils (‰)" (17). The respiration of CO_2, which is depleted in ^{13}C, is the main cause of these slight ^{13}C enrichments of biomass relative to substrates. However, a correlation of these ^{13}C enrichments with trophic levels is complex due in part to the heterogeneity of $^{13}C/^{12}C$ among different organic molecules within the same biomass: δ^{13}C(polysaccharides) > δ^{13}C(protein) ≈ δ^{13}C(nucleic acids) > δ^{13}C(lipids) (13,14). See a recent review (18) for more details.

$^{15}N/^{14}N$ Ratios

The bacteria cells of *Erwinia agglomerans* have nearly constant δ^{15}N values (+7.43 to +7.75‰), but those of the three *Bacillus globigii* vary from +7.15 to +9.21‰ (Fig. 1*a,c*). The cause of this rather large variation (2.06‰) is not known, although *C/N* ratios of these cells also vary from 4.49 to 5.07 (Table 1). All cultured bacteria have δ^{15}N values slightly greater than that of the soy broth (+5.32 ± 0.02‰, n = 2). The *Bacillus globigii* spore has the lowest δ^{15}N value (+4.89 ± 0.01, n = 2).

Systematic enrichments of ^{15}N with trophic levels in the food chain (e.g., phytoplankton, zooplankton, and fish) have long been long recognized (19). The value of $\varepsilon(^{15}N/^{14}N)$ appears to be fairly constant among species (approximately +3‰) (20). In this study, $\varepsilon(^{15}N/^{14}N)$ values were +1.83 to +3.89‰ for *Bacillus globigii* and +2.11 to +2.43‰ for *Erwinia agglomerans* (Fig. 1*a,c*), consistent with the above general trend.

D/H Ratios

The three media waters have distinctive δD values: δD (Edmonton, Canada) = −155.3 ± 0.5‰ (n = 2); δD(ORNL) = −38.6 ± 0.1‰ (n = 2), and δD(ORNL, D_2O-spiked) = +99.6 ± 1.2‰ (n = 2). The isotopic composition of local meteoric water is controlled by the source of water vapor, the trajectory, and the precipitation history of air masses leading to their well-established geographical differences (21 to 23). For this reason, the Edmonton, Canada, deionized water is far more depleted in deuterium than the ORNL deionized water. The soy broth used has δD = −68.4 ± 1.0‰ (n = 2). The δD values of both *Bacillus globigii* and *Erwinia agglomerans* cultured bacteria vary widely from −97.7 to −32.2‰ and −77.5 to −5.2‰, respectively, in a positive correlation with the δD values of the media waters (Fig. 1*b,c*). However, the variations in the δD values among each strain (65.5 and 72.3‰ for *Bacillus globigii* and *Erwinia agglomerans*, respectively) are much smaller than that of the media waters (255‰), sug-

gesting that the soy broth also contributed hydrogen to the bacterial cells. It is documented in the literature (24,25) that hydrogen, which is not bound to carbon (e.g., —OH, —COOH, —NH_2), exchanges its isotopes relatively quickly with ambient water, liquid, or moisture. We attempted to minimize this exchange by washing the cultured bacteria with the same water used for growth, drying quick, and storing the bacteria in a vacuum desiccator until analysis. However, it is still likely that isotopic exchange between "exchangeable" hydrogen of the bacteria and ambient water occurred. The proportion of "exchangeable" hydrogen is, however, usually small (≈20% of the total hydrogen of dehydrated organic materials) (26).

The fact that variations in δ*D* values of three bacteria samples of each strain (65.5 and 72.3‰ for *Bacillus globigii* and *Erwinia agglomerans*, respectively) were much smaller than that in δ*D* values of the three waters used (255‰) suggest that only small fractions of hydrogen in bacteria were acquired from water and that the remaining large fractions came from the substrate:

$$(D/H)_{\text{Bacteria}} = X \cdot \alpha_{\text{Bacteria}-\text{water}} \cdot (D/H)_{\text{Water}} + (1 - X) \cdot \alpha_{\text{Bacteria}-\text{substrate}} \cdot (D/H)_{\text{Substrate}} \quad (3)$$

where X is the fraction of hydrogen in bacteria derived form water. The value of $(D/H)_{\text{Water}}$ changed, while $(D/H)_{\text{Substrate}}$ remained the same. Then, the above equation is reduced to:

$$X = \frac{\Delta\delta D_{\text{Bacteria}}}{\alpha_{\text{Bacteria-water}} \cdot \Delta\delta D_{\text{Water}}} \quad (4)$$

where Δδ*D* is the difference in δ*D* values between different waters or between different bacteria cells of the same strain. If we assume that a *D*/*H* isotopic fractionation between bacteria cells and water ($\alpha_{\text{Bacteria-water}}$) can be approximated by unity, we can calculate the fraction of hydrogen in the cells derived from water (X). We obtained 26 and 28% for *Bacillus globigii* and *Erwinia agglomerans*, respectively. Our values on bacteria agree well with results of similar experiments on quails (18 to 32% of non-exchangeable hydrogen) (26). These results show that substrates largely (70 to 80%) control the δ*D* values of bacteria cells and other heterotrophs. On the other hand, Sessions et al. (27) reported that anaerobic methanotroph *Methlococcus capsulatus* acquired only 31% of hydrogen from CH_4 and the remaining from water.

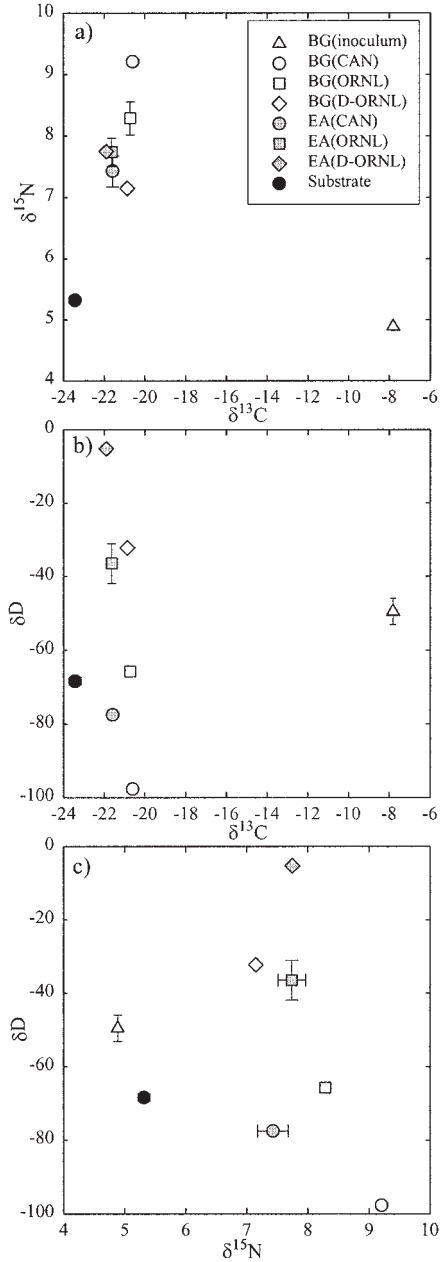

FIG. 1—*Hydrogen, carbon, and nitrogen isotopic compositions of bacteria spores and cells (Bacillus globigii, BG, and Erwinia agglomerans, EA) and soy broth substrate:* (a) $\delta^{13}C$ *vs.* $\delta^{15}N$, (b) $\delta^{13}C$ *vs.* δ*D, and* (c) $\delta^{15}N$ *vs.* δ*D.*

Forensic Applications

We have demonstrated that microbial agents cultured under controlled conditions inherited the isotopic compositions of growth media and substrates in predictable manners. Because the isotopic composition of organic substrates and media waters most likely varies depending on the source and manufacturing processes of raw materials and the geographic locations, even bacteria of the same strain can be readily distinguished on the basis of their isotopic compositions. This may also be the case for different batches of biological and chemical agents produced in the same laboratories, if raw materials and/or processes differ. The use of "stable-isotope fingerprints" of multi-elements (H, C, N, O, S, etc.) should make it possible to distinguish most of, if not all, chemical and biological agents. Figure 2 shows this clearly on each of the two strains of bacteria grown in this study.

It is of great concern whether chemical and biological agents retain their original isotopic signatures during storage and transportation and after dispersal in the public ("isotopic fidelity"). Except for the "exchangeable hydrogen," the isotopic composition of chemical agents most likely remains intact until its degradation. When kept dormant, biological agents are also expected to remain intact. To mitigate this potential problem, refractory organic molecules in biological agents (i.e., lipids) can be specifically targeted for the purpose of the "stable-isotope fingerprints." For this purpose, modern techniques for "compound-specific" isotopic analysis of organic molecules (28) are particularly suitable for samples as small as 10's of nanograms. Bacterial vegetative cells and spores of pathogens used as biological weapons for bioterrorism and biological warfare are often finely milled and coated by clays and other compounds to make highly "weaponized" aerosols. If compounds are used, which contain carbon, hydrogen, etc., these have to be removed from bacterial cells before the isotopic analysis. The effects of these chemical decontamination and γ-ray irra-

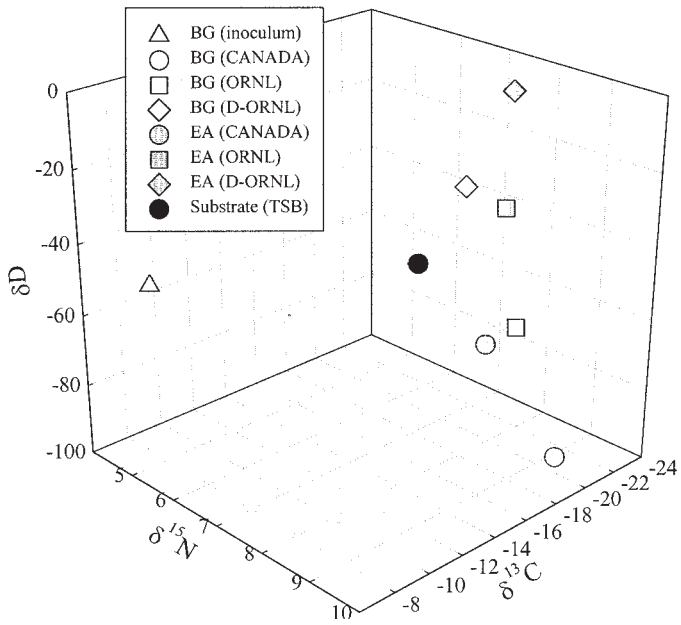

FIG. 2—*Three-dimensional plot of hydrogen, carbon, and nitrogen isotopic compositions of bacteria spores and cells (Bacillus globigii, BG and Erwinia agglomerans, EA) and soy broth substrate.*

diation on the isotopic composition of biological agents have to be examined.

For forensic applications, it is very desirable to establish a database of the "stable-isotope fingerprints" not only of many chemical and biological warfare agents, but also of substrates and starting materials. Once such a database is established and archived, the source of biological and chemical materials used in terrorism attacks could be identified by matching with those in the database. If bacteria is grown from an original batch of spores stolen from biological laboratories, the isotopic signatures of the newly grown bacteria cells will change from those in the database of the "stable-isotope fingerprinting" system. Even in such cases, it is still possible to evaluate growth conditions and media based on the newly acquired isotopic signatures of pathogens. For example, when culturing media and materials are confiscated, the suspect could be identified either positively or negatively based on the isotopic analysis of the confiscated materials. It is also possible to narrow down manufactures and vendors, from which the growth media were purchased, and geographic locations of the laboratory, based on the isotopic signatures of biochemical agents and the knowledge of their isotopic fractionation during growth and synthesis. This information can be used along with the results of conventional methods (immunoassays, polymerase chain reaction, etc.) to determine the perpetrators and prevent future bioterrorist attacks.

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