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Stable Isotope Ratios as a Tool in Microbial Forensics—Part 3. Effect of Culturing on Agar-containing Growth Media*

ABSTRACT: Stable isotope ratios of hydrogen and oxygen in microbes have been shown to be functions of the corresponding isotope ratios of the water with which the culture medium was prepared, and thus to contain a potential geographic signal. Water can evaporate from agar (solid) media during culturing, changing its isotope ratios. Here we describe the effect of drying on the isotope ratios of water extracted from agar media and the H and O stable isotope ratios ratios of *Bacillus subtilis* spores cultured on agar. The δ^2H vs δ^{18}O relationship of water in Petri dish agar was surprisingly constant during evaporation regardless of the ambient relative humidity, making it possible to calculate the approximate isotope ratios of the original water, even in significantly evaporated agar. The H stable isotope ratios of spores cultured on agar remained relatively unchanged as the agar dried, but the O ratio became significantly enriched.

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

The organic molecules of plants, animals, and microbes record aspects of their environments in the stable isotope ratios of their elements (e.g., ^{2}H/^{1}H, ^{13}C/^{12}C, ^{15}N/^{14}N, ^{18}O/^{16}O, ^{34}S/^{32}S). Isotope ratio analysis of organic matter can therefore reveal information about the growth environment and/or diet of the organism. The physiological differences between the C_3 and C_4 photosynthetic pathways result in large differences in the ^{13}C/^{12}C values of carbohydrates, allowing one to trace the flow of this organic carbon through food webs (1–3) as well as to detect adulteration of foods and alcoholic beverages (4–6). Characteristics of a plant's growth environment affect the precise value of the ^{13}C/^{12}C ratio, allowing its use as an indicator of the geographic origins of plant products such as cocaine and heroin (7,8).

The oxygen and hydrogen stable isotope ratios of precipitation and surface water carry a strong geographic signature and are thus of particular potential forensic interest (9). Because the stable isotope composition of local waters is recorded in the oxygen and hydrogen isotope ratios of plant cellulose (10,11) and in the bones, hair, blood and tissues of animals (12–15), stable isotope ratio analysis has been used to trace the origins of many organisms, including migratory populations of birds (16,17), butterflies (18), and elephants (19,20).

The stable isotope ratios of microorganisms, too, are a function of their substrate nutrients and water (21–23). Previous studies of cultured microorganisms have shown that microbial carbon and nitrogen stable isotope ratios are a function of the growth medium nutrients, while oxygen and hydrogen stable isotope ratios are a function of both nutrients and water. Stable isotope ratios therefore provide a potential forensic link to media or source water in the event that a microbiological agent was recovered in the course of a criminal investigation. The oxygen and hydrogen stable isotope ratios would be of particular interest in such a case, because of their potential to provide insights into the geographic water sources used to culture the microbes.

The studies cited above used organisms cultured in liquid growth media. Under normal culture conditions, evaporation from liquid cultures is minimal during the growth period. However, organisms can also be cultured on media that have been solidified through the addition of the polysaccharide gelling agent agar. The preparation of agar-containing media ("agars") and the conditions under which organisms are incubated on agar can permit significant evaporation from the medium during the growth of the organism, particularly if incubation is prolonged, as when the goal is to produce spores of the genus *Bacillus*.

When water evaporates from a reservoir, the residual water becomes isotopically enriched (e.g., (24,25)). Thus evaporation of medium water during the course of growth of a bacterial culture might affect the O and H stable isotope ratios of the organisms produced later in the culture period relative to those first produced in the culture period, changing the overall relationship of their isotope ratio values to those of the original culture water. Such a change could confound the interpretation of isotope ratio data of microbial spores. In this study, we have examined the O and H stable isotope ratios of *B. subtilis* growing on agar-containing media, and compared the results to those obtained with liquid cultures.

Methods

Our experimental organism was *Bacillus subtilis* strain 6051 (ATCC, Rockville, MD). Cultures were grown on Schaeffer's Sporulation Medium solidified by the addition of 15 g agar powder per liter [SSA; (26)]. In addition to the agar, SSA contained 8 g Difco Nutrient Broth powder per liter plus the following

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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. Nutrient agar, used in the evaporation studies, contained 8 g Difco Nutrient Broth powder and 15 g agar powder per liter.

To make SSA, the nutrient broth powder was dissolved in 976 mL water, the KCl, $MgSO_4$, and NaOH solutions were added, the agar powder was added, and the medium was autoclaved. The autoclaved solution was cooled to approximately 55°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. The warm medium was immediately poured into sterile Petri dishes and allowed to solidify. Nutrient agar was prepared by adding 8 g Nutrient Broth powder and 15 g agar to one liter water and autoclaving.

We prepared four containers of water of different oxygen and hydrogen isotope ratios by adding various amounts of $^2H^1HO$ and $H_2^{18}O$ to local deionized water. We used waters from these containers throughout our experiments in preparing media and washing spores. The containers had airtight lids and were kept in the cold room.

Overnight cultures were grown in liquid SSM and diluted tenfold in fresh sterile SSM before being used to inoculate the agar medium in Petri dishes (agar plates). To inoculate the agar plates, 0.5 mL of the diluted overnight culture was pipetted into the center of the plate and spread with a sterile bent glass rod. Inoculated plates were incubated at 37°C for at least 48 h (incubation times are noted in the text below).

Spores were harvested by pipetting a few mL of the water used to make the medium onto the agar surface and gently suspending the spores with a sterile bent glass rod, avoiding damage to the agar surface. The spore suspension was pipetted into a centrifuge tube and the agar plate was rinsed 2–3 times. Spore suspensions were centrifuged for 10 min at 8,000 rcf, resuspended in 20 mL of their culture water, re-centrifuged, and resuspended 20 mL water. 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 (27). Finally, the spore pellet was resuspended in 1 mL water, frozen, and lyophilized for analysis.

Samples of the water contained in agar media were taken by cutting an approximately 2 × 0.5-cm rectangular slice of agar from an agar plate. The agar slice was immediately placed in a 0.5-dram vial and frozen. Water was cryogenically extracted from the samples.

Stable Isotope Ratio Analysis

Stable isotope ratios were measured on an isotope ratio mass spectrometer and values were expressed in "delta" notation relative to internationally recognized standards. We calibrated laboratory standards to the international standards, and then included the laboratory standards as internal standards in every analytical run. Stable isotope compositions are expressed in "delta" notation as δ values in parts per thousand (‰), where $\delta‰ = (R_A/R_{Std} - 1) * 1000‰$, and R_A and R_{Std} are the molar ratios of the rare to abundant isotope (e.g., $^{18}O/^{16}O$) in the sample and the standard. The standard used for both oxygen and hydrogen is Vienna Standard Mean Ocean Water [VSMOW (28)].

For O and H stable isotope analysis of solid samples, 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. 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 medium 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‰ for H and 0.6‰ for O. Confidence intervals for measurements of individual samples are reported as 2× this standard deviation, or 6‰ for H and 1.2‰ for O.

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 (purchased from University of Indiana) in a Pyrex tube at 500°C (29). 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‰

Water samples were prepared for oxygen isotope ratio analysis by equilibration with CO_2 as described by Fessenden et al. (30). Isotopic analysis was done on a Finnigan-MAT Delta S IRMS (Bremen Germany) equipped with an elemental analyzer (Carlo Erba 1108, Milan Italy). The standard deviations of repeated measurements of a standard water sample made over several months was 0.22‰.

Results

O and H Stable Isotope Ratios in Water Extracted from Evaporating Petri Plate Agar

The oxygen and hydrogen isotope ratios in evaporating water depend on both the isotope ratios of the original water and the conditions under which the water evaporates. The relationship of the δ^2H and the $\delta^{18}O$ values of precipitation and most surface waters is fairly well described by the Global Meteoric Water Line, although the y-intercept value for local meteoric precipitation can vary (9):

$$\delta^2H = 8\,(\delta^{18}O) + 10$$

As water evaporates, the residual liquid becomes increasingly enriched in the heavier isotopes of both oxygen and hydrogen, increasing the delta values. If the δ^2H and $\delta^{18}O$ values of a pool of evaporating water are repeatedly measured as the water evaporates, the slope of the line describing the relationship between them ranges from somewhat less than 4 at 0% relative humidity to approaching 8 as relative humidity approaches 100% (25,31). Slopes are shallower if there is a significant boundary of stagnant air over the evaporating reservoir, as in the case of water evaporating from soil columns (32). We could find no literature references in which the isotope ratios of water evaporating from agar media in Petri dishes had been measured.

We therefore prepared Nutrient Agar, poured it into Petri dishes in the standard manner (which includes placing the cover on the dish) and left the poured plates at room temperature for up to two weeks. Each day or so, we sampled agar from two plates and discarded them. Agar samples were frozen until analysis. At the end of the sampling period, we extracted the water from the frozen agar slices and determined the δ^2H and $\delta^{18}O$ values. We also asked colleagues around the United States to duplicate the experiment and send us frozen samples. Experimental sites were Salt Lake City, UT; Elon, NC; Fairhope, AL; State College, PA; and Livermore, CA.

We measured the rates of evaporation and relative humidity at three different sites: Salt Lake City, Elon, NC, and Fairhope, AL.

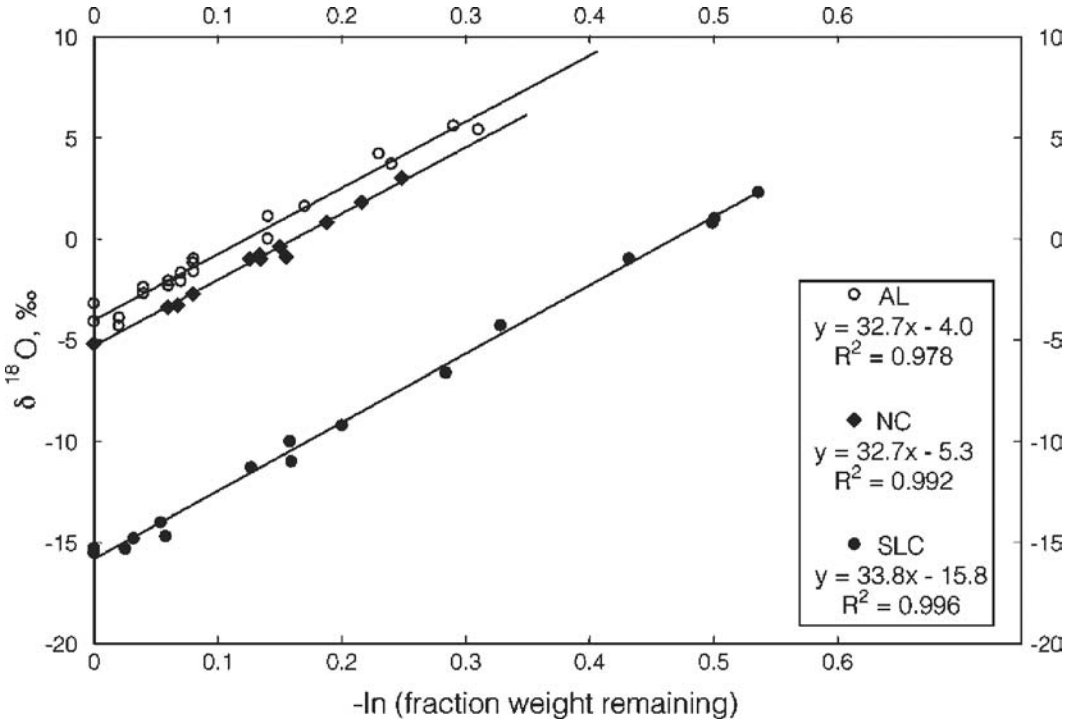

FIG. 1—*The oxygen stable isotope ratio of agar water versus the extent of evaporation of the water, measured at three geographic locations with varying relative humidity. The change in the stable isotope ratio of extracted agar water was a constant function of the extent of evaporation regardless of the ambient humidity.*

Rate of evaporation was determined by taring the empty Petri dishes before pouring the agar medium into them, then re-weighing as soon as the medium solidified to obtain initial weights. When an agar plate was harvested to obtain a sample for water extraction and stable isotope ratio analysis, it was re-weighed and the fraction of initial weight lost calculated. The rate of weight loss from the plate (due to evaporation) varied with ambient relative humidity (Table 1). The change in oxygen stable isotope ratios as a function of weight loss (expressed as the negative natural logarithm of the fraction of agar weight remaining) at the three sites is shown in Fig. 1. The slopes of the lines, representing the rate of increase in $\delta^{18}O$ value with extent of evaporation, were statistically indistinguishable.

Plots of the δ^2H versus $\delta^{18}O$ values from all five test sites are shown in Fig. 2, and the statistics of the regression curves are presented in Table 2. The slopes of all of the lines except for that of the curve obtained in Livermore, CA, were statistically indistinguishable. Furthermore, the y-intercept values of the various curves were linearly correlated with the δ^2H values of the laboratory water used to make the Nutrient Agar in each location (Fig. 3).

A potential reason that the slope of the Livermore evaporation curve was statistically different from the slopes of the other evaporation curves is that the water vapor in the air at Livermore might not be in isotopic equilibrium with the tap water. Livermore is relatively close to the California coast; it is likely that marine air masses containing relatively enriched water vapor could frequently prevail there. Yet the δ^2H value of the Livermore water was a relatively depleted –100‰, suggesting a montane origin for the precipitation. Back-exchange between water vapor molecules and liquid water has been demonstrated to occur (31) and in this situation the back-exchange could make the water in the agar medium isotopically more enriched than it would have been in an atmosphere at isotopic equilibrium with the liquid water.

To test the disequilibrium hypothesis as the explanation for the odd Livermore agar sample, we set up an evaporation experiment in our laboratory using water (Evian bottled water) that was isotopically heavier than our local water ($\delta^2H = -72.5$‰ vs –125‰ and $\delta^{18}O = -10.2$‰ vs –15.8‰) and would therefore be out of equilibrium with ambient water vapor. This situation is the reverse of the hypothetical Livermore scenario in that the vapor is depleted relative to the liquid rather than enriched. If back-exchange between water vapor and the residual liquid in the agar medium is significant, then we would predict a slope of less than about 2.5. The measured slope of this evaporation curve was 2.2, with 95% confidence intervals from 2.0 to 2.4, supporting the hypothesis.

TABLE 1—*Rates of evaporation from nutrient agar in covered Petri dishes*

Site	Rate of Evaporation (% Weight Loss oer Day)	Relative Humidity During Experiment
Salt Lake City, UT	3.2%	<20%
Elon, NC	2.7%	17–27%
Fairhope, AL	1.5%	37–56%

TABLE 2—*Statistics of δD vs $\delta^{18}O$ evaporation curves.*

Location	Estimated Slope	95% Conf. Interval	R^2	Estimated y-intercept	95% Conf. Interval
Salt Lake City, UT	2.52	2.37–2.66	0.991	−82	−84–80
Elon, NC	2.52	2.29–2.76	0.979	−18	−18–17
Fairhope, AL	2.48	2.15–2.81	0.933	−12	−13–11
State College, PA	2.67	2.51–2.83	0.988	−44	−46–43
Livermore, CA	3.18	2.80–3.56	0.971	−55	−59–50

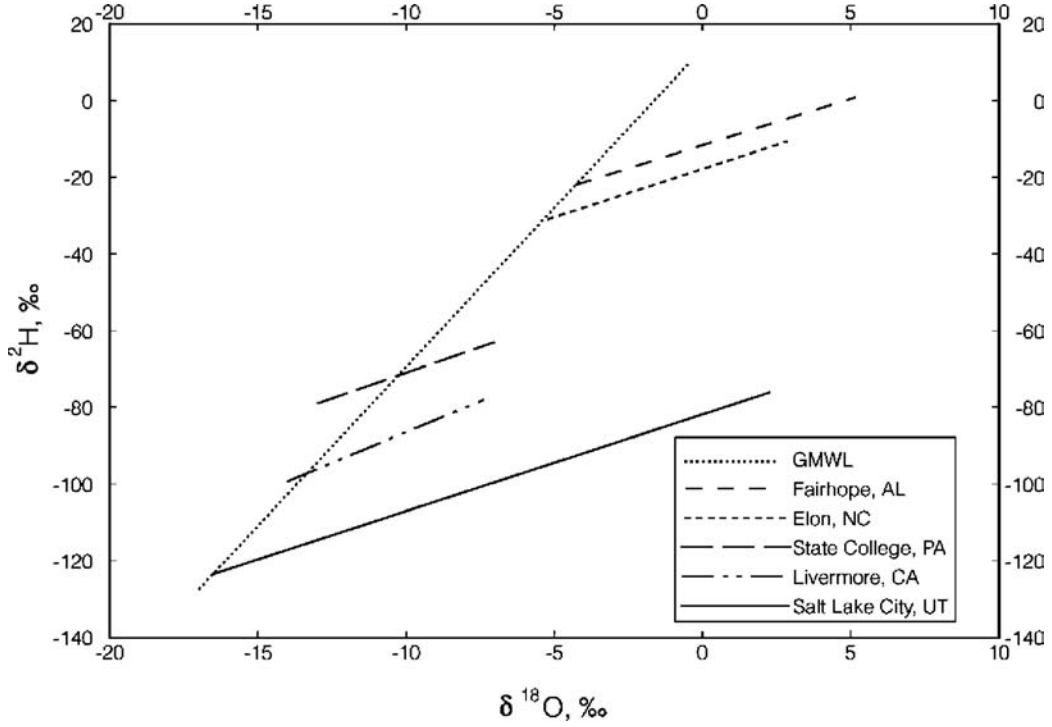

FIG. 2—*Thee hydrogen versus oxygen stable isotope ratios of water extracted from agar media as it evaporated at five different geographic locations. The slopes of the regression lines are statistically indistinguishable, except for the curve obtained in Livermore, CA (see Table 2). The Global Meteoric Water Line (GMWL) is shown for reference.*

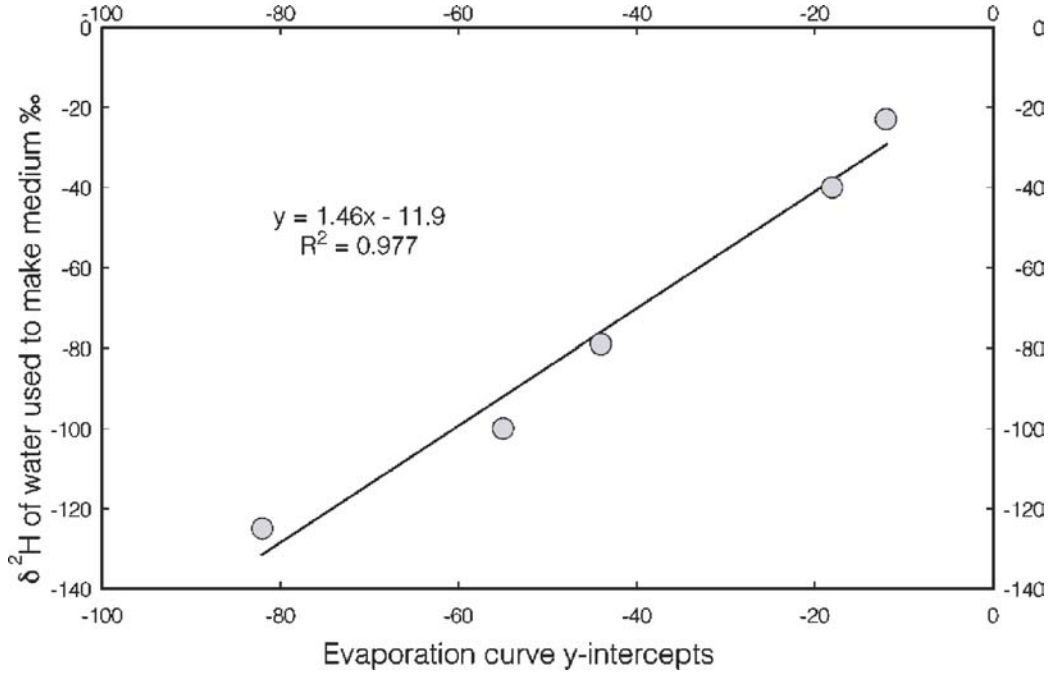

FIG. 3—*The y-intercepts of the regression equations of the lines shown in Fig. 2 are linearly correlated with the δ^2H values of the water originaly used to make the agar medium at each corresponding location.*

A Model for Predicting the δ^2H and $\delta^{18}O$ Values of Water Used to Make Agar Media

The results from our evaporation experiments suggested we might be able to predict the δ^2H value of water used to make an agar medium even if that medium had undergone substantial evaporation. We therefore took the δ^2H and $\delta^{18}O$ values of every individual agar plate we tested, including those from the Evian-water disequilibrium experiment and a second set of plates made with our local deionized water and evaporated in our laboratory ($n = 105$), to make a predictive model using the statistics program JMP (SAS Institute). The program returned the following regression model:

$$\delta^2H \text{ source water} = 1.4\,(\delta^2H \text{ plate water}) - 3.25(\delta^{18}O \text{ plate water}) - 9.5$$

$$R^2 = 0.986$$

$$P < 0.001$$

We used this equation to predict the δ^2H value of the source water of each of the 105 plates and also calculated the difference between the predicted value and the measured value. The average discrepancy was 3.9‰ with a standard deviation of 2.2‰. When we used the predicted values for δ^2H of the source water and the GMWL relationship ($\delta^2H = 8\delta^{18}O + 10$) to calculate a predicted δ^{18}O value for the source water, the average discrepancy between the predicted and measured values was 1.0‰ with a standard deviation of 0.65‰.

An alternative method of making the same prediction would be to take the individual δ^{18}O and δ^2H values measured for water extracted from an agar plate, and extrapolate back from those values along a line with a slope of 2.55 (the average slope of 4 experiments, excluding the Livermore data) to the Global Meteoric Water Line. The point at which the two lines intersected would be a close approximation of the original water used to make the medium. This approach can be visualized looking at Fig. 2. When this method was used to predict the δ^2H value of the source water of each of the 105 plates, the average discrepancy was 5.2‰ with a standard deviation of 3.1‰. This approach also give a predicted value for δ^{18}O of the source water. The average discrepancy between these predicted δ^{18}O values and the measured values was 1.1‰ with a standard deviation of 0.85‰.

Growing Organisms on Agar Media

When microbial organisms are grown on agar, the plates are inoculated and then incubated for varying amounts of time in order for the microbes to replicate many times over. If the goal of the exercise is to produce spores of the genus *Bacillus*, plates may be incubated for a week or more. During this time, the agar medium could undergo substantial evaporation, depending on the ambient relative humidity.

We made quantitative predictions of the expected isotope ratios of spores by approximating an entire harvest of spores as a two-component mixture consisting of 90% spores produced on Day 1 water and 10% spores produced on Day 7 water. We used agar plate water data from a Salt Lake City evaporation experiment and the equations relating medium water isotope ratios to those of *B. subtilis* spores produced in liquid SSM (22) to predict the isotope ratio of such a mixture of spores (Table 3). This exercise predicted an observable increase in the δ^{18}O value of spores harvested after a brief incubation (e.g., on Day 1) and spores harvested after prolonged incubation. Interestingly, the exercise also predicted that there would not be a simultaneously observable increase in δ^2H values.

TABLE 3—*Predicting isotope ratios of spores incubated for prolonged times on evaporating agar: Model 90% Day 1 and 10% Day 7 spores.*

	Water Isotope Ratios		Predicted Spore Isotope Ratios	
	δ^{18}O, ‰	δD, ‰	δ^{18}O, ‰	δD, ‰
Source water	−15.8	−124	5.0	−92
Plate water after 1 day incubation	−14.8	−120	5.7	−91
Plate water after 7 days' incubation	−6.6	−100	11.9	−85
90% Day 1 and 10% Day 7 mixture			6.3	−90

To test these predictions, we prepared a series of SSA plates (identical in composition to liquid SSM except for the addition of the agar solidifying agent), inoculated them with *B. subtilis*, and allowed the plates to incubate for varying numbers of days before harvesting spores. In addition, we sent medium and organisms to colleagues in Houston and Livermore, who duplicated the experiment and sent us spores harvested at varying times.

The results of these experiments fit the prediction pattern shown in Table 3 (Fig. 4). In each case, the δ^{18}O value of the spores increased with incubation time, while the δ^2H value did not. The lower rate of increase of the δ^{18}O values seen in the Houston cultures was presumably a consequence of the area's substantially greater (than Salt Lake City) average relative humidity. Higher humidity within the laboratory would result in lower rates of evaporation (see Table 1) and less enrichment of the agar water and spores.

The above result indicated that oxygen isotope ratios of spores produced on agar media were sensitive to evaporation, while hydrogen isotope ratios were much less so. Hydrogen isotope ratios therefore provided a better link between spores and the original culture water. To test whether the relationship between stable hydrogen isotopes of water contained in an agar medium and spores produced on that medium was the same as that in liquid medium, we used four isotopically distinct waters to prepare SSA. Immediately before inoculating the plates with *B. subtilis*, we aseptically removed slices of agar from the plates for water extraction and analysis. Organisms were then spread onto the sampled plates, incubated for varying amounts of time, and harvested. The results were clear. Both the slope and the intercept of the curve describing the relationship between δ^2H values of water and spores were indistinguishable from the liquid relationship (Fig. 5, Table 4).

Discussion

Isotope Ratio Analysis of Water Extracted from Agar Media

Our results showed that the isotopic behavior of water evaporating from agar-containing bacteriological media in Petri dishes was different from that of water evaporating from open pools. The ambient environmental relative humidity did not affect the slope of the δ^2H vs δ^{18}O relationships, as it does in evaporation from pools, but it did appear to affect the rate of evaporation from the Petri dishes. A simple mechanistic explanation is that in a covered Petri dish, water evaporates from the agar into a semi-closed atmosphere, where net movement of vapor is by diffusion. The air within the Petri dish is likely to be near 100% humidity, irrespective of geographic origins of the sample. The net water vapor flux is outward from the Petri dish, although there is both an outward and inward diffusion of vapor. Given mass-dependent diffusivity differences, we expect that the changes in the δ^2H of residual water would be less than those of the δ^{18}O of residual water and that the pattern would be similar across geographic locations if water in the Petri dish was

TABLE 4—*Statistical comparison of spore versus medium water dD curves produced from spores grown on solid and liquid Schaeffer's Sporulation Medium. (Solid medium curve shown in Fig. 5; liquid medium data from reference 23.)*

Medium (solid or liquid)	Slope of Spore vs. Medium Water Relationship, with 95% Confidence Interval	Intercept of Spore vs Medium Water Relationship, with 95% Confidence Interval
Solid medium	0.28 ± 0.01	−59.6 ± 2.0
Liquid medium	0.28 ± 0.02	−56.2 ± 3.5

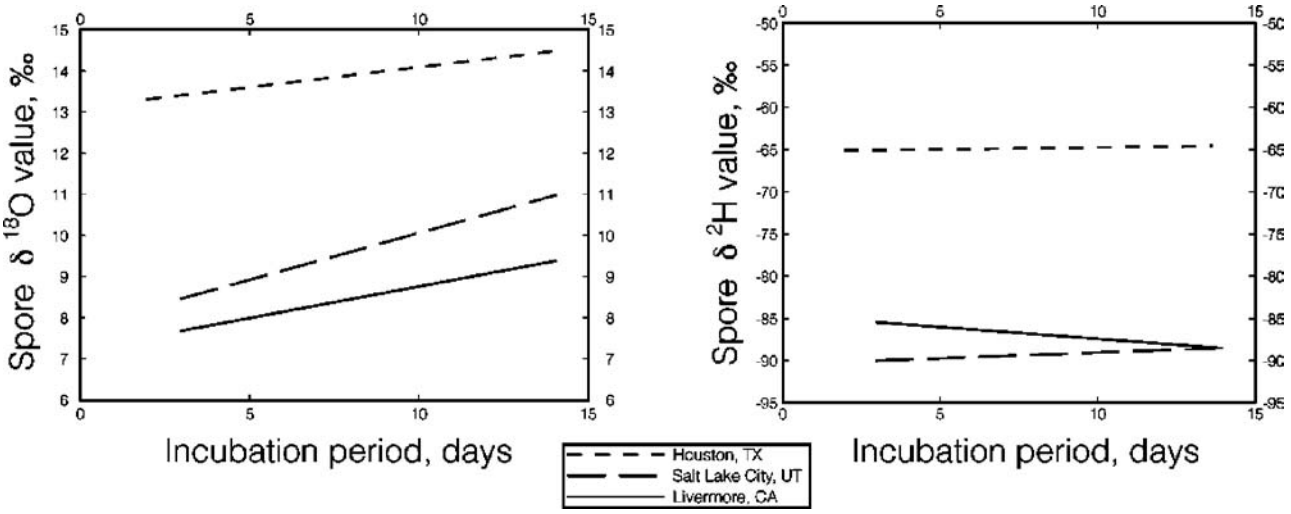

FIG. 4—*Oxygen and hydrogen stable isotope ratios of spores harvested from agar plates after incubating for varying lengths of time. Experiments were performed at three geographic locations.*

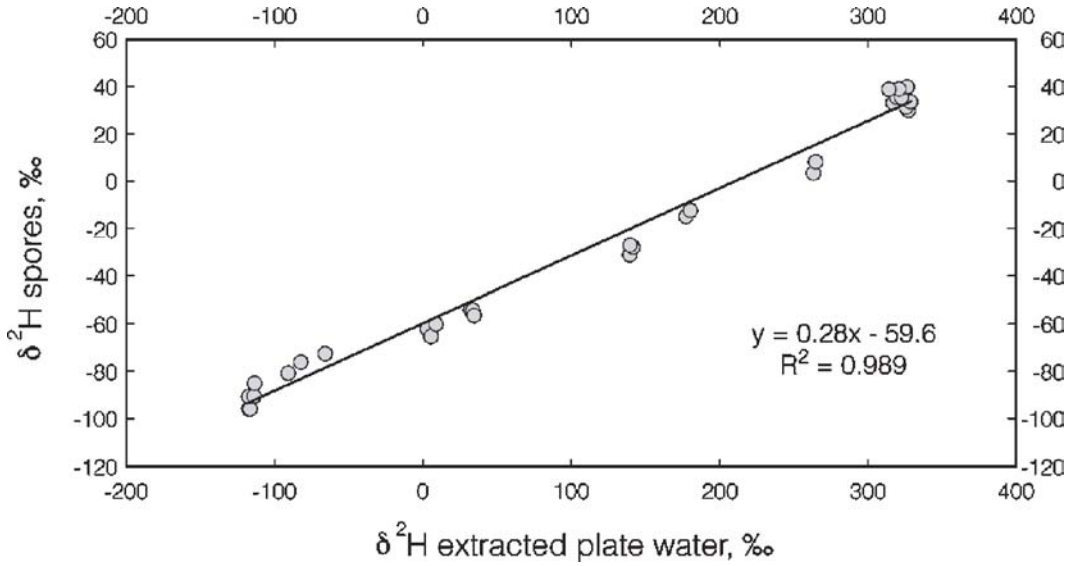

FIG. 5—*The relationship between the δ^2H values of water extracted from agar plates immediately prior to inoculation and the δ^2H values of spores produced on those plates after incubation for varying lengths of time. The relationship was insensitive to incubation period.*

at or near isotopic equilibrium with atmospheric water vapor. The consistent relationship between δ^2H and $\delta^{18}O$ values of agar water allowed us to construct a model for predicting the isotope ratios of the original culture water used to make agar media. This model could be of some forensic use if it ever became important to trace the origins of a medium-containing Petri dish, since water isotope ratios bear a geographic signature.

In evaporation from open pools there is back-exchange between water molecules in the vapor and water phases (e.g., Refs 24 and 25). This effect would not be noticeable if ambient vapor and liquid water were in isotopic equilibrium, but when they are not, back-exchange could change the isotope ratio of water in the liquid reservoir. In our disequilibrium evaporation experiment, the slope deviated from 2.5 in the negative direction. In this case, the ambient vapor was isotopically lighter than the water.

In the Livermore agar observations, the slope deviated in the positive direction, which is what would be predicted if the vapor in the air were isotopically heavier than the local tap water. Much of the tap water in that area of California originates as precipitation from higher elevations in the Coastal Range or Sierra Nevada and could reasonably be expected to be isotopically lighter than vapor masses from the Pacific Ocean, particularly in late summer when this experiment was conducted.

Disequilibrium is relevant for forensic analysis because plates could be prepared at one facility and then transported to another for use. This situation results when laboratory personnel order pre-poured plates from geographically distant medium companies, and could also result if a bioterrorist prepared plates at a laboratory facility, inoculated them, and then immediately transported them to another locale. Our results suggest that disequilibrium would have a minimal effect on the analysis of agar water isotope ratio data. Even with the deviation in the Livermore slope, the average discrepancy between the statistical model prediction and the measured value of the hydrogen isotope ratio of the source water was only 2.7‰ with a standard deviation of 1.4‰. This result suggests that in the event a Petri dish were seized, it should be possible to predict the isotope

ratio of the water used to make the original medium even if it had been prepared at a distant site.

It would also be possible to compare the source water signature of an unused agar plate to the isotope ratio of spores discovered elsewhere. Since the hydrogen isotope ratios of spores growing on agar media are not measurably affected by evaporation of the agar during growth, the hydrogen isotope ratio of seized spores would bear a predictable relationship to the source water used to make the agar. Thus the hydrogen isotope ratio of source water, extrapolated from a seized, potentially evaporated Petri plate, could be compared to that of spores to determine whether the spores could have been produced on plates or in liquid medium made with that (or isotopically similar) water.

Effect of Culturing on Agar on Spore Isotope Ratios

Evaporation of water from agar media that occurs before inoculation will affect the isotope ratio of water that the organisms initially use during growth, and the isotope ratios of spores produced initially will reflect that agar-water isotope ratio. Additional effects of agar-water evaporation on the isotope ratios of subsequently-produced spores depend on the amount of evaporation that occurs during culturing. The ambient relative humidity, the incubation temperature, and the length of time plates are incubated before harvest are parameters that could affect the rate and extent of evaporation.

From our observations, spore oxygen isotope ratios were more sensitive to evaporation during culturing than were spore hydrogen isotope ratios. Part of the greater sensitivity was a result of the greater dependence of spore oxygen than hydrogen isotope ratios on water. Spores derive ca. 70% of their O atoms from water but only 30% of their H atoms from water (22,23). In the absence of information as to whether spores were produced on agar or not, the hydrogen isotope ratios of spores were a more reliable link to the source water than the oxygen isotope ratios. The relationship between the isotopes of water in the medium at the time of inoculation and the time of harvest are the same for solid media as for liquid.

We had previously constructed an integrated model linking the hydrogen isotope ratios of spores, water and nutrients using data from cultures of *Bacillus subtilis* grown in liquid (23). We tested that model with data from spores produced on SSA and incubated for 2, 3 or 4 days. The predicted δ^2H value of spores from the model was –94.6‰ and the average measured value of the spores was –94.1‰. Thus the integrated model seems to be valid for spores grown on agar media as well as for those grown in liquid. Based on all of these results it appears that hydrogen isotope ratio data can be interpreted and analyzed in a similar manner whether the spores were produced in liquid or on agar-containing media.

Organisms incorporate atoms into their organic molecules from both nutrients and water, The isotope ratios of various elements thus constitute signals that can potentially reveal information about their growth environments, if the isotopic relationships between the growth environment and the sample are understood. The isotope ratios of water used to culture microorganisms are of particular forensic interest, as they contain a potential geographic link to the location at which the culturing took place.

It appears that the relationships between hydrogen and oxygen isotopes in growth medium water and spores are the same whether the medium was solid or liquid. The significant variable in growth on solid media appears to be evaporation, which has a greater impact on the oxygen isotope ratios of both the agar-water and the resulting spores. If spores are cultured on agar media under conditions in which evaporation from the plates occurs before inoculation or during incubation, the oxygen isotope ratios of the resulting spores can be significantly enriched compared to the value they would have had following culturing in a liquid medium made with the same original water. This potential for enrichment introduces uncertainty into the interpretation of spore oxygen isotope ratio values, unless it is known that the spores were cultured in liquid medium.

Hydrogen isotope ratios of spores appear to be relatively insensitive to evaporation and thus constitute a more stable signal linking spores to growth water regardless of the mode of culturing. The hydrogen isotope ratio of spores is a consequence of both water and nutrients in the medium, but unlike water isotope ratios, nutrient medium signatures would not be expected to rule out any given geographic area as the source of the culture, since microbiologists tend to order nutrient media from a few suppliers. Nutrient medium isotope ratios would however provide a fingerprint for matching with that of media seized from a suspect laboratory.

A survey of over 500 bacteriological media and medium components showed that although the isotope ratios of media vary, the expected range of δ^2H values for common media is about 50‰ (33). The δ^2H values of surface waters across the United States vary by more than 100‰. It follows that even though nutrients contribute roughly twice as many hydrogen atoms to spores as does culture water, the contribution of each to the total isotopic variation in spores is about equal. Further, the δ^{13}C and δ^2H values of nutrient media are significantly correlated (33), enabling an investigator to constrain the possible isotope ratios of the medium based on the δ^{13}C value of a sample. Thus even with no knowledge of the nutrient medium used to produce spores, it should be possible to determine a range of δ^2H values for the water used to produce a given sample and thus to determine whether a sample could have been produced with water from a given geographic region.

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