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¹⁴C Analysis of Protein Extracts from Bacillus Spores

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Highlights

- $\delta^{14}\text{C}$ of *Bacillus* spores reflects ^{14}C content of the media in which they were grown
- Carbon sources for the media were alive within ~ 1 year of the date of manufacture
- Water-soluble spore proteins are amenable to ^{14}C bomb-pulse dating

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Abstract

Investigators of bioagent incidents or interdicted materials need validated, independent analytical methods that will allow them to distinguish between recently made bioagent samples versus material drawn from the archives of a historical program. Heterotrophic bacteria convert the carbon in their food sources, growth substrate or culture media, into the biomolecules they need. The F¹⁴C (fraction modern radiocarbon) of a variety of media, *Bacillus* spores, and separated proteins from *Bacillus* spores was measured by accelerator mass spectrometry (AMS). AMS precisely measures F¹⁴C values of biological materials and has been used to date the synthesis of biomaterials over the bomb pulse era (1955 to present). The F¹⁴C of *Bacillus* spores reflects the radiocarbon content of the media in which they were grown. In a survey of commercial media we found that the F¹⁴C value indicated that carbon sources for the media were alive within about a year of the date of manufacture and generally of terrestrial origin. Hence, bacteria and their products can be dated using their ¹⁴C signature. *Bacillus* spore samples were generated onsite with defined media and carbon free purification and also obtained from archived material. Using mechanical lysis and a variety of washes with carbon free acids and bases, contaminant carbon was removed from soluble proteins to enable accurate ¹⁴C bomb-pulse dating. Since media is contemporary, ¹⁴C bomb-pulse dating of isolated soluble proteins can be used to distinguish between historical archives of bioagents and those produced from recent media.

Keywords:

Accelerator Mass Spectrometry, Spore, Bacillus, ¹⁴C bomb-pulse dating, isotope forensics

1.0 Introduction:

Investigators of bioagent incidents or interdicted materials need validated, independent analytical methods that will allow them to distinguish recently made bioagent samples from material drawn from the archives of a historical program. The tactics used in WWI led to the inclusion of biological weapons in the 1925 Geneva Protocol [1,2]. Both Axis and Allied nations conducted bioweapons research leading up to and during WWII and research on biological weapons was conducted by both East and West during the cold war [1,2]. The Biological and Toxin Weapons Convention came into force in 1975 with 22 ratifying countries. There are currently 170 ratifying or acceding countries. Spores in a forensic case would most probably have been produced since WWII, but could be near a century old.

Radiocarbon dating is not generally used in forensics studies. The slow radioactive decay of radiocarbon (^{14}C or carbon-14) (radioactive half-life of 5,730 years) makes the isotope more suitable for archeological work because its decay is minimal within the time periods of interest in forensic cases. Above ground testing of nuclear weapons from the middle 1950s until the implementation of the Limited Test Ban Treaty in 1963 nearly doubled the $^{14}\text{CO}_2$ of the atmosphere above natural production, producing the ^{14}C bomb pulse [3-7]. The level of $^{14}\text{CO}_2$ has decreased since the end of atmospheric testing with a mean life of about 16 years. The decrease is due to bomb-pulse ^{14}C mixing with large marine and terrestrial carbon reservoirs. The excess ^{14}C has not decayed, but has just migrated out of the atmosphere. These temporal variations of artificially high levels of atmospheric ^{14}C have been captured in organic material world-wide and provide a means to determine a synthesis date for biomolecules. Since all living things possess isotopic signatures of their food sources, the ^{14}C signature is naturally

incorporated into all living things. The ^{14}C bomb-pulse thus provides an isotopic chronometer of all living things since 1955 (Fig. 1) [8,9].

The isotopic content of new plant growth reflects the fraction modern radiocarbon ($F^{14}\text{C}$) of atmospheric CO_2 because plant carbon mass comes directly from atmospheric CO_2 . The $F^{14}\text{C}$ of herbivores lag the atmosphere slightly because their primary carbon source is on the order of weeks to months old. Omnivores and carnivores lag the atmosphere further because their carbon sources are another step removed in the trophic food chain. The date of formation of a tissue or specific biomolecule can be estimated from the bomb-curve by considering these lags in incorporation and relating the $F^{14}\text{C}$ with the date [8,9]. Using an annual average of the carbon intake over a growing season can account for much food-chain lag and produce a smooth curve without the noise seen in the data sets (Fig. 1) [3-9]. Additionally, since food averages the $F^{14}\text{C}$ of atmospheric CO_2 over a growing season, it makes sense to graph the average of the atmospheric data over the appropriate growing season [8,9]. Caution must be exercised when dating an elevated sample because the pulse is double valued. Placing a sample on the ascending or descending side of the pulse can often be accomplished if other information is available [8-10].

Bacteria convert the carbon in their food sources, the media in which they are grown, into the biomolecules they need to function, just like plants and animals. The $F^{14}\text{C}$ of *Bacillus* spores reflects the radiocarbon content of the media in which they were grown. When an organism is metabolically inactive, as in the case of frozen cells or isolated spores, its stable isotopic content is unchanged while its radioisotope concentration decreases with time as the radioisotope decays. In the case of a long-lived isotope like ^{14}C , decay does not significantly change the $F^{14}\text{C}$ content over a few decades. The isotopic content of organisms can be measured very precisely by

isotope ratio mass spectrometry [11] as well as AMS. *Bacillus* spores whose stable isotopic content falls within the spectrum of naturally occurring values can provide useful forensic information [11].

Over more than three decades, stable isotopic studies of the major biological elements C, H, N and O have been used to construct food source relationships and establish migratory patterns [12-21]. Additionally, the ^{14}C bomb-pulse has been used for forensic dating organic material and teeth of recent origin [10,22-31]. Although bomb pulse dating was not developed using microorganisms, the same general principles of ^{14}C sample definition and analysis that apply to higher-order organisms also apply to heterotrophic bacteria. For example, Cherrier, et al (1999) [32] measured $\Delta^{14}\text{C}$ and $\delta^{13}\text{C}$ of nucleic acids extracted from marine bacteria to associate natural bacteria populations with local dissolved organic carbon pools.

Our initial approach to date archived spore samples had mixed results. The lack of archived media and buffers used in production of the samples prevented confirmation that the measured $F^{14}\text{C}$ values of archived spore preparations mirrored the food sources of the bacteria or if there was contamination from buffers or solvents used in the spore preparations. Some of the samples produced consistent $F^{14}\text{C}$ values after a variety of pretreatment techniques designed to remove residual buffer salts; others experienced substantial shifts in $F^{14}\text{C}$ values. About half of the archived spore samples had $F^{14}\text{C}$ values <1 indicative of a significant fossil carbon contribution. It is possible that the media contained fossil or significant marine-derived carbon, but it is more likely buffers containing carbonate and acetate from fossil sources were used. Furthermore, virtually all detergents regularly used in spore isolation contain significant levels of carbon in a variety of molecular forms. Detergents can resist removal due to their hydrophobic attractions with proteins. Incomplete rinsing can leave a substantial detergent residue that may

skew the $F^{14}C$ value. Additionally, nearly all organic solvents aside from ethanol are manufactured from petroleum sources containing fossil carbon. It is our belief that the hydrophobic nature of the spore coat physically entrapped fossil carbon from detergents and organic solvents used in the preparation of the archived samples.

Problems associated with the removal of process contamination are outside the scope of traditional radiocarbon dating. Due to these difficulties a more specific sample definition was required rather than analyzing whole spores. Spore components spatially removed from the suspect contamination needed to be isolated, purified, and dated. A method for producing high purity DNA samples suitable for dating was developed with collaborators investigating human cell turnover [33], but the large starting sample size required (~10 g) make it impractical for spore dating where evidence is often limited.

Proteins were selected because they comprise a significant fraction of the carbon mass of all biological samples and a variety of separation techniques are well established. A density gradient separation technique has been used to separate pathological senile plaques and neurofibrillary tangles composed of proteins for bomb-pulse dating [24]. Furthermore, experience with tracing covalently bound protein adducts suggested that very little contaminant carbon (ppm) will bind to the soluble proteins [34] such as those located in the interior of the spore.

Common protein isolation techniques aim to keep the protein in the native structure and are not concerned with purity of the protein with respect to small molecule contaminants. These techniques were therefore not directly appropriate for the isolation of pure protein samples for radiocarbon dating which is destructive and consumes samples. We separated the proteins in the liquid phase and avoided using gel extraction techniques due to the incomplete nature of these

techniques. Additionally, gels have fossil carbon containing polymerizing components that can contaminate the proteins and decrease the yield of protein available for analysis. Ultrafiltration techniques used to purify bone collagen for traditional radiocarbon dating [22,27,35,36] and eye lens proteins for bomb pulse dating [37] were adapted for use with spore proteins.

2. Materials and methods

2.1. Spore Sample Species

Spore samples from the *Bacillus* genus (BSL1, biosafety level 1) were obtained from the Lawrence Livermore National Laboratory (LLNL) archive as well as generated on site as described below. *B. thuringiensis kenyae* spores (*Bt ken*, control spores) generated onsite with defined media and carbon free purification were used as standards; The test samples included *B. thuringiensis israelensis* (*Bti*), *B. globigii* (*Bg*), and *B. thuringiensis kurstaki* (*Btk*) from the LLNL archive. The archived spores were produced and purified by means unknown to the researcher performing the extraction, in order to mimic real world specimens. The archived spores were contaminated with petroleum-derived carbon from solvents and detergents used during processing. In some cases detergents used to wash dead *Bacillus* fragments from spores were not removed by rinsing prior to drying the spores with volatile petroleum-derived solvents.

2.2. Control Spore Sample Generation

Growth and sporulation of control cultures: *B. thuringiensis kenyae* (LLNL internal source) was grown in nutrient broth (EMD Biosciences) by inoculating 5 mL of sterile media with a single colony which was grown on plates of solid brain heart infusion media (Teknova). Cultures in nutrient broth were incubated at 30°C with shaking at 225 rpm, overnight. To induce spore formation, cells were harvested from the nutrient broth and transferred to G medium [38] (Table 1). Cells were harvested by centrifugation at 3000 x g for 10 min and resuspended in 5 mL sterile

G medium. Cells were then washed by repeating the centrifugation and re-suspension in 5 mL G medium. This suspension was used to inoculate a 200 mL sporulation culture in G medium. Sporulation cultures were incubated at 35°C with shaking at 225 rpm, after incubation for 4 d. Spores were confirmed by phase contrast light microscopy on a Zeiss inverted microscope with a 100x oil immersion lens (Figure 2). The spores were then harvested by centrifugation at 8000 x g for 15 min at 4°C and re-suspended in 60 mL DNase free water. Spore samples were then washed repetitively, by centrifugation at 3000 x g for 10 min and re-suspending in 10 mL of DNase free water, repeating the centrifugation as above. The sample was then resuspended in 4 mL of DNase free water and split into 4 – 1 mL aliquots that were separated by centrifugation at 10,000 x g for 10 min at 10 °C and repeated. These samples were frozen at -20 °C. Thawed spores were further purified by centrifugation at 10,000 x g at 4°C for 10 min, and subsequent re-suspension in ddH₂O, repeating an additional two times. Spore solutions (1 mL each) were lyophilized in clean, dry and sterile pre-weighed vials and stored dry. Typical masses of 7-15 mg were achieved per tube.

2.3 Spore protein purification

A variety of sample pretreatment methods were employed to clean the available archived spores and recently generated *Bt. ken* spores, lyse the spores, and collect soluble proteins and insoluble debris for ¹⁴C analysis. All techniques employed were chosen to be easily adaptable to a biosafety laboratory. Because AMS is an isotope ratio measurement, understanding the carbon inventory of the sample is critical to converting an isotope ratio into a date. Standard radiocarbon sample pretreatment methods are designed to remove common classes of carbon contaminants from samples typically obtained from soil, sediments, ash, water or ice. These techniques generally avoid adding carbon to avoid skewing the carbon inventory and included washes with

acidic and basic solutions followed by rinses with water. The standard radiocarbon pretreatment techniques did not remove all carbon contaminants from all the available archived whole spore preps, probably due to physical entrapment of contaminants in spore agglomerations. Some archived spore samples retained $F^{14}C$ values corresponding to ages thousands of years old, indicating significant fossil carbon contamination was not removed by the applied clean up method.

Separation of water-soluble and insoluble fractions was accomplished using the following procedure. Positive displacement pipets (Gilson) were used to avoid carbon contamination from filter tips and pipette cross contamination. Sterile filtered 0.1 M ammonium sulfate was added to vials of spores with a known mass of 2-5 mg, diluting to a final spore concentration of 20 mg/mL. A blank of ammonium sulfate alone was included as well as a protein (BSA) control. Some spore samples were not miscible after vortexing potentially due to contaminating organics. An equal volume of 1.0 M NaOH was then added to the samples diluting to 10 mg/mL, and the samples were vortexed. Samples were then centrifuged at 10,000x g for 5 min at room temperature. The supernatant was removed and saved for analysis as wash 1. The pelleted samples were re-suspended to 10 mg/mL in ddH₂O by vortexing and pipetting with a positive displacement pipet (Gilson). The 2-5 mg sample aliquot was further processed by centrifugation at 10,000 x g for 5 min at room temperature and collecting the supernatant for analysis as wash 2, then resuspending the sample pellets in 400 μ L of ddH₂O, repeating the centrifugation and re-suspension two additional times combining the supernatants (wash 2-4) for further analysis. Samples were finally resuspended on ice in 3% hydrogen peroxide and incubated on ice for one hour. To these solutions 400 μ L of 1 M KCl (aq) was added and vortexed. The samples were then collected by centrifugation as above and the supernatant was added to the wash 2-4. All

collected washes were combined and called the soluble extract. The pelleted solid was then re-suspended in 400 μ L 0.1 M potassium phosphate, pH 7.0. In order to mechanically disrupt the solid spore samples, bead beating was performed using a mini bead beater-1 (BioSpec Products). Samples in 0.1 M potassium phosphate (BDH) were added to 75-100 mg of triple water washed 425-600 μ m glass beads (Sigma) in a 2 mL bead beater tube with o-ring aerosol tight cap and cycled for 180 sec at 4800 rpm. Samples were then iced for 3 min and disrupted by bead beating an additional two times with incubation on ice in between. To reduce foam, samples were then centrifuged for 1 min at 10,000x g. Liquid supernatant was removed by pipetting and transferred to a new 2 mL low bind tube (Eppendorf). Beads were then washed with 400 μ L of 0.1 M HCl followed by 400 μ L of 0.1 M NaOH. Buffer composition of the spore lysate was 0.05 M KHPO_4 and 0.05 M KCl (Acid Base reaction). Collected soluble (washes) and solid (lysate) samples were concentrated to approximately 500 μ L by tangential ultrafiltration unit with a molecular weight cut off of 5K (Vivaspin500, Sartorius), which had been extensively washed (4x, 1 mL ddH₂O) to remove filter wetting agents. Solid spore extracts, controls, blanks and soluble washes were then lyophilized in pre-weighed vials, and the masses of the samples were determined.

2.4 Spore viability and protein concentration

Viability of any live BSL1 control spores was assayed by plating 5 μ L of the lysate/spore extract solution on to BHI agar plates and growing overnight at 35 °C in incubator oven. No growth was observed. The concentration of accessible protein was determined by Coomassie plus assay (Pierce) with bovine gamma globulin standard (Pierce). This assay was run in triplicate according to manufacturer's procedure for a microplate assay.

2.5 AMS sample prep and measurement

A variety of media samples were obtained from local biology laboratories and purchased from commercial vendors for ^{14}C analysis. Aliquots containing approximately 1 mg C were placed in quartz combustion tubes and dried in a vacuum centrifuge. Isolated fractions of water-soluble proteins and water-insoluble solids from spores were analyzed for $F^{14}\text{C}$ value by AMS. Water-soluble and water-insoluble fractions were transferred to quartz combustion tubes and lyophilized to dryness. NIST SRM 4990C (Oxalic Acid II) and IAEA C-6 (sucrose) were dissolved in water and aliquots containing 20-100 μg carbon were co-lyophilized with the samples as controls to monitor carbon contamination during drying. Excess copper oxide (CuO) was added to each dry sample, and the tubes were brought to vacuum with a turbo pump station employing an oil-less diaphragm backing pump and sealed with a H_2/O_2 torch. Tubes were placed in a furnace set at 900°C for 3.5 h to combust all non-mineral carbon to CO_2 . The evolved CO_2 from combusted samples was purified, trapped, and reduced to graphite on iron catalyst in individual reactors [39, 40]. All $^{14}\text{C}/\text{C}$ measurements were completed with graphite targets analyzed at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory on the HVEE FN-class AMS system [37, 41].

For the $\delta^{13}\text{C}$ fractionation correction a sample value of -20 ± 2 ‰ [42] was used for all samples based on the measurements of selected sample splits of large CO_2 samples. Corrections for background contamination introduced during sample preparation were made following standard procedures [43]. All data were normalized with six identically prepared NIST SRM 4990B (Oxalic Acid I) standards. AMS spectrometer performance was monitored using NIST SRM 4990C, IAEA C-6, and TIRI wood [44] as quality control secondary standards. The measurement error was determined for each sample and ranged between ± 2 -10‰ (1 SD). The $F^{14}\text{C}$ nomenclature was used for reporting post-bomb data defined in Eq. 2 of Reimer et al.

(2004)[45]. It is enrichment or depletion of ^{14}C relative to an oxalic acid standard normalized for isotope fractionation.

3.0 Results:

We performed a survey of commercial growth media derived from terrestrial agricultural source materials in order to determine the relationship between the ^{14}C age of the media and specified stock or expiration dates. For any measured $F^{14}\text{C}$ value (Fig. 1 y-axis), there are two intercepts with the curve, one on the rising part (1955-1963) and the other with the falling part (1963-present) of the bomb pulse curve. The uncertainty in the $F^{14}\text{C}$ measurement maps to a chronological uncertainty of ± 2 years as depicted in the error bars of Fig. 3. As one might expect, we found that the ^{14}C ages of our media samples were within 0-5 years of labeled expiration or stock dates (Fig. 3) which is consistent with the expectation that the harvest dates for the materials are within a year or so of manufacture and that the shelf life of media is 5 years or less. Since growth media are the primary source of carbon isotope signatures incorporated by the bacteria during culture it is expected that ^{14}C measurements can be used to associate bacterial cultures with specific growth media. It may even be possible to determine date of culture if bacteria are cultured on contemporaneous food and samples of both the bacteria and their growth media can be obtained.

Preparations of *B. thuringiensis kenyae* spores (*Bt ken*, control spores) were generated locally with defined media and carbon free purification as needed for controls for processing evaluations. The process for generation of control spores is described in the methods section. The $F^{14}\text{C}$ values of the G-media and nutrient broth used are shown in Table 2. The nutrient broth

contained carbon that was contemporary (within a year of being in the atmosphere as CO₂) at its time of purchase while the G-media was several years old. One would expect the F¹⁴C values of spores to be between the isotope ratios of the media used to produce them.

Table 3 contains mass recovery and isotope analysis data of control spores. The starting mass was not measured precisely but is accurate within +/- 0.5 mg. The masses of soluble and insoluble fractions reported are carbon masses as measured by CO₂ pressure during AMS sample preparation. It was clear that a significant fraction of the spore mass was not carbon. The protein separations from control spores were conducted independently over a period of one year by different researchers. Although not completely reproducible, the water-soluble component of the proteins yielded reasonably consistent values in most cases. The average F¹⁴C of the soluble fraction of control Bt ken spores was 1.078 ± 0.032 (1 SD) for a chronological uncertainty of ± 6 years. This is more variability than typically seen in bomb pulse dating [22-27,31,33,37] and was attributed to initial use of a shared lyophilizer to dry control spore preparations. Pump oil and solvents can contaminate samples with ¹⁴C-free carbon, causing depressions in F¹⁴C by adding carbon mass that is devoid of ¹⁴C. As noted, some spore samples were not miscible in initial extraction components indicating significant surface contamination, however, in subsequent steps slurries were obtained.

The archived spore samples tended to be more variable than the controls, probably due to heterogeneous contamination on the surfaces of the spores (Table 4). The F¹⁴C of the solids from archived spores had many measurements significantly depressed with fossil carbon. All four of the archived spores had average F¹⁴C below contemporary levels with three of the spore samples possessing large standard deviations (Table 5) due to samples containing significant amounts of fossil carbon. . However, by using mechanical lysis and a variety of non-carbon containing

chemical washes with carbon free peroxides, acids and bases, contaminant carbon was removed from soluble proteins to enable accurate ^{14}C analyses.

4.0 Discussion

The recovered carbon masses of both control and archived samples were generally between 10-25% of the starting mass of spores. The majority of the mass resided in the lysate solids, which contained the spore coats. In general, ~25% of the starting spore mass of the controls was recovered carbon and 70-95% of that carbon was insoluble solids. Although more variable, the carbon recovery from archive spores was generally closer to 10% of the starting spore mass with 60-90% of the carbon residing in insoluble solids. The carbon recovery of water-soluble protein was 1-3% of the starting spore mass, so a spore sample of 2-5 mg is necessary to yield 20-150 μg carbon for routine ^{14}C analyses by AMS.

The $F^{14}\text{C}$ values of the soluble-protein washes concentrated with the tangential ultrafiltration filters to remove small molecule contamination were more reproducible than those of lysate solids. The solids appeared to have some petroleum derived carbon deposited on them that resisted removal. This could be due to the extensive crosslinking and hydrophobic nature of spore coats. The $F^{14}\text{C}$ values of the solids were more variable and lower than the soluble sample in all but one of the controls (Table 3) and in most of the archived samples (Table 4). Some samples of Btk008 and Bg007 had similar $F^{14}\text{C}$ values for solids and soluble fractions, indicating that the initial washing removed small molecule contamination on the surface of the spores. All the other archived samples had significantly depressed $F^{14}\text{C}$ values in the lysate solids.

The soluble fractions of the control spores possessed $F^{14}\text{C}$ values consistent with that of the G-media used to induce sporulation and the nutrient broth used to grow the bacteria. The precision of the isotope measurement depends upon spectrometer stability over ~4 hours and the number of ^{14}C atoms counted in the detector, so smaller samples tend to have larger

measurement uncertainties. A contemporary 30 μg carbon sample contains about 1.5×10^6 ^{14}C atoms, of which 10% are countable when system efficiencies are included [41]. The measurement uncertainty was 0.4-1.9% (1 standard deviation) for the soluble controls ranging in mass from 198-33 μg , respectively.

The use of the bomb pulse to assign a date of nutrient cessation is valid only between 1955 and the present. Prior to 1955, the $F^{14}\text{C}$ of CO_2 in the atmosphere decreased slightly during the 20th century due to combustion of fossil fuels. Determining whether the measured $F^{14}\text{C}$ value of a sample corresponds to synthesis during the rise (1955-1963) or fall (post 1963) of the bomb pulse cannot be ascertained from the $F^{14}\text{C}$ value alone, other information is needed. Recent reviews of bomb pulse dating in biology [8] and forensics [9] address placing a sample on the rising or falling portion of the bomb curve. Determination of $F^{14}\text{C}$ to 1% precision can provide a date estimation of less than 1 year when the ^{14}C concentration changed quickly. More recently the small annual change in atmospheric $^{14}\text{CO}_2$ yields an age estimate of ± 2 -4 years for a 1% $F^{14}\text{C}$ measurement, limited by natural and anthropogenic atmospheric variations. The AMS measurement precision is the same for old and new samples, the shallow slope of the curve (Figure 1) results in a larger chronological uncertainty for recent biological material.

A protocol for extracting water-soluble proteins without adding carbon was developed and used to link the $F^{14}\text{C}$ of proteins extracted from *Bacillus* spores with that of the media used to grow the bacteria. The procedure is limited by the purity of the isolated sample and elimination of extraneous sources of carbon. The shallow slope of the atmospheric $^{14}\text{CO}_2$ record in recent years limits the chronological precision of the ^{14}C dating method to ± 2 -4 years after 2000. The technique links the $F^{14}\text{C}$ values of *Bacillus* spores with the media used to produce them. Since most commercial media are made from contemporary terrestrial carbon sources, the

$F^{14}C$ values of soluble spore proteins identifies the age of the media used to produce them. Although interdicted bioagents could be a century old, they are more likely to have been produced since WWII [1,2]. If the $F^{14}C$ of water-soluble spore proteins corresponds to recent atmospheric CO_2 , it is likely that the spores were recently produced from contemporary media. If the $F^{14}C$ of water-soluble spore proteins is significantly elevated (e.g., $F^{14}C > 1.15$), the media used to produce them was manufactured between 1958-1990, with smaller chronological windows reflecting the actual measured $F^{14}C$ values. The ^{14}C analysis of spore proteins places the media source material of the spores in a chronological context.

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Figure and Table Captions

Figure 1. Northern hemisphere growing season average of atmospheric ^{14}C concentration in CO_2 from 1940-2007. The ^{14}C concentration curve is a compilation of extensive tree ring and atmospheric records [3-7]. The F^{14}C (i.e., fraction modern) nomenclature is designed for expressing bomb-pulse ^{14}C concentrations [45].

Figure 2. Monitoring the formation of spores for control samples by phase contrast microscopy. A. *Bacillus thuringiensis* pre-spore formation. B. *Bacillus thuringiensis* in sporulation.

Figure 3. Bomb-pulse intercepts and expiration date of commercial media. The expiration dates of media are typically a few years after manufacture. This was the case for recent and old samples. Error bars denote ± 2 standard deviations.

Table 1. Limited media: Used to induce spore formation. Prepare 1.5 L at a time. Prepare solution with potassium phosphate, ammonium sulfate, yeast extract, and glucose. Autoclave and cool the solution. Then add sterile filtered metal solutions of the volume and concentration listed. Confirm the solution is pH 7 (use sterile technique) on pH paper.

Table 2. F^{14}C Values of Feed Stock for Control Spores. Bacteria were grown on nutrient media and switched to G media to induce sporulation.

Table 3. Mass Recovery and F^{14}C Data for Proteins from Control Spores Prepared from November 2009 – October 2010. Bacteria were grown on nutrient media and switched to G media to induce sporulation.

Table 4. Mass Recovery and F^{14}C Data for Proteins from Archived Bti, Btk, and Bg Spores Prepared from November 2009 – October 2010.

Table 5. Average F^{14}C Values of Solids and Water-Soluble Fractions of Control and Archived Spores. The averages and standard deviations of the F^{14}C values are calculated from values in

Table 4. The estimated age range of the soluble fraction is based on the published atmospheric record [3-7] and recent measurements at LLNL.

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Figure 1

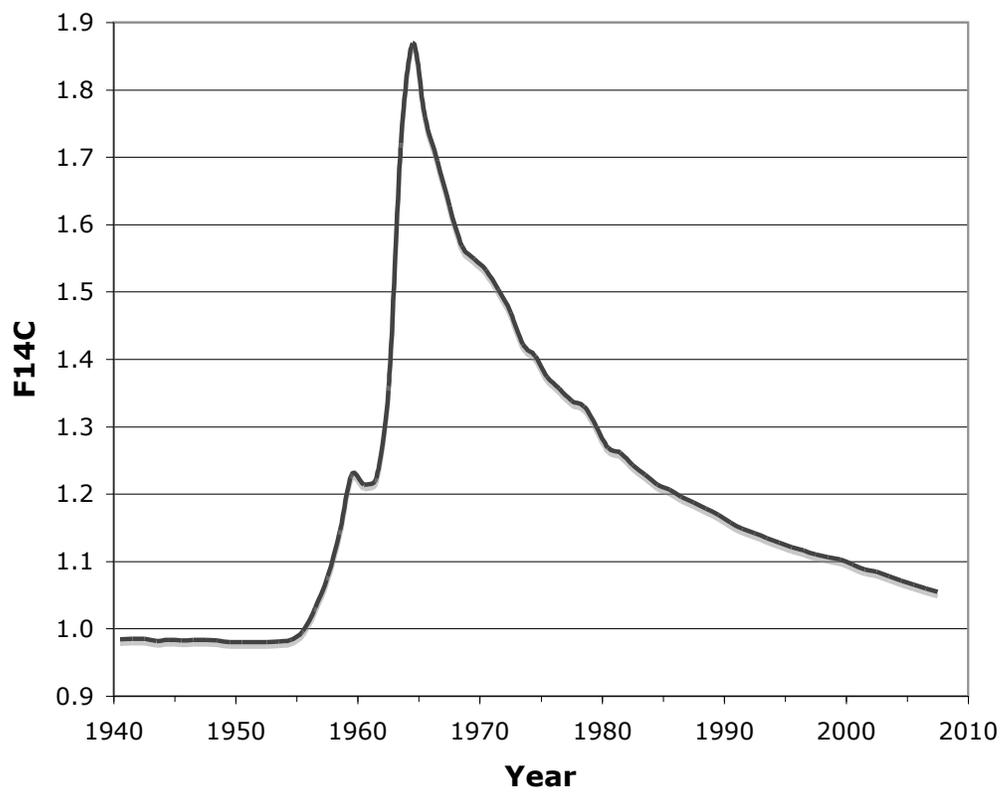


Figure 2

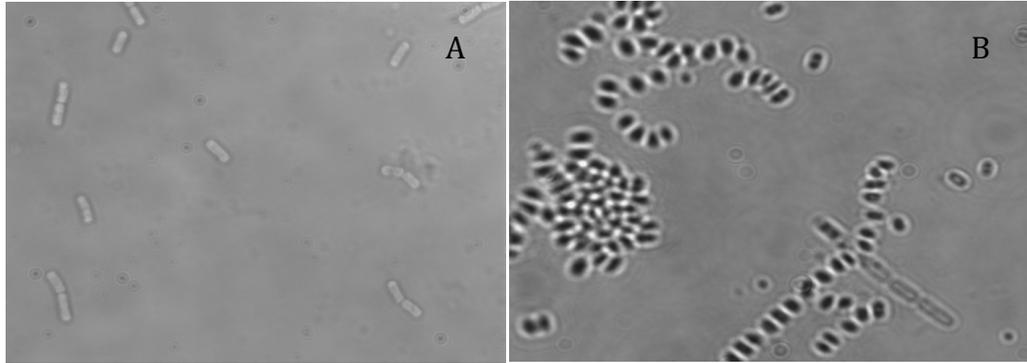


Figure 3

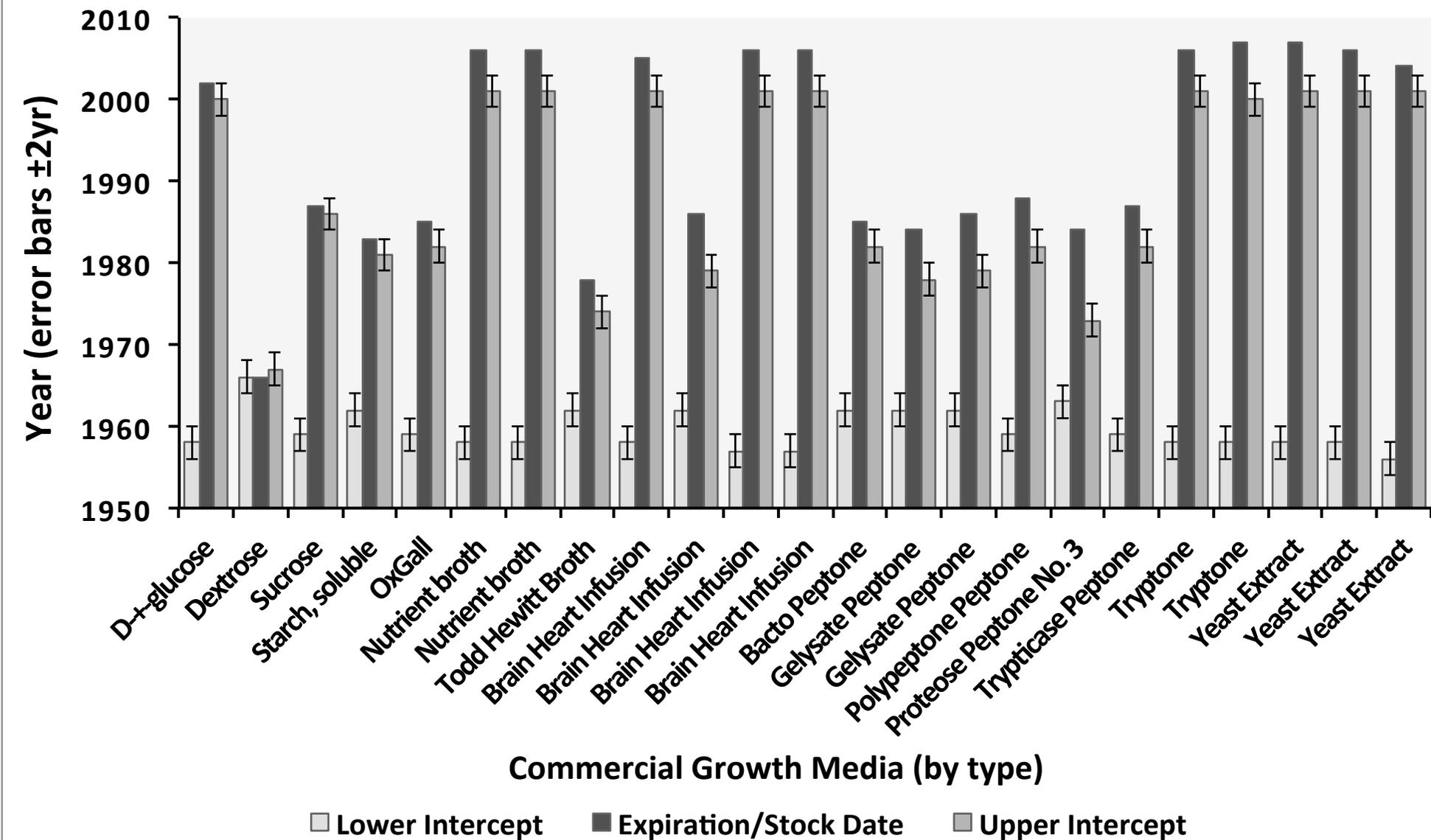


Table 1.

G medium	Name	%	Grams per (1.5 L)	Conc. (mM)	Vol (mL)
1.5 L	KH ₂ PO ₄	0.05	0.75		
Batch	(NH ₄) ₂ SO ₄	0.2	3		
	Yeast extract	0.2	3		
	glucose	0.1	1.5		
	FeSO ₄	0.00005	0.00075	1	4.936
	CuSO ₄	0.0005	0.0075	10	4.699
	ZnSO ₄	0.0005	0.0075	10	4.645
	MnSO ₄	0.005	0.075	10	49.67
	MgSO ₄	0.02	0.3	1000	2.492
	CaCl ₂	0.0025	0.0375	10	0.338

Table 2.

Media Sample	F ¹⁴ C Value \pm 1 SD
G-media	1.105 \pm 0.004
Nutrient Broth	1.055 \pm 0.004

Table 3.

Spore Prep ID	Date Processed	Approximate Starting Mass (mg)	Solid Mass Recovered (mg)	Soluble Mass (mg)	Solid F ¹⁴ C (± 1 SD)	Soluble (F ¹⁴ C) (± 1 SD)
Bt ken	Nov 2009	2	0.419	0.112	0.477 ± 0.002	1.08 ± 0.01
Bt ken	Jan 2010	3	0.647	0.033	1.011 ± 0.005	1.03 ± 0.02
Bt ken	Jan 2010	3	0.600	0.105	1.038 ± 0.003	1.10 ± 0.01
Bt ken	Mar 2010	4	t/f	0.198	t/f	1.089 ± 0.004
Bt ken	Oct 2010	3	0.251	0.065	1.064 ± 0.004	1.104 ± 0.007
Bt ken	Oct 2010	3	0.233	0.088	1.095 ± 0.004	1.109 ± 0.006
Bt ken	Oct 2010	3	0.128	0.042	1.040 ± 0.004	1.034 ± 0.009

t/f= lost due to combustion tube failure

Table 4.

Spore Prep ID	Date Processed	Approximate mass processed (mg)	Solid Mass Recovered (mg)	Soluble Mass (mg)	Solid F ¹⁴ C (±1 SD)	Soluble F ¹⁴ C (±1 SD)
Bti003	Nov 2009	2	0.221	0.042	0.318 ± 0.002	1.04 ± 0.01
Bti003	Mar 2010	3	0.102	0.079	0.887 ± 0.005	1.057 ± 0.004
Bti003	Mar 2010	3	0.126	0.037	0.836 ± 0.004	1.059 ± 0.004
Btk006	Jan 2010	3	0.195	0.051	0.861 ± 0.003	1.03 ± 0.01
Btk006	Mar 2010	3	0.219	0.047	0.846 ± 0.004	1.069 ± 0.005
Btk006	Mar 2010	3	0.167	t/f	0.930 ± 0.004	t/f
Btk006	Oct 2010	3	0.126	0.014	0.898 ± 0.003	1.074 ± 0.026
Btk008	Nov 2009	3	0.421	0.040	0.445 ± 0.002	1.05 ± 0.01
Btk008	Jan 2010	3	t/f	0.084	t/f	1.16 ± 0.01
Btk008	Mar 2010	3	0.353	0.112	0.963 ± 0.003	1.047 ± 0.004
Btk008	Oct 2010	3	0.226	0.067	1.134 ± 0.004	1.147 ± 0.007
Bg007	Nov 2009	2	0.467	0.102	0.462 ± 0.002	1.05 ± 0.01
Bg007	Jan 2010	3	1.063	0.105	1.092 ± 0.004	1.02 ± 0.01
Bg007	Mar 2010	3	0.423	0.198	1.049 ± 0.004	1.041 ± 0.005
Bg007	Oct 2010	3	0.293	0.067	1.084 ± 0.004	1.085 ± 0.007

t/f= lost due to combustion tube failure

Table 5.

Spore Prep ID	Average Solid ($F^{14}C \pm 1$ SD)	Average Soluble ($F^{14}C \pm 1$ SD)	Estimated Age Range of Soluble Fraction
Bt ken control	0.954 ± 0.235	1.078 ± 0.033	1997-2009
Bti003	0.680 ± 0.315	1.052 ± 0.010	2005-2010
Btk006	0.884 ± 0.038	1.058 ± 0.024	2002-2012
Btk008	0.847 ± 0.359	1.101 ± 0.064	1990-2010
Bg007	0.922 ± 0.307	1.049 ± 0.015	2005-2012