**REVIEW
ARTICLE****Preservation records of micro-organisms:
evidence of the tenacity of life**

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Historical perspective

Occasionally there appear in the literature, comments about the long-term preservation and revival of life forms. Revivals from storage structures such as seeds or spores after short periods of time are well-documented in the scientific literature. However, occasionally one of these revivals will stand out and demand attention. One of the most famous of these finds was the successful germination of lotus (*Nelumbium nucifera*) seeds from the bottom of an old dried-up lake in Manchuria after 800 years (Ohga, 1923; Sussman & Halvorson, 1966). The first person to investigate storage of micro-organisms was Antonie van Leeuwenhoek, back in the late 1600s. Keilin (1959) states 'he [Leeuwenhoek] clearly demonstrated that these animalcules can be kept dry for several months without showing any visible sign of life'. Debate about whether a preserved organism had actually been dead and brought back to life, or had simply continued to live with an undetectable metabolism during the storage period occupied a large number of the ensuing years. Thus the concept of anabiosis (restoring to life from a deathlike condition – American Heritage Dictionary) was introduced. Two other almost synonymous terms 'cryptobiosis' and 'latent life' became popular scientific usages at various times.

This initial development of the field was followed by a relatively quiet period, until Lipman (1928, 1931) claimed to have revived micro-organisms from ancient rocks and coal hundreds of millions of years old, and Dombrowski (1960, 1961a, b, 1963) claimed to have revived bacteria which were approximately 650 million years old from Precambrian salts. These claims created quite a stir, and dispute of their findings was swift in coming. Interestingly, Lieske (1932) also claimed to have revived micro-organisms from coal, but because the work was not in English it did not attract much attention. Gallipe's (1921) claim of reviving micro-organisms from meteorites also suffered a similar fate because it was not in English. It

was indeed a pity that the two early claims which attracted attention, those of Lipman and Dombrowski, were so spectacular, involving storage for many hundreds of millions of years, as the disbelief of the scientific community stifled further research on the topic for many years. This was because there were plenty of claims in the literature of micro-organisms surviving 10 or 20 years and then these 'ridiculous' claims of millions of years, with nothing in between.

After the Dombrowski claim, a sprinkling of other revivals began to dot the literature and to fill up the void between the extreme claims and those of a much shorter time span. These revivals quietly increased in number as the years went by. Micro-organisms were revived from the sea-bed, rocks, salt deposits, permafrost, glaciers and ice-cores, mastodon intestines, bricks from ancient temples, old tins of meat, herbarium samples, and beer from sunken ships (for examples, see Fig. 1). Indeed many of these claims involved an air of mystery and adventure as microbiologists revived life-forms from a bygone age, and from specimens obtained from exotic locations. Most of these claims were not given serious consideration, and were passed off as merely modern day contaminants. Thus most of the accounts of preservation records disappeared from modern discussions, almost as if researchers feared ridicule if they mentioned such ludicrous claims. It is now time to review the accumulated evidence.

This article summarizes these claims in a preserved micro-organism database (Table 1). This database, which lists microbial preservations and revivals after storage periods of greater than 50 years, shows that these revivals are much more common than had been previously thought. The database, developed after input from the scientific community (Kennedy & Reader, 1992; Ross, 1993), contains more than 5000 micro-organisms [the majority of the 5000 micro-organisms are from five sites in the Department of Energy (DOE) Deep Microbiology Sub-program, and the number of species revived per specimen varies widely from 1 to over 200], recovered from 62

Fig. 1. (a) Mastodont bones recovered from the Burning Tree mastodon site on Burning Tree golf course, Newark, Ohio. Intestinal remains found with these bones yielded cultures of bacteria. Photograph courtesy of Gerald Goldstein, Ohio Wesleyan University. (b) Bottles of Porter Ale from 1825 and 1993 brewed with the same yeast. Photograph courtesy of

separate specimen origins, and involves the work of 37 independent research teams. Also included in the database are references to further work done on the revived micro-organisms and references refuting the claims, or examples where researchers have failed to isolate viable micro-organisms from similar material.

A study of Table 1 reveals that most of the revivals are from rocks, sediments, salt deposits and soils. The next biggest grouping after this is from human or extinct animal intestinal or faecal remains, and from man-made structures such as bricks (Table 2). Looking at the frequency of the find versus the preservation age (Fig. 2), a distinctly bimodal distribution can be seen, with no revivals in the 100 000–1-million-years grouping. The reason for this gap in revivals in this grouping is not known. However, the bimodal distribution suggests that two types of different preservation environments exist: those below ground (rocks, soils, sediments, etc.) and those above ground (animal and human remains and man-made structures, etc.). To reflect these two different environments, world records for the preservation of different types of micro-organisms are presented in two tables (Tables 3 and 4). Another trend seen in Table 1 is that spore-forming species such as *Bacillus* are well-represented. This ties in with the greater survival ability of the spores compared to vegetative cells. In fact the reverse observation (that non-spore-forming micro-organisms even appear in Table 1) may cause the greater surprise.

A special note should be made of bioinclusions. Bioinclusions are organized biological materials, e.g. whole cells, bacteria, or DNA, that are completely enclosed in an organic, usually geological, matrix. There are a host of finds of cells in calcite, salts, phosphate, spring sinters, and from tooth and bone material (Schaffer, 1993). However, only one of these finds shows even a remote possibility of revival of live micro-organisms (Bargar & Fournier, 1990).

One interesting facet of the revival claims presented in Table 1 is the controversy surrounding the claims. The early investigators in this field, Lipman and Dombrowski, came in for heavy criticism, mainly because claiming that life could survive for hundreds of millions of years was so revolutionary. Also greeted with great hostility was the claim by Gallipe and Lipman of isolating living micro-organisms from meteorites, which sparked debate on the seeding of life on earth from off-planet sources. These meteorite claims, not backed up by further, more modern, repetitions, also heaped doubt on Lipman's other finds. However, the large number of claims from independent research teams shown in Table 1 indicates that it is time to re-examine the limits of survival of micro-organisms.

Even considering that some of the claims listed in the

database may be due to modern day contaminants, the collection of the data is so large and made by so many independent teams that it is now possible to say that revivals from ancient specimens are a reality. The question that is still open to debate is, are these micro-organisms truly ancient or is there some other explanation for the revivals?

Rationale for claiming preservation age

Currently there is no way of definitively proving the age of preservation of any revived micro-organism, and as a result the investigators usually rely on circumstantial evidence to back up their claims or postulations of authenticity. There are also plenty of detractors who are willing to dispute this circumstantial evidence. This is healthy in one respect in that all scientific theories should be scrutinized and proved before being taken as fact. On the other hand it has meant that publication is often disregarded as a possibility, and cultures discarded for fear of ridicule in the scientific community. The following is a list of arguments used to bolster claims of long preservation age.

Inaccessibility of the environment from which the specimen originated

Long-preserved micro-organisms are often isolated from very inaccessible places. Examples include the isolation from a 2800-m-deep bore hole, and from within bricks of temples. The argument used in this case is that the likelihood of micro-organisms penetrating into these locations during the supposed storage period is very small. For example, it is difficult to imagine a surface bacterium boring through 2800 m of rock and soil to contaminate a drill sample. The Achilles' heel of this argument is water ingress. Water may indeed seep into porous structures during long time periods, and this fact is often used to refute claims of revivals from rock or coal. The porous nature of some rocks may mean that microbial migration, even without the assistance of free water, may be a factor. The migration rate of micro-organisms through rock structures needs considerable further study. Another problem occurs when it is assumed that the specimen has remained cool and dry for long periods of time, such as the revival of micro-organisms from bricks or herbarium soil samples which have not been exposed to the weather. It may be that during the long storage time a temporary period of high temperature and humidity could have been experienced, which revived the micro-organism, which subsequently went through a reproduction cycle. Similarly when isolating specimens from permafrost it is difficult to prove that the soil water at no time melted throughout thousands of years. This inaccessibility of the original specimen also does not

Table 1. The preserved micro-organism database, containing a list of the micro-organisms that could possibly have been preserved for long periods of time

The database so far contains more than 5000 micro-organisms recovered from 62 separate specimen origins, and involves the work of 37 independent research teams. Abbreviations: MY = million year(s); CC = stored in a culture collection. Most authors do not make definite claims of authenticity about the age of the micro-organisms.

Estimated length of storage (years)	Micro-organisms	Origin of specimen and comments	References
650 MY	Micro-organisms	Precambrian rocks from the Algonkian, Canada	Lipman (1928, 1931) Farrell (1933)
650 MY	<i>Bacillus circulans</i>	Isolated from Precambrian salts from Irkutsk	Dombrowski (1960, 1961a, b, 1963) Sussman & Halvorson (1966) Rippel (1935) Muller & Schwartz (1953)* De Ley <i>et al.</i> (1966)*
600 MY	<i>Kakabekia umbellata</i>	Ammonia-rich soils from Harlech, Wales	Siegel & Guimarro (1966) Siegel & Siegel (1968)
430 MY	Encapsulated diplococcus-like bacteria	Silurian salts from New York State	Reiser & Tasch (1960)
430 MY	Bacteria	Silurian deposits	Dombrowski (1960, 1961a, b, 1963) Sussman & Halvorson (1966) Rippel (1935)* Muller & Schwartz (1953)* De Ley <i>et al.</i> (1966)*
390 MY	Bacteria	Middle-Devonian salts	Dombrowski (1960, 1961a, b, 1963) Sussman & Halvorson (1966) Rippel (1935)* Muller & Schwartz (1953)* De Ley <i>et al.</i> (1966)*
300 MY	Diplococci	Permian mine salt	Reiser & Tasch (1960)
300 MY	Bacteria	Permian Zechstein and Kali salts. Halophilic pseudomonads similar to the Zechstein salt micro-organisms were also found from salt springs at Bad Nauheim.	Dombrowski (1960, 1961a, b, 1963) Sussman & Halvorson (1966) Rippel (1935)* Muller & Schwartz (1953)* De Ley <i>et al.</i> (1966)*
225–270 MY	<i>Halobacterium saccharovorum</i> , <i>Halobacterium salinarium</i> <i>Haloarcula</i> sp.	From 1200 m depth in the Permian potash mine operated by Cleveland Potash Ltd at Boubly, Cleveland, England	Norton <i>et al.</i> (1993) Rippel (1935)* Muller & Schwartz (1953)* De Ley <i>et al.</i> (1966)*

Table 1. (cont.)

Estimated length of storage (years)	Micro-organisms	Origin of specimen and comments	References
200 MY and varying ages; CC	Thermophilic anaerobes, diversity of chemoheterotrophic bacteria, fungi	Samples were collected as part of the Department of Energy (DOE) Deep Microbiology Subprogram, which was set up in 1984 and to date has a collection of over 5000 revived micro-organisms. Samples included in the collection are from five sites: the Savannah River Site in Aiken, SC; the Hanford site in Richland, Washington; the Idaho National Engineering Laboratory in Idaho Falls; the Nevada Test Site West of Las Vegas; and one further site in Virginia which was explored in cooperation with Texaco. The record for the deepest sample from which bacteria have been isolated by the DOE is 2800 m from Triassic (200-MY-old) sediments sampled from beneath the state of Virginia. Micro-organisms are stored at the DOE Subsurface Microbial Culture Collection (SMCC) at Florida State University (Dr David Balkwill curator); 97 % of the micro-organisms in the SMCC are bacteria, the rest are fungi.	Colwell <i>et al.</i> (1992) Department of Energy (1991, 1992) Frederickson (1992) J. K. Frederickson (personal communication) Russell <i>et al.</i> (1992) Balkwill (1993)
195–225 MY	<i>Halobacterium saccharovorum</i> , <i>Halobacterium salinarium</i> <i>Haloarcula</i> sp. <i>Halococcus</i> sp.	From 170 m depth in the Triassic rock salt mine operated by Imperial Chemical Industries at Winsford, in Cheshire, England. Six out of 350 samples contained living halobacteria. W. D. Grant and colleagues showed that if sodium chloride crystals were grown in a culture of halobacteria, not only were microbes entrapped in fluid inclusions, but they remained alive inside the crystal for several months.	Norton <i>et al.</i> (1993) Postgate (1994) Rippel (1935)* Muller & Schwartz (1953)* De Ley <i>et al.</i> (1996)*
190 MY 25–40 MY	Bacteria <i>Bacillus</i>	Jurassic salt from a Humble Oil Salt Dome DNA from a <i>Bacillus</i> sp. from the gut of a stingless bee entombed in amber was sequenced. Although no viable bacteria were obtained, this work provides possible evidence that DNA can be stored for millions of years.	Reiser & Tasch (1960) Ross (1993) Cano <i>et al.</i> (1993)
24 MY	<i>Aspergillus</i> , <i>Cladosporium</i> , <i>Penicillium</i> , <i>Trichoderma</i> , yeasts, moulds	Miocene core samples from 600–3000 m depth, Fujioka, Japan	Sugiyama (1966, 1967, 1968) Sugiyama & Goto (1969)
15–200 MY	Bacilli and cocci	Pennsylvania anthracite coal	Lipman (1931) Farrell & Turner (1932)* Burke & Wiley (1937)* Turner (1932)*

Table 1. (cont.)

Estimated length of storage (years)	Micro-organisms	Origin of specimen and comments	References
5 MY	<i>Aspergillus</i> , <i>Cladosporium</i> , <i>Penicillium</i> , <i>Trichoderma</i> , yeasts, moulds	Pliocene core samples from 800–3200 m depth, Kambara, Japan	Sugiyama (1966, 1967, 1968) Sugiyama & Goto (1969)
3–4 MY	Bacteria	Sediments in the sea of Japan	Matthews (1993)
1 MY	Bacteria	Constantly frozen sedimentary rocks and buried soils in the Kolyma lowland, USSR	Zvyagintsev <i>et al.</i> (1985)
1 MY	Rod-shaped bacteria	Caves in the US Dry Valley Drilling Project in Antarctica	McFarlane (1992) Cameron & Morelli (1974)
14000–45000	Bacteria-like particles	US Geological Survey research drill hole Y13, Lower Geyser Basin, Yellowstone National Park, Wyoming. Three crystals have been found with bacteria-like particles present in large numbers. The viability of the particles has yet to be tested, but a video has been made showing the particles in motion. To avoid destroying the three crystals, non-destructive testing techniques such as the Nicolet scanning infrared microprobe, Raman spectroscopy and sulphur and phosphorus analysis using the Brookhaven cyclotron are being investigated. Readers with other ideas on methods of non-destructively proving whether these inclusions are bacteria are encouraged to contact Dr Bargar.	Bargar (1992a, b) Bargar & Fournier (1988, 1990, 1991) Bargar <i>et al.</i> (1985) Glen (1986)
11600–17000	Mycelial fungi, nonspore-forming bacteria, spore-forming bacteria	From 300 m depth in a drill hole of a glacier in Central Antarctica in the Vostok Station region	Abyzov & Belyakova (1982) Abyzov <i>et al.</i> (1982a, b, 1988, 1990)
11000	<i>Enterobacter cloacae</i> , <i>Enterobacter agglomerans</i>	Extinct mastodon intestines (pink smelly material from the region near the mastodon's bones) preserved in a bog	Park (1991) Ross (1993) Folger (1992)
7000	<i>Thermoactinomyces vulgaris</i>	Sediments from Elk Lake, Minnesota	Parduhn & Watterson (1985)
5800	<i>Bacillus stearothermophilus</i>	Sediments from off the coast of Ensenada, Mexico to off the coast of Catalina Island, California	Bartholomew & Paik (1966)
2300	Clostridia and azotobacter	Brick from the temenos (wall) surrounding the Great Temple of Amon at Karnak, Luxor, Egypt, examined in December 1962	Abd-el-Malek & Ishac (1966) Sneath (1962*, 1964*)
1950–2750	Thermoactinomycete endospores	Seamere drilling cores from East Anglia	Unsworth <i>et al.</i> (1977) Postgate (1990)
1850–1890	Thermoactinomycete endospores	Debris from beneath the Roman Fort Vindolanda, just south of Hadrian's wall near Carlisle, England	Unsworth <i>et al.</i> (1977) Postgate (1990)

Table 1. (cont.)

Estimated length of storage (years)	Micro-organisms	Origin of specimen and comments	References
1000–3000	Bacteria, fungi, algae	Frozen subsoil from a depth of 2–7 m	Kapterev (1936, 1938) Sussman & Halvorson (1966) Oparin (1957) James & Sutherland (1942)*
1000–1400	Bacteria	Adobe bricks from the interior of pre-Inca pyramids, near Lima, Peru	Lipman (1934)
1000	Thermoactinomyces and <i>Bacillus</i> endospores	Sediments of Lake Thirlmere and Lake Windermere in the Lake District of England	Cross & Attwell (1974) Postgate (1990)
800–1000	Bacteria	Adobe bricks from the pre-Aztec pyramids in Mexico	Lipman (1934)
320	Bacillus	Dry soil samples on the roots of pressed plants in the Herbaria of the British Museum of Natural History and of Kew gardens obtained from botanical voyages dating from 1640.	Sneath (1962, 1964)
200	Bacteria	Arizona Pueblo walls	Lipman (1934)
180–250	<i>Clostridium tertium</i> , <i>Clostridium sordelli</i> , <i>Clostridium novyi</i>	Abdomens of well-preserved mummies found in the catacombs of the town of Klatovy in Bohemia. In contrast, the faeces from the intestines from a peat burial (<i>circa</i> 1000 AD) showed no revivable micro-organisms.	Patocka & Sefrna (1944) Sussman & Halvorson (1966) Sneath (1962,* 1964*)
166; CC	Porter Beer brewers yeast Y35 (<i>Pichia</i>), Gram-positive cocci, Gram-negative bacilli	A bottle of Porter beer from a sunken sailing barge which sank off the Little Hampton coast in 1825. The yeast is used in the commercial production of Porter Beer. The record for revival of yeast from beer prior to this discovery was 15 years, as reported by Tanner (1944).	Coghlan (1991) Tanner (1944)
112–150	Bacteria and green algae	California mission (San Luis) structures, from the interior of a dry brick from a room used as a prison, then sealed and forgotten for a century	Lipman (1934) Postgate (1990, 1994)
118; CC	<i>Bacillus licheniformis</i> NCTC 5399 <i>Bacillus subtilis</i> NCTC 5398 <i>Bacillus brevis</i> NCTC 5400 <i>Bacillus brevis</i> NCTC 5401	Tin of roasted meat (veal) canned in the 19th century, and taken on Parry's Arctic expedition (1820–1830); isolated in 1938. NCTC 5398 has undergone five subcultures, and NCTC 5399 has undergone two subcultures to date. NCTC 5400 and NCTC 5401 were discarded in the 1950s.	Wilson & Shipp (1938) Gibson (1938) L. Hill (personal communication)
100–1000; CC	Yeast, <i>Rhodotorula rubra</i> , <i>Rhodotorula</i> sp. <i>Cryptococcus albidus</i> , <i>Cryptococcus</i> sp. <i>Candida</i> sp. <i>Sporidiobolus salmonicolor</i> , unidentified species	Greenland ice core, from depth of 62–1406 m, deposited with Dr Clete Kurtman in the culture collection for the National Centre for Agricultural Utilization Research, Peoria, Illinois 61604	W. Starmer (personal communication)

Table 1. (cont.)

Estimated length of storage (years)	Micro-organisms	Origin of specimen and comments	References
83	<i>Schizophyllum commune</i>	Fresh mushroom fruit-bodies collected at Winnipeg in October 1909. Revived in 1945 and 1962. Preservation date assumes that the organism is currently alive in the remaining vials.	Bisby (1945) Ainsworth (1962)
75	<i>Hemitrichia clavata</i>	Herbarium collection	Sussman & Halvorson (1966)
68	<i>Lycogala flavofuscum</i>	Herbarium collection	Sussman & Halvorson (1966)
68	<i>B. anthracis</i>	Samples of anthrax spores prepared by L. Pasteur in 1888 which were still viable in 1956	Sneath (1962, 1964) Jacotot & Virat (1954)
60	<i>B. anthracis</i>	Soil stored in a bottle at the University of Wisconsin. The soil came from the La-Crosse outbreak of anthrax in August 1902.	Wilson & Russell (1964)
52	Tobacco mosaic virus†	Dried tobacco leaves	Spector (1956)
circa 50	<i>Escherichia coli</i>	Frozen pony faeces from Scott's Antarctic expedition of 1911	Sneath (1962, 1964) Boyd & Boyd (1963)
circa 50	<i>B. anthracis</i>	During World War II, trials of <i>B. anthracis</i> as a potential agent of biological warfare were carried out on Gruinard Island, which lies off the west coast of Scotland. Tethered sheep were exposed to clouds of <i>B. anthracis</i> spores released by detonation of small bombs. Soil samples were tested annually from 1948 to 1968, 1972, and 1979. The survey in 1979 disclosed the persistence of virulent spores in the soil. In a 1983 paper it was considered that the area of contamination was small enough to consider decontaminating the site. Although Gruinard Island is off-limits to the public, foreign tourists unable to read the warning signs occasionally stray onto the island. If the spores are still viable today, they will have survived approximately 50 years.	Gest (1987) Manchee <i>et al.</i> (1981, 1983)
Age unknown	Diplococcus-like form	Mississippian salt of Nova Scotia	Reiser & Tasch (1960)
Age unknown	Bacteria	Gulf Tertiary Salt Dome samples	Reiser & Tasch (1960)
Age unknown	Bacteria	Coal	Lieske (1932)
Age unknown	Living 'organites'	Crushed amber, meteorites, quartz, basalt. Meteorites have been shown to be porous and absorb water. Louis Pasteur is reported to have attempted to revive micro-organisms from meteorites but he failed and thus did not publish the results. Pasteur constructed a special boring apparatus which enabled him to take specimens from the inner parts of meteorites.	Gallipe (1920, 1921) Keilin (1959) Farrell (1933)* Roy (1935)* Oparin (1957)*
Age unknown	Filamentous bacteria	Travertine rock from Terrace Mountain, Yellowstone, USA	Lipman (1935)

Table 1. (cont.)

Estimated length of storage (years)	Micro-organisms	Origin of specimen and comments	References
Age unknown	Bacteria	Found in oil from wells drilled to several thousand feet	Zobell (1945)
Age unknown	Bacteria	Samples of frozen subsoil	Kriss (1940) James & Sutherland (1942)*
Age unknown	Thermophilic spore-forming bacilli	Arctic ocean bottom mud samples at 50–500 m depth	Bartholomew & Paik (1966)
Age unknown	Thermophilic bacteria	Deep ocean bottom cores taken about 10 and 30 miles off Point Firmin, California, at depths of 2880 and 4320 feet, respectively	Bartholomew & Rittenberg (1949)
Age unknown	Thermophilic bacilli	Gathered in 1934 from permafrost of the desert islands (Island Dixon, Island of Solitude, and Island of Vise) of the Kara Sea. Also gathered from silt at a depth of 159 m, while taking part in an expedition aboard the ice breaker Sedov.	Egorova (1938) McBee (1950) James & Sutherland (1942)*
Age unknown	Bacteria	Mucosa and other tissues of the trunk of a frozen Sanga Yurakh mammoth in Siberia. The sample was sent to the investigator for analysis.	Omeliansky (1911) Sneath (1962, 1964) Sussman & Halvorson (1966) Oparin (1957)
Age unknown, origin unknown	Coccus and rod-shaped bacteria	Isolated from 18 meteorites from separate geographical locations. Meteorites have been shown to be porous and absorb water. Louis Pasteur is reported to have attempted to revive micro-organisms from meteorites but he failed and thus did not publish the results. Pasteur constructed a special boring apparatus which enabled him to take specimens from the inner parts of meteorites.	Lipman (1932, 1936) Farrell (1933)* Roy (1935)* Oparin (1957)*

* Reference refutes claim, or fails to isolate micro-organisms from similar material.

† A virus is included in the database. It can be argued that a virus is not alive and therefore is by definition incapable of being revived.

preclude the idea that modern contaminants may have entered during sample handling and revival.

Contamination precautions

Many researchers take very elaborate precautions to avoid contamination. Lipman must be one of the most rigorous exponents of this. Typical precautions involve the surface sterilization of material, autoclaving rocks to sterilize the outside layers only, and duplicate extractions and controls. None of the elaborate precautions seems to have impressed the critics, who delight in pointing out new possible sources of contamination. It is very difficult to prove that you have avoided all possible sources of contamination, as all sterilization processes, by their very nature, have a small but finite probability of letting through a contaminant. There is some hope that things

will improve as several authors have set out guidelines for recovering micro-organisms from isolated environments (Colwell *et al.*, 1992; Russell *et al.*, 1992; Fitch & Anders, 1963; Sall, 1964). These guidelines are often specific to one type of specimen, e.g. sampling subsurface sediments, and much more work on these guidelines is required. Arguments based solely on contamination precautions seem to be the weakest and easiest to question.

Morphological difference to other organisms handled, or those nearby in the natural environment, and the similarity of repeat samplings

One of the better ways of confirming that the isolated organism is not a laboratory contaminant is if the isolated organism is different to those encountered in the laboratory. Also if the same organism is repeatedly cultured

Table 2. The origin of specimens which contain micro-organisms that could possibly have been stored for long periods of time

Specimen origin	Number of specimen origins	Number of independent research teams*
Rocks/sediment	19	10
Salt deposits	10	3
Soils/subsoils/permafrost	10	9
Bricks	6	3
Human/extinct animal intestines or faeces	5	5
Coal	2	2
Meteorites	2	2
Ice/glaciers	2	2
Bottles or cans	2	2
Laboratory specimens	2	2
Oil	1	1
Dried leaves	1	1
Total	62	37

*Column does not add up to 37 because some research teams studied specimens from more than one origin.

Fig. 2. The frequency of finds as a function of preservation age, showing a bimodal distribution. The number of specimens in each age grouping (■), and the number of independent research teams reviving micro-organisms in each age grouping (▨), are shown. Age groupings in years (MY = million years) are as follows: (1), 50–100; (2), 100–1000; (3), 1000–10000; (4), 10000–100000; (5), 100000–1 MY; (6), 1 MY–10 MY; (7), 10 MY–100 MY; (8), > 100 MY.

from different but closely related samples, e.g. a short distance apart in rock samples, then this adds weight to the hypothesis that it is not a contaminant. These techniques were used in the isolation of the Porter Beer yeast (Coghlan, 1991). Another example is the revival of *Enterobacter cloacae* from mastodon intestines (Park, 1991; Goldstein, 1991). To help verify that the *E. cloacae* was not a contaminant from the surrounding soil, the researcher took 12 soil samples from near the mastodon bones and showed that these cultures did not contain *E. cloacae*.

RNA evidence

The theory applied in this case is that mutation and selection have been occurring since ancient times at a constant rate. Therefore by investigating the genetic information of a supposed ancient micro-organism, it should be different to those of its modern day descendants. By quantifying the difference, and assuming a constant rate of mutation, then the age of the revived micro-organism can be estimated. Estimates of the evolutionary mutation rate range from 0.1 to 1.0 amino acid changes per 10^6 years (Darnell *et al.*, 1986). Based on this mutation rate, this mutation method is only applicable to micro-organisms that have been preserved for millions of years, preferably hundreds of millions of years. The most popular part of the information structure within micro-organisms for analysis of relatedness is, however, not DNA, but rRNA (16S for prokaryotes, 18S for eukaryotes). RNA is studied because DNA can contain introns, or nonsense DNA sequences, which are not present in rRNA. Hence RNA represents higher 'quality' genetic information than that stored in the original DNA sequence. rRNAs are longer than other RNA molecules and hence genealogical similarities or separations can be more effectively traced through comparisons of rRNA sequences (Darnell *et al.*, 1986). This RNA approach suffers from several weaknesses. Firstly there is considerable debate over the constancy of the molecular clock, and mutations may not take place at a uniform rate. Calibration of the molecular clock using dated phylogenies of insect hosts (Harvey & May, 1993) offers some hope that this technique will grow in credibility. Secondly and by far the most problematic aspect of this type of evidence is that there is no verified standard to which to compare microbial mutations. We do not yet have a verified ancient micro-organism which we can say has undergone X number of mutations. Once one micro-organism is sufficiently accepted as being truly ancient, using perhaps a combination of the other verification techniques, then a standard can be set up and the RNA technique applied more uniformly. Again contamination with modern RNA is also a problem that should be considered. The most recent work of applying RNA analysis to ancient viable micro-organisms is being conducted on halophiles revived from British salt mines (Norton *et al.*, 1993).

Table 3. World records for the potential survival of various micro-organisms from specimens of rock, soils, amber, salts and ice (below-ground environments)

Micro-organism type	Genus and species	Record for preservation (years)*	Origin of specimen and comments	References
Virus	–	–	None recovered	–
Bacteria†	<i>B. circulans</i>	650 MY	Precambrian salts from Irkutsk	Dombrowski (1960, 1961a, b, 1963)
	<i>K. umbellata</i>	600 MY	Ammonia-rich soils from Harlech, Wales	Siegel & Guimarro (1966) Siegel & Siegel (1968)
	Encapsulated diplococcus-like bacteria	430 MY	Silurian salts from New York State	Reiser & Tasch (1960)
Yeast	Not specifically identified	24 MY	Miocene core samples from 600–3000 m depth, Fujioka, Japan	Sugiyama (1966, 1967, 1968) Sugiyama & Goto (1969)
Filamentous fungi	<i>Aspergillus</i> , <i>Cladosporium</i> , <i>Penicillium</i> , <i>Trichoderma</i>	24 MY	Miocene core samples from 600–3000 m depth, Fujioka, Japan	Sugiyama (1966, 1967, 1968) Sugiyama & Goto (1969)
Algae	Not specifically identified	1000–3000	Frozen subsoil	Kapterev (1938)

*MY = million years.

† Because of the great age involved and difficulties with verification, the top three candidates are listed.

Table 4. World records for the potential survival of various micro-organisms from specimens other than rock, soils, amber, salts and ice (above-ground environments)

Micro-organism type	Genus and species	Record for preservation (years)	Origin of specimen and comments	References
Virus	Tobacco mosaic virus	52	Dried leaves	Spector (1956)
Bacteria	<i>E. cloacae</i>	11 000	Extinct mastodon intestines	Park (1991)
	<i>E. agglomerans</i>			Lepper <i>et al.</i> (1991) Goldstein (1991) Ross (1993) Coghlan (1991)
Yeast	Porter Beer brewers yeast Y35	166	A bottle of Porter Beer from a sunken sailing barge, which sank off the Little Hampden coast in 1825	
Filamentous fungi	<i>S. commune</i>	83	A fresh mushroom fruit-body collected at Winnipeg in October 1909	Bisby (1945) Ainsworth (1962)
Algae	Green algae	112–150	The interior of a dry brick from a room used as a prison in a Californian mission structure, then sealed and forgotten for a century	Lipman (1934) Postgate (1990)

Growth capacity on medium similar to that of the natural environment from which the specimen originated

The notion behind this verification technique is that if the micro-organism truly comes from an ancient specimen, then it should readily grow in similar conditions to those from which it was isolated. Thus if the ancient micro-organism was isolated from rocks or soil with no oxygen present, then it should grow under similar conditions. This may, however, not be the case. Many of the micro-organisms isolated from deep-sea drillings have been anaerobic thermophiles (Bartholomew & Paik, 1966; Bartholomew & Rittenberg, 1949). The natural environment beneath the sea-bed is too cold to support growth of these micro-organisms. Researchers state that conditions when the rocks were laid down must have been such that they supported thermophilic growth. Hence the question of whether the micro-organism should or should not grow in conditions similar to those from which it was revived is not resolved.

Morphology consistent with the fossil record

The rationale for this means of verification is that if the revived micro-organism does not have the morphology of a modern day micro-organism, but does have the morphology of a micro-organism preserved in the fossil record, then this represents evidence of preservation age. The most famous case of this was the discovery of *Kakabekia*-like forms of bacteria from ammonia-rich soil samples from Harlech, Wales. These micro-organisms did not fit any modern day category but were morphologically similar to *Kakabekia umbellata* Barghorn, which has been identified as a Precambrian microfossil (Siegel & Guimarro, 1966; Siegel & Siegel, 1968).

Radiocarbon dating

To date no one has attempted to use radiocarbon dating to verify the age of ancient revived micro-organisms; however, the potential exists for this technique to be of great value. Radiocarbon dating makes use of the fact that all living things absorb the isotope ^{14}C from their nutrient sources. When the organism dies, ^{14}C is no longer absorbed and hence starts to decay. Because the half-life of ^{14}C and the amount of ^{14}C in the atmosphere throughout geological time are reasonably accurately known, the time since death (or the date of the last intake of ^{14}C -containing nutrients) can be estimated. If it can be assumed that these preserved micro-organisms do not undergo significant metabolic activity during the preservation period, then theoretically the preservation age could be calculated. There are, however, some significant limitations to be overcome. The first problem is that the half-life of ^{14}C is 5700 years and this means that the technique can only be used to date material less than 50000 years old (approximately eight half-lives). This excludes most of the finds from rocks or salt deposits because they are too old; however it does offer the possibility of proving that the micro-organisms are not greater than 50000 years old and hence disproving the rock and salt finds. A second

problem is that when the micro-organism is revived and cultured it will absorb ^{14}C -containing nutrients again, invalidating the technique. Hence the technique needs to be conducted on pre-revived samples. The smallest amount of sample required for ^{14}C analysis is approximately 1 mg. Finding 1 mg of pre-revived micro-organism could be a significant obstacle. The technique shows most promise for providing circumstantial evidence for revivals from salt deposits. If enough salt could be dissolved with water containing no ^{14}C traces then conceivably 1 mg of micro-organism could be obtained. If this microbial sample then contained no ^{14}C , it would be evidence that the micro-organisms found in salt deposits had been preserved for greater than 50000 years. Some very interesting experiments await the eager, innovative researcher.

The most successful verifications to date combine as many of the above arguments as possible.

Theoretical considerations to the limits of preservation (arguments against long-term survival)

There are five main theoretical arguments used to refute long-term survival claims.

Extrapolation of survival data

Several researchers have tried to estimate the maximum time that a micro-organism can be preserved, based on extrapolation of current survival data. Perhaps the most significant and ground-breaking analysis was created by Sneath (1962, 1964), who used data from the preservation of micro-organisms in herbarium soil to estimate a death-rate in stored micro-organisms. This represents the extrapolation of data from survival times at constant temperature. These calculations led Sneath to conclude that 'reports of small numbers being found whatever the age of the deposits are difficult to reconcile with survival curves. We would expect many survivors in recent strata, virtually none in old ones'. The alternative approach was that taken by Becquerel (1950), who extrapolated survival time versus temperature data to estimate that at absolute zero micro-organisms could survive for millions or billions of years (Sneath, 1962). Assenov (1982) extrapolated data obtained at high temperatures (which are used for sterilization) to show that the energy of excitation must limit the extreme duration of anabiosis to 10^4 – 10^5 years. The problem with all these analyses is that they are extrapolations. These extrapolations are very far from where the data resides, and as such the extrapolation is risky.

Decay time of biological molecules

The decay of biological molecules with time is thought to limit the time that a micro-organism can be preserved. This argument comes from the large amount of data that are available on the preservation of DNA and other cellular molecules extracted from old materials. Paabo *et*

al. (1989) state that a peptide bond can last for up to 10^8 years in fossil shell and bones, and that subcellular detail implying the survival of ribosomes and chromatin is evident in insects from 40-million-year-old amber. However the story is different for DNA. Like all macromolecules extracted from old tissue remains, ancient DNA is heavily modified. This is one of the major difficulties in sequencing old DNA. These modifications are so extensive that less than 1 % of the DNA molecules extracted from museum specimens or archaeological finds can be expected to be undamaged. In fact the presence of short chains of DNA is thought of as convincing evidence that the DNA is ancient and not a modern contaminant (Paabo *et al.*, 1989). Thus the argument goes, if the DNA from museum samples degrades so readily, how can micro-organisms be expected to survive for millions of years? Perhaps one solution to this quandary is that the DNA from museum and archaeological samples comes from dead tissue. In contrast, ancient revived micro-organisms are alive during storage, and their metabolism continues, even though at a very very slow rate. Another possible explanation is that inclusion of DNA within minerals can prevent extreme degradation, the rationale being that because DNA is phosphate-based, it should be less prone to dissolution when contained with phosphate minerals, e.g. when found with bones and teeth (Anon, 1994).

Transport within samples

One argument used to discount revivals is transport of micro-organisms into samples. This, as mentioned earlier, can be by water, or simply the motile micro-organisms penetrating the specimen. Very little data exist on this phenomenon. Perhaps the most reliable data to date comes from ZoBell, who demonstrated that motile bacteria could travel 2–40 ft per year in deep oil formations (Postgate, 1994). If such transport is possible then the distance travelled by micro-organisms in millions of years could be considerable. However, micro-organism transport within rocks is probably very different to transport within oil formations. More data are clearly needed.

Susceptibility to radiation

Another fact thought to go against the long-term survival of micro-organisms is their susceptibility to radiation. Sneath (1962) states that 90 % of bacterial spores will be killed by an accumulated radiation dose of about 10^5 R. Most revived micro-organisms are protected by rocks, which themselves contain inherent radioactivity. Sneath calculates that depending on the rock type micro-organisms could be expected to live between 10^4 and 10^8 years, based on the radiation mechanism of death. Also all living cells contain radioactive sources, such as K^{40} , but if this was the only source of radiation, then Sneath estimates that cells could be expected to last 10^9 years. These calculations are thought to be more reliable because they are based on the well-known half-lives of radioactive isotopes. However, it should be noted that the radio-

activity of the rock type and the resistance of the micro-organism to radiation varies considerably. For example, Matthews (1993) states that *Micrococcus radiodurans* can withstand 6.5×10^6 R of radiation. Hence these calculations could conceivably contain significant error.

***In situ* reproduction**

Reproduction in the preserved environment is also thought to be a possibility. It can be argued that the micro-organisms thought to be present in the deep drill hole rocks could not have migrated into them, but that this does not mean that the micro-organisms are millions of years old. The micro-organisms could merely be modern progeny of the original micro-organisms that were trapped in the rocks when they were laid down. Thus the rock microbial population could have been happily reproducing during the supposed storage period. Going against this argument is the fact that the nutrients present in most rock environments are very poor and will not readily support growth. Postgate (1994) sites *in situ* reproduction to be the most compelling explanation for the mastodon find of Goldstein. The extent of *in situ* reproduction remains an unanswered question.

Of all the arguments against long-term storage, the possibility of *in situ* reproduction is by far the strongest. Reproduction in very old samples is clearly one of the areas that needs considerable investigation.

Scientific and industrial significance of long-preserved micro-organisms

The scientific implications of preserved micro-organisms are very far reaching. These micro-organisms represent a frozen snapshot of the past, and they could have significant impact on evolutionary theories. The reason for this is that most work to date on evolutionary theory has concentrated on the fossil record, or on tiny fragments of DNA or other cellular components that have been extracted from preserved material. If these micro-organisms are truly ancient then the entire genome of the pre-evolved micro-organism now becomes available for study. Most of the finds to date have not been deposited in culture collections, mainly because of the criticism that some of the discoveries have attracted, or the significance of the find has not been truly appreciated. Thus the potential of these micro-organisms for further study has been lost. However, the Department of Energy (DOE) Subsurface Microbiology Programme has established a significant culture collection of over 5000 micro-organisms and this offers great hope for the future. The potential of this resource has yet to be exploited by the scientific community, and it is hoped that this database will provide a mechanism to hasten developments in this area.

Since most of the preserved micro-organisms recovered to date have been from rocks, these micro-organisms represent another scientific bonus in terms of the study of geological microbiology. Questions such as the distri-

bution of these micro-organisms, their role in the formation of the strata, and their impact on oil deposition and recovery can all be addressed with access to these micro-organisms.

There are also industrial implications to these preserved micro-organisms. The first industrial application of long-preserved micro-organisms was the revival of yeast from a 166-year-old bottle of Porter Beer. This yeast was then combined with an 1850 Porter Beer recipe and a new commercial product, Flag Porter, was formed. Sales were reported to be 50 000 bottles per year (Ross, 1993).

However, potentially the most significant industrial application of preserved micro-organisms comes from screening them for the production of novel compounds. It is possible that since these micro-organisms are in a pre-evolved state they could synthesize products that were subsequently lost because the environment has changed. Perhaps the more significant possibility is that the chemicals produced by ancient micro-organisms are slightly chemically modified versions of those that we have today. This slight difference may be enough to convey different and useful functional properties to the molecules.

The DOE Subsurface Microbiology Culture Collection (SMCC) has recognized this potential stating 'Because new genetic information is embodied in the collection, the isolates are attractive candidates to screen for new microbial products'. Already the programme has identified an unusual pigment that could have applications in the food, biomedical, cosmetic, textile and chemical industries (Balkwill, 1993). Time will tell whether this potential will be translated into beneficial products.

Ethical issues surrounding long-preserved micro-organisms

There is an ethical issue involved in reviving these preserved micro-organisms. This issue involves the release of these micro-organisms into the environment. Even though these micro-organisms were freely present in nature a long time ago, and are currently present in a stored form in the environment, they represent a genetic pool that is not actively reproducing in the environment today. Since they are a novel genetic pool, should special precautions be taken in the handling of these micro-organisms, precautions similar to the handling of other novel genetic pools, such as genetically engineered micro-organisms? As commercial applications become greater, the growth of these micro-organisms on a large scale will be required, and then the problem of handling and releasing them will need to be addressed. One particularly unpleasant scenario would be the revival of disease-causing micro-organisms which had died out previously. Although not gone from our present reproducing environment, the revival of spores of *Bacillus anthracis* after prolonged storage on Gruinard off the west coast of Scotland, decades after the organism was released there in biological warfare experiments during 1942–1943 (Dixon, 1993), represents one example of this. Other examples

(Dixon, 1993) include the eighteenth century smallpox outbreak triggered by the disinterring of a smallpox victim buried 30 years earlier (Razzell, 1976), and the preservation of viable smallpox virus and other pathogens in the graves of people buried in the Arctic up to 150 years ago (Lewin, 1985). Perhaps treating revived micro-organisms as genetically engineered micro-organisms is being overly cautious, but it may pay in the early stages of the development of this field to treat as such, until the body of evidence decrees otherwise and suitable regulations are in place.

Concluding remarks

The literature shows that claims of revivals from preserved environments are not isolated incidents, but are common. To date this collection of preservation records contains the accounts of over 5000 micro-organisms being revived after storage for greater than 50 years, many after millions of years. These revivals from 62 separate specimen origins, by 37 independent research teams, represents the beginning of a new field of research on the longevity of life. The difficult question is whether these micro-organisms are truly long-term survivors, or whether other artifacts could explain the revivals. Currently it is very difficult to prove the point one way or the other, but the observations presented in this report indicate that further attention should be given to this burgeoning field. The records in this database put us on the threshold of understanding the limits of storage of life. It is likely that these limits are much longer than was previously thought, and that micro-organisms, given the right conditions, can exert a very tenacious grip on life.

The authors are very interested in knowing of other examples of micro-organisms being preserved for long periods of time. Those with additional information are encouraged to contact them directly, so that a complete up-to-date list of possible revivals can be maintained.

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