Bacterial life in ancient salt

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Living bacteria and dormant stages of bacteria known as spores, have been found in many sediments with reported ages of up to hundreds of millions of years. It is difficult to prove that these ancient bacteria were laid down during sedimentation (autochthonous origin) since it is also difficult to exclude the possibility that they entered the sediments later in time (allochthonous origin), or that they are sample contaminants.

Viable spores, with proven autochthonous origin, have recently been isolated from water inclusions in the late Permian Salado evaporite, New Mexico (USA). This evaporite has been dated at 250 Ma. However, current rates of bacterial life decay (the decrease in the number of viable spores with time) do not support the age of 250 Ma. Instead, these decay rates indicate an age for the Permian Salado salt layer of less than 35,000 years.

Age estimates based on bacterial decay rates are qualitative or semi-quantitative at best. Nevertheless, improved age estimates for the Salado evaporite might be possible by estimating the average temperature of the Salado evaporite and determining the decay rate of the bacterium *Bacillus marismortui* under conditions representative for water inclusions.

Present-day bacteria

Bacteria are present virtually everywhere on Earth, many types surviving and even flourishing under extreme conditions such as pressurised water above 110°C, ice below -10°C, acids at pH values down to 2 and bases at pH values up to 13, and environments without oxygen, feeding on essentially everything including those inorganic nutrients available around mid-ocean hot springs. They even survive for long periods of time as spores without any nutrients.[1]

The presence of live bacteria in sediments has been the subject of study for almost a century. Autochthonous origin, the availability of nutrients over a long period of time, and contamination of the samples during sampling and handling are still subjects of dispute. During the '70s, viable spores of a thermophilic (heat loving) bacterium were discovered in lake sediments with radiocarbon ages of 7 ka or more.

The likelihood of the sediments being contaminated with this bacterium later in time was excluded, as the sediments had never exceeded 35°C, the minimum temperature required for germination of this specific type of thermophilic bacterium. Another well-known example is the isolation of bacteria from the gut of a frozen Siberian mastodon, radio-dated at 11 ka. These and other studies point to a maximum life-span for autochthonous bacteria in recent sediments of around 10,000 radiocarbon years, assuming the absence of significant metabolic activity.[1]

Ancient bacteria

A point of controversy is the age of bacteria trapped in pre-Pleistocene Phanerozoic sediments. Once again the presence of bacteria is reported deep below the Earth's surface and in several types of deposits, including coal and oil layers and evaporites with reported ages up to 650 Ma. Evidence for autochthonous origin has not generally been accepted, as it is difficult to exclude allochthonous origin or contamination during sampling and handling.[1–3] For example, the presence of bacteria in remote oil wells has been established, whereas the origin of these bacteria remains unsolved. These may have been trapped during the formation of the oil or have been transported by aquifer activity, or they may have migrated over tens to hundreds of millions of years.[4]

Recently, viable *Bacillus sphaericus* spores were isolated from an extinct bee species enclosed in amber with a reported age of 25–30 Ma.[5] The careful selection of the sample and thorough precautions taken against contamination has made this a convincing find.[2]

Even more recently, ancient bacteria have been isolated from autochthonous water inclusions in salt samples from the Permian Salado evaporite (New Mexico) with a reported age of 250 Ma.[2,6] Based on 16S ribosomal RNA comparison, these ancient bacteria show remarkable similarity to *Bacillus marismortui*, (named after the Dead Sea) and *Virgobacillus panthothenticus*. The difference with *V. panthothenticus* is small, with an homology of 97.1% (although Vreeland *et al.* indicate 97.5% similarity). Of the 1,560 base pairs, 45 are different, which corresponds to 1×10^{-10} substitutions per base pair each year. This substitution rate is small but possibly acceptable.[7] However, the 99% homology with *B. marismortui* is even more remarkable. If these ancient bacteria are indeed relatives of *B. marismortui*, which is yet to be established, how can this difference be so small? The free living *B. marismortui* have supposedly experienced 250 million years of mutations and a much larger difference to their isolated counterparts would be expected,[2,6] although an efficient DNA repair mechanism during germination could partly explain the similarity.[8]

In this respect, the ultimate life-span of isolated bacteria is a key parameter which will be discussed in more detail below.[9]
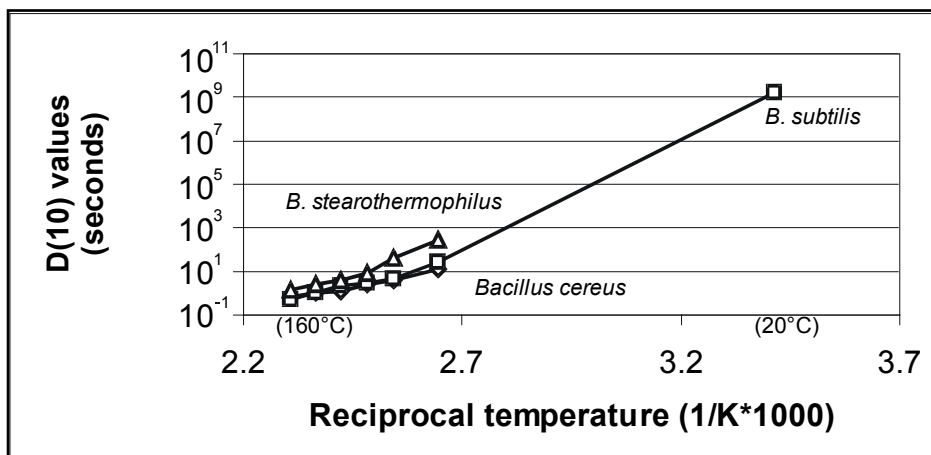
Figure 1. *Arrhenius plot for bacterial life decay.*

Bacterial survival

In theory, bacteria can live indefinitely as long as a sufficient supply of nutrients are available. They multiply asexually, whereby one cell simply splits into two cells, and both mother and daughter cells (which in fact are indistinguishable) are rejuvenated and the population remains constantly viable so long as optimal nutrition is available and the levels of excreted toxic substances is low. The life of bacteria is limited in the absence of nutrients, but it is impossible to determine their life expectancy. Nevertheless, the number of viable individuals steadily decreases over time as the level of nutrients decrease and the levels of toxic excretions increase. The decay rate of spores is usually expressed as the decimal reduction time, D(10), which is the time required, at a given temperature, for the number of viable bacteria in a population to be reduced to 10%. This rate is comparable with that from other exponential decay processes such as first-order chemical reactions or radioactive decay.

Bacteria can typically be stored for about one year if dried under vacuum, and for several years at liquid nitrogen temperature (-196°C).[10] They can survive for much longer if they can maintain basal metabolic activity. Bacterial cultures have been found in Siberian permafrost at an average temperature of around -10°C. The permafrost is estimated at between one thousand and one million years. The suggestion has been made that despite the low temperature, the bacteria must have been active, otherwise they could not have survived for so long.[11] Supporting evidence for low-temperature activity is the recent discovery of bacteria in snow and ice in the surface layers of Antarctica, which indeed have metabolic activity at -12°C to -17°C.[12]

It is interesting to note that the Antarctica researchers discovered members of *Deinococcus*, a genus of bacteria that was already known in the preserved food industry for its ability to withstand ionising radiation, including X-rays and radioactivity.[13] This is remarkable, as such radiation extremes are not present in the natural environment, and it was unclear what advantage this ability could give. The answer could be that in the Antarctica snowfields, *Deinococcus* species are subject to intensive UV irradiation which causes ionisation.[12] This example again illustrates the remarkable ability of some bacteria to survive under extreme conditions.

Most bacteria find it difficult to survive when subjected to high temperature, radiation (including UV irradiation), acidic or basic environments, oxidising agents (including oxygen), certain inorganic compounds, mechanical stress and wet conditions.[10] Nevertheless, many of these

Bacteria are now known to survive incredible environmental extremes. Some have been found in geothermal areas where the temperature can reach 110°C and others at the Antarctic where they have endured sub-freezing temperatures and also intense ultraviolet radiation.

conditions can be favourable for bacteria if they occur in a water inclusion in an evaporite, even if water is present. Although salt is detrimental to most bacteria, some such as *B. marismortui* can survive as spores or even flourish under saline conditions below 25% w/v.

In summary, some bacteria can withstand severe conditions as long as metabolic activity can take place. In the absence of nutrients, the life-time of active bacteria could be years or decades, but survival over thousands of years in the absence of metabolic activity under otherwise optimal conditions is already challenged. Can bacteria imprisoned in a water inclusion in salt survive over 250 Ma?

The survival of spores

Some bacteria, including *Bacillus* species, can form spores in the absence of nutrients or when under stress. Spores are inactive dormant stages which tide bacteria over a harsh period of time, include cysts, exospores and myxospores which are less stable than the *Bacillus* endospores[10] considered here. The transition of a bacterium to a spore is a complex process resulting in a nucleus of stable compacted DNA and enzymes, wrapped in an impermeable keratin-like skin. There is hardly any water, but a large amount of the conservation complex calcium-dipicolinate is present to keep the enzymes inactivate and to stabilise the nucleic acids. The DNA is also protected by a group of unique DNA-binding proteins, called α/β-type SASP.[13] Most bacteria cannot survive heating at 80°C for 10 minutes (pasteurisation), but a few spores do, some even lasting for hours in boiling water.

How long can spores survive? Research conducted on the soil residues on plant roots stored under dry conditions in a herbarium gives us some indication. After 200–300 years, some spores from *B. subtilis* and *B. licheniformis* were still viable. In addition, *B. coagulans* and *B. circulans* were identified in 50- to 100-year-old samples. This was equivalent to a D(10) of 50 years for *B. subtilis* and *B. licheniformis*, and means that after 1,000 years only a few viable spores from the original large number of bacteria can still be expected from 1,000 kg of soil.[10]

D(10) times have been reported for various temperatures, including sterilisation conditions (Table 1). The sterilisation stability of *B. stearothermophilus* is exceptional and probably not representative for most other *Bacillus* species. Unfortunately, data for *B. marismortui* under conditions characteristic for water inclusions in salt is not available. *B. marismortui* does not survive laboratory sterilisation precautions,[6] but this feature cannot be translated into a D(10) time. Figure 1 is a plot of the D(10) data from Table 1 on a logarithmic scale as a function of the reciprocal temperature (Arrhenius plot), and provides the trend of D(10) values as a function of temperature.

The 'bacterial age' of the Salado evaporite

Bacillus marismortui forms spores when the salt concentration exceeds 25% w/v. Under saturated saline conditions, spores are not expected to 'wake up' regardless of the nutrients available. The number of viable spores in the Salado deposit has declined steadily with time, from possibly 100 million bacteria per ml originally[2] to only a few viable spores in just three out of 66 inclusions, with a total volume of 0.365 ml (for the 66 inclusions) having been evaluated to date.[6] Assuming a minimum of three viable spores in 0.365 ml liquid means that seven periods of decimation have passed. Taking a D(10) of 50 years results in a 'bacterial age' of 350 years for the Salado evaporite. Additional supporting evidence for the young age of this evaporite is the previously mentioned similarity between the 16S ribosomal RNA of the ancient bacteria and *B. marismortui* or *V. panthothenticus*.

The difference with the 250 Ma reported for the Salado evaporite is almost six orders of magnitude! An unreasonable D(10) of 35 Ma instead of just 50 years is required to reach a total of 250 Ma. Conversely, assuming the evaporite has an age of 250 Ma and a decimation time of 50 years implies that the original bacteria have experienced 5 million decimation periods. It is impossible to find a living organism after 5 million decimations.

An age of 350 years for the Salado deposit is within historic time and of course not realistic. There are a number of uncertainties, that must be considered.

- The small number of viable spores (three as a minimum) has hardly any statistical significance and results in a wide range of possible ages, with greater ages more probable than shorter ones.
- The D(10) of *B. marismortui* under conditions representative of water inclusions is unknown and needs to be established independently.
- The times of decimation for different species might vary by one order of magnitude (Table 1).
- A temperature change of 25°C has an effect of two orders of magnitude on the time of decimation (Figure 1).
- An average evaporite temperature at or below 0°C over time is highly improbable.
- An average temperature above 20°C might be more realistic and result in shorter D(10) times and lower ages.

The calculated 'bacterial age' of 350 years is therefore highly uncertain and can easily vary by two, or possibly three, orders of magnitude with greater ages more probable than younger ages. This results in an estimated range of 200 to 35,000 years, corresponding to four log cycles of 50 years at 20°C or seven log cycles and a D(10) of 5,000 years at 0°C, respectively. It is difficult to imagine conditions leading to 350,000 years, but even this estimate differs by more than two orders of magnitude with the 250 Ma reported for the Salado evaporite. This gap may even increase when claims of 650 million-year-old bacteria in

evaporites are confirmed.

Surprisingly, the presence of viable autochthonous bacterial spores in the Salado evaporite has not led to a challenge of its geological age. Parkes assumes virtual bacterial immortality rather than challenging the geological time scale. Even survival of bacteria in meteorites and the origin of life on Earth via inter-planetary transport of bacterial spores ('panspermia') are being considered.[2] More recently, Lindahl has denied the high age of these bacteria based on the impossibility of DNA surviving longer than 100,000 years, stating that the recovered bacteria are common extant ones.[14]

Conclusions

The presence of viable spores of bacteria in water inclusions in the Permian Salado evaporite, in combination with bacterial decay rates, is incompatible with the age of 250 Ma reported for this evaporite.

The 'bacterial age' of the Permian Salado evaporite has here been estimated semi-quantitatively and is probably within the range of 350 and 35,000 years, under the assumption that decay rates available from literature are representative for inclusion conditions.

Estimates of the average temperature of the evaporite over time and measurement of times of decimation for *Bacillus marismortui* within a wide range of temperatures are key parameters for improving the 'bacterial age method' estimate.

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