RESEARCH NOTE

Is the ancient permafrost bacteria able to keep DNA stable?

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Introduction

Permafrost underlies about 20% of the earth's land surface (Ershov 1998), and a majority of the biosphere is constantly below +5°C (Ashcroft 2000). Microorganisms have been recently found in ice-sediment communities in the surface layers of perennial and permanent lake ice (Psenner and Sattler 1998) in a high Arctic glacier (Skidmore *et al.* 2000) and Greenland (Sheridan *et al.* 2003). The isolation of microorganisms from ancient deposits, including permafrost, and the question of whether or not the microorganisms are as old as the deposits, are still area of controversy. It is intriguing that many representatives of ancient bacteria are close to modern bacteria at a molecular level. In some cases, geological data proves the old age of these organisms, while molecular data presents evidence for their modernity. It is unclear how bacteria remain viable for long periods. Despite the fact that the mechanism is unknown, bacteria from amber are reported to survive for 40 million years or longer (Greenblatt *et al.* 2004). Living (or at least viable) bacteria apparently occur deep in solid-frozen ground (permafrost) in the cold regions (Gilichinsky and Wagener 1995). We isolated and characterized bacterial strains from ancient (Neogene) permafrost sediment that was permanently frozen for 3.5 million years (62°56′N, 133°59′E). According to the preliminary DNA analysis, bacterial cells collected from the relict permafrost appeared to be *Bacillus* sp. strains, which are close to modern bacteria at a molecular level. Therefore, a number of questions arises. For example, are isolated bacteria as old as the permafrost itself or could contamination with more recent bacteria have occurred? Do the bacteria grow in the permafrost? And to what extent are 'normal' metabolic processes taking place? Or are they inactive and cryopreserved?

Further, DNA in the ancient bacteria is expected to decay due to a number of reasons including thermal fluctuations, nucleotide deamination, radiation in the soil, etc., and the bacterium is expected to lose its viability within several hundred years (Lindahl 1993). Therefore, the nature of extreme longevity of microorganisms has no clear explanation.

Materials and methods

Sample collection and treatments

Samples were collected and treated as we described previously (Zhang *et al.* 2013).

Sample analysis

Samples of different dilutions in sterile conditions were added to Petri dishes containing yeast peptone dextrose (YPD) (Becton, Dickinson and Company, Sparks, USA), de Man, Rogosa and Sharpe (MRS) (Becton, Dickinson and Company, Sparks), and noble agar (NA) (Becton, Dickinson and Company, Sparks) media. Samples were also added into a liquid meat–peptone broth under anaerobic and aerobic conditions.

For the sequence analysis, DNA of one of the isolated strains was extracted by using a Fast DNA kit for soil (BIO 101, Vista, USA) based on a physical extraction method using glass beads. The procedure was conducted according to the manufacturer's instructions. The 16S rRNA genes were amplified by a polymerase chain reaction (PCR) with primer sets specific for eubacteria (27F; 5′-AGAGTTTGATCCTGGCTCAG-3′, 1492R; 5′-TGACTGACTGAGGYTACCTTGTTACGACTT-3′) (Amann

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et al. 1995). Band sizes (∼1500 bp) were used as the template DNA for sequencing. The sequence was compared with similar sequences of reference organisms by basic local alignment search tool (BLAST) search, and the closest relative was *B. macroides* with 97% homology. The 16S rRNA gene sequence was deposited in the DDBJ/EMBL/GenBank database under accession numbers AB178889 and entry ID 20040510203204.24251 (Brushkov *et al.* 2009).

Results

Isolation of microorganisms from permafrost samples

Samples were collected in July 2009 at an altitude of 83 m above sea level at the Mammoth mountain exposure in the central Yakutia (62°56′N, 133°59′E), exposition north, and at a depth of 1.5 m from the surface of the Neogene formation as we described previously (Zhang *et al.* 2013).

The age of the permafrost in the Mammoth mountain area exceeds three million years, dated by paleoclimatic reconstructions (Baranova *et al.* 1976; Bakulina and Spector 2000). The exposure is destroyed by the river (more than 1 m per year); therefore, the sampled sediments were in a state of permafrost.

The active layer during a recent unusually warm summer might have reached sampling depths, compromising the integrity of samples. But this seems unlikely as the exposure (a consequence of river erosion of a few cm up to 0.7 m per year) only occurred very recently. Prior to the erosion producing the exposure, the sample would have been some 9 m deep and unlikely to be thawed over a long period of time. Presently, the mean temperature of the deposits is about −4°C, and the temperature remains below zero. The systematic composition of seeds, pollen and leaves is related to middle Miocene (Baranova *et al.* 1976), about 11–16 million years ago. Recent studies show that an intensive cooling began in late Pliocene, 3–3.5 million years ago (Hansen *et al.* 2013). The temperature in January was estimated by Bakulina and Spector (2000) to be −12 to −32°C, and from about +12 to +16°C in July; thus the age of permafrost at the Mammoth mountain probably reaches up to 3.5 million years. Microscopic pictures of soils sampled from permafrost show mostly single cells (much less groups of a few cells), not colonies (figure 1).

Bacterial strain isolated from the frozen samples was *Bacillus* sp. strain F and identified as *B. cereus* with 100% homology of 16S rRNA. In addition, a 3-kb plasmid containing five open reading frames was isolated (Fursova *et al.* 2013). After the whole sequencing, comparative sequence analysis revealed that the plasmid shows between 99.00 and 99.99% identity to the plasmid pAH820_3 of *B. cereus* AH820 (available at GenBank under accession number ACC NC_011657.1). Unique was the fact that full compliance with the plasmid DNA isolated from ancient bacterial cultures with the modern *B. cereus*. However, theoretical calculations propose 2% genomic variation per million years. It can be also described as 1×10^{-8} nucleotide substitutions per nucleotide site per year for mitochondrial DNA.

Discussion

Parkes *et al.* (2000) review convincing cases of bacteria in diverse environments that have remained viable over inordinate lengths of time. Unfrozen water, held tightly by electrochemical forces onto the surface of mineral particles, occurs in even hard-frozen permafrost. Bacterial cells are not frozen at temperatures of −2 and −4°C (Clein and Schimel 1995).

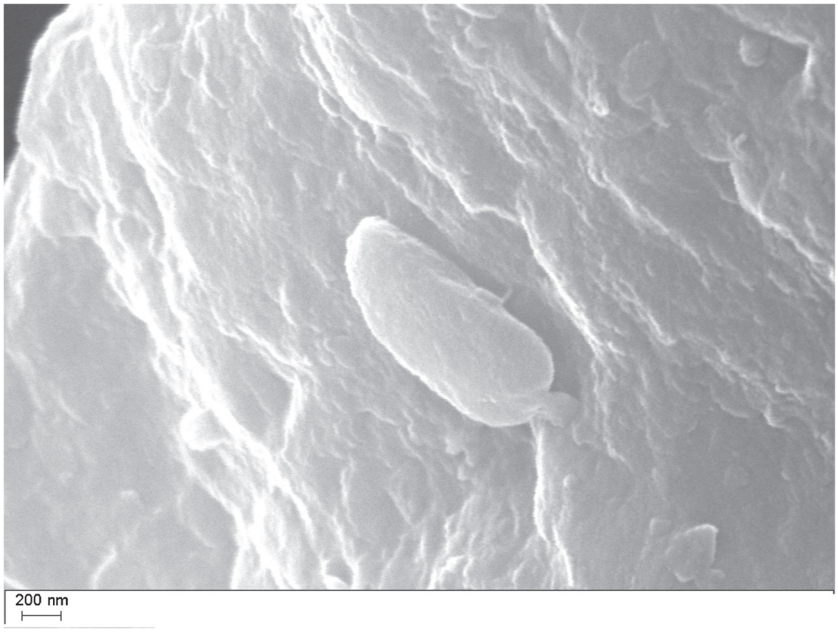

Figure 1. Electronic scanning microscope picture of bacteria in ancient Neogene permafrost, exposure of the Mammoth mountain.

The thin liquid layers provide a route for water flow, carrying solutes and small particles, possibly nutrients or metabolites, but its movement is extremely slow (Burt and Williams 1976). A bacterium of greater size (0.3–1.4 μm) than the thickness of the water layer (0.01–0.1 μm at temperatures -2 and -4°C) is unlikely to move, at least in ice (Ershov 1998). Rates of gas diffusion in the frozen deposits are about one metre per 10,000 years. Samples for this study were taken from the depth of about 30–40 metres from the earth surface, thus theoretically, air could be there at least 0.5 million years ago; however, air does not necessarily carry bacteria. In this study, we present data obtained from previous Siberian permafrost microbiological studies, and suggest a possible explanation of controversial molecular data received from our study and other studies of the ancient bacteria.

'Modern' bacteria in ancient deposits: DNA stability and evolution rates

Proteins are far from stable (Jaenicke 1996). Bacteria are affected by ageing (Johnson and Mangel 2006). The genome is subject to mutations. The half-life of cytosine does not exceed a few hundred years (Levy and Miller 1998). Ancient DNA of mummies, mammoths, insects in amber, and other organisms appear fragmented and destroyed (Willerslev and Cooper 2005). Despite the nature of mutations, we think the degree of variability in mutation rates is still an open question. It looks very unlikely that the rates are similar for different bacteria, for different environments, for different genes, and for prokaryotes and eukaryotes. The rate of substitution for 16S ribosomal DNA has been found to be remarkably uniform between about 1×10^{-8} and 5×10^{-8} substitutions per site per year (Clark *et al.* 1999). The phylogenetic substitution rates in mitochondria are $\sim$0.5% per million years for avian protein-coding sequences and 1.5% per million years for primate protein-coding and d-loop sequences (Ho *et al.* 2005). Two hundred and fifty-million years old bacteria living in salt deposits (Vreeland *et al.* 2000) were tested by Nickle *et al.* (2002), who performed relative rate tests using 16S rDNA with the same result; the branch leading to isolate 2-9-3 is not extraordinarily short, as would be expected of an organism that has not been evolving for millions of years. Bacteria from amber also show 'modern' genomic features (Parkes *et al.* 2000). Since DNA decays quickly, and the rate estimations are so different, we come to the question, are the ancient bacteria really old?

It is interesting that some bacteria show stable genomes and appear to have recent origin. For example, no sequence polymorphisms were detected in six gene fragments from 36 isolates originating from the three classical biovars, indicating that *Yersinia pestis* evolved from *Y. pseudotuberculosis* within the last 1500–20,000 years (Achtman *et al.* 1999). It should probably be taken into account that rates of spore-forming bacteria evolution might be significantly slower. Estimations of the dormant state length vary from 10^2 to 10^4 years between times of growth. Some studies (Parkes *et al.* 2000) show that generation times of bacteria isolated from subseafloor sediments are roughly hundreds to thousands of years. Bacteria isolated from amber often show a high homology to the younger sequences, sometimes explained by the fact that the amber samples were from different geoclimatic regions of the Earth (Veiga-Crespo *et al.* 2004). Although contamination is always an issue, a number of studies show the same results: many isolated ancient bacteria have 'modern' genomes. It seems very unlikely that contamination or error occurred in all cases.

Microorganisms in permafrost

An important characteristic of permafrost is that some water, held tightly by electrochemical forces onto the surfaces of mineral particles or under the influence of capillary forces, occurs in even hard-frozen permafrost (Brouchkov and Williams 2002). The thin liquid layers provide a route for water flow, which normally occurs from warmer to colder parts along a temperature gradient (Derjaguin and Churaev 1986). Abyzov's (1993) investigations at the Vostok station revealed bacteria, fungi, diatoms and other microorganisms that were probably carried to Antarctica by winds. The ages of these individuals could be more than half a million years. Abyzov (1993) also has shown the presence of viable bacteria in ice that were hundreds of thousands of years old and at a depth of a thousand metres, which could not have been contaminated from the surface or from below the surface in recent times. Although most microorganisms do not grow at temperatures below 0°C, certain bacteria and fungi can be physiologically active. Water is the solvent for the molecules of life, and availability of water is a critical factor affecting the growth of all cells. As the temperature falls to -2 or -3°C, the remaining water in layers is so thin that a bacterium could not fit. Metabolic activity, especially the ability of microorganisms to grow for a long time are greatly limited in the environmental conditions within the permafrost. The single bacterial cell is trapped and not even free to move or expand within the unfrozen water layer.

Stability of ancient DNA over the millions of years

Modern genomics allows reexamination of old questions of evolutionary biology. Now it seems possible to consider the issue of mutation rates at the level of complete genomes. Our isolate is related to the *Bacillus* group, and *B. anthracis*, for example, represents one of the most molecularly known monomorphic bacteria. It is interesting that the chromosome of *B. anthracis* is collinear with the closest near neighbour strains.

The molecular basis of thermal stability of biological materials is a significant, as yet unsolved problem (Jaenicke 1996). In general, the stability of a polymer is defined as the free energy change, G, for the reaction from folded to unfolded form under physiological conditions. Most proteins are characterized by values of $G = 5$–15 kcal/mol. In terms

of thermodynamics, the fraction of residues in random coil regions divided by the fraction of residues in ordered regions (k) would appear as the following:

$$k = e^{-(G/RT)}, \quad (1)$$

where G is the free energy change for the thermal transition of one residue from an ordered region to a randomly coiled one, kcal/mol; T – temperature, K; R – gas constant, ~0.001989 kcal/mol · K.

Calculations show that even for $G = 30$ kcal/mol, the time of existence of molecular bonds in the polymer chain is less than 300 years, which is close to experimental data (Levy and Miller 1998). These calculations are very approximate and extreme, but they show how unstable proteins and DNA are.

We can presume that alive microorganisms in permafrost, if they are of considerable age, have special mechanisms of repair for cell structures (Brouchkov and Williams 2002) or preserve those otherwise prone to collapse because of the duration of their existence. If so, their mutation rates must be extremely low. This explains genome homology between ancient permafrost bacteria and modern strains. A number of publications as well as data presented in this paper are in agreement with that point of view (Parkes *et al.* 2000; Nickle *et al.* 2002; Greenblatt *et al.* 2004, etc.).

Obviously, with minimal nutrition and lack of movement, the main factor causing spontaneous mutations is not genomic recombination (the probability of processes occurring inside a cell close to anabiosis is very low), nor chemical compounds (whose flows are significantly reduced), but electromagnetic radiation. Eliminating the possibility of UV rays due to the great depth of the sample, the contributing mutagens are X-rays and gamma radiation, which exist in earth's natural radiation background. Therefore, X-rays and gamma radiation become the only energy source affecting molecular bonds. So, how is the survival of cell possible after long incubation periods? However, it is possible that the answer lies in the fact of the matter; i.e., the factor that causes mutations, short-wave radiation, is also a contributing factor to DNA repair. In other words, the energy flux quanta passing through the bacterial cell 'bricked' in the ice and causing damage to genetic material is captured, accumulated, transformed and used by cellular mechanisms, carrying out repair to restore the primary structure of DNA identical to the original matrix.

Currently, it is known that bacteria and fungi possess the enzyme deoxyribose-pyrimidine-lyase, which is an enzyme belonging to the photolyase class—the DNA repair enzymes that repair damage caused by exposure to ultraviolet light. The repair mechanism requires visible light, preferentially from the violet/blue end of the spectrum, and is known as photoreactivation (Selby and Sancar 2006). So, speculatively, we cannot exclude the probability of a similar mechanism's existence, which can carry out DNA repair using ultra short electromagnetic waves. This is a prospective area for future investigation of permafrost organisms to understand the key to maintain life in a frozen state for millions of years.

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