Viruses in ancient ice wedges in the Central Yakutia, Siberia

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ABSTRACT
The study of the viral component of ancient microbial communities from permafrost is important for the understanding evolution of microbial communities, possibility of their variations due to climate change, changes in the physical-chemical state of permafrost and practical questions of biosafety. For the first time the virus particles in native samples of ancient ice wedges of the Mammoth Mountain in Siberia have been discovered. Defined morphological diversity of viruses that can be attributed to five main types: miovirus, sifovirus, podovirus, spherical and filamentous. Specific characteristic of these viruses are small size and fever genome.

RÉSUMÉ
L'étude des virus qui sont présents dans des communautés microbiennes anciennes du pergélisol est cruciale pour la compréhension des questions fondamentales telles que l'évolution des communautés microbiennes, la possibilité de leur changement suite aux changements du climat, de l'état physico-chimique du pergélisol aussi bien que les questions pratiques concernant la sécurité biologique. Les virus ont été découverts pour la première fois dans des glaces éternelles de la montagne Mammouth. La définition de leur diversité morphologique faite, les virus peuvent être classés en cinq types principaux : miovirus, sifovirus, podovirus, virus sphériques et virus filamenteux. Leur spécificité consiste en une petite taille du génome.

1 INTRODUCTION

Permafrost microorganisms in comparison with ancient salt or amber isolates are widely distributed (Vishnivetskaya et al., 2006; Steven et al., 2008; Yergeau et al., 2010; Margesin&Miteva, 2011). For more than a century there have been reports of living organisms in permafrost, some of which are certainly might be millions of years old, if they have age which is similar to the age of permafrost itself. Living (or at least viable) bacteria apparently occur deep in solid-frozen ground (permafrost) in the cold regions (see the review by Gilichinsky and Wagener, 1995). Sometimes permafrost as well as microorganisms in it is dated quite well (Katayama et al., 2007). Viruses in permafrost were not broadly reported, however, their presence might be associated with psychrophile bacteria and other organisms (Morita 1997). There is a number of questions related in life in ancient permafrost. For example, are isolated bacteria as old as the permafrost itself or can 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? 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 (Williams and Smith, 1991; Brouchkov & Williams, 2002). The thin liquid layers provide a route for water flow, which is normally from the warmer to the colder parts (Derjaguin and Churaev, 1986). The water may carry solutes and small particles and thus perhaps, bacteria, but its movement is extremely slow (Burt and Williams, 1976): at a few degrees below °C it may thus take thousands of years to move a meter. A bacterium of greater size than the thickness of the water layer is likely to move much more slowly than the water. The microorganisms are about 0.3 to a few microns in size, while the thickness of the water films tends to be less. One concludes that microorganisms in permafrost have been isolated, certainly from the ground surface, trapped among the mineral particles and ice.

The longest, continuously frozen permafrost in the Northern hemisphere is variously estimated as between one and three million years old (Foundations of geocryology, 1998). Abyzov's investigations at the Vostok station (Abyzov, 1993) revealed bacteria, fungi, diatoms and other microorganisms which were probably carried to Antarctica by winds. The ages of these individuals could be more than half a million years. Abyzov (1993) has showed the presence of viable bacteria in the ice which was hundreds of thousands of years old and at a depth of thousand meters which could not have been contaminated from the surface or from below in recent time.

Although most microorganisms do not grow at temperatures below 0°C, certain bacteria and fungi can be physiologically active and Friedmann (1994) notes metabolic activity in permafrost bacteria at -20°C. Others reporting evidence concerning bacterial activity in soils below 0°C, include Kalinina, Holt and McGrath (1994); and Clein and Schimel (1995). Water is the solvent for the molecules of life, and availability of water is a critical factor affecting the growth of all cells. But the particular water which is unfrozen in permafrost, although at less than 0°C and in the presence of ice, differs from 'ordinary' water. It is attached to the soil mineral particles surfaces. As the temperature falls to -2 or -3°C, the remaining water

is in layers so thin that a bacterium could not be fitted in. Metabolic activity and especially the ability of microorganisms to grow for a long time are greatly limited in the conditions of the environment within the permafrost.

The single bacterial cell is trapped and not even free to move or expand within the unfrozen water layer. Probably some microorganisms grow if only because of the substantial degree of microbial activity at temperatures below 0ºC. But for the most part it appears unlikely. Microscopic pictures of frozen soils show single cells mostly (much less groups of a few cells), not colonies (Figure 1), and that fact is another argument for dormancy microorganisms in permafrost (Melnikov et al., 2011). Studies of viruses are of interest for permafrost, however, they are almost unknown (Allen, 2010).

2 METHOD OF WORK AND ISOLATION

2.1 Overview

Samples were collected in at an altitude of 83 m above sea level at the Mammoth mountain exposure (Figure 2) 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 (Figure 3). A deep hole of approximately 100 cm was horizontally dug into the frozen Neogene horizon. After sterilizing the surface of this sampling hole by flame, pieces of frozen sediment (icy sand) were collected from a horizontal depth of 75–100 cm, cleaved with a sterilized axe, and collected in sterile 50 mL vials by using sterile spatulas. The mean temperature of the icy sand at the time of sampling was −4 °C.

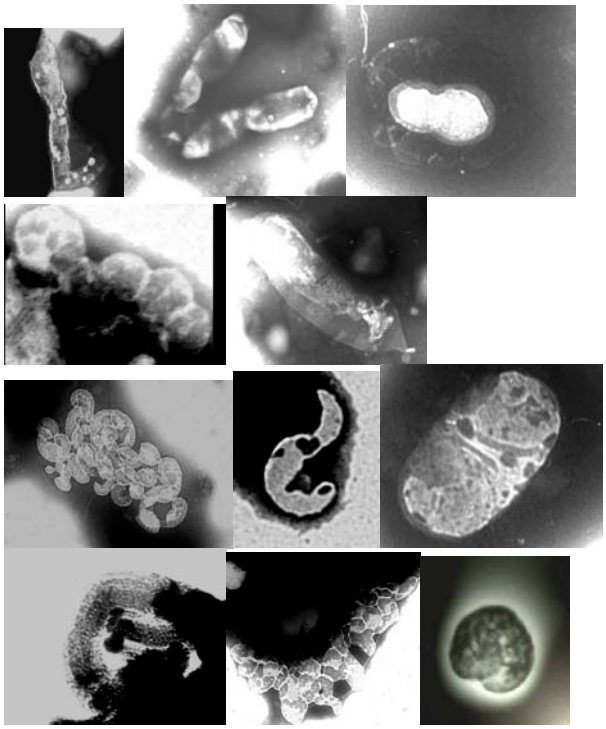
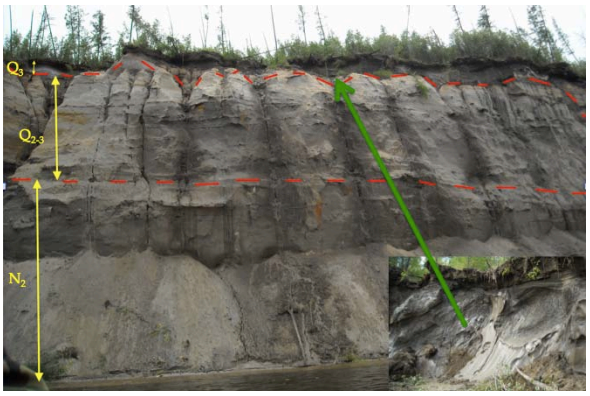
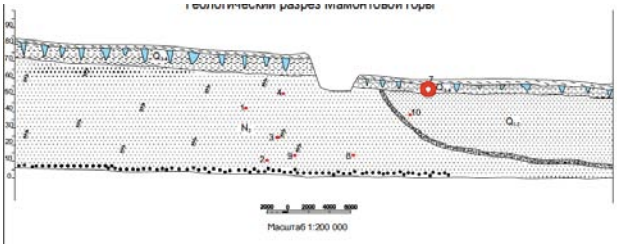


Figure 1. Variety of microorganisms isolated from ice wedge of the Mammoth Mountain (Filippova et al., 2014)





Figures 2 & 3. Section of Mammoth Mountain

Samples were immediately embedded in frozen natural permafrost material, then stored in a cryogenic mixture of NaCl and water to keep the material constantly frozen. The samples were kept frozen during transport from Yakutia to the laboratory in Moscow where samples were stored at−20 °C. Thus, the collected material was constantly kept frozen and never subjected to thawing. A composite sample was produced under sterile conditions immediately before analysis. At this stage of modern science development, it is possible to determine accurately the age of the amber fossils (Lambert&Poinar, 2002), as well as to determine the age of frozen soils.

The age of the permafrost in the Mammoth mountain area exceed 3 million years that was dated by paleoclimatic reconstructions (Bakulina&Spector,2000; Baranova et al., 1976). The exposure is destroyed by the river (more than 1 meter per year); therefore, the sampled sediments were obviously in a state of permafrost. The latter are fine-grained sands, and their age corresponds to the middle Miocenbe, 10–12 million years. The sediments have been intensively studied and did not thaw out because of the cold climate of Yakutia (Markov, 1973; Foundations of Geocryology, 1998; Bakulina and Spector, 2000). Samples of different dilutions in sterile conditions were added to Petri dishes containing liquid ISP1 media for 20-30 days at 20°C. A few isolated strains were described before (Brouchkov et al., 2012; Zhang et al., 2013) from the sample. Observations of the appearance of the negative parts of lysis in the area of active growth of colonies was performed visually using a magnifying glass during the whole period of incubation. Material was collected from the zones of lysis by the bacteriological

hook for subsequent electron microscopy analysis. Colonies with negative portions were separated on an agar slant medium and incubated for 2 days at 28°C. Culturing the isolates was done in liquid medium ISP1. The medium was dispensed into 250 ml flasks at 50 ml, and sterilized in an autoclave at a pressure of 1 atm. for 30 minutes. 1 ml cell suspension of 1-2 x overnight culture was placed in the flasks with a sterile nutrient medium. Cultivation was conducted by submerged cultivation on a rotary shaker while aeration and stirring is carried out simultaneously by rotating at a speed of 180 rev / min. Incubation was carried out at a temperature of 26-28°C for 48 hours.

Phage lysate preparation. Liquid submerged lysogenic culture was centrifuged at 9000 g. The resulting supernatant was filtered using a syringe membrane filter, pore size 0.2 µ to release phage lysate from cell fragments of the host bacterium.

The method of phages collection. Phage lysate was used to accumulate phages in the indicator culture liquid or the bacterial culture of phage host. 500 ml of the filtered phage lysate was added in the submerged culture of the indicator strain of lysogenic bacteria or bacterial isolate after 7 hours, then culture was incubated under the same conditions for 20 - 24 hours.

The resulting culture fluid was centrifuged at 9000 g. The supernatant containing phage particles and cell fragments were centrifuged at 100000 g for release from the bacterial cell fragments. The result is a phage concentrate.

2.2 Study of lytic properties of phage

2.2.1 Selection of the indicator culture

One day cultures of Bacillus subtilis ATCC 6633, as well as strains B.mycoides, B. megatherium and Paenibacillus sp., isolated from the Antarctic Lake Untersee, were used to study the lysing activity of the phage.

2.2.2 Study of lysis activity

Concentrated material containing phages in amount of 5µ, and also diluted by 10^{-1}, 10^{-2}, 10^{-3} was applied to freshly prepared bacterial lawns. Thereafter it was incubated at 28°C for a day. Lysing activity was estimated by appearance of the transparent zones - zones of lysis.
Methods for microscopic study included phase-contrast protocols by Zetopan microscope with phase-contrast device.

The method of electron-microscopic study. For tests 10 ml of melted sample was taken. After standing about 0.5-2 hours at room temperature, enlightened upper portion was selected to produce samples for electron microscopy. Electron microscopic studies were performed on the electron microscope JEM-100CXII (JEOL, Japan). Samples were viewed with magnification × 40,000.

2.2.3 The method of isolation of phage DNA.

Isolation of DNA from the concentrated lysate: 0.5 ml of the precipitated sample of phage were centrifuged at 14,000 rpm for 2 minutes to separate from cell fragments. Then equal volume of phenol equilibrated with buffer to pH = 8.0 for 10 seconds was added. Then after a 5 minute centrifugation aqueous (top) fraction was taken to a new tube. Then equal volumes (250mcl / 250mcl) phenol and chloroform mixed for 10 seconds was added there.

After 3 min of centrifugation the resulting mixture the overhead fraction was taken to a new tube, and chloroform was added in a volume equal to the volume of obtained the fraction. The solution was mixed for 10 seconds. Then the resulting mixture was again centrifuged for 2 min. The top fraction was separated, and sodium chloride was added to a final concentration of 0.5M. Then isopropanol in a volume of 0.7 part of the total volume of the mixture was added and mixed. After centrifugation for 5 minutes the precipitate was separated, and 0.5 mL of 70% ethanol solution was added, stirred, then centrifuged again for 5 minutes. The resulting supernatant was removed under vacuum, and then dried at 37°C for 10 minutes. The dry material was dissolved in 105 µl of ampoule water. To determine the DNA concentration 5 µl sample was transferred to spectrophotometer. Spectra were recorded at a wavelength of 260 nm and 280 nm.

Electrophoresis in agarose gel: For preparing a substrate 2% agarose was used for gel solution preparation in the final TE buffer. A dye (ethidium bromide to a final concentration of 2 mg/ml) was added and mixed thoroughly. The sample of DNA and marker fragments of the phage DNA was applied in an amount of 2 µl in appropriate wells. Electrophoresis was performed for 15 minutes at 120 V.

3 RESULTS

3.1 Identification of virus-like particles in the sample from ice wedge by electron microscopy

Viral particles of different morphology by the electron microscopy of melted ice samples were found (Figure 4).

3.2 Identification of lysogenic bacterial forms.

The number of colony forming viable organisms in the samples was an average of 10^{2}-10^{3} CFU / ml. Increasing the incubation periods has revealed 2-3 colonies of similar type, in the area with active growth where there is a negative sites ranging size 1.5 - 2 mm, whose number is increasing with the aging of the colonies.

The appearance of the sterile areas in the peripheral zone of the old colonies suggests that these areas are the result of the release of the phage from lysogenic bacteria cells and subsequent lysis of some of them (Figure 5). The release of the phage can be due to physiological state of the cells, i.e., with aging, there is an accumulation of metabolic products which can induce the phage output. It was noted that during the period of normal saline (2 -3 days) appearance of sterile areas were not observed. It is known that cells lysogenic cultures of microorganisms resistant to contained phage and only a small portion of

them can be sensitive and lysed. Aging and death of the cell population may contribute to the release of the phage lysogenic cultures. For studying the source of the appearance of bald spots on the colonies electron microscopic examination was carried out. The results revealed filamentous virus particles (Figure 6). Colonies of this bacteria were isolated and maintained on an agar medium ISP1. The study of the morphology of cells lysogenic bacteria showed that their cells are rod-shaped, often grouped into chains in the stationary growth phase, the formation of spores. This can be attributed to the bacteria like Bacillus.

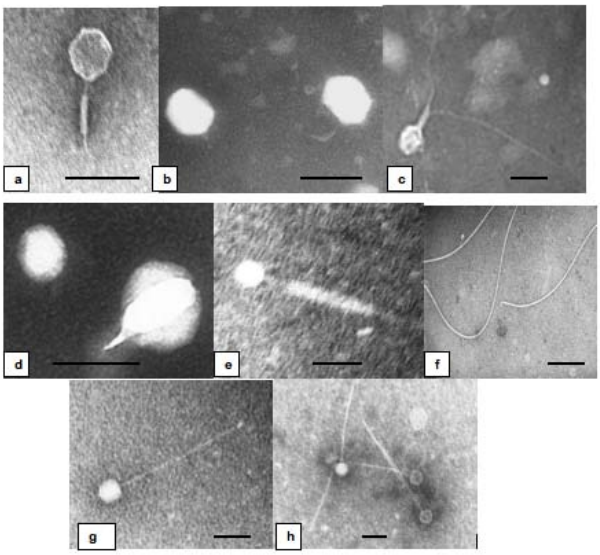


Figure 4. Morphological diversity of viruses attributed to five main types: miovirus (a,c,e), sifovirus(g,h), podovirus(d), spherical(b) and filamentous(f). Scale line 0,05 mkm

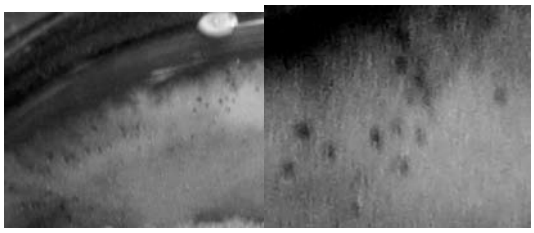


Figure 5. The negative (sterile) zone in the region of active growth of bacterial colonies (after 20-30 days of incubation at 200C)

3.3 The accumulation of phage and identifying its lytic action

Lytic activities zones were found at the site with initial filtrate, which may indicate its small litic activity or lack of sensitivity indicator culture (Figure 7).

For getting a concentrated viral material performed its accumulation in a submerged indicator culture conditions. For this obtained viral material was used to inoculate 7 hours of immersion indicator culture then the culture was continued for another 24 hours.

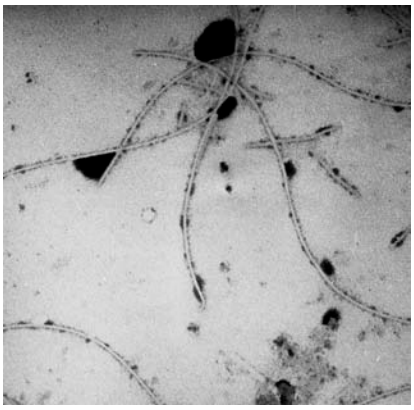


Figure 6. Filamentary particles of the negative portions lysogenic bacterial cultures. Scale line 0.12mkm

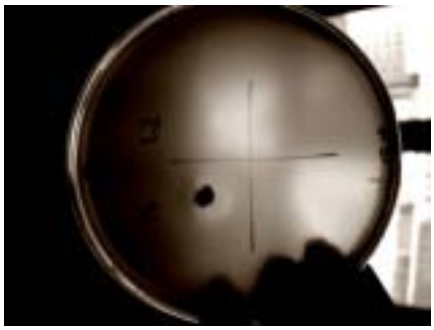


Figure 7. Area of lytic action

3.4 Isolation of phage DNA

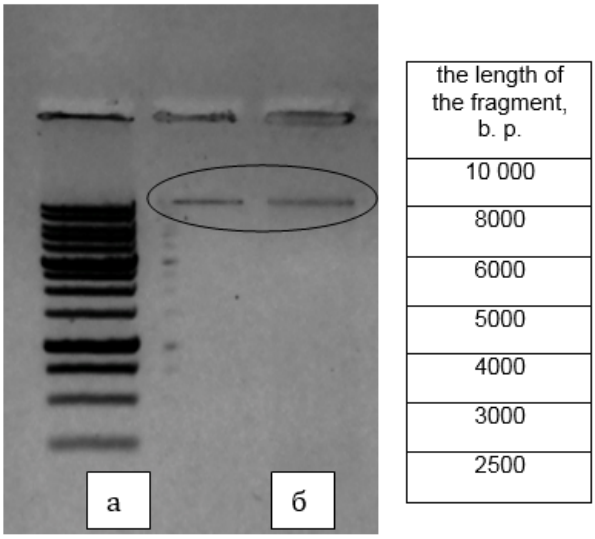
After culturing the resulting fagolizat (culture liquid containing cellular material and phage particles) was placed in a refrigerator to 4 ° C and held up to 14 days in order to optimize lysis (Figure 8). Then fagolizat was centrifuged for separating cellular material and concentration of phage particles. Thereafter, DNA was isolated and the electrophoretic separation of virual DNA from impurities bacterial DNA was made. It has been found that the size of the test filamentous phage not greater than 10000 bp (base pairs).

4 CONCLUSIONS

The oldest permafrost in Eurasia is likely to be in the Yakutia, where glaciers were not formed and whose age can reach 3 million years, when the surface temperature was perhaps similar to modern as it follows from paleoclimatic studies (Ershov, 1998; Lisiecki & Raymo, 2005; Hansen et al., 2010). The upper part of the Mammoth mountain section is so-called "ice complex", which is a syngenetic ice wedges located in the icy alluvial sediments. These deposits are younger, they are late Pleistocene (Vasil'chuk, 1991), but still represent a kind of "time capsule", which have ancient microorganisms which

have penetrated into the cracks during its formation with surface waters. It gives a unique opportunity to study microbial communities, their ability to survive, various cell-cell interactions and symbiotic relationship with viral particles, whose role in the survival of ancient microbial communities is still unknown.

Viral particles found in samples of ice wedges of the Mammoth Mountain can be attributed to five main morphotypes: miovirusy, sifovirusy, podovirusy, spherical and filamentous. Bacterial isolates were also detected, which cells are carriers of phages. A characteristic feature of these phage also are their small sizes and simple genome. Their distinguishing feature is the shape of the virion. Finding filamentous phage in the colonies of the ancient forms of bacteria indicates the possibility of the phenomenon of lysogeny in the geological history.



the length of the fragment, b. p.
10 000
8000
6000
5000
4000
3000
2500

Figure 8. Electrophoregram DNA isolated from fagolizat: a) marker fragments from phage DNA; b) prototype

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