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PHAGE DETECTION IN THE RHIZOSPHERE OF HIGHER ANTARCTIC PLANTS USING TRANSMISSION ELECTRON MICROSCOPY

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This work is focused on electron microscopy of soil samples from root zone of higher Antarctica plants *Deschampsia antarctica* and *Colobanthus quitensis* for detection of bacteriophages. Here we compared several techniques and proposed optimal method for isolation of phage suspensions from the substrate. For the first time, the biodiversity of bacteriophages and virus-like particles (VLPs) in the root zone of Antarctic plants has been evaluated. According to morphological description, the dimensions and taxonomic position of several bacteriophages were elucidated. Possible reasons for degradation of virus particles observed during the microscopy studies have been suggested.
Key words: bacteriophages, soil viruses, phage ecology, transmission electron microscopy

Електронномікроскопічні дослідження різосфери вищих антарктичних рослин на присутність бактеріофагів.
Мась О.В., Андрійчук О.М., Поліщук В.П.
Реферат. Дана робота присвячена електронномікроскопічному аналізові зразків ґрунту прикореневої зони вищих антарктичних рослин *Deschampsia antarctica* та *Colobanthus quitensis* з метою виявлення бактеріофагів. Порівняно та підібрано оптимальний метод виділення вірусних суспензій з субстрату. Вперше проаналізовано різноманіття вірусів бактерій та вірусоподібних часток у прикореневому ґрунті антарктичних рослин. За результатами електронної мікроскопії виявлено велику кількість фагових та вірусоподібних часток. За морфологічним описом визначено таксономічну приналежність окремих виявлених бактеріофагів та їхні розміри. Розглянуто можливі причини деградації вірусних часток, виявлених при ЕМ дослідженні.

Электронномикроскопические исследования ризосферы высших антарктических растений на присутствие бактериофагов.
Мась Е.В., Андрийчук Е.Н., Полищук В.П.
Реферат. Данная работа посвящена электронномикроскопическому анализу образцов почвы прикорневой зоны высших антарктических растений *Deschampsia antarctica* и *Colobanthus quitensis* с целью обнаружения бактериофагов. Проведено сравнение и подобран оптимальный метод выделения вирусных суспензий из субстрата. Впервые проанализировано разнообразие вирусов бактерий и вирусоподобных частиц в прикорневой почве антарктических растений. Согласно морфологическому описанию, определены таксономическая принадлежность отдельных обнаруженных бактериофагов и их размеры. Рассмотрены возможные причины деградации вирусных частиц, обнаруженных при микроскопических исследованиях.

1. Introduction

This work is focused on searching bacteria viruses in Antarctica soil. Samples were collected in the location of 'Academician Vernadsky' station. This station is based on Marina Cape of Galindez Island, just 7 kilometers from the western coast of Antarctic peninsula (65.245678° SLat, 64.257825° WLong). This region is characterized with relatively mild climatic conditions as the

Southern ocean acts a heat accumulator. Mean summer temperature is about 0°C but may vary from 1-2°C down to -6°C and lower in the night. Mean winter temperature is approximately -20…-25°C. During the winter, the temperature decreases when southern wind brings cold air from the continental part of Antarctica. At this time of the year flaws of wind of 30-35 m/s and above are also common. The region where the 'Academician Vernadsky' station is based sees precipitations 300 days a year (Martazinova et al., 2011/2012).

It is logical to assume that such atypical geoclimatic conditions would limit massive spread and development of biota. In part this hypothesis is confirmed by nearly total absence of vascular plants in the region. Only two species of higher plants are registered for the continent of Antarctica: *Deschampsia antarctica* and *Colobanthus quitensis*. However, many moss, lichen and algae species have been found (Polischuk et al., 2009). Works focused on analyzing quantitative abundance and species diversity of bacteria in Antarctica provided evidence that even such inclement geoclimatic conditions cannot significantly prevent bacteria preservation and spread. Depending on biotope, numbers of bacteria in the environment range from 10^4 to 10^9 per gram of substrate. This figure is just 1-2 orders less comparing to similar values for bacterial flora in moderate climate (Abedon, 2011).

Moreover, one can think that climatic and geological conditions of Antarctica, including properties of soil and soil substrates (pH, ionic strength, etc.), influence adsorption of virions on soil particles (Kimura et al., 2008) and hence pose serious problems for virus preservation and spread in such environment. In spite of the said above, some researchers were able to detect phage particles of various morphotypes in soil and moss 'rhizosphere' (Zhilenkov et al., 2007).

In turn, this fact provide evidence of, at first, occurrence and spread of bacterial hosts of these viruses, and at second, successful coexistence of such bacteria and their respective phages (i.e., productive viral infection) overcoming adverse environmental conditions not favoring bacterial growth.

In our research we used soil sampled from the root zone of higher plants as such biotopes were demonstrated to have the highest relative numbers of microflora (Tashyrev, 2009). In addition, bacterial species composition typical for the rhizosphere of *Deschampsia antarctica* has been described earlier (Barrientos-Díaz L. et al., 2008). Identified microorganisms belonged to the groups of typical representatives of soil microflora capable of degrading pesticides, reducing heavy metals and even invading plants (phytopathogenic bacteria): *Pseudomonas sp., P. tolaasii, P. trivialis, P. panacis, Flavobacterium sp.*, and *Arthrobacter sp*.

2. Materials and methods

Bacteriophages were isolated from samples of soil from the root zone (rhizosphere) of *Deschampsia antarctica* and *Colobanthus quitensis* plants which were collected in 2012 at the region of 'Academician Vernadsky' station, Galindez Island.

In a search for efficient method of phage isolation, we have used several techniques:

- 5 g of soil were homogenized with 50 ml of 0,1M Tris HCl, pH 7,4, and then incubated at room temperature for 30 min. Resultant suspension was centrifuged at 5000 g for 15 min, and then the supernatant was centrifuged at 37,000 rpm for 1,5 h. Precipitate was dissolved in 0,5 ml of 0,1M Tris HCl, pH 7,4;
- 2 g of root zone soil were homogenized with 20 ml of 0,1M Tris HCl, pH 7,4, mixed on magnetic stirrer for 15 min, and then mixed on orbital shaker for 20 min using glass beads (5 mm in diameter). Resultant suspension was centrifuged at 2000 g for 5 min, and then filtered through membrane filter with pores of 0,22 mcm in diameter to minimize soil substances and organic matter;
- 5 g of rhizosphere soil were homogenized with 50 ml of physiological saline, mixed on orbital shaker for 20 min. Resultant suspension was centrifuged at 4000 g for 20 min, and then

filtered through membrane filter with pores of 0,45 mcm in diameter obtaining 30 ml of the filtrate.

After such preparation and filtration all samples were further qualitatively checked for bacteriophages using transmission electron microscopy. Formvar-coated grids were immersed in 10 mcl of each suspension for 5 min and then negatively contrasted with 1% phosphotungstic acid for 1 min. these grids were then analyzed using JEOL JEM-1400 microscope.

3. Results and discussion

Soil biotopes of Antarctica are rather well studied from microbiologist's point of view. In particular, it was demonstrated that relative bacteria number and diversity in soil may be fairly considerable and depend on sampling location and soil properties (Abedon, 2011). For cold climates it was shown that gram-negative bacteria, α, β and γ-proteobacteria (*Pseudomonas spp.* and *Vibrio spp.*), as well as phylum *Cytophaga–Flavobacterium–Bacteroides* were detected most often. In case of gram-positive microorganisms, coryneforms (*Arthrobacter* and *Micrococcus sp.*) were most common. *Oscillatoria*, *Phormidium* and *Nostoc* genera typically were widespread *Cyanobacteria* representatives in Antarctica. Psychrophilic yeasts *Cryptococcus spp.* have also been isolated from soil samples. Research on density and species diversity of bacterial populations in biotopes of Dry Valleys confirmed that such locations might be characterized with 10^6-10^8 prokaryotic cells per gram of substrate (Barrientos-Díaz L. et al., 2008).

For Galindez Island (where Ukrainian Antarctic station 'Academician Vernadsky' is based) it was demonstrated that most of isolated microorganisms belonged to 'classic' taxons typical for various regions with moderate climate including *Bacillus, Actinomyces, Streptomyces, Pseudomonas, Methylobacterium, Enterobacter, Staphylococcus*, and *Brevibacterium* genera, etc. In this region, total quantity of chemorganotrophic aerobic microorganisms was 2-3 times smaller comparing to regions with moderate climate and constituted 10^5-10^8 cells/g of sample. Quantity of microorganisms in different biotopes decreased in the following order (cells/g of sample): soil (1×10^6-5×10^7) → aboveground part of *Deschampsia antarctica* plants (10^6-10^8) → underground part of mosses (1×10^6-5×10^8) → sludge of freshwater reservoir (10^5-10^7) → aboveground part of mosses (10^3-10^6) → lichens (10^3-10^6) (Tashyrev, 2009). Microbiological analysis of rhizosphere soil of *Deschampsia antarctica* Desv. plants allowed identification of bacteria belonging to *Pseudomonas sp., P. tolaasii, P. trivialis, P. panacis, Flavobacterium sp., Arthrobacter sp*. These groups of bacteria consist of type members of soil microflora capable of degrading pesticides, reducing heavy metals and even invading plants.

There is virtually no information on spread and diversity of bacterial viruses in soil biotopes of Antarctica. This may be due to the influence of various factors affecting viruses in soil. Different characteristics of soil and environment, as well as virus own properties, effect the adsorption characteristics of viral particles in soil. In particular it is known that viruses adsorb on soil particles and degree of adsorption commonly exceeds 90% for various viruses. Such factors as type of clay materials, cation-exchange capacity, ionic strength, soil-bound and unbound organic matter, and pH influence the efficiency of virus adsorption on soil particles (Kimura et al., 2008).

Morphology and biochemical characteristics of viral particles (for instance, isoelectric point, etc.) also make significant contribution in this phenomenon. Soil is a heterogeneous substrate consisting of particles with different charge(s) and varying hydrophilic/ hydrophobic properties which may have a dramatic effect of the adsorption of virus particles. This factor, in our opinion, plays a decisive importance in a 'bioavailability' of bacteriophages for their biological hosts – soil microorganisms. Based on such reasoning, the selection of optimal technique for phage isolation (elution) from soil samples was considered a critical step to this work.

Earlier, phages were detected in samples of surface layer of soil neighboring moss rhizoids. This research resulted in isolation and morphological descriptive classification of phages belonging to the families *Podoviridae* (C1 morphotype), *Siphoviridae* (B1 morphotype), and

Myoviridae (A1 morphotype). However, the authors have not managed to select any sensitive bacterial host capable of maintaining virus replication in laboratory conditions (Жиленков и др., 2007).

In the view of said above, this work was focused on isolation of phage particles from rhizosphere soil samples and their subsequent analysis using transmission electron microscopy (TEM). Bacteriophages were isolated from the soil samples collected from the root zone of higher plants *Deschampsia antarctica* and *Colobanthus quitensis* in Antarctica during the expedition of 2012 at the location of Ukrainian Antarctic station 'Academician Vernadsky', Galindez Island.

Comparative analysis of different techniques used for isolation (elution) of bacteriophages from soil samples provided evidence that methods avoiding high-speed centrifugation and including steps of sample washing on orbital shaker and following filtration through sterilizing membrane filters with pores of 0,45 and 0,22 mcm in diameter were proved most efficient for virus elution as confirmed by TEM.

During the microscopy analysis of soil eluates particles with clearly discernible tails were considered as viral particles when virus-like particles (VLPs) were those having spherical or icosahedral form resembling phage heads. Other morphotypes of rod-type or filamentous morphology were not taken into account due to the difficulties with their unambiguous detection on the background of soil debris/organic matter.

Following the analysis of TEM from as far back as 1959, it was stated that phages with non-contractile tails (representatives of *Siphoviridae* family) make up to 61% (i.e., absolute majority) among tailed bacterial viruses belonging to *Caudovirales* order (Ackermann, 1998, 2001, 2003). Based on this reasoning we expected that particles of B1 morphotype (representatives of *Siphoviridae* family) would prevail in virus populations eluted from Antarctic soil samples. In addition, based on literature data and previous results (Puhach O., 2011-2012, Zhilenkov et al., 2007), we also expected rather low numbers of viral particles in microscope's field of view. On the contrary, electron microscopy of our samples allowed detecting many viruses undoubtedly belonging to *Myoviridae* family. Moreover, such virus morphotypes clearly dominated quantitatively over viruses with other type(s) of morphology, and in particular they outnumbered siphoviruses.

Using classification proposed by Ackermann (1998), we have identified the following virus and virus-like particles:

i) phages characterized as morphotype A1 (Fig. 1 a, b, c, d; Fig. 2 o, p, q, r, t) with icosahedral head and contractile tail which may be attributed to *Myoviridae* family of *Caudovirales* order. Many of such viral particles had contracted tails and/or empty heads. Typical dimensions of these particles were 75×160 ± 4 nm; 88×94 ± 3nm;

ii) phages characterized as morphotype B1 (Fig. 1 e, f, g, h, i) with icosahedral head and long non-contractile tail which may be attributed to *Siphoviridae* family of *Caudovirales* order. Typical dimensions of these particles were 65×131 ± 4 nm, 50×200 ± 3nm;

iii) elongated (prolate) and spherical virus-like particles (VLPs) with dimensions of 101×86 ± 4 nm; 50 ± 3 nm (Fig. 1 k, l; Fig. 2 x, y, z);

iv) degraded phages and structure elements of virions (separated tails, contracted tail sheaths, heads without nucleic acid) (Fig. 1 c, d, i; Fig. 2 o, p, t, u, v, z).

In addition, we have identified viral particles visually similar to representatives of *Podoviridae* family of *Caudovirales* order (with icosahedral head and short tail) (Fig. 1 j; Fig. 2 w, x). However, few numbers of such particles did not allow making sound conclusions on their tentative taxonomic relations.

We have also detected viral particles of 'correct' icosahedral form approximately 50 nm in diameter (Fig. 2 s) which were fairly difficult to attribute to any certain family basing purely on visual characteristics.

Phages with empty heads (without nucleic acid) were quite abundant, as well as separated tails and contracted tail sheaths. This fact may be explained by effects of various factors at the stages of virus interaction/isolation/microscopy. It has been established, for instance, that the

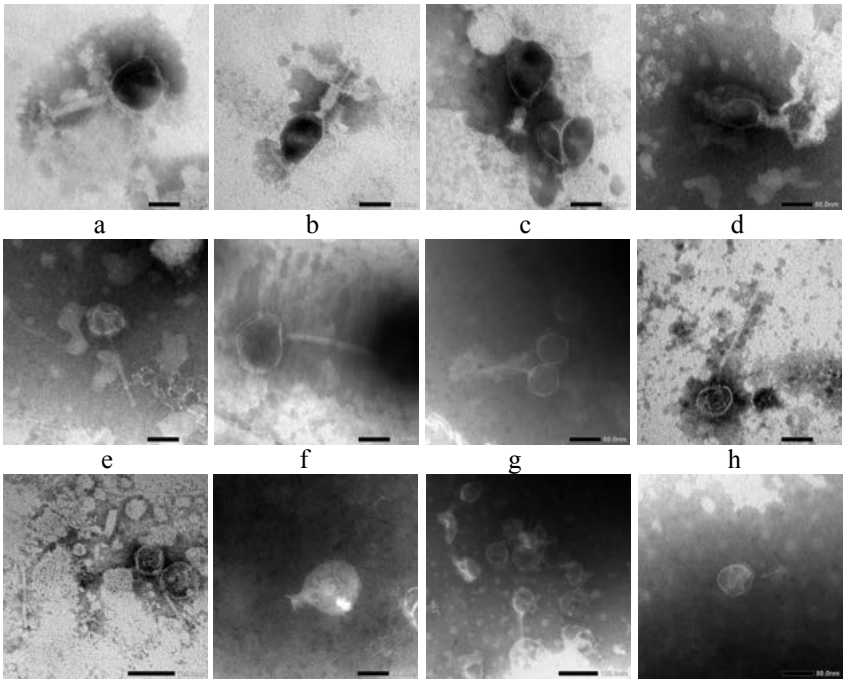

Fig. 1. Morphology of virus and virus-like particles detected during electron microscopy of soil samples from rhizosphere of *Deschampsia antarctica.*

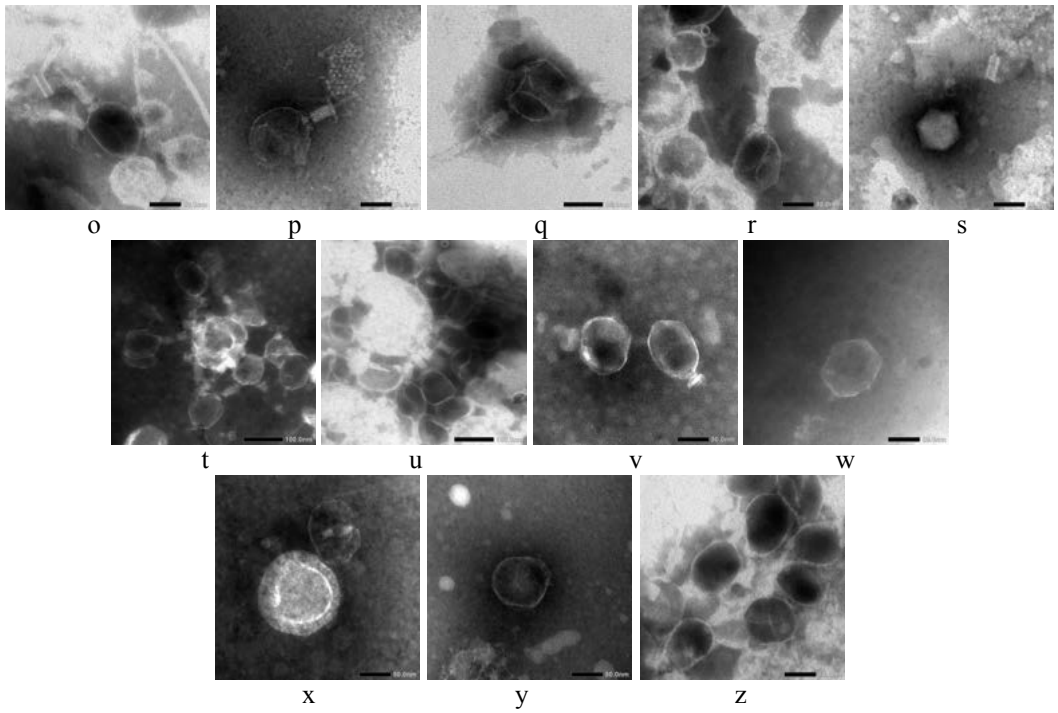

Fig. 2. Morphology of virus and virus-like particles detected during electron microscopy of soil samples from rhizosphere of *Colobanthus quitensis.*

majority of bacteriophages demonstrated tolerance towards pH fluctuations in the range of pH 5-8. At low temperatures, however, this range of virus tolerance for pH might be somewhat wider, pH 4-10. Strong acidulation or alkalinization induced progressive degradation of viral particles with the release of nucleic acid, tail separation, tail sheath contraction, and particle breakdown into structural elements – protein head capsid, tail sheath, tail tube, and tail fibers. Similar effects were also shown for temperature stress. For instance, momentary or repeated freezing-thawing often led to viral particle degradation, DNA release, contraction of tail sheath, sheath separation from the tail, tail separation from the head, etc. Surprisingly, basal plate and fibers often preserved (Tihonenko, 1968).

Occurrence of many (partially or nearly fully) degraded bacteriophages in the fields of view of the microscope (Fig. 1 c, d; Fig. 2 o, p, t, u, z) may provide evidence of both non-optimal methodic conditions for isolating viruses from a substrate, effect(s) of substrate factors (pH, etc.), and conditions of substrate samples' transportation/conservation (influence of temperature, i.e. momentary or repeated freezing-thawing) affecting structural stability of viral particles. In Antarctica, temperature may vary from +1-2°C at daytime to -6°C at night, causing repeated freezing-thawing of superficial soil layers which in turn may affect virus preservation in substrate.

From ecological point of view, preservation of bacteriophages' populations in such extreme conditions remains a major issue. Increased tolerance of virions to physical factors, high rates of virus production (relative virus yield per infected cell) and/or virions' capability to bind to soil particles for keeping intact outside host cells all are crucial elements for maintaining virus numbers in the environment.

Therefore, the result of successful phage isolation from soil and obtaining structurally intact viral particles depends on characteristics of viruses, their tolerance toward varying physical conditions of the environment, as well as on proper technique used for phage elution. This information is of special significance when studying viral biota of geographical regions dramatically differing from moderate climatic zones.

Future work will involve isolation of Antarctic bacteria from soil samples and selection of natural microorganisms susceptible to previously identified bacteriophages for establishing laboratory 'virus-host' model system.

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