RESEARCH PAPER

A NEW BACTERIOPHAGE OF THE FAMILY *SIPHOVIRIDAE* ISOLATED FROM THE SODDY-PODZOLIC SOILS OF THE PRIOKSKO-TERRASNY NATURE RESERVE

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ABSTRACT. Bacteria of the genus *Streptomyces*, one of the main microorganisms of soils, and their bacteriophages are important inhabitants of soil ecosystems. Important though they are, not much is known about their functional patterns and population dynamics. A question of particular interest, which is still to be understood, is how bacteriophages regulate the population dynamics of *Streptomyces* and how this regulation affects the soil ecosystem as a whole. Isolation and study of new *Streptomyces* bacteriophages can help to understand these problems. In this paper, we describe isolation of a new bacteriophage from the soils of the Prioksko-Terrasny Reserve. The analysis of morphology of the new phage allows us to conclude that it belongs to the family *Siphoviridae*.

KEYWORDS: Streptomyces, phage, soddy podzolic soils, Prioksko-Terrasny Reserve

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INTRODUCTION

On the basis of their infection mechanism, bacteriophages, the viruses of bacteria, are divided into two groups. The first group is virulent bacteriophages, whose replication and propagation occurs via the lytic cycle (e.g., it involves lysis of the host bacterial cell as an obligatory step). The second group is temperate bacteriophages; their life cycle is lysogenic, meaning that they may incorporate in the bacterial genome in the form of a prophage. The prophage is not active; it does not cause lysis of the bacterial cell and can, therefore, be replicated along with the bacterial genome during the process of bacterial cell division (Abedon 2008). Bacteriophages are found in all the bacterial habitats. Some of the bacterial/phage ecosystems (e.g., aquatic and animal intra-intestinal habitats) are well-studied; others, like soil bacterial/phage communities, are not – the data on the latter are scarce.

Meanwhile, it is assumed that phage communities play a significant role in the soil ecosystems. For example, a metagenomic analysis of permafrost soils showed that bacteriophages could infect bacteria involved in the carbon cycle, thereby affecting biogeochemical processes (Truble et al. 2018; Emerson et al. 2018). The analysis of viriome of Chinese soils indicated that bacteriophages might also contribute to the cycle of phosphorus in the soil ecosystems (Han et al. 2022). In particular, quite a large number of agricultural-soil viruses were shown to have genes for the synthesis of phosphorus-containing compounds. The authors suggested that a part of soil phosphorus could be redirected to the synthesis of viral genomes, thereby affecting the strategies for acquiring phosphorus by plants and soil bacteria (Han et al. 2022).

The estimates based on direct counting indicate that soils can contain a large number of phage particles, up to ~10^{10} per gram of soil (Ashelford et al. 2003). It is suggested that in soils, the incidence of viral infection of bacteria is higher than in aquatic ecosystems (Kuzyakov and Mason-Jones 2018). The higher rate of infection may result from the adsorption of bacteriophages and their bacterial hosts on clays. In soil ecosystems, bacteriophage particles interact directly with the chemical components of the soil, and these interactions can have a great impact on the survival of phages. Depending on the phage and the clay type, the effect can be either negative or positive. In the case of actinophages, for example, the range of pH, in which they can survive, depends on the clay that the phage is adsorbed upon (Sykes and Williams 1978). As

active physical and chemical media, clays modulate the acts of host cell recognition and infection that occur on their surface. The negatively charged phages and bacteria bind to the clay cation groups, resulting in a stronger interaction between viral particles and the susceptible bacterial cells (Dashman and Stotzky 1984; Lipson and Stotzky 1984). On certain clays, the binding of phages to the clay particles was shown to be very strong (Sykes and Williams 1978). It was also demonstrated that clay binding could prolong the infectivity of eukaryotic viruses and reoviruses (Lipson and Stotzky 1985) – confirmed by a later observation that particles of infectious bacteriophages could persist in the soil for a long time (Williams et al. 1987). One could, therefore, argue that, in the absence of host bacteria, virulent phages can be preserved in the soil for relatively long periods and will resume their reproduction as the population of their hosts grows. Such a preservation was reported, for example, by Marsh and Wellington, who isolated virulent actinophages from a soil which was air-dried for several months and then rewetted with sterile distilled water (Marsh and Wellington 1994). The authors observed a spike in the number of phage particles after a 24-hour incubation, which could be either a gradual elution of phages tightly bound to the clay components or release of the new lysogenic and pseudolysogenic progeny. In the eluate, temperate phages, which produce turbid plaques on the indicator strain, were more numerous than virulent phages, producing clear plaques (Marsh and Wellington 1994). Evidently, bacteriophage particles are less protected outside the host, making lysogeny a more optimal strategy for the survival of phages in the soil ecosystems. Pseudolysogeny, which is observed in virulent bacteriophages, seems to contribute to the survival of phages as well.

There is only a small number of studies directly examining the in *situ* lytic activity of phages in soils. Pantastico-Caldas et al. (1992) reported that the presence of SP10C, a virulent phage of *Bacillus subtilis*, in the soil decreased the host population by an order of magnitude. On the other hand, the temperate actinophage KC301 did not affect the size of *Streptomyces* populations in both sterile and non-sterile soils (Marsh and Wellington 1992; Marsh et al. 1993). The experiments showed that the infectivity of KC301 was high enough to inhibit the vegetative growth of *Streptomyces lividans*, yet the inhibitory effect was compensated for by the sporulation of *Streptomyces* (Marsh and Wellington 1994). Other observations of the population dynamics of soil bacteria indicated that the effects of phages could be both adverse or beneficial. Many of the phage effects are related to the state of lysogeny in bacteria and the phenomenon of transduction, with the expression of phage genes or maintenance of lysogeny changing the metabolism of the host bacterial cell due to lysogen conversion.

Thus, soil bacteriophages are able to influence microbial mortality, food web dynamics, and biogeochemical cycling of soil elements (Emerson 2019).

This work examines bacteriophages from the soils of the Prioksko-Terrasny Biosphere Reserve, Moscow Region. The reserve is a semi-pure forest area (The Prioksko-Terrasny Nature Biosphere Reserve 2022), with brown forest, sod-weakly podzolic and sod-medium podzolic soil cover being the common soil types on the territory of the reserve (Kurganova et al. 2020). The reserve has a unique nursery of European bison, permanent dwellers of this area since the Middle Ages which are now listed in the Red Data Book of the Russian Federation (Zemlyanko et al. 2017). The soils of the Prioksko-Terrasny European bison nursery can, therefore, be considered as a model of native forest soils of ancient Eurasia. These forest soils are dominated by Actinomycetes; in particular, representatives of the genus *Streptomyces*. They are producers of various physiologically active substances and are able to effectively suppress the growth of other microorganisms (such as phytopathogenic fungi) within their econiche (Xu L.H et al. 1996; Pedziwilk 1995). The literature contains detailed data on the dynamics

Fig. 1. A paddock for young European bison where soil samples were collected (soil type, sod-podzolic)

and structure of soil *Streptomyces* populations, as well as their interactions with other bacteria (Dobrovol'skaya et al. 2015). At the same time, little is known on the modulation of soil *Streptomyces* populations by bacteriophages. How the communities of *Streptomyces* and their bacteriophages function as a whole, what the molecular mechanisms regulating their dynamics in particular soils are, and what role this bacterium/phage complex plays in the ecosystem – all these questions are still to be answered. In this paper, we have characterized the morphotype of new *Streptomyces* bacteriophages, which have been isolated from the soil samples taken on the territory of the Prioksko-Terrasny European bison nursery (Fig. 1).

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Streptomyces venezuelae (Ac-589 type VKM) was used as the bacteriophage host. The strain was grown on a modified MS medium (mannitol, 10 g/L; peptone, 20 g/L; glucose, 2.5 g/L; agar, 20 g/L; tap water) at a temperature of 30°C. For agar double layers, MS was used with 0.5% and 1.5% agar for the top and bottom layers, respectively.

Sampling and processing

The sod-podzolic soil samples were collected from the top layer of soil cover in one of the paddocks of the young European bison nursery of the Prioksno-Terrasny Reserve (marked with orange crosses on the map of Figure 2). The samples (2 ml of soil) were collected into test tubes under sterile conditions and supplemented with 10 ml of a phage buffer (200 mM NaCl, 25 mM Tris-HCl (pH=8)). Extraction was carried out for two hours on a mini-rotator BioRS-24 (mini-rotator biosan) at a low rotation speed, followed by centrifugation of the samples (10,000 rpm; 90 seconds) to precipitate the soil particles. The supernatant was passed through a bacterial filter (0.2 µm); the filtrate was supplemented with a couple of drops of chloroform and stored is at 5-7°C.

Isolation and cultivation of phages

The phage filtrate (0.1 ml) was added to 2 ml of an overnight Streptomyces venezuelae culture (8–12 hours of growth), and the mixture was added to 0.5% MS agar (5 ml) melted in a water bath at 43°C. The inoculated agar was poured onto a layer of 1.5% MS agar solidified in a Petri dish (the double agar layer method; Kauffman and Polz 2018). After an overnight incubation at 30°C and visualization of plaques, phages from individual plaques were microbiologically purified by the three-passages-and-titration protocol according to Gracia. The overall scheme of procedures, from the soil sampling to the isolation of phages, is shown in Figure 3.

Preparing phage samples for the electron microscopy examination

To prepare samples for electron microscopy, bacteriophage particles were extracted with the phage buffer from fresh lawns of streptomycetes almost completely lysed by the phages. After incubation for a couple of days at 6°C, the phage extract was separated from the cell debris by centrifugation at 5000 rpm for 15 min and filtered through a bacterial filter (0.2 µm). The filtrate was concentrated using a vivaspim-500 concentrator, which increased the number of phage particles in the samples by a couple of orders of magnitude.

Electron Microscopy Examination of Phage Virions

The electron microscopy (TEM) examination was performed according to a standard technique. A sample aliquot was placed on a 400-mesh carbon-coated copper grid, washed with distilled water to remove unattached particles, and contrasted with an aqueous solution of 1% uranyl acetate. The samples were analyzed using a JEM-1200EX electron microscope (Jeol, Japan) at an accelerating voltage of 80 kV and magnification of 40–50 × 103. The results were micrographed on Kodak film SO-163 (Kodak, USA), 6.5 × 9 cm.

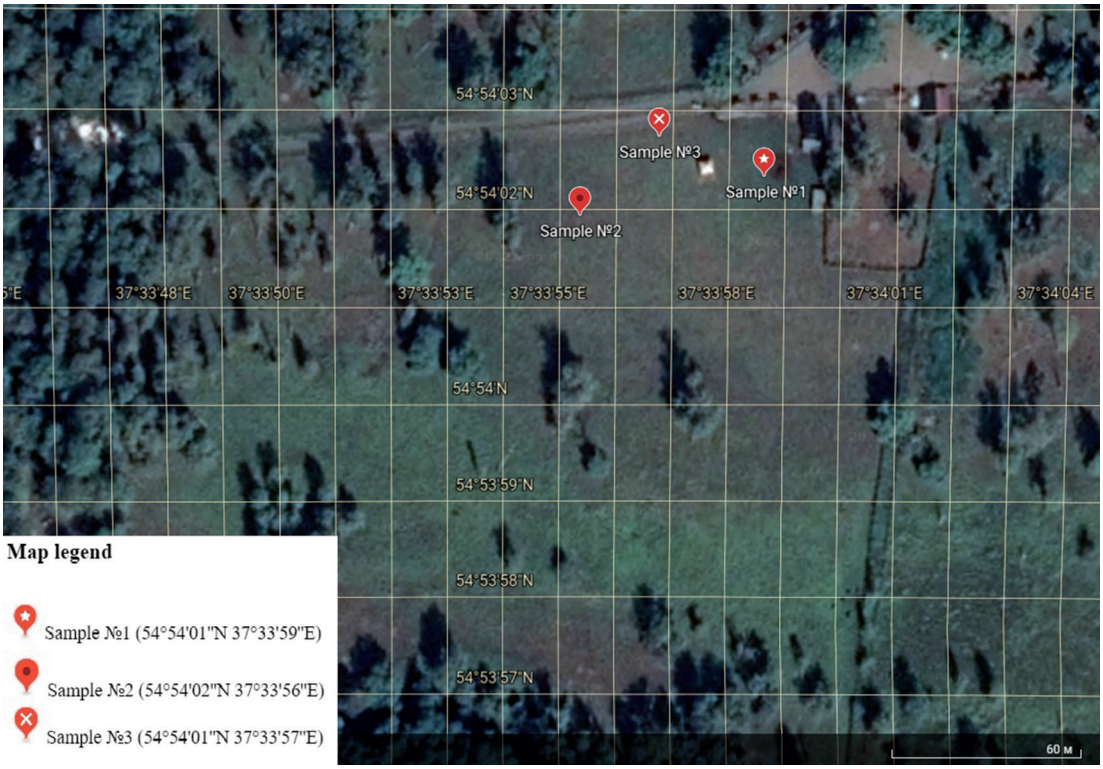

Fig. 2. The map of soil sampling sites. Sampling locations are marked with red label. Coordinates of sampling location are indicated in brackets on the map legend. The space image was taken from the Google Earth

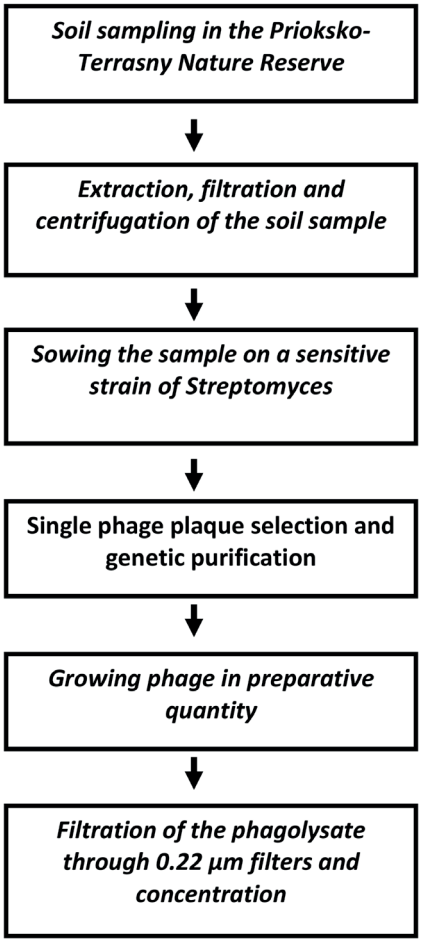

Fig. 3. A scheme of procedures for the isolation of Streptomyces phages from soils

RESULTS

As a result of the conducted experiments, we have isolated and microbiologically purified, through a series of successive titrations, an earlier unknown bacteriophage. The bacteriophage, which was isolated from a single plaque, was able to form new plaques within 12 hours since the moment of culture infection.

The maximal titer of the bacteriophage cultivated in large volumes (500 ml) under laboratory conditions was 10^{12} PFU/ml. When the phage was cultivated from a single plaque for 16 hours, the titer usually was in the range of 10^7-10^8 PFU/ml and could drop to 10^6 PFU/ml in the process of storage. The bacteriophage titer was calculated according to formula 1:

$$A = \frac{x \times 10^n}{v} \quad (1)$$

where x was the number of plaques on *a* Petri dish; *v*, the sample volume; n, the degree of sample dilution; *A*, the number of phage particles expressed in PFU/ml. The average titer was calculated as the arithmetic mean of the previously obtained titers (Table 1).

From the analysis of 100 bacteriophage plaques, we have described their characteristic morphology. The plaque diameter varied greatly: from 1 to 8 mm (diameter variability (CV) = 47.9%). The average diameter of bacteriophage plaques was calculated as 4.09 ± 2.08 mm (Table 2, Figure 3). More details on the variation of the plaque diameter are given in Table 2.

The plaques were transparent and had irregular edges, with the jags of viral infection protruding deeper into the field of bacterial culture. All over their perimeter, the plaques were interspersed with islets of phage-resistant colonies (Fig. 4-6).

Table 1. The quantitative parameters of bacteriophage cultivation

	Number of Streptomyces phage plaques on Petri dishes							
growing time and volume	n,V	12 h, 500 ml		16 h, 10 ml		16 h, 10 ml		16 h, 50 ml a month later
the degree of dilution of the sample cup no. 1 (n)	6	100000	2	10000	4	998	2	1350
the degree of dilution of the sample cup no. 2 (n)	6	100000	4	210	4	1115	2	1002
the degree of dilution of the sample cup no. 3 (n)	8	1215	4	230	4	1033	4	58
the degree of dilution of the sample cup no. 4 (n)	8	1250	6	6	6	14	4	51
the degree of dilution of the sample cup no. 5 (n)	10	12	6	1	6	10	6	0
V cup samples no. 1, ml	0,1		0,1		0,1		0,1	
V cup samples no. 2, ml	0,1		0,1		0,1		0,1	
V cup samples no. 3, ml	0,1		0,1		0,1		0,1	
V cup samples no. 4, ml	0,1		0,1		0,1		0,1	
V cup samples no. 5, ml	0,1		0,1		0,1		0,1	
number of phages of sample no. 1		$1{,}00\times10^{12}$		$1{,}00\times10^{7}$		$9{,}98\times10^{7}$		$1{,}35\times10^{6}$
number of phages of sample no. 2		$1{,}00\times10^{12}$		$2{,}10\times10^{7}$		$1{,}12\times10^{8}$		$1{,}00\times10^{6}$
number of phages of sample no. 3		$1{,}22\times10^{12}$		$2{,}30\times10^{7}$		$1{,}03\times10^{8}$		$5{,}80\times10^{6}$
number of phages of sample no. 4		$1{,}25\times10^{12}$		$6{,}00\times10^{7}$		$1{,}40\times10^{8}$		$5{,}10\times10^{6}$
number of phages of sample no. 5		$1{,}20\times10^{12}$		$1{,}00\times10^{7}$		$1{,}00\times10^{8}$		$0{,}00\times10^{6}$
average number of phages, PFU/ml		$1{,}13\times10^{12}$		$2{,}48\times10^{7}$		$1{,}11\times10^{8}$		$2{,}65\times10^{6}$

Table 2. Size characterization of the phage plaques

Plaque diameter, mm	Frequency, pi
1	15
2	12
3	12
4	15
5	25
6	6
7	7
8	8
The total number of plaques examined	100
Mean $\bar{x}$	4,09
Dispersion σ^2	4,34
Standard deviation σ	2,08
Coefficient of variation (CV)	47,99
Standard error (SE)	0,2

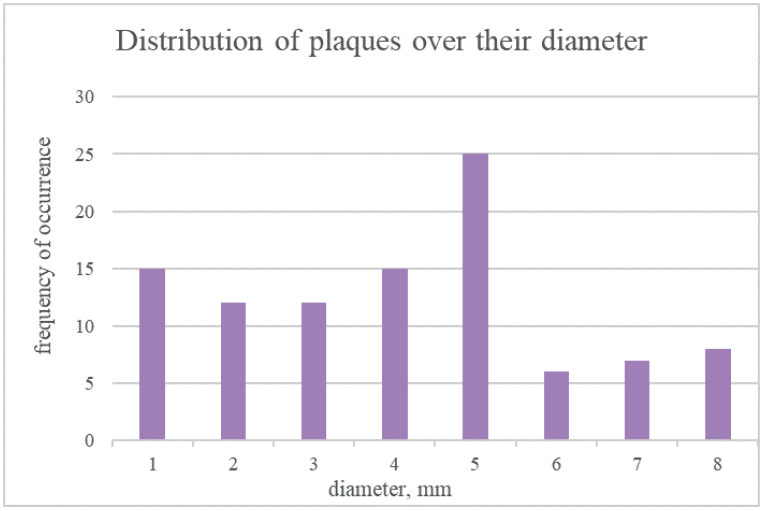

Fig. 4. The distribution of plaques of the new *Streptomyces* phage over their diameter

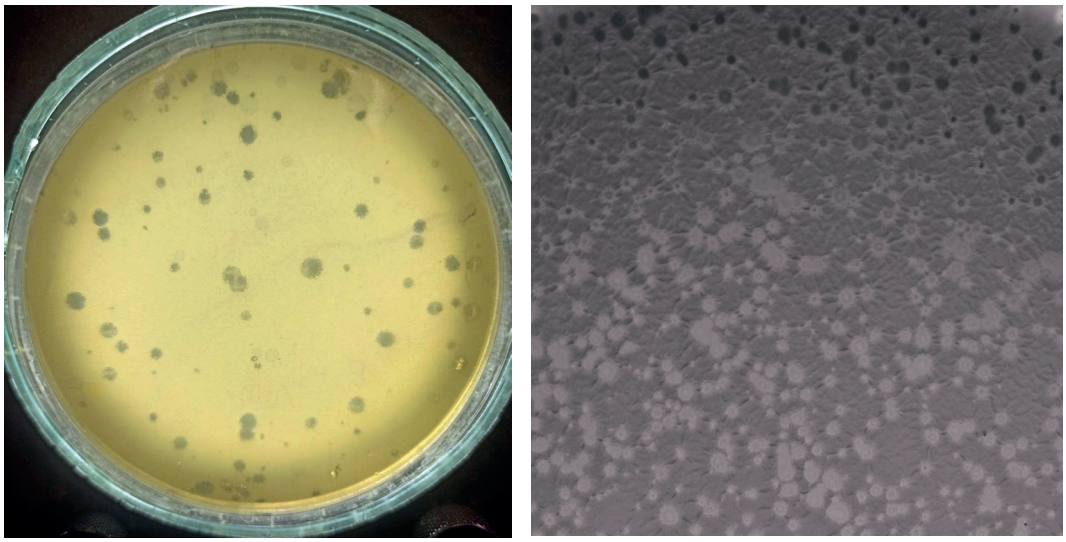

Fig. 5-6. Plaques of the new bacteriophage of *Streptomyces*

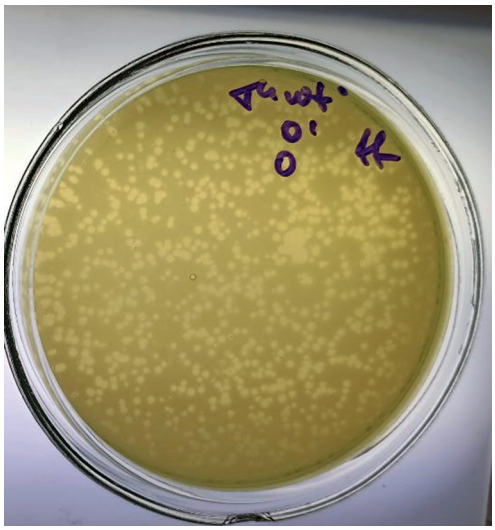

Fig. 7. Plaques of the lytic bacteriophage T4

Comparing the plaque morphology of the new bacteriophage with that of other phages (lysogenic phage λ and lytic phage T4; see Fig. 7) – as well as taking into account the character of the plaque overgrowth with the phage-resistant bacterial clones – we concluded that the new bacteriophage was, probably, lytic (Mitarai et al. 2016). There is, however, another possibility which cannot be write off. Some of the bacteriophages of *Streptomyces* are known to be plasmid-phages. In their prophage stage, plasmid-phages are not integrated into the host chromosome, but remain in the cytoplasm in the form of a plasmid (Zhong et al. 2010). Not much is known about the plaque morphology of plasmid-phages and how it differs from that of lytic phages. Therefore, an accurate assessment of the type of life cycle of the new phage can only be made after additional experiments.

The results of electron microscopy examination of the new phage are shown in Figure 8. The phage has an isometric icosahedral head, approximately 50 nm in size, and a seemingly non-contracting tail, approximately 110 nm long and 10 nm thick. According to Bradley's classification (Fig.9), the phage particle belongs to the B1 morphotype (Bradley 1967; Ackermann 2006; Ackermann 2012; Ackermann and Prangishvili 2012), which is characteristic of bacteriophages of the family *Siphoviridae*. The basal plate is quite thick (about ~15 nm) – and there should be 6 long fibrils extending from it (not visible on the micrograph).

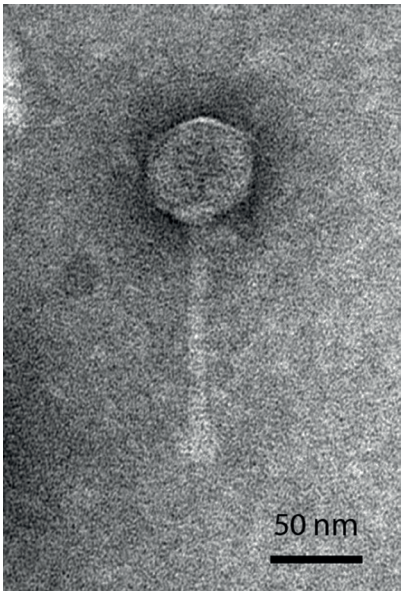

Fig. 8. An electron micrograph of the new bacteriophage of *Streptomyces*. The micrograph indicates that the phage belongs to the family *Siphoviridae*

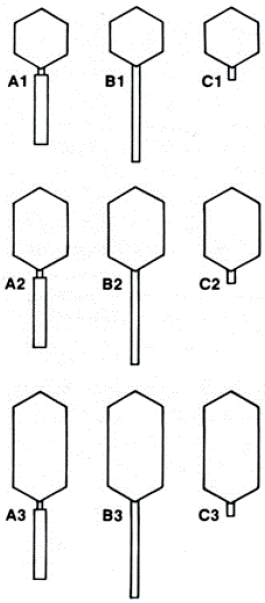

Fig. 9. Morphotypes of the families *Myoviridae* (A1 - A3), *Siphoviridae* (B1 - B3) and *Podoviridae* (C1 - C3) according to Bradley's classification (Bradley 1967)

DISCUSSION

The methods aimed at the study of soil viruses, which have been developed in this work, are the first step towards examining the role of viruses in soil ecosystems. In most soils, viruses are plentiful, reaching 10^{10} particles per gram of dry soil (Ashelford et al. 2003). This abundance, which is quite striking in comparison to other ecosystems, is likely a combined result of both replication of viruses and their accumulation over time. Determining the contribution of these two processes to the abundance of viruses in soils is a hot topic of research. The bacteriophage isolated and described in this paper can be used as a model object for studying preservation of bacteriophages in natural soils. Certain areas of the Prioksko-Terrasny Reserve are good testing grounds for such experiments. What is currently needed is modification and adaptation of the molecular-genetic methods for the analysis of viral and, more specifically, bacteriophage soil communities. Metagenomic projects aimed at the study of soil viruses are rare, despite the evidence that the genetic diversity of soil viruses may exceed that of marine ones. Soil viruses can control life activity of their bacterial hosts (through infection, lysis, lysogenization and lysogenic conversion), and might also affect their evolution (through horizontal transfer of genes by bacteriophages).

It has already been demonstrated that soil microbiocenoses are built on complex relationships between bacteria, fungi, and plants. Soil bacteriophages are also participants in these relationships (Ashfield-Crook et al. 2018, 2020). For example, *Streptomyces*, antagonists of fungi, are known to facilitate the development of the root system of plants by releasing fungicidal substances. In particular, the fungicidal activity of *Streptomyces* was reported to be crucial in the prevention of weight losses caused by the pathogenic fungus *Fusarium solani* in wheat shoots. Furthermore, inoculation of soil with phages had a negative effect on the growth of the shoots. Apparently, the phage-induced suppression of *Streptomyces* propagation may contribute to the opportunistic fungal infections in plants (Ashfield-Crook et al. 2018). The data of Ashfield-Crook et al. (2018) clearly demonstrated that a virulent phage of *Streptomyces* was capable to control the life activity of the bacterium: the experimental introduction of the phage into this system led to a decrease in the *Streptomyces* titer and provided conditions for an uncontrolled propagation of a parasitic fungus.

Similar to many other phages of *Streptomyces*, the new bacteriophage described in this paper may be polyvalent: rather than being specific to any particular strain of *Streptomyces*, it can infect various strains of this genus. The bacteriophage may, therefore, be capable of controlling the development of several or even many species of *Streptomyces*. By reducing their titer and facilitating the propagation of fungi, the bacteriophage could, therefore, contribute to the decomposition of plant residues in the forest podzolic soils of Central Russia, leading to their enrichment with nutrients necessary for the growth of trees.

In perspective, it seems promising to further study the newly discovered bacteriophage and the relative phages – both in the laboratory and natural setups. The isolated bacteriophage can be cultivated to high titers – and model experiments, in which the formation and dynamics of soil microbiocenoses could be studied under periodic inoculation of the soil with the phage filtrate (with monitoring the spontaneous emergence of resistant *Streptomyces* mutants), would be interesting. It would be also of interest to investigate the distribution of the new bacteriophage and the related phages in the soils of the Prioksko-Terrasny Reserve – both in the European bison nursery and other sectors of the reserve. The purpose of such studies could be to assess, by indirect methods, the influence of the European bison nursery on the development of forest soils, which would not only provide information on the rate and specifics of soil formation, but might also give an insight into the development of the soils in the future.

The methods of microbiological examination of *Streptomyces* bacteriophages, which were modified and adapted in this work for the analysis of soil samples, can be useful in further research in the field. The quantitative determination of phage titer in soil samples, for example, can be used to monitor the titer of bacteriophages (and, thus, to control the propagation of their *Streptomyces* hosts) in the soil. In the future, we plan to obtain data on the genome of the isolated phage, which would allow us to make a PCR-test – to replace microbiological methods for monitoring the phage in the reserve soils with genetic ones.

CONCLUSIONS

Based on the data obtained, we assume that the new bacteriophage of *Streptomyces* isolated and described in the paper belongs to the order *Caudovirales*. The bacteriophage has an isometric head in the shape of an icosahedron, approximately 50 nm in size, and a seemingly non-contracting tail, approximately 110 nm long and 10 nm thick. The phage forms transparent plaques with an average diameter of 4.09 ± 2.08 mm. A supposition is made that the phage belongs to the family *Siphoviridae*, representatives of which often infect *Actinomycetes*. Further studies of the phage and its genome, as well as the mechanisms and specifics of the infection process, will help to understand the relationships between the phage and its host bacteria and the role of these relationships in the natural ecosystems.

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