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EXPERIMENTAL ARTICLES

Diversity and Dynamics of Bacteriophages in Horse Feces

E. E. Kulikov[a], A. S. Isaeva[a,b], A. S. Rotkina[a,b], A. A. Manykin[a,c], and A. V. Letarov[a, 1]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117312 Russia*

[b] *Skryabin Moscow State Academy of Veterinary Medicine and Biotechnology, ul. Skryabina 23, Moscow, 109472 Russia*

[c] *Ivanovskii Research Institute of Virology, Russian Academy of Medical Sciences, ul. Gamalei 16, Moscow, 123098 Russia*

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Abstract—The complex cellulolytic microbial community of the horse intestines is a convenient model for studying the ecology of bacteriophages in natural habitats. Unlike the rumen of the ruminants, this community of the equine large intestine is not subjected to digestion. The inner conditions of the horse gut are much more stable in comparison to other mammals, due to the fact that the horse diet remains almost unchanged and the intervals between food consumption and defecation are much shorter than the whole digestive cycle. The results of preliminary analysis of the structure and dynamics of the viral community of horse feces, which combines direct and culture methods, are presented. In horse fecal samples, we detected more than 60 morphologically distinct phage types, the majority of which were present as a single phage particle. This indicates that the community includes no less than several hundreds of phage types. Some phage types dominated and constituted 5–11% of the total particle count each. The most numerous phage type had an unusual morphology: the tails of its members were extremely long (about 700 nm), flexible, and irretractable, while their heads were 100 nm in diameter. Several other phage types with similar but not identical properties were detected. The total coliphage plaque count of the samples taken from three animals revealed significant fluctuations in the phage titers. During the observation time, the maximum titer ranged within four orders of magnitude (10^3–10^7 plaque forming units (PFU)/g); the minimum titer ranged within two orders of magnitude. The samples contained two to five morphologically distinct and potentially competitive coliphage types, specific to a single *Escherichia coli* strain.

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Before widespread use of direct methods for phage count in various natural samples, the numbers and the likely role that phage particles play in the functioning and autoregulation of microbial communities were seriously underestimated [1]. For instance, in saline and freshwater reservoirs, the numbers of bacterial cells are on average ten times smaller than those of phage particles. In various aquatic communities, between 10 and 70% of bacterial mortality is associated with phage infection [1–3]. It has been demonstrated that bacteriophages are the main factor regulating the numbers of bacterial cells when the population density reaches a threshold (the "kill the winner" concept; see [1] and references therein). However, unlike the consumption of bacteria by protozoa, phage lysis does not result in organic matter mineralization; it redistributes organic matter in microbial food chains. The input of up to 26% of organified carbon into the dissolved organic matter pool available for prokaryotic organisms is due to viral infection (see [1, 3] and references therein).

The structure of natural viral communities is still not clearly understood. It is believed that the composition of uncultivable viral communities, in which dominant viruses comprise up to 5–7% of the total number of viral particles, is extremely variable [4]. The large intestine of warm-blooded animals represents an excellent model for studying the ecology of bacteriophages in natural microbial communities. One of the main benefits of this model is the presence of *Escherichia coli* and fairly high titers of *E. coli*-specific bacteriophages in bacterial populations.

The cellulolytic microbial community of the large intestine of horses is distinguished by its high complexity and includes several hundred species of bacteria, archaea, fungi, and protozoa. This community thrives under very stable conditions, since the horse diet

[1] Corresponding author. E-mail: arthq@mail.ru

remains almost unchanged, whereas the intervals between food consumption and defecation are shorter than the whole digestive cycle (ca. 72 h) [5].

The results of sequencing of random DNA clones of the total viral community from horse feces have been published [6]. On the basis of the data obtained, the authors assume that the "metagenome" of this viral community includes several hundreds of individual phage genomes. Data on the coliphage numbers in horse feces have been repeatedly published [7, 8]. It was found that the differences between individual titers could comprise several orders of magnitude; however, no investigations have been carried out into these considerable variations.

Differences in phage concentrations between individual animals may be due to their permanent features (for instance, the structure of the surface molecules of their intestines, which determines colonization by *E. coli* strains), or the temporal dynamics of phage populations.

The aim of the present work was to develop a method for monitoring the numbers and population composition of coliphages from horse feces, as well as the structure of the uncultivable viral community.

MATERIALS AND METHODS

Microbial strains. The *Escherichia coli* strains C600 (Promega, United States), Be/1 (derivative of strain B; laboratory collection), and NM522 (Clontech, United States) were used for bacteriophage titration. The strains C600 and NM522 are derivatives of *E. coli* K12. The strain NM522 contains the F' plasmid responsible for its sensitivity to F-specific bacteriophages.

Monitoring of coliphage titers. Horse feces were sampled immediately after defecation. Approximately 20 g of the material was transferred into sterile preweighed flasks with 100 ml of buffer solution (pH 7.5) containing Tris-HCl, 50 mM; 0.9% NaCl; 0.01% NaN_3; and 0.05% Tween 20. The flasks were sent to the laboratory and weighed. The samples were incubated on a shaker for 20 min in order to ensure bacteriophage desorption. After that, 50 ml of the suspension was centrifuged in a fixed-angle rotor at 10000 *g* for 10 min. The supernatant was sampled for coliphage inoculation and for the isolation of the total viral community for subsequent electron microscopic examination. Before the experiments were carried out, we had examined ten animals kept in the same stable in equivalent conditions. Three animals were selected, two mares (five and six years old) and a gelding (eight years old). All the horses were clinically healthy and had not been subjected to antibiotic therapy for three months. No symptoms of gut dysfunction were detected. During the preliminary examination of the animals, coliphage titers on strain C600 ranged from 10^3 to 10^5 plaque forming units (PFU) per one gram of wet sample. Henceforth, samples were taken from each animal every two days for sixteen days in June–July 2005. The coliphage titers on strains C600, NM522, and Be/1 were determined. The titration was carried out by the two-layer method using the LB medium containing the following (per 1000 ml of distilled water): tryptone (Amresco, Spain), 10 g; yeast extract (Difco, USA), 5 g; NaCl, 5 g; and bacto agar (Difco, United States), 15 g. The LB medium supplemented with 0.6% bacto agar was used as the upper layer.

Coliphage enrichment culture on petri dishes. The preliminary titrated fecal extract was inoculated by the two-layer method so that one petri dish contained $(1–3) \times 10^4$ PFU. After overnight incubation at 37°C, the upper agar layer was detached, homogenized with a glass spatula, and extracted with an equal volume of the buffered saline for 2 h. Thereafter, the extract was cleared by centrifugation under the same conditions as the fecal extract.

The phage purification was performed by centrifugation in viscosity gradient. The centrifuge tubes were supplemented with 3 ml of 40% glycerol solution in SM buffer (pH 7.5) containing Tris-HCl, 50mM; $MgSO_4$, 10 mM; 0.6% NaCl. This solution was then overlaid with 3 ml of 5% glycerol dissolved in the same buffer and up to 15 ml of the phage-containing extract. The tubes were centrifuged at 100 000 *g* for one hour in a bucket rotor. Over the phage sediment, 100 µl of 40% glycerol was left. It was then overlaid with subsequently added 100-µl portions of SM buffer at 15 min intervals until the volume of the solution reached 500 µl. We left the sediment coated with buffer for 12 h and then resuspended it and cleared it by centrifugation in an Eppendorf table centrifuge for 2 min at maximum speed.

We have developed a new method for precipitation of phage particles through the glycerol viscosity gradient on a freon-113 pad as an alternative technique, which may increase the number of intact phage particles. The floating density of the phages is lower than that of freon-113 (1.57 g/cm^3). This fact makes it possible to harvest phage particles at the interface separating two liquid phases.

The portions (20 ml) of fecal extract were layered over the gradient containing (starting from the tube bottom) freon-113, 2 ml; 40% glycerol solution in SM buffer, 5 ml; and 5% glycerol solution in the same buffer, 3 ml; they were then centrifuged in a bucket rotor at 100 000 *g* for 1.5 h. After centrifugation, lysate and glycerol solution were removed with a pipette. As a result, about 1 ml of 40% glycerol was left over the freon layer. Glycerol was slowly dissolved with 200-µl portions of SM buffer which were added at 5 min intervals until the volume of the solution was 3 ml, and then with 1 ml portions of SM buffer, so that the solution was brought to the volume of 6 ml. The solution was then supplemented with the buffer up to the brim and recentrifuged at 100 000 g for 30 min. The supernatant was removed with a pipette. About 1 ml of the buffer

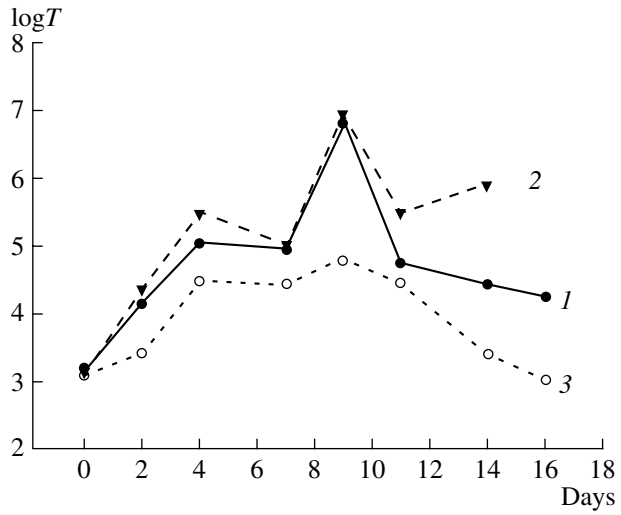

Fig. 1. Dynamics of coliphage titers determined by inoculation on the *E. coli* strains: (*1*), C600; (*2*), NM522; and (*3*), Be/1, isolated from one of the animals.

was left over the freon layer. The preparation was left overnight in a refrigerator to ensure better resuspension. The remaining large aggregates were removed by centrifugation in the table centrifuge at maximum speed for 5 min. The obtained purified preparations were examined under an electron microscope.

Electron microscopy. The preparations were obtained by bacteriophage adsorption on copper grids covered with carbon-stabilized formvar support, ionized before the application. The adsorption time was 10 min. The preparations were stained with 1% uranyl acetate for 30 s, washed on top of water drops 30 s, and dried. The preparations were examined under a Jeol 100S microscope (Japan) at ×20000 magnification.

RESULTS

Within 18 days of observation, substantial fluctuations in the phage titers were detected for all the studied strains (Fig. 1). For each animal, the curves of various bacteriophage titers distinctly correlated between themselves; however, we did not detect any correlation between the animals (data not shown). In the course of the experiment, the fluctuation amplitude of the phage titers was 2 orders of magnitude for two animals and four orders of magnitude for the third one (Fig. 1). The titers of the bacteriophages able to lyse strain C600, as a rule, were higher than those of the Be/1-specific phages; this difference was of an order of magnitude or more. The phage titers of the strains C600 and NM522 coincided at most points; however, at several points, the titer of strain NM522 was approximately an order of magnitude higher than that of the C600 strain (Fig. 1). This can be attributed to the fact that *E. coli* NM522 has receptors not found in C600, for example, F-pili. Furthermore, NM522 is impaired with respect to some restriction/modification systems, which are active in C600.

It should be noted that phage titers determined on different days are difficult to compare, since many phages form small plaques, which are difficult to mark and which, therefore, can be missed during counting. Since, along with the fluctuations of the PFU levels, the spectrum of plaque morphology changes as well, the counting accuracy might change over time. Thus, despite the fact the data spread did not exceed 10% for repeated inoculation with the same extract, only differences of at least $\pm 0.5 \log T$ between the titers of various specimens were treated as reliable ones. Titer fluctuations ranging from one to four orders of magnitude, including sharp changes when two successive specimens featured a 100-fold difference, undoubtedly account for significant ecological events in the intestinal microbial community.

The extract samples supplemented with glycerol (15%) were stored at –70°C for further investigation. The repeated determination of phage titers after two months did not reveal any differences from the initial values.

The maximum coliphage titer for strain C600 was 10^7 PFU/g. Values approximating those of the coliphage titers in the fecal material had been previously reported [7], but the question regarding the number of phage types which are present in the intestines of one host at one time has not been addressed. The study of viral morphology with transmission electron microscopy (TEM) is the most suitable method for particle identification. Massive investigation by molecular biological techniques has been hampered due to the abundance and complexity of the procedures for the analysis of individual phages, while sequencing of the random fragments of the phage community metagenomes is a very expensive procedure which cannot provide unambiguous results.

The quantitative assessment of coliphage composition requires isolation of a large series of individual isolates from each sample under study, as well as their subsequent characterization. Unfortunately, we lacked the technical resources needed for the investigation of the series consisting of tens of preparations by transmission electron microscopy. Moreover, this approach is not suitable for subsequent monitoring. Therefore, we applied the method of semiquantitative evaluation of enrichment cultures on petri dishes. Under these conditions, phage plaques begin to overlap and form a pattern of "reticular" lysis. However, over a substantial period of time, the descendants of each phage particle develop independently. Thus, unlike liquid enrichment cultures, the competition between various phages for the host is considerably reduced. As a consequence, the total replacement of slow-growing phages by rapidly growing ones does not occur. The coliphage mixtures obtained using this method were studied by transmission electron microscopy. We took more than a hundred

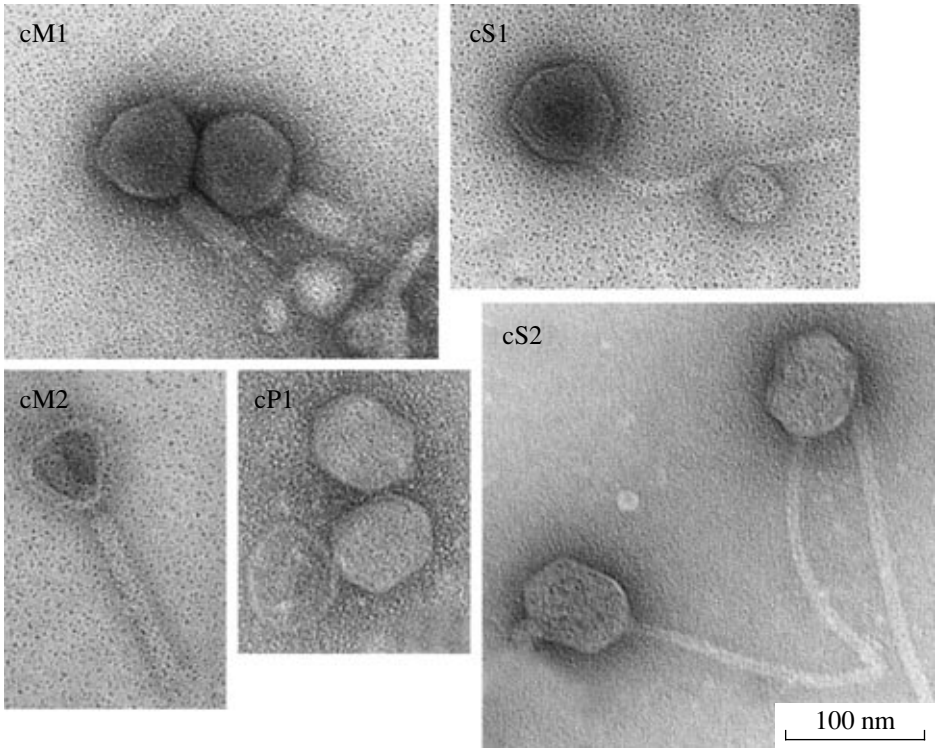

Fig. 2. Morphological types of the coliphages discovered in the enrichment cultures grown in petri dishes (for explanation, see text). Designations: cS, coliphage belonging to the family *Siphoviridae*; cM1, *Myoviridae*; and cP, *Podoviridae*. The morphotype numbering corresponds to that of the Laboratory catalog.

photographs of all the particles we found. Phages representing different morphotypes have been isolated. Phages indistinguishable by the size of the morphological elements of the particles at a given magnification and resolution were considered identical. It is obvious that the percentage of coliphage types differs from that in the initial extract. However, given the presence of one or two predominant morphotypes, we can detect their replacement. Another shortcoming of this approach is its applicability only to specimens with phage concentrations not less than 5×10^4 PFU/ml; however, according to our data, the typical titer values are higher than this limit. Moreover, when the occasion requires, the specimens may be concentrated by ultracentrifugation or ultrafiltration.

We studied the morphological spectra of the coliphages detected in two animals. It was found that no less than three morphologically distinct phage types were present in each of the obtained coliphage mixtures (Fig. 2). The two morphological types cS1 and cM1, which were predominant in the enrichment cultures grown in petri dishes, were identical in the two studied specimens. However, in the sample taken from the first animal, coliphages of the (c) type (cS2 and cP1) were abundant; in the samples taken from the second animal, we detected some shades of the second phage with a retractable tail (M2). Hence, the population of the coliphages simultaneously present in horse feces includes several phage types.

We have also tested our purification method in order to find out if it is appropriate for the isolation of the uncultivable viral community from horse feces. Examination of the purified specimens of the uncultivable viral community by transmission electron microscopy has revealed a great quantity of phage particles of various morphological types (Fig. 3). The level of purification was quite satisfactory for TEM. However, the total number of particles was not very high; their concentration in the initial material ranged from 10^8 to 10^9 particles/g. Thus, we needed to take about 20 photographs for the quantitative analysis of the uncultivable community composition. For this reason, to carry out comparative investigations of the preparations of uncultivable viral communities from several specimens, it would be appropriate to change somewhat the procedure in order to improve the efficiency of particle adsorption on the grid support.

We have characterized the uncultivable community composition in the two samples obtained from different animals. The particles which could not be distinguished according to their morphological properties or size (e.g., two particles of the S8 type; Fig. 3) under the given observation conditions were assigned to the same morphological type. As to small phages (like P9 and P10 types; Fig.3) whose morphological properties were

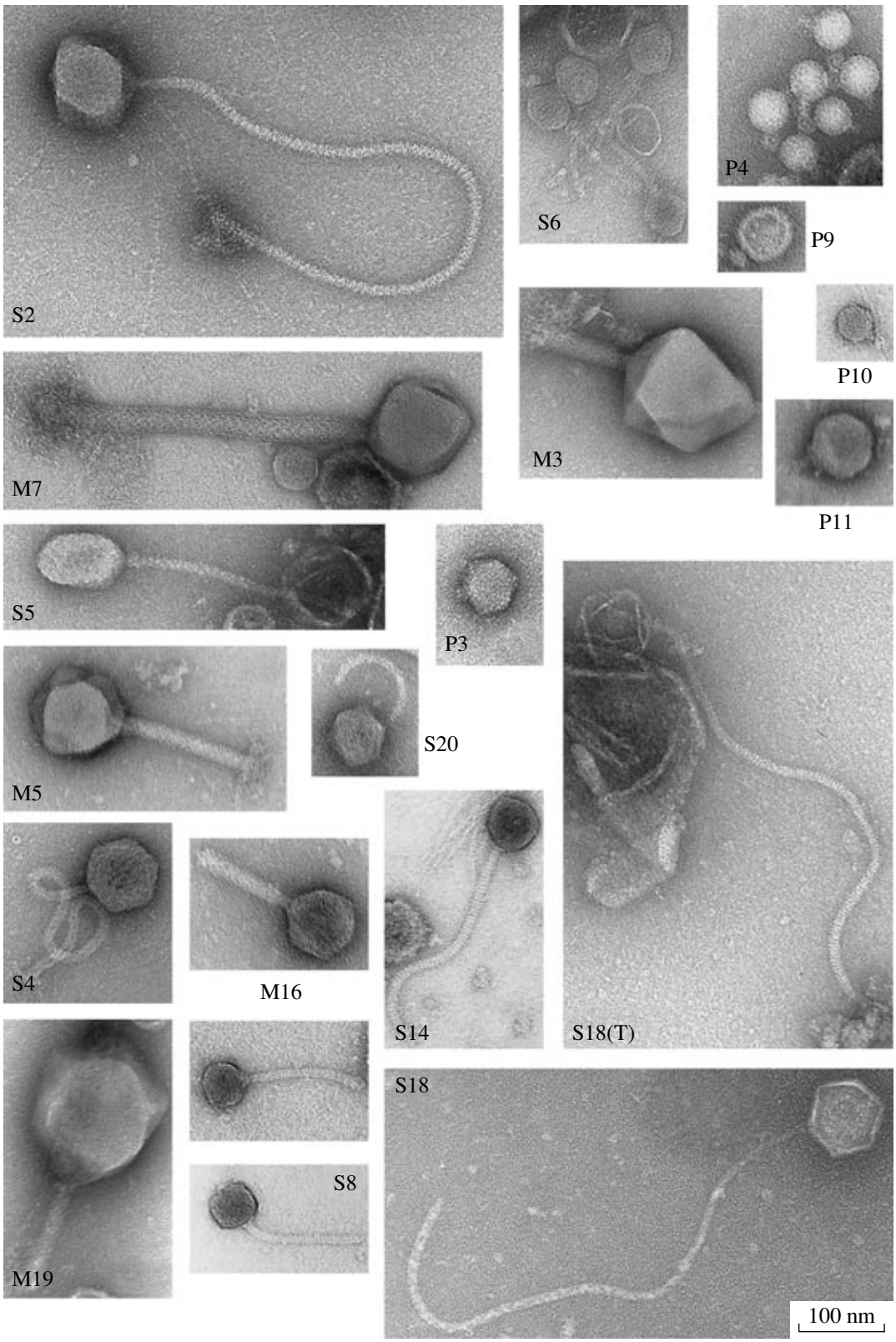

Fig. 3. Some morphological types of the bacteriophages detected in the uncultivable viral community from horse feces. Designations: see Fig. 2.

poorly recognized, the genetically distinct viruses could have been classified as belonging to the same type. However, in the case of large, morphologically unusual phages (Fig. 3), it seems unlikely. We took about 100 individual photographs of phage particles or shades for each sample. A large majority of these phages belong to the order *Caudovirales* (tailed phages). Only a few particles presumably belonging to the family *Microviridae* were detected. Particles similar to animal viruses were detected only in one photograph; they were incorporated in a small aggregate. The studied uncultivable viral community is compositionally diverse. This is demonstrated by the fact that the majority of the morphological types were represented by one particle. However, some types were represented by several particles. Some of them were found in aggregates; other particles were observed side by side only in one photograph (e.g., types P4 and S6; Fig. 3). In that case, their abundance in the sample may not reflect the true community composition, since it can be due to the accidental discovery of an aggregate. Other predominant types were detected in several distant parts of grids, indicating their abundance in the sample. Interestingly, the studied communities included many phages with very long flexible nonretractable tails, resembling a fire-hose. Only in one animal were four different types of such phages (S2, S4, and S18; Fig. 3) detected. Phages of the S18 type had tails of about 700 nm length. Members of this morphotype prevailed in both animals and comprised about 10% of all the counted particles. It seems likely that the particles of the S18 phage are sufficiently labile, because most of them were present as shades (S18(T); Fig. 3). However, we have found several intact particles. The abundance of large bacteriophages with retractable tails and head diameter ranging from 100 to 190 nm has drawn our attention. None of these phages was represented by more than two particles. Figure 4 shows the distribution of the described phage morphotypes in one animal depending on their head diameter. A total of 61 types of morphologically distinct phages were found in this animal. The bar chart shows that the phage classes with capsid diameters ranging from 55 to 75 nm prevailed; however, the proportion of large phages was rather high. The morphotype S18, prevailing in both animals, is the only type of bacteriophage with the capsid diameter of about 100 nm.

Among the described phage types of the total uncultivable community, we have not detected any morphological types identical to those of the previously described coliphages. This can be attributed to low *E. coli* titers in comparison to the total numbers of bacteria in horse feces. Among the 272 rRNA clones analyzed in [9], which contained fragments of the 16S rRNA genes of bacteria isolated from various sections of horse intestines, no rRNA clones were identified as *E. coli* clones. The total number of proteobacterial rRNA clones was less than one percent.

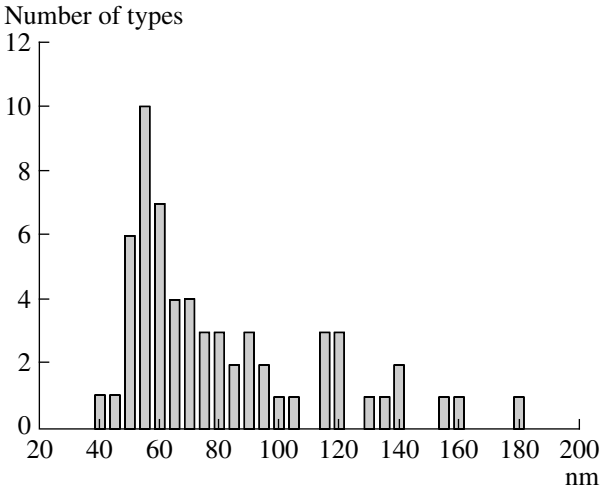

Fig. 4. Distribution of the number of described bacteriophage morphotypes from the uncultivable viral community isolated from horse feces throughout the classes depending on their capsid diameters. Class width, 5 nm.

DISCUSSION

Our study showed that the coliphage numbers in horse feces were subject to significant temporal fluctuations (on a scale of up to four orders of magnitude). This may explain the significant differences in the titers of the phages, which were previously found in various animals. Coliphages of three or more different types were present at the same time (it should be taken into account that morphologically identical phages may be genetically different). It is still unknown whether different coliphage species inhabit their own ecological niches, or if they compete with each other for a single niche, resulting in continuous change of phage species. It is probable that some phages are released due to spontaneous induction of lysogenic bacteria, whereas others exist in this microbial system due to the continuous reproduction in the lytic cycle.

It is still unknown why these fluctuations occur. We believe that the reciprocal regulation of the viral and bacterial populations is the most plausible explanation. However, we should not completely rule out the possibility that these fluctuations reflect changes in the state of the macroorganism. The simultaneous monitoring of the numbers of coliphages and *E. coli* cells, coupled with the study of the autostrain sensitivity to phages, as well as of the phage titers of various autostrains, might help to elucidate these mechanisms. The methodical approaches developed during our preliminary study will be of use in carrying out such an experiment in near future.

Our method for purification of the uncultivable viral community is generally appropriate for phage characterization by transmission electron microscopy. The advantage of this method is that there is no need for

centrifugation in the cesium chloride gradient, which destroys some phage types.

The data obtained on the diversity and occurrence of morphological types are in agreement with the experimental observations of Cann et al. [6]. Judging from the low occurrence of most morphotypes, as well as by their diversity (we have isolated more than 60 morphologically distinct types), the phage community includes up to several hundred various phage types. The proportion of predominant types ranges from 5 to 10% of the total number of particles. The abundance of phages with extremely long tails (S2, S4, S14, and S18; Fig. 4) has attracted our attention. It is probable that, in the viscous chyme of the large intestine, relatively large phages have a significant selective advantage if their adsorption apparatus is capable of "scanning" the space around their capsids in search of a host.

It should be emphasized that the traditional method used for determining the virus diversity in an uncultivable community by transmission electron microscopy yields no less biologically important information than sequencing of restricted (up to 1000) random clone samples of viral DNA (termed "metagenomes"). The availability of databases containing high-quality microphotographs of phages from various natural communities, provided with reliable size scales, would make comparative investigations easier. We believe that expensive studies of metagenomes must certainly be accompanied by the development of databases, which would contain photomicrographs of the same specimens. Purification of the predominant phage types from the uncultivable community performed under control of transmission electron microscopy and followed by genetic characterization would allow us to compare the distribution of these phages in various habitats, e.g., in the intestines of the animals of one species found in various parts of the world.

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