ISOLATION AND CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* BACTERIOPHAGES — POTENTIAL AGENTS FOR PHAGE THERAPY

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Pseudomonas aeruginosa — is one of the pathogens characterized by the critical number of multidrug-resistant (MDR) strains. Phage therapy is considered an alternative to antibiotics, especially in treatment of infections caused by MDR strains. The aim of this study was to isolate and characterize *P. aeruginosa* phages that could potentially be suitable for treating infectious diseases. To isolate the *P. aeruginosa* phages, enrichment cultures were used. The lytic activity spectrum was confirmed by spot testing on 40 *P. aeruginosa* strains. Whole-genome sequencing was performed using Illumina MiSeq instrument. Phylogenetic analysis was done using VICTOR tool. Isolated phages vB_PaeA-55-1w and vB_PaeM-198 from *Autographiviridae* and *Myoviridae* families, respectively, had a broad spectrum of lytic activity (about 50% each), including lysis of MDR strains. The genomes vB_PaeA-55-1w and vB_PaeM-198 comprise double-stranded DNA of 42.5 and 66.3 kbp in length, respectively. Open reading frames were annotated for both phages (52 for vB_PaeA-55-1w, and 95 for vB_PaeM-198), no integrases and toxins were detected. On a phylogenetic tree, vB_PaeA-55-1w phage was clustered with phages from the *Phikmvvirus* genus (*Autographiviridae* family), which are also used in phage therapy. vB_PaeM-198 phage was clustered with phages from the *Pbunavirus* genus (*Myoviridae* family). vB_PaeA-55-1w and vB_PaeM-198 phages could be considered as candidates for phage therapy and may be used to treat infections caused by MDR *P. aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, virulent bacteriophages, phage therapy, *Autographiviridae*, *Myoviridae*, whole genome sequencing, phylogenetic analysis

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ВЫДЕЛЕНИЕ И ХАРАКТЕРИСТИКА БАКТЕРИОФАГОВ *PSEUDOMONAS AERUGINOSA* — ПОТЕНЦИАЛЬНЫХ АГЕНТОВ ДЛЯ ФАГОВОЙ ТЕРАПИИ

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Одним из патогенов, характеризующихся критическим показателем доли штаммов с множественной лекарственной устойчивостью (МЛУ), является *Pseudomonas aeruginosa*. В качестве альтернативы антибиотикам при терапии инфекций, вызванных штаммами с МЛУ, рассматривают фаготерапию. Целью исследования было выделить и охарактеризовать бактериофаг *P. aeruginosa*, потенциально пригодный для терапии инфекционных заболеваний. Выделение проводили методом накопительных культур. Спектр литической активности устанавливали спот-тестированием на коллекции из 40 штаммов *P. aeruginosa*. Полногеномное секвенирование выполняли на платформе MiSeq (Illumina). Филогенетический анализ геномов проводили с помощью VICTOR. Выделенные бактериофаги vB_PaeA-55-1w и vB_PaeM-198, принадлежащие к семействам *Autographiviridae* и *Myoviridae* соответственно, обладали широким спектром литической активности (около 50% каждый), в том числе вызывали лизис штаммов с МЛУ. Геномы vB_PaeA-55-1w и vB_PaeM-198 представлены двухцепочечной ДНК длиной 42,5 и 66,3 т.п.н. соответственно. В составе геномов аннотировано 52 (vB_PaeA-55-1w) и 95 (vB_PaeM-198) открытых рамок считывания, среди них гены интеграз и токсинов не обнаружены. На филогенетическом древе vB_PaeA-55-1w располагался в кластере совместно с бактериофагами рода *Phikmvvirus* семейства *Autographiviridae*, в том числе с используемыми в фаготерапии, а vB_PaeM-198 входил в кластер, включающий бактериофаги рода *Pbunavirus* семейства *Myoviridae*. Бактериофаги vB_PaeA-55-1w и vB_PaeM-198 можно рассматривать в качестве кандидатов для применения в фаготерапии, в том числе и для лечения инфекций, вызванных штаммами *P. aeruginosa* с МЛУ.

Ключевые слова: *Pseudomonas aeruginosa*, вирулентные бактериофаги, фаготерапия, *Autographiviridae*, *Myoviridae*, полногеномное секвенирование, филогенетический анализ

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According to the World Health Organization, antibiotic resistance is rising to dangerously high levels in all parts of the world [1]. Gram-negative bacteria, including *Pseudomonas aeruginosa*, occupy the first places in the global priority list of antibiotic-resistant bacteria posing the greatest threat to human health [1]. The bacteria of this species are ubiquitous, their genetic plasticity and environmental adaptability are high. The wide variety of pathogenicity mechanisms often makes the infection general in cases of infestation with *P. aeruginosa* strains [2]. *P. aeruginosa* causes a wide range of diseases, from intoxication to extensive pyoinflammatory processes and septic shock [2]. According to AMRmap portal, the share of *P. aeruginosa* isolates, which occupy the top lines in the list of most common nosocomial pathogens in Russia, was 16.83% among all such pathogens isolated in 2015–2020 [3]. Besides, about 30% of *P. aeruginosa* strains circulating in the population are multidrug-resistant (MDR), i.e., they are resistant to at least one antibiotic drug out of three or more antibiotic groups, and about 15% of the circulating *P. aeruginosa* strains show extreme drug resistance (XDR), i.e., they are resistant to at least one antibiotic from all groups of antibiotics, with the exception of 1–2 groups; these factors drive the related patient mortality up [4].

An urgent task currently is to develop alternative, non-antibiotic treatments for infectious diseases caused by MDR and XDR pathogens. One of the most promising alternatives are virulent bacteriophages, which are the basis of phage therapy [5]. The promise is in the capability of virulent bacteriophages to lyse both antibiotic-sensitive and antibiotic-resistant strains of bacteria. In addition, bacteriophages do not cause toxic and allergic side effects, have no contraindications [6] and can be prescribed to pregnant women in combination with other medications [7].

A number of reports and preclinical and clinical trials [8, 9] confirm the success of phage therapy against *P. aeruginosa* infections both in animals and humans. There are several commercial therapeutic drugs designed to counter infections caused by *P. aeruginosa* that are produced in Russia: *Pseudomonas aeruginosa* bacteriophage, Intesti-bacteriophage, Polyvalent purified pyobacteriophage (Microgen; Russia).

Despite the availability of bacteriophage preparations active against *P. aeruginosa* and the successful experience of their use, phage collections need to be constantly updated, as the specifics of modern phage therapy require. Since virulent bacteriophages have a rather narrow specificity and usually target only several strains, updating the collections means extending them with phages that cause lysis of current bacterial strains. Moreover, there are reported cases [10] when bacteria mutate and acquire resistance to bacteriophages [10]. Isolation of new bacteriophages and their inclusion in the composition of therapeutic drugs solves this problem.

In connection with the above, the purpose of this work is to isolate and characterize *P. aeruginosa* bacteriophages that can be used to treat infectious diseases.

METHODS

Bacterial strains

The study used *P. aeruginosa* strains (n = 40) selected from the collection of bacterial strains of the laboratory of molecular genetics of microorganisms of the Federal Research and Clinical Center of Physical-chemical Medicine of Federal Medical Biological Agency of Russia. The strains of the collection were characterized by the profile of drug susceptibility (to ceftriaxone, gentamicin, ciprofloxacin, and meropenem), as well as by genotypes according to the results of multilocus sequence typing (MLST) [11]. The bacteria were cultivated for 18–24 h in the lysogeny broth (LB) nutrient medium (Oxoid; UK) at 37 °C.

Isolation of bacteriophages

The bacteriophages were isolated by enriching cultures from natural sources (sewage, water samples from various rivers) with *P. aeruginosa* strains PA55 and PA198 (Table 1). A sample of water (50 ml) was drawn through a Millipore filter with a polyvinylidene fluoride membrane, pore diameter of 0.45 μm (Merck Millipore; USA), and then two-fold LB broth was added to it. Subsequently, 300 ml of an overnight culture of the host strain was added and incubated on a shaker at 37 °C for 18 h. After cultivation, bacterial cells were centrifuged for 10 minutes at 3500 g to achieve precipitation. The supernatant was put through a Millipore filter with a polyethersulfone membrane and a pore diameter of 0.22 μm (Merck Millipore; USA). Individual bacteriophages were obtained by sequential (threefold) isolation from individual negative colonies. Further on, bacteriophages were grown in 50 ml of LB broth containing 300 μl of the bacterial strain overnight culture. The concentration of the bacteriophage in the phage lysate was estimated by the standard Grazia titration method [12].

Bacteriophage lytic capability range determination

Bacteriophage lytic capability range was determined by spot testing. Phagolysates with a titer of 3×10^6 PFU/ml (plaque-forming units per ml) were used in the tests with the aim to prevent non-specific lysis. An overnight culture (10^{10} CFU/ml) of the tested bacterial strain was sequentially diluted in LB broth to a cell concentration of 10^6 CFU/ml, then 0.1 ml thereof was mixed with 5 ml of semisolid LB agar (0.6% agar) and added to a Petri dish containing a thin layer of LB agar (1.5% agar). After solidification of the semisolid agar, a drop (5 μL) of the studied bacteriophage was applied to the dish's surface. Petri dishes were incubated at 37 °C for 18–24 h. Lytic capability was assessed visually: the bacterial strain was considered sensitive to the bacteriophage in case there appeared a transparent spot or separate negative colonies. In the absence of such a lysis spot or if it was opaque the bacterial strain was classified as resistant.

Table 1. Characteristics of *P. aeruginosa* bacteriophage host strains

Strain	MLST sequence type	Antibacterial susceptibility pattern			
		ceftriaxone	gentamicin	ciprofloxacin	meropenem
PA198	ST508	R	S	S	R
PA55	ST2690	R	S	S	S

Table 2. Genetic characteristics of the collection strains and range of the hosts of vB_PaeA-55-1w and vB_PaeM-198 bacteriophages

MLST sequence typing	Total number of strains	Share of strains lysed by vB_PaeA-55-1w, %	Share of strains lysed by vB_PaeM-198, %
ST12	5	0	100
ST17	1	0	0
ST186	1	100	100
ST198	2	100	50
ST207	2	100	0
ST233	1	100	100
ST235	1	0	0
ST244	4	100	75
ST266	1	100	100
ST357	1	100	0
ST395	1	0	0
ST483	1	0	0
ST498	1	0	0
ST499	2	100	100
ST508	1	0	100
ST569	1	0	0
ST589	1	0	100
ST654	3	33,3	0
ST1094	1	100	100
ST1292	1	0	0
ST1527	1	0	0
ST2427	1	0	0
ST2690	1	100	100
Unique type 15-5-11-8-4-4-1*	2	0	50
Unique type 15-2-11- 3-3-38-3*	2	100	50
Unique type 17 -5-12-3-14-4-7*	1	0	0

Note: * — the allele numbers of genes included in the standard MLST pattern (*arcC-aroE-glpF-gmk-pta-tpi-yqi*) [21] are indicated for each unique sequence type.

Isolation of bacteriophage DNA and whole genome sequencing

Total bacteriophage DNA was isolated by phenol-chloroform extraction method [13] with preliminary enzymatic treatment of phage lysates with RNase A, DNase I, and proteinase K (Thermo Fisher Scientific; USA) in accordance with the manufacturer's instructions.

The library was prepared with 250 ng of genomic DNA. Covaris S220 System (Covaris; United States) enabled DNA fragmentation to 400–500 bps. Quality of the fragmented samples was assessed with Agilent 2100 bioanalyzer (Agilent; USA) in accordance with the manufacturer's instructions. The NEBNext Ultra II DNA Library Prep Kit (New England Biolabs; USA) was used to prepare genomic libraries, and the NEBNext Multiplex Oligos kit for Illumina (96 index primers, New England Biolabs; USA) was used to index the libraries. Quantitative analysis of the libraries was performed with the help of Quant-iT DNA Assay Kit, High Sensitivity (Thermo Scientific; USA). Sequencing was done with the MiSeq system and MiSeq Reagent Nano Kit v2 (500 cycle) (Illumina; USA) in accordance with the manufacturer's recommendations.

Bioinformatic analysis of bacteriophage genomes

Prokka v1.14.6 software [14] was used to assemble the whole genome sequences of bacteriophages. Rapid Annotation using Subsystem Technology (RAST) enabled bacteriophage genome annotation [15]. The functions of some open reading frames (ORF) have been predicted using BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and HHpred (https://toolkit.tuebingen.mpg.de/#/tools/ hhpred). Transport RNAs (tRNAs) were searched for with ARAGORN software [16]. The obtained genomes were deposited into the GenBank database under numbers MZ553931 and MZ553930 for bacteriophages vB_PaeA-55-1w and vB_PaeM-198, respectively.

The taxonomy of the studied bacteriophages was determined based on the homology of their genomic sequences with the sequences of bacteriophages registered in the GenBank database using the BLASTN service (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Naming of the bacteriophages followed ICTV (International Committee on Taxonomy of Viruses) recommendations [17] and depended on their taxonomic position.

Phylogenetic analysis of the genomes was performed with the help of VICTOR online tool (Genome-BLAST Distance Phylogeny); the settings were as recommended for prokaryotic viruses [18]. The branches were processed with FASTME [19] according to the D0 formula, visualization done with FigTree [20]. For bacteriophage vB_PaeA-55-1w, we used the following bacteriophage genomes (GenBank database numbers) for comparison: NC_054890, NC_047953, NC_047967, NC_048201, NC_047965, NC_026602, NC_027375, NC_047956, NC_047957, NC_031014, NC_016764, NC_030923, NC_028836, NC_013638, NC_004665,

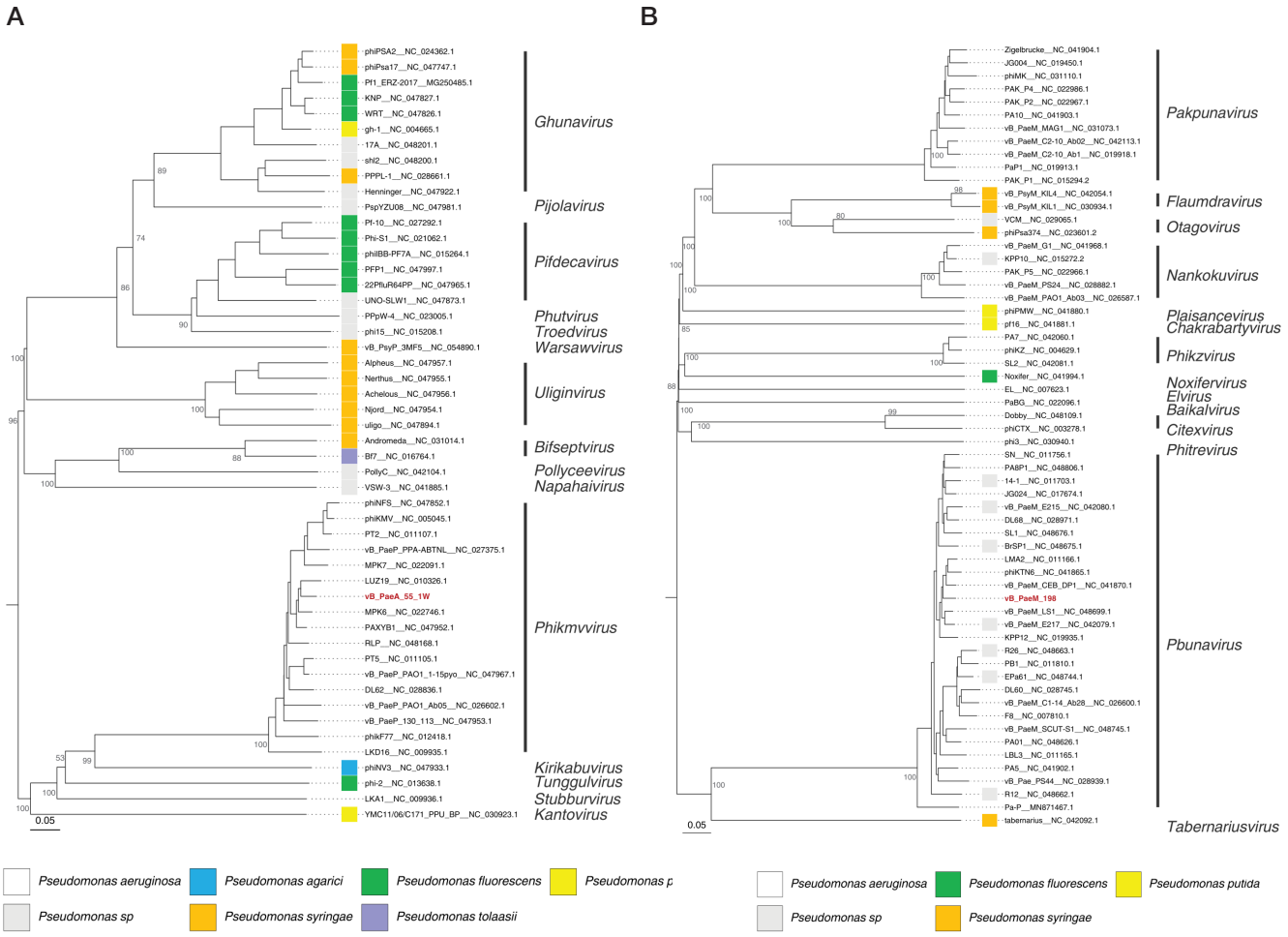

Fig. 1. Phylogenetic analysis of the whole genome sequences of Pseudomonas spp bacteriophages **A**. Phylogenetic tree of the Autographiviridae family bacteriophages (*n* = 50). **B**. Phylogenetic tree of the Myoviridae family bacteriophages (*n* = 60). Bacteriophages vB_PaeA-55-1w and vB_PaeM-198 are marked in *red*

NC_047922, NC_015264, NC_012418, NC_047827, NC_009936, NC_009935, NC_010326, NC_022746, NC_022091, NC_047955, NC_047852, NC_047954, NC_047933, NC_047952, NC_027292, MG250485, NC_047997, NC_015208, NC_005045, NC_047747, NC_024362, NC_021062, NC_042104, NC_028661, NC_023005, NC_047981, NC_011107, NC_011105, NC_048168, NC_048200, NC_047894, NC_047873, NC_041885, NC_047826. For bacteriophage vB_PaeM-198, the genomes were as follows: NC_048675, NC_048744, NC_048626, NC_048662, NC_048663, NC_048676, NC_048745, NC_011703, NC_026587, NC_026600, NC_042113, NC_019918, NC_028745, NC_028971, NC_048109, NC_041870.1, NC_042080, NC_042079, NC_007623, NC_007810, NC_041968, NC_019450, NC_017674, NC_042054, NC_030934, NC_015272, NC_019935, NC_041865, NC_011165, NC_011166, NC_048699.1, NC_031073, NC_031073, MN871467, NC_041994, NC_041903, NC_041902.1, NC_042060, NC_048806, NC_022096, NC_015294, NC_022967.1, NC_022966, NC_022986, NC_019913, NC_011810, NC_041881, NC_030940, NC_003278, NC_004629, NC_031110, NC_041880, NC_028882, NC_028939, NC_023601, NC_042081, NC_042081, NC_042081, NC_011756, NC_042092, NC_029065, NC_041904.

Modular structure of the genomes was determined based on the annotation and when establishing homology of the nucleotide sequences of individual ORFs with BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

RESULTS

Isolation of bacteriophages and characterization of the lytic capability range

To isolate bacteriophages active against the *P. aeruginosa* species we selected strains PA55 and PA198 from the bacterial strains collection of the Federal Research and Clinical Center of Physical-Chemical Medicine (Table 1). These host strains allowed isolating two bacteriophages, which were later named vB_PaeA-55-1w and vB_PaeM-198. As for their lytic capability, vB_PaeA-55-1w phage caused lysis of 19 strains of the collection (47.5%) and vB_PaeM-198 lysed 20 strains (50%) (Table 2). It should also be noted that out of 17 MDR strains vB_PaeA-55-1w lysed 8 (47%) and vB_PaeM-198 — 6 (35%).

Whole genome sequencing of the bacteriophages

Detailed characterization of the studied bacteriophages relied on the whole genome sequencing data and annotation thereof. Genomes of the bacteriophages were double-stranded DNA 42.5 kbp (vB_PaeA-55-1w) and 66.3 kbp (vB_PaeM-198) long. Bacteriophage vB_PaeA-55-1w encoded 52 ORFs, while vB_PaeM-198 encoded 95 ORFs. None of the analyzed bacteriophages contained tRNA in the genome.

Taxonomic position of the bacteriophages and their closest relative were established by comparing the obtained genome-wide sequences with the genomes available in the Genbank database. Bacteriophage vB_PaeA-55-1w belonged to the

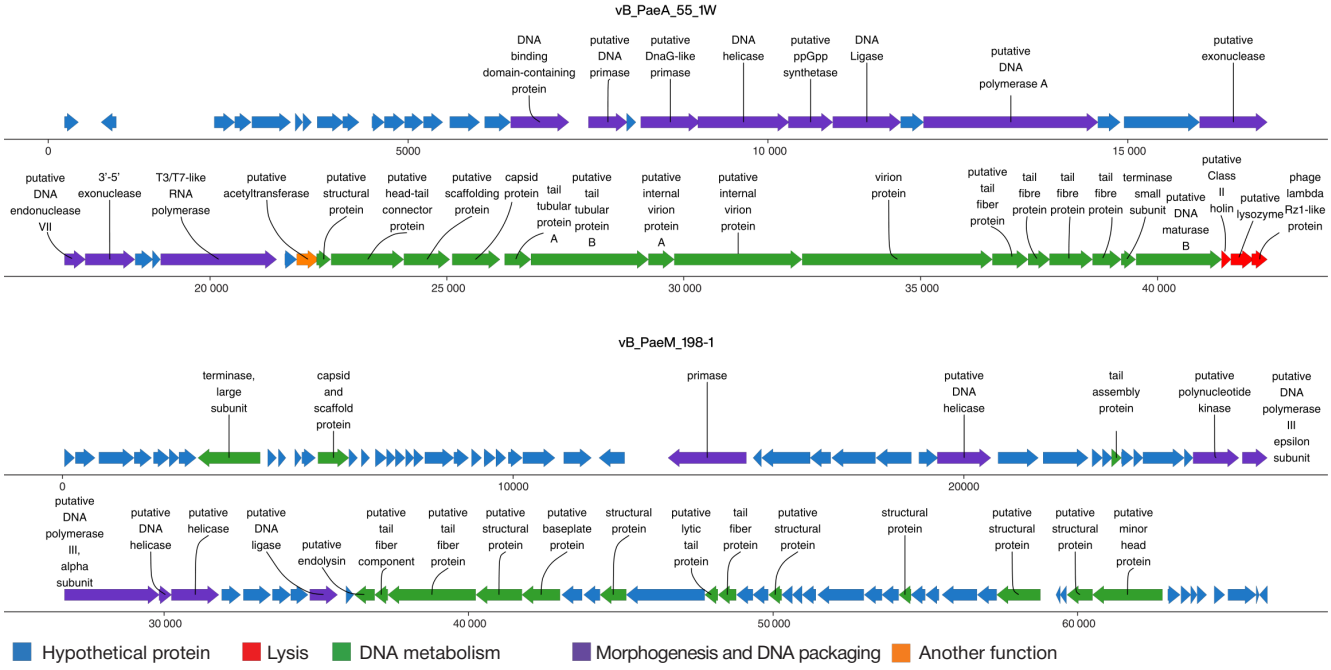

Fig. 2. Main structural modules of the genomes of vB_PaeA-55-1w and vB_PaeM-198 *P. aeruginosa* bacteriophage

Phikmvvirus genus of the *Autographiviridae* family, and the closest genome corresponded to the *Pseudomonas* phage MYY9 (95% similarity, 97.59% alignment length, Genbank number — MW406975.1). Bacteriophage vB_PaeM-198 belonged to the *Pbunavirus* genus of the Myoviridae family and was highly similar to *Pseudomonas* phage phiKT28 (99% similarity, 96.34% alignment length, Genbank number — KP340287.1).

Phylogenetic analysis of *Pseudomonas spp.* bacteriophages

To position the studied bacteriophages within their respective families we relied on the reference genomes recommended by ICTV [17]. Phylogenetic analysis of the *Autographiviridae* family included 50 genomes of *Pseudomonas spp*. bacteriophages (Fig. 1A), that of the *Myoviridae* family — 60 genomes (Fig. 1B).

Two large clusters can be identified on the phylogenetic tree of *Pseudomonas spp*. bacteriophages belonging to the *Autographiviridae* family (Fig. 1A). The first cluster includes bacteriophages that have *P. aeruginosa* species as hosts, and *Pseudomonas agarici, Pseudomonas putida* and *Pseudomonas fluorescens* bacteriophages (one of each). It should be noted that phylogenetic analysis results match taxonomic classification of these viruses. Within the first cluster, *P. aeruginosa* bacteriophages are grouped separately and belong to the *Phikmvvirus* genus, including the investigated vB_PaeA-55-1w bacteriophage. The only exception is *Pseudomonas* phage LKA1, which has its own branch on the phylogenetic tree. The taxonomy has this bacteriophage belonging to another *Stubburvirus* genus. As for *Pseudomonas agarici*, *Pseudomonas putida* and *Pseudomonas fluorescens* in the first large cluster, each of them also occupies an individual branch of the phylogenetic tree and belongs to a separate taxon, namely, genera *Kirikabuvirus*, *Tunggulvirus*, and *Kantovirus*, respectively. The second large cluster on the phylogenetic tree is comprised of the bacteriophages that have *Pseudomonas syringae*, *Pseudomonas tolaasii*, *Pseudomonas putida*, *Pseudomonas fluorescens* as hosts, as well as *Pseudomonas genus* bacteria with unestablished species identity. Phylogenetic subgroups of the second cluster are also consistent with genera of their bacteriophages, namely *Pifdecavirus, Ghunavirus, Troedvirus, Pollyceevirus, Phutvirus, Napahaivirus, Pijolavirus, Pifdecavirus, Bifseptvirus, Uliginvirus.* It should be noted that several genera within the family had hosts belonging to different species: *Bifseptvirus* — hosts of *P. syringae* and *P. tolaasii*; *Ghunavirus* — hosts of *P. fluorescens*, *P. putida*, *P. syringae*.

P. aeruginosa bacteriophages of the Myoviridae family were found in different parts of the phylogenetic tree and corresponded to seven genera: *Baikalvirus, Citexvirus, Elvirus, Nankokuvirus, Pakpunavirus, Pbunavirus* and *Phikzvirus* (Fig. 1B). The studied vB_PaeM-198 bacteriophage shares the position on the phylogenetic tree with 26 other *Pbunavirus bacteriophages*. *P. fluorescens, P. putida, P. syringae* bacteriophages of the *Myoviridae* families belong to 6 different genera: *Chakrabartyvirus, Flaumdravirus, Noxifervirus, Otagovirus, Plaisancevirus* and *Tabernariusvirus*.

Modular structure of the vB_PaeA-55-1w and vB_PaeM-198 bacteriophages

To describe the genomic organization of bacteriophages vB_PaeA-55-1w and vB_PaeM-198, we analyzed functional modules of the genomes. It should be noted that the number of genes the functions of which were identified was higher for the vB_PaeA-55-1w bacteriophage (n = 30/52, 58%) than for vB_PaeM-198 (n = 24/95, 25%) (Fig. 2). The analysis also yielded localization of the modules of nucleic acid metabolism and morphogenesis and packaging. The location of the lysis module was established for the vB_PaeA-55-1w bacteriophage. Targeted search revealed no known bacterial toxins and various integrases in the vB_PaeA-55-1w and vB_PaeM-198 genomes.

DISCUSSION

Due to antibiotic crisis associated with the spread of MDR and XDR bacteria, *P. aeruginosa* bacteriophages are used in therapeutic practice more and more often. Infections caused

by *P. aeruginosa* are most often treated with phages of the *Autographiviridae* and *Myoviridae* families [22, 23]. For this study, we isolated *Autographiviridae (*vB_PaeA-55-1w) and *Myoviridae* (vB_PaeM-198) bacteriophages from natural sources; these bacteriophages offer a wide range of lysing capability (47.5% and 50%, respectively), which is comparable to the *P. aeruginosa* bacteriophages of the corresponding families [24, 25]. It should be noted that the bacteriophages lysed various strains, which can make therapy more efficient with a phage cocktail that includes both of the studied bacteriophages.

It should also be emphasized that the bacteriophages caused lysis of the strains that belong to different sequence types, including ST235 (*n* = 1), ST244 (*n* = 4), and ST395 (*n* = 1). Isolates belonging to these sequence types are among the most widespread throughout the world; they are often associated with outbreaks of infectious diseases, and they have higher resistance to antibacterial drugs [26]. It seems interesting to further study the lytic capability of vB_PaeA-55-1w and vB_PaeM-198 on a collection of *S. aureus* strains of the high epidemic risk sequence types.

Current requirements for therapeutic drugs prescribe describing them in detail, and in the case of bacteriophages, it is also necessary to determine their genomic sequences [27] and thus confirm the virulent nature thereof through showing there are no integrase genes in their genomes. Temperate bacteriophages are not used for therapy because they can facilitate transfer of genes of bacterial toxins and determinants of antibiotic resistance in the bacterial population [27]. Besides, in order to assess therapeutic safety of a bacteriophage its genome is searched for genes of known toxins [27].

The studied bacteriophages (both families) were shown to have typical modular genome structure [24, 28], including a nucleic acid metabolism module, a morphogenesis and packaging module. In addition, localization of the lysis module was established for vB_PaeA-55-1w (*Autographiviridae*). In case of the vB_PaeM-198 (family *Myoviridae*) bacteriophage, no genes highly similar to the known genes of lysines or cholines were found. Both studied bacteriophages were found to have no lysogeny module with integrase genes, which confirms their virulent nature, nor were they established to contain known genes of toxins, which makes them potentially usable in therapy.

Based on the results of phylogenetic analysis of *Pseudomonas spp*. bacteriophages belonging to the *Autographiviridae* and *Myoviridae* families, it was shown that *P. aeruginosa* bacteriophages, regardless of their family, form separate clusters on phylogenetic trees that correspond to genera *Phikmvvirus* and *Stubburvirus* (family *Autographiviridae*), as well as *Elvirus, Nankokuvirus, Pakpunavirus, Pbunavirus, Phikzvirus, Phitrevirus* (*Myoviridae* family). This fact indicates that *P. aeruginosa* bacteriophages are species-specific. On a separate note, bacteriophages previously described and used in phage therapy (phiKMV, PPA-ABTNL, MPK6, RLP), including ΦNH-4 (*Pbunaviruses*) and PAK_P1 (*Pakpunavirus*), have shown their efficacy in animal models [29, 30], and they also cluster together with the studied vB_PaeA-55-1w and vB_PaeM-198 bacteriophages.

CONCLUSIONS

Based on the analysis performed, bacteriophages vB_PaeA-55-1w and vB_PaeM-198 can be recommended for use in phage therapy, including the protocols designed to combat infections caused by MDR strains of *P. aeruginosa*.

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