RESEARCH ARTICLE

Biological Properties of Bacteriophages *Pectobacterium carotovorum* subsp. *carotovorum*

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Abstract

Experimentally passing time, the balance of bacteriophage with indicator crop of *Pectobacterium carotovorum* subsp. *carotovorum* is determined. Morphology of plaque-forming units of isolated bacteriophages was studied by agar-layer technique. The lytic activity of isolated bacteriophages by Appelmann method was from 10^{-4} to 10^{-8} . The lytic activity of isolated bacteriophages by Gratia method was from $(1.0 \pm 0.1) \times 10^5$ to $(3.0 \pm 0.1) \times 10^9$ plaque-forming units/ml. The specificity of isolated bacteriophages *Pectobacterium carotovorum* subsp. *carotovorum* was examined on strains of other species of the family Enterobacteriaceae: *Escherichia coli*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Erwinia amylovora*, *Pectobacterium carotovorum* spp. *atrosepticum*, and also *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Aeromonas hydrophila*, *Xanthomonas campestris*. The stability of isolated bacteriophages to the effect of physical and chemical factors was studied.

Introduction:

As per an expectation, by the year 2050, the population will grow to 9.6 milliards of people, which will lead to an increase in demand for food products. It was counted that for satisfying this requirement world food supply can grow to 70%. It was counted that at least 10% of world food output is lost due to plant diseases. There are more than 200 types of pathogenic bacteria. *Pectobacterium* is one of the most important, has a wide spectrum of hosts and worldwide distribution (Buttimer *et al.*, 2017). Phytopathogenic bacteria *Pectobacterium* spp. of the family Pectobacteriaceae are gram-negative, pectinolytic, necrotrophic bacterial plant pathogen, causing soft rot, damping off and cauline consumption, and leading to significant economic waste all over the world (Li *et al.*, 2018; Charkowski, 2015; Yasuhara-Bell *et al.*, 2017; Charkowski, 2018; Bellieny-Rabelo *et al.*, 2019; Alic *et al.*, 2018; Alic *et al.*, 2017). The spread of pathogens happens mainly by means of obscure infected seed tuber (Humphris *et al.*, 2015). Bacteria *Pectobacterium* produce ferments, destroying cell wall, which allows to in pour and macerate plant tissue (Lim *et al.*, 2017). Pathogenic microorganisms control requires new green alternatives to existing methods. Nowadays

there are no effective ways to control them. Phage cocktails modulate the becoming of soft rot (Carstens *et al.*, 2018). Caused by the increase in stability to antibiotics and their use restriction the alternatives antibacterial are being developed as bacteriophages (Kabanova *et al.*, 2019; Kim *et al.*, 2019). Bacteriophages appliance as the agents of biocontrol recently got a wide circle of interest as a harmless alternative for the environment (Soleimani-Delfan *et al.*, 2015; Carstens *et al.*, 2019).

Bacteriophages are spread in all mediums, containing bacteria, in soil, wastewater, plants (Smolarska *et al.*, 2017). From the specimens of potatoes, water, the soil of potato rhizosphere, from rootstalk of Japanese radish lytic bacteriophages were isolated, which infect *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *P. wasabiae* (Czajkowski *et al.*, 2015; Lee *et al.*, 2017; Hirata *et al.*, 2016). The thermal inactivation point of an isolated lytic bacteriophage specific to *carotovorum* from the specimens of clayed soil was 64°C (Marei *et al.*, 2017). The effectiveness of phages isolated from the specimens of soil and water was evaluated by the application of individual phage strains or cocktail of phages after inoculation by strain *P. carotovorum*. Phages lysed 20 strains *P. carotovorum*, but not the control

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strains *Pseudomonas fluorescens* (Muturi *et al.*, 2019). Nowadays bacteriophage ZF40 is the only available carried phage of phytopathogenic bacteria *Pectobacterium carotovorum* subsp. *carotovorum* (Korol *et al.*, 2016). Bacteriophages *Pectobacterium carotovorum* subsp. *carotovorum* is a comparatively understudied phage group (Lim *et al.*, 2015). Bacteriophage use as the detection is advanced in biotechnology. Detection of bacterial cells with the help of bacteriophages is carried out at low concentrations and is a reliable, fast, exact, sensitive, and economical method (Singh, 2018). Phages of phytopathogenic species *Pectobacteriaceae*, causing damping-off and soft rot on potato are advanced as biocontrol agents for loss prevention of seed and food potato tuber (Voronina *et al.*, 2019).

The research aim is the isolation of bacteriophage *Pectobacterium carotovorum* spp. *carotovorum* and study of their biological properties (morphology of plaque-forming units, lytic activity, specificity, stability to physical and chemical factors).

Materials and methods:

Soil potato plant residues were collected from Ulyanovsk, Orenburg, Astrakhan, West-Kazakhstan, Aktyubinsk, Samara regions. Two strains of bacteria *Pectobacterium carotovorum* spp. *carotovorum* were used. The specificity of bacteriophages was studied on strains of other family species *Enterobacteriaceae*.

We used strains *Pectobacterium carotovorum* spp. *carotovorum* from All-Russian collection of microorganisms, isolated strains *Pectobacterium carotovorum* spp. *carotovorum*, and also bacteriophage strains (seven) isolated by a team of contributors. As heterogenic genus and species of bacteria, bacteria strains were used, which we got from the museum of the department of microbiology, virology, epizootiology, and veterinary-sanitary expertise of FSBEI of Higher Education of Ulyanovsk SAU: *Escherichia coli*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Erwinia amilowora*, *Pectobacterium carotovorum* spp. *atrosepticum*, and also *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Aeromonas hydrophila*, *Xanthomonas campestris*.

Isolation, a study of biological properties of bacteriophages (morphology of plaque-forming units, lytic activity, specificity, stability to physical and chemical factors) were carried out by methods, which were offered by Vasilyev *et al.* (2019).

Results and Discussion:

During the use of a method of bacteriophage search without affection on them of inducer studied phytopathogenic bacteria did not show lysogenic properties. During the use of the isolation method with the

use of inducer on bacteria, we acted upon by ultraviolet rays. Studied bacteriophages showed negative results. Further results were connected with the search of potato and soil samples which were damaged by soft rot. As the search result, we discovered seven bacteriophages, specifically for the strains *Pectobacterium carotovorum* spp. *carotovorum*. The selection of isolated isolates was conducted by passaging on indicator crops with undone negative colonies. Experimentally it was established that passaging time was 3,5 hours at the cultivation temperature of 28°C.

Table-1: Biological properties of isolated bacteriophages

Bacteriophage name	Morphology of negative colonies.	Lytic activity	
		A	B
1 PCC – 1 ULSAU	0.5±0.1 – 1.0±0.1	10 ⁻⁵	(2.0±0.1) x 10 ⁸
2 PCC – 2 ULSAU	1.0±0.1 – 1.5±0.1	10 ⁻⁵	(7.0±0.1) x 10 ⁸
3 PCC – 3 ULSAU	1.5±0.1 – 2.0±0.1	10 ⁻⁵	(5.0±0.1) x 10 ⁸
4 PCC – 31 ULSAU	2.0±0.1 – 2.3±0.1	10 ⁻⁷	(1.0±0.2) x 10 ⁸
5 PCC – 37 ULSAU	2.0±0.1 – 3.7±0.1	10 ⁻⁸	(3.0±0.1) x 10 ⁹
6 PCC – 74 ULSAU	1.0±0.1 – 1.3±0.1	10 ⁻⁴	(1.0±0.1) x 10 ⁵
7 PCC – 105 ULSAU	2.0±0.1 – 2.6±0.1	10 ⁻⁸	(7.0±0.2) x 10 ⁸

A-by Appelman; B-by Gratia

Table-2: Stability of selected phages to the temperature effect in a range of 52-70°C during 30 minutes duration

Bacteriophage name	Temperature factor °C									
	52	54	56	58	60	62	64	66	68	70
PCC – 1 ULSAU	+	+	+	+	+	+	+	-	-	-
PCC – 2 ULSAU	+	+	+	+	+	+	-	-	-	-
PCC – 3 ULSAU	+	+	+	+	+	-	-	-	-	-
PCC – 31 ULSAU	+	+	+	+	+	-	-	-	-	-
PCC – 37 ULSAU	+	+	+	+	+	+	-	-	-	-
PCC – 74 ULSAU	+	+	+	+	-	-	-	-	-	-
PCC – 105 ULSAU	+	+	+	+	+	+	-	-	-	-

Table-3: Stability of isolated bacteriophages to the influence of trichloromethane

Bacteriophage name	Time interval of influence, minutes			
	45	50	55	60
PCC 1 ULSAU	+	+	+	+
PCC 2 ULSAU	+	+	+	+
PCC 3 ULSAU	+	+	+	+
PCC 31 ULSAU	+	+	+	+
PCC 37 ULSAU	+	+	+	+
PCC 74 ULSAU	+	+	+	+
PCC – 105 ULSAU	+	+	+	+

The balance of bacteriophage with indicator crops was determined by the Gratia method with various ratios of bacteriophages with indicator crops. It was established that the balance of bacteriophage and indicator crop is 1:1 (0.2 ml: 0.2 ml).

Morphology of plaque-forming units of isolated bacteriophages: isolates have round, oval, transparent, half-transparent and turbid negative colonies with even borders with the diameter (Plate-1a; Table-1)

Lytic activity of isolated bacteriophages by Appelman

Plate-1: a) Plaque-forming-units of bacteriophage PCC-105 ULSAU;
b) Spectrum of lytic activity of bacteriophage PCC-37 ULSAU

method was from 10^{-4} to 10^{-8} . Lytic activity of isolated bacteriophages by Gratia method was from $(1.0 \pm 0.1) \times 10^5$ to $(3.0 \pm 0.1) \times 10^9$ plaque-forming units/ml. For the research spectrum of lytic activity, we used Otto method, which consisted of applying bacteriophage droplets on the lawn of the indicator crop (Plate-1b). As the research, we used 16 (1 reference strain, 1 strain from the department museum, and 14 isolated) strains of bacteria species *Pectobacterium carotovorum*. As a result of the conducted research, we found out that studied phages have various ranges of lytic activity. The wide range towards studied cultures was at PCC – 37 ULSAU and PCC – 1 ULSAU. The specificity of bacteriophages *Pectobacterium carotovorum* was conducted on solid beef extract agar using a method of applying bacteriophage drops onto studied genus of the family Enterobacteriaceae: *Escherichia coli*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, *Proteus vulgaris*, *Proteus mirabilis*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Erwinia amilowora*, *Pectobacterium carotovorum* spp. *atrosepticum*, and also *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Aeromonas hydrophila*, *Xanthomonas campestris*. Discovered bacteriophages were inactive to representatives of bacteria of other genera of the family Enterobacteriaceae.

The stability of discovered bacteriophages to various temperatures was studied according to the following method: 10 test- tubes with phage were heated on a heated bath at the temperature from 52° to 70°C with the interval of 2°C during 30 minutes. Test tubes with control were not heated. We made a lawn on indicator crop of daily cultures on the plates with beef extract agar. The plates with the lawn were dried during 20-30 minutes. Dried plates were separated into sectors. Into the first sector we dripped the control (unheated test tube) into the other we dripped heated phage. The plates were incubated at the temperature of 28°C for 16-18 hours. As a result of research, it was established that bacteriophages inactivate at the temperature of $60-64^\circ\text{C}$ (Table-2).

The stability of phages to the influence of trichloromethane was carried out according to the

following scheme: balance of phage and trichloromethane was 10:1, influence time 10-60 minutes with a 5-minute interval during continuous shaking of test tubes and clarify during 10 minutes. Then we selected supernatant and dropped bacteriophage that was cured by trichloromethane on the lawn of indicator daily crop by Otto method «dripping». The presence of lyzed zones showed the stability of isolated bacteriophages to trichloromethane (Table-3).

While studying bacteriophage titer storing at the temperature of $2-4^\circ\text{C}$ for six months it was established that bacteriophage titers did not change.

Conclusion:

As the research result on the detection and study of biological properties of bacteriophages of pathogenic bacteria *Pectobacterium carotovorum* spp. *carotovorum* it can be concluded that the most perspective is the method of bacteriophage isolation from the objects of the environment. Passaging phage time and indicator crop were 3.5 hours. Optimal balance of bacteriophage and indicator crop is 1:1 ($0.2\text{ ml} : 0.2\text{ ml}$), which is analogue for all bacteriophages. Isolated bacteriophages on beef extract agar have round, transparent, half-transparent and turbid negative colonies with even border, with the diameter of $0.5 - 1$ to $2.0 - 3.7\text{ mm}$. Lytic activity by Appelmann method was from $10^{-4} - 10^{-8}$, by Gratia method was from $(1.0 \pm 0.1) \times 10^5$ to $(3.0 \pm 0.1) \times 10^9$ PFU/ml.

Lytic activity of isolated bacteriophages towards other strains of heterological culture is absent. Isolated bacteriophages inactivate at the temperature of $60-64^\circ\text{C}$. The bacteriophage is stable to trichloromethane influence. While storing during six months titers of isolated bacteriophages did not change. Isolated bacteriophages will be the part of ecologically safe bacteriophage preparation for eliminating of phytopathogenic bacteria *Pectobacterium carotovorum* spp. *carotovorum*.

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