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Bacteriophages of *Pseudomonas syringae*: features of isolation and study of main biological properties

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Abstract. The article presents the results of studies on isolation and study of biological properties of bacteriophages specific for phytopathogenic bacteria *Pseudomonas syringae*. 8 new virulent bacteriophages were isolated and selected from environmental objects. The main biological properties of isolated phages, including lytic activity, were studied (according to Appelman, the indicator was equal to from 10⁻⁵ to 10⁻⁸; by diffusion method in «soft agar» - from 1.0±0.1*10⁶ to 2.0±0.1*10⁹ (PFU/ml), «spot test» - spot test confirmed the results obtained by diffusion method in «soft agar» - n6 – n9) and its diapason, which ranged from 21.4% (Ps.s-13 UIGAU) to 85.7% (Ps.s-7 UIGAU, Ps.s-27 UIGAU), morphology of plaque-forming units (morphology of negative colonies), specificity within genus and species, changes in lytic activity index during storage, features of impact of physical and chemical factors (trichloromethane at the ratio of 1:10, the impact of which during 5-85 minutes does not influence on biological activity of phages when determining by indirect method; urea, whose 30% solution reduces the yield of plaque-forming units of each bacteriophage studied by about 77.6-85.5 %; temperature in the range of -5 to 10°C and from 50 to 100°C). The study of biological properties allowed us to systematize biological features of each of isolated clones of virulent bacteriophages and select the 4 most promising bacteriophages for further research. The research is carried out with the support of Russian Foundation for Basic Research, the project "Fundamental foundations for the development of phage preparation specific to *Pseudomonas syringae*, and applied aspects of its application for phagoidentification and bioprocessing of food and agricultural raw materials" No 19-44-730014.

1. Introduction

Pseudomonas syringae bacteria are the cause of diseases of all plants cultivated by humans, as well as wild plants. Among the symptoms that they cause, there are tumor neoplasms, rot, growth cessation and death of a part of the plant without rotting, chlorosis, necrosis [1]. These symptoms are caused by a disturbance in normal metabolism of plant cells caused by certain substances secreted by bacteria, such as enzymes that can attack plant tissue components, toxins, and phytohormones [2].

The effectiveness of using bacteriophages in agriculture to reduce food spoilage caused by various bacteria is probably the least studied. Knowing biological characteristics of pathogen, it is possible to create environmentally safe biopreparation based on specific bacteriophages, which will allow decontamination of plants at different stages (seed material, during growing season and during storage) [3]. Bacteriophages that are part of the biopreparation lyse only bacterial infectious agents that are their hosts, do not inhibit growth of probiotic strains and plant normoflora [4]. The development and appliance of biological preparation based on a bacteriophage will allow controlling



the common pathogen *Pseudomonas syringae*. The presence of such drug will allow us to develop an innovative biologized, environmentally safe plant protection system, especially in seed production. The developed biological product will allow us to recommend system of plant protection from *Pseudomonas syringae* and provide farmers with healthy seed material of productive varieties of vegetable crops, which will increase productivity and economic efficiency of vegetable growing in the country [5].

2. Materials and methods

124 samples were studied for the presence of *Pseudomonas syringae* bacteriophages – 31 samples of wastewater and 31 samples of soil from various territories of the Ulyanovsk, Samara, Orenburg, Saratov regions (allotments, vegetable gardens) of the Russian Federation, 31 samples of water from the Volga, Sviyaga, Ural Rivers; 31 samples of plants (leaves and fruits: cucumber - *Cucumis sativus*; tomato - *Solanum lycopersicum*; beans – *Phaseolus* wheat - *Triticum*) with signs of bacterial disease (brown mucosa, frostbite, fruit damage and leaf spotting of plants) caused by *Pseudomonas syringae*.

Bacterial strains: *Pseudomonas syringae*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Pseudomonas putida*, *Pseudomonas stutzeri*, *Pectobacterium carotovorum*, *Xanthomonas campestris*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Echerichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, received from the museum of the Department of microbiology, virology, epizootology and veterinary and sanitary expertise of FSBEI HE Ulyanovsk SAU.

Isolation and study of biological properties of phages were carried out using methods of E. Katter [6], R.J. Clokie et al. [7], N. I. Girilovich et al. [8], K. V. Martynova [9], N I Molofeeva [10].

Statistical processing of research results was carried out using the Statistica Desktop 13 Russian software package (for Windows; StatSoft Russia (TIBCO USA), Microsoft Excel 2010)

3. Results

As the result of conducted studies, 8 bacteriophages specific to *Pseudomonas syringae* bacteria were isolated from environmental objects. Empirically, it was established that the reservoir of distribution of bacteriophages specific to above-mentioned bacteria is relatively wide. Six bacteriophages were isolated from soil samples, one from vegetative parts of cucumber and one from wastewater sample. Thus, we believe that the objects for isolation of potentially virulent bacteriophages *Pseudomonas syringae* are soil samples.

Several protocols of bacteriophage isolation were selected empirically and tested: the method of induction from collection bacterial strains of *Pseudomonas syringae*, isolation method from environmental objects without "seeding", isolation method from environmental objects "with seeding". The stage of purification and concentration of bacteriophages was selected from the following methods that we tested: ultrafiltration-filtration through membrane filters "Millipore" (0.22 microns); purification by physical method-heating during 30 minutes at the temperature of (60±1) °C; purification-by chemical method-with trichloromethane at the ratio of 1:10 (the exposure time is 30 minutes in schuttel apparatus, including settling for 5 minutes – the use of a supernatant). The optimal method of purification of bacteriophages is the method of concentration by ultrafiltration.

It was experimentally established that isolated bacteriophages, indicator crops for which were *Pseudomonas syringae* bacteria, formed externally similar plaque-forming units of rounded shape with clear transparent centers, without secondary growth with a diameter of 0.5 to 5.0 mm. The calculation of plaque-forming units (lytic activity) of isolated bacteriophages was determined by diffusion into a two-layer agar-by "sandwich" method and by method of successive dilutions according to Appelman. In isolated and selected bacteriophages of *Pseudomonas syringae*, various parameters of lytic activity were recorded - according to Appelman, it was from 10^{-5} to 10^{-8} ; by method of diffusion into two-layer agar-from $1.0 \pm 0.1 \cdot 10^6$ to $2.0 \pm 0.1 \cdot 10^9$ (PFU/ml), «spot test» - spot test confirmed the results obtained by method of diffusion of bacteriophages into two-layer agar-by the «sandwich» method- $n^6 - n^9$. The range of lytic action of isolated *Pseudomonas syringae* bacteriophages was determined using Otto method.

Analyzing the data in table 1, where the results of studies are presented, it becomes clear that the range of lytic activity of isolated and selected phages varied from 21,4% (Ps.s-13 UIGAU) to 85.7% (Ps.s-7 UIGAU, Ps.s-27 UIGAU) (table 1).

Table 1. Range of lytic activity of isolated and selected phages within *Pseudomonas syringae* species.

No	Phage name	Percentage of lysed 14 bacterial strains	Strains of bacteria <i>Pseudomonas syringae</i>													
			B-10917	№3	36	56	66	11	7	23	4	33	35	37	38	77
1	Ps.s-1 UIGAU	42.9	+	+		+				+	+		+			
2	Ps.s-7 UIGAU	85.7	+	+	+	+	+	+			+	+	+	+	+	+
3	Ps.s-8 UIGAU	64.3	+	+		+			+	+	+	+	+	+		
4	Ps.s-13 UIGAU	21.4	+	+									+			
5	Ps.s-15 UIGAU	50.0	+	+	+		+		+	+			+			
6	Ps.s-27 UIGAU	85.7	+	+	+	+		+	+	+	+	+	+		+	+
7	Ps.s-30 UIGAU	50.0	+	+	+	+			+	+			+			
8	Ps.s-77 UIGAU	50.0	+	+	+		+		+	+			+	+		

The specificity of isolated bacteriophages was determined by Otto method. It was established that all eight bacteriophages of *Pseudomonas syringae* did not form lysis zones on the lawn of bacterial strains: *Pseudomonas spp.*, *Pectobacterium carotovorum*, *Xanthomonas campestris*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Echerichia coli*, *Proteus vulgaris*, *Proteus mirabilis*, that is, they were specific within the species.

Study on determining the effect of physical action (temperature parameter of cultivation) on biological activity of isolated and selected bacteriophages by indirect indicator-presence of lysis zones on the lawn of indicator crop: bacteriophages were cultivated for 30 minutes at the following temperature parameters - at the temperature range from -5 to 10 °C and from 50 to 100 °C with the interval of 5 °C. As control, sterile MPB was taken. Then bacteriophages were seeded, subjected to temperature exposure to MPA by Otto method. The crops were cultivated under the conditions of a thermostat for 18 hours at a temperature of 28 °C. The presence of lysis zone in the form of "path" indicated the stability of phages to the influence of temperature conditions. It was determined that all isolated and selected bacteriophages specific to *Pseudomonas syringae* are not resistant to temperature effects at temperatures ranging from -5 to 10 °C and from 50 to 100 °C, resistant to the temperature of 50 °C; temperature 55 °C – it does not negatively influence on the development of all studied bacteriophages, except for the phage Ps.s-27 UIGAU; cultivation of bacteriophages at a temperature of 60 °C inactivate phages Ps.s-1 UIGAU, Ps.s-8 UIGAU, Ps.s-15 UIGAU, Ps.s-30 UIGAU, Ps.s-77 UIGAU.

The study of influence of physiological action (effect of trichloromethane) on the phages isolated and selected by us in the framework of the project was carried out in accordance with the following parameters: the ratio of phage and trichloromethane is 10: 1, the exposure time is 5-70 minutes with 5-minute interval with constant shaking of test tubes in schuttel apparatus and settling during 1/5 of

exposure time interval. Then, using a pipette, the supernatant was collected and treated bacteriophage was seeded on MPA by Otto method. The seeds were cultivated under the conditions of a thermostat for 24 hours at a temperature of 28 ± 1 °C. The presence of lysis zone indicates that trichloromethane does not influence on biological activity of phage, which is established by indirect method – presence of lysis zones on the lawn of indicator crop. The research results are presented in table 9. It was found that interaction of trichloromethane with studied bacteriophages for 5-85 minutes does not have a negative effect on biological activity of studied phages.

The resistance of bacteriophages to the action of concentrated urea solutions. Bacteriophages in an amount of 0.5 ml were added to 4.5 ml of Marten broth (pH 7.6) containing 30% urea. The mixture was kept at 37 °C for 18-20 hours and titrated by agar layer method-diffusion into two-layer agar-«sandwich method». It was experimentally determined that 30% urea solution reduces the output of plaque-forming units of each studied bacteriophage by approximately 77.6-85.5 %.

All isolated bacteriophages were stored in closed glass flasks at a temperature of 2-4 °C. They represented clear liquid of straw-yellow color without the presence of sediment. Every 3 months, the flasks were opened and lytic activity was determined by Appelman method, method of diffusion of bacteriophage into a two-layer agar-"sandwich" method and "spot test". The obtained data indicate that the isolated and selected bacteriophages of *Pseudomonas syringae* were stored in a hermetic state for 3 months without the use of preserving substances at a temperature of 2-4 °C maintain the indicators of lytic activity that were recorded when they were placed in storage. It was established that bacteriophages Ps. s-7 UIGAU, Ps. s-13 UIGAU during storage for 6 months do not change titer of lytic activity, unlike other bacteriophages, which titer decreased by about single order.

Studies were carried out to increase lytic activity after storage. It was established that six-fold passaging of isolated negative colonies (plaque-forming units) on meat-peptone agar with subinoculation on meat-peptone broth, using method of screening of bacteriophages by method of bacteriophage diffusion into two-layer- agar - "sandwich" method increases the lytic activity of bacteriophages by single order, that is, up to the value determined when laying phages for storage.

4. Discussion

For bacteriophages isolation from environmental objects, a panel of *Pseudomonas syringae* bacterial cultures created earlier was used as indicator strains. Considering that lysogeny is widespread among all systematic groups of microorganisms and we aimed to test this postulate on the studied field and reference strains of *Pseudomonas syringae* bacteria, we could not identify the prophage in the studied bacterial crops. Using various methods of bacteriophage isolation, it was possible to isolate 8 virulent phage isolates from the objects of external environment by «sub-seeding» method. Phage selection was carried out by ten-fold passaging of isolated negative colonies (plaque-forming units) on meat-peptone agar with subinoculation on meat – peptone broth, using method of screening bacteriophages by diffusion into two-layer agar-by the «sandwich» method. The method of purification of bacteriophages - concentration by ultrafiltration was selected empirically.

In research, the parameters of lytic activity were determined by the method of serial dilutions, agar layers (spot test and seeding in upper layer of soft agar). according to Appelman, was from 10^{-5} to 10^{-8} ; by diffusion method in «soft agar» - from $1.0 \pm 0.1 \cdot 10^6$ to $2.0 \pm 0.1 \cdot 10^9$ (PFU/ml), «spot test» - spot-test confirmed the results, obtained by diffusion method in «soft agar» - $n^6 - n^9$. The range of lytic activity of isolated and selected phages varied from 21.4% (Ps. s-13 UIGAU) to 85.7% (Ps.s-7 UIGAU, Ps.s-27 UIGAU). Eight *Pseudomonas syringae* bacteriophages were established to be specific within the species. The biological activity of isolated and selected bacteriophages specific to *Pseudomonas syringae*, determined by indirect method – the presence of lysis on the lawn of bacterial crop when studying the influence of temperature factor, was not recorded in the range of cultivation temperatures from -5 to 10°C and from 65 to 100°C, it was determined that the temperature of 50 °C – does not influence bacteriophage growth and development; the temperature of 55 °C also doesn't have a negative influence on the development of all studied bacteriophages except the phage Ps.s-27 UIGAU; cultivation of bacteriophages at the temperature of 60 °C inactivate phages Ps.s-1 UIGAU,

Ps.s-8 UIGAU, Ps.s-15 UIGAU, Ps.s-30 UIGAU, Ps.s-77 UIGAU. It was established that exposure of trichloromethane for 5-85 minutes does not affect biological activity of *Pseudomonas syringae* phages determined by indirect method – the presence of lysis on the lawn of bacterial crop. It was determined that a 30% urea solution reduces the output of plaque-forming units of each bacteriophage studied by approximately 77.6-85.5 %. The obtained data indicate that the isolated and selected bacteriophages of *Pseudomonas syringae* during 3 months of storage in a clogged state without use of preservatives at a temperature of 2-4 °C have the indicators of lytic activity that were recorded during storage. It was established that bacteriophages Ps. s-7 UIGAU, Ps. s-13 UIGAU during storage for 6 months do not change titer of lytic activity in contrast to other bacteriophages, titer of which decreased by about one order. The criteria for the selection of phage isolates for further studies – specificity, range of lytic action, and lytic activity-are determined. 4 bacteriophages specific to *Pseudomonas syringae* were selected: Ps. s-7 UIGAU, Ps. s-13 UIGAU, Ps.s-27 UIGAU, Ps.s-30 UIGAU for further studies.

5. Conclusion

A collection of 8 virulent bacteriophages of *Pseudomonas syringae* isolated from objects of phytosanitary control was created.

The main biological properties of isolated phages (lytic activity by consecutive dilution methods, agar layer method (spot test and seeding in the upper layer of soft agar) and its changes during storage under conditions of 2-4 °C, the range of lytic action, specificity to *Pseudomonas syringae*, effect on bacteriophages of following factors – the effects of temperature, trichloromethane and urea.

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