RESEARCH ARTICLE

Isolation and Study of The Main Properties of Bacteriophage *Pseudomonas syringae*

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Introduction:

Bacterial diseases damage almost all agricultural crops and often are the reason yield inhabitation. Nowadays it is known that over 200 bacteria species, which pertain to disease agents of plants are unmistakable (Wojtus *et al.*, 2019; Pinheiro *et al.*, 2019; Sistrom *et al.*, 2015; Ravindran *et al.*, 2015).

Plant diseases are the reason for the serious economical loss in the agricultural sector all over the world. Monitoring of plant state and early detachment of pathogenic microorganisms are of great importance for the reduction of the spread of disease and support to the effective methods of control procedure (Martinelli *et al.*, 2015; Balestra *et al.*, 2013; Cameron & Sarojini, 2014; Di Lallo *et al.*, 2014). In spite of numerous research from native and foreign scientists, dedicated to agents of species *Pseudomonas syringae*, the degree of their specialty and character of related circuitry are understudied (Yang *et al.*, 2016; Frampton *et al.*, 2015; Meaden *et al.*, 2015). The similarity of cultural –biochemical characteristics and antigenic structure and absence of species specificity at most phytopathogenic bacteria of the fluorescent type allowed to combine phylum (Roberts *et al.*, 2019; Wagner &

Abstract

For the isolation of bacteriophages from the objects of ambient (sewage waters, soil from different regions) 98 tests were used and as indicator strain reference of bacteriophage strain *Pseudomonas syringae* N°3 and N°10917 strain, determined by Otto method. As a result of research 8 bacteriophages were isolated. BFU of bacteriophages *Pseudomonas syringae* is round-shaped with clearly defined transparent centers without secondary growth with the diameter from 0.5 to 5.0 mm. Further, the study results biological properties of isolated bacteriophages like: lytic activity by Appelmann and Gratia, specificity of bacteriophage, balance of bacteriophage and culture are identified. All bacteriophages were found to have rigid specificity. The results of this research can be used for the development of bio preparation on the basis of bacteriophages, which can be applied as the effective method during the indication and identification of bacteriophages *Pseudomonas syringae* in the objects of sanitary control, and also as a mean of plant protection from bacterial diseases.

Rajkov, 2019; Ravindran *et al.*, 2015). So, to collective species *Pseudomonas syringae* we refer a number of agents affecting bacteriosis of various plants (Yin *et al.*, 2019; Chen *et al.*, 2018; Frampton *et al.*, 2014; Gardan *et al.*, 1999; Sistrom *et al.*, 2015). This points to the complexity of the problem, the solution of which would allow to get a valuable summary for the development of control measures with many bacteriosis (Yedidia *et al.*, 2003).

Bacteriophages are omnipresent and spread in different ecosystems. For bacteria, living in and on host –plants, phages can exercise significant influence on the interlocking of vegetational bacteria. The potential mechanism, organizing this interlocking, includes lysing of bacteria cells, lateral gene transfer between bacterial genomes, and change of bacterial phenotype. (Koskella & Taylor, 2015; Kandel *et al.*, 2019). The evolution of resistance to the parasite is fundamentally important for disease ecology, however, we cannot predict when and how the resistance will develop (Koskella & Taylor, 2018; Atanasova *et al.*, 2015). To a large extent it is due to contextually determined by the crucial nature of host with parasite because advantage from the stability will be dependent on the abiotic environment. Depending on context is the

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advantage of stability to phages, which led to different evolutionary results in different mediums. These results highlight the importance of the study of stability evolution to the parasite in environmentally sensitive mediums. (Park *et al.*, 2018; De Smet *et al.*, 2017). It is known that chemicals affect adversely on economical balance and reduce the quality of production security-wise (Cement *et al.*, 2018; Kim *et al.*, 1998). Development of environmentally responsible bio-preparation of «dot-effect» on the basis of bacteriophages will allow us to get not only plant protection from bacteriosis, caused by *Pseudomonas syringae*, and to offer the laboratories the effective methodology of indication and identification of above-mentioned microorganisms in the objects of sanitary inspection (Yu *et al.*, 2016a). Generally, polyvalent phages can be easily isolated from the ambient environment with the use of various successive hosts, and this approach must make the study of ecological significance easier, and allow new fields of application (Yu *et al.*, 2016b; Addy *et al.*, 2016).

The aim of the research is isolation and study of the main properties of bacteriophages specific for bacteria *Pseudomonas syringae*.

Materials and methods:

For the work we used 2-reference strains of bacteria *Pseudomonas syringae* B-10917 (isolated from the collection BRC RNCIM SRC «Kurchatov institute») and strain Ps.s N° 3 (isolated from the museum collection of the department «MVE and VSE» Ulyanovsk SAU).

We used harvest broth for cultivation of microorganisms of dry GRM-broth (FSBI State Research Centre of Applied Microbiology and Biotechnology, Russia, Obolensk city), GRM-agar, agar (FSBI State Research Centre of Applied Microbiology and Biotechnology, Russia, Obolensk city), trichloromethane stabilized 0,6-1 % ethanol (chloroporm) a.r.g. TY 2631-066-44493179-01. For bacteriophage specificity, we used strains of the following bacteria: *Pseudomonas aeruginosa* – 1 strain, *Pseudomonas fluorescens* – 2 strains, *Pseudomonas putida* – 2 strains, *Pseudomonas stutzeri* – 1 strain and other Garm negative fitopathogenic bacteria (*Pectobacterium carotovorum*– 1 strain, *Xanthomonas campestris*– 1 strain) and enterobacteria (*Saimonella enteritidis* – 1 strain, *Salmonella typhimurium* – 1 strain, *Yersinia enterocolitica* – 1 strain, *Shigella sonnei* – 1 strain, *Klebsiella pneumoniae* – 1 strain, *Echerichia coli* – 1 strain, *Proteus vulgaris* – 1 strain, *Proteus mirabilis* – 1 strain).

We did 98 tests: these are sewage waters and soils of various territories of the Ulyanovsk, Samara, Astrakhan, and Volgograd, Orenburg regions (summer cottages, gardens) of the Russian Federation and West Khazakhstan Province, Aktyubinsk region, Kyzylorda region of Republic of Kazakhstan, also streams of the Sviyaga (Ulyanovsk), the Volga (Ulyanovsk), Syr-Darya (Kyzylorda), the Ural and Chagan (Uralsk). We also took samples of plants(leaves and

fruits): cucumber - *Cucumis sativus*; tomato - *Solanum lycopersicum*; beans – *Phaseolus*; wheat - *Triticum*) with characters of bacterial disease (fulvous mucilaginous discharge, freezer burn, fruit and leaf blight of plants) caused by *Pseudomonas syringae*. Isolation and study of biological properties of phages were carried out by methods of Ganyushkin (Vasilyev, 2011), I.P. Revenko (Vasilyev, 2019), Katter & Vasilyev (Balestra *et al.*, 2013; Vasilyev, 2019).

Lytic activity was determined by Gratia and Appelmann methods (Vasilyev, 2011). Statistical processing of research results was carried out with the help of program pack Statistica Desktop 13 Russian (for Windows; StatSoft Russia (TIBCO USA), Microsoft Excel 2010). The arithmetic mean (M), selecting bias (m), confidence coefficient (by Student, t), and degree of probability (P) were calculated. Differences were considered reliable at $p=0.05$ (Table 3).

Results and Discussion:

The research was carried in various stages. The first stage of our research was to isolate bacteriophages specific for bacteria *Pseudomonas syringae* from the ambient environment. We prepared dilution from the research objects in meat-peptone broth (MPB) 1:10, added bacteria strains B-10917 and P.s.- 3 in a concentration of 10^4 KBT /ml by 1.0 ml. We put flasks with tests into a thermostat for 24 hours at 28°C. Then we filtered them through padded gauze filter for clearing from mechanical impurities. After that, the flasks content was dispensed into sterile test tubes, centrifuged at 3000r/min during 30 min., further centrifuged was put into sterile test tubes at the ratio of 1:10 trichloromethane and shook during 20 min., then it was left for 10 min. for trichloromethane deposition. And after that supernatant was selected into a sterile test tube. All this work was carried out to clear bacteriophage from microbial cells. Studied filtrate was tested for the presence of phage by Otto method, by applying «path» on culture lawn, which was applied onto meat-peptone agar (MPA) in Petri dishes beforehand and «dried» in thermostat during 35-40 min. As a control sterile MPB was applied to the culture lawn aimed to get trustworthy results on the detection of phage presence in the studied substrate. Plating was incubated under the conditions of thermostat during 18-24 hr at $28\pm 2^\circ\text{C}$. The positive result of the experiment was the presence of lyzed zone of the «path» form on culture lawn (Plate-1).

Table 1: Characteristics of objects for bacteriophage isolation

#	Bacteriophage	Indicator crop	Source of isolation
1	Ps.s-1 ULSAU	B-10917	Cucumber leaves
2	Ps.s-7 ULSAU	P.s.-3	Soil
3	Ps.s-8 ULSAU	P.s.-3	Soil
4	Ps.s-13 ULSAU	P.s.-3	Soil
5	Ps.s-15 ULSAU	P.s.-3	Soil
6	Ps.s-27 ULSAU	P.s.-3,	Soil
7	Ps.s-30 ULSAU	B-10917	Sewage water
8	Ps.s-77 ULSAU	B-10917	Soil

As the result of conducted research w isolated 8

bacteriophages, specifically for *Pseudomonas syringae* strains. The research results are shown in Table-1.

Further, we bred the selection of isolated bacteriophages by tenfold passaging of isolated plaque-forming units (PFU) on beef-extract agar-agar with sub inoculation on beef-extract agar-agar. (Andriychuk *et al.*, 2016). From the lysed zone we unwinded PFU into test-tube with beef-extract agar-agar and added 0.5 ml of indicator crop, into the second tube with beef-extract agar-agar we put only 24-hr indicator crop with the volume of 0.2 ml. The first tube was the treatment, and the second tube was the control. The tubes were put into the thermostat and were incubated at $28 \pm 2^\circ\text{C}$ till the formation of well-defined growth of indicator crop in the control tube (the growth of indicator crop was checked every 30 min.). During the experiment it was established that passage time (incubation) of bacteriophages specific for bacteria *Pseudomonas syringae*, varies from 3 hr to 5 hr. The incubation time of bacteriophages is shown in Table-2.

Table 2: Incubation time of bacteriophages

a-	Ps.s-1ULSAU	Ps.s-7ULSAU	Ps.s-8ULSAU	Ps.s-13ULSAU
b-	3.0 h	3.5 h	3.0 h	3.5 h
a-	Ps.s-15ULSAU	Ps.s-27ULSAU	Ps.s-30ULSAU	Ps.s-77ULSAU
b-	4.0 h	3.5 h	3.0 h	5.0 h
a- Incubation time; b- hour				

The next stage of our work was the study of the main biological properties of isolated bacteriophages.

Lytic activity of bacteriophages is the property of phage to cause lysis of bacterial crop on toughen and culture fluid, it is stated by maximum phage dilution, showing lytic activity. Lytic activity of isolated bacteriophages was determined by Appelmann and Gratia methods (Vasilyev, 2017) based on the obtained research results, statistical processing of results was conducted using the program package Statistica Desktop 13 Russian (for Windows; StatSoft Russia (TIBCO USA), Microsoft Excel 2010). The results are shown in Table-3. Lytic activity of studied bacteriophages of bacteria *Pseudomonas syringae* was from 10^{-4} to 10^{-8} by Appelmann; from $1.0 \pm 0.1 \cdot 10^9$ to $2.0 \pm 0.1 \cdot 10^9$ (PFU/ml) by Gratia.

Table-3: Lytic activity of bacteriophages

#	Phage name	Lytic activity	
		by Appelmann	by Gratia
1	Ps.s-1 ULSAU	10^{-8}	$1.0 \pm 0.1 \cdot 10^9$
2	Ps.s-7 ULSAU	10^{-8}	$2.0 \pm 0.1 \cdot 10^9$
3	Ps.s-8 ULSAU	10^{-6}	$1.0 \pm 0.1 \cdot 10^8$
4	Ps.s-13 ULSAU	10^{-5}	$2.0 \pm 0.1 \cdot 10^8$
5	Ps.s-15 ULSAU	10^{-5}	$5.0 \pm 0.1 \cdot 10^7$
6	Ps.s-27 ULSAU	10^{-8}	$1.0 \pm 0.1 \cdot 10^9$
7	Ps.s-30 ULSAU	10^{-7}	$2.0 \pm 0.1 \cdot 10^8$
8	Ps.s-77 ULSAU	10^{-4}	$1.0 \pm 0.1 \cdot 10^6$

The spectrum of lytic activity of bacteriophages, that is

Plate-1: Bacteriophage detection
Ps.s-27 ULSAU (9) u Ps.s-30
ULSAU (10) on culture lawn ?-
10917

range of lysis of homological and bacteriophage bacteria is a special feature and can be applied to identification (Wojtus *et al.*, 2019; Vasikyev, 2017; Rombouts *et al.*, 2016). In this study, we used 2 reference- strains and 13 fields strains of bacteria *Pseudomonas syringae*. Spectrum of lytic activity varied from 42.9% (Ps.s-1 ULSAU) to 71.4% (Ps.s-7 ULSAU). Morphology of plague-forming units we studied in toughen breeding ground for the differentiation into low and virulent, according to I.P. Revenko (Vasilyev, 2017). Experiments established that isolated bacteriophages of bacteria *Pseudomonas syringae* formed similar PFU round-shaped with well-defined transparent centers without secondary growth with the diameter from 0.5 to 5.0 mm. Species specificity of phages is used in practice for differentiation of bacteria. This phage ability is determined by their affinity to receptors of lysed bacteria. The determination of the species specificity of isolated bacteriophages *Pseudomonas syringae* was carried out on beef extract agar-agar method of applying phage onto the lawn culture. As the result of the study of specificity of 8 bacteriophages *Pseudomonas syringae* towards representatives of other species and types of bacteria: *Pseudomonas aeruginosa*- 1 strain, *Pseudomonas fluorescens*- 2 strains, *Pseudomonas putida*- 2 strains, *Pseudomonas stutzeri*- 1 strain, *Pectobacterium carotovorum*- 1 strain, *Xanthomonas campestris* - 1 strain, *Salmonella enteritidis* - 1 strain, *Salmonella typhimurium*- 1 strain, *Yersinia enterocolitica* - 1 strain, *Shigella sonnei* - 1 strain, *Klebsiella pneumoniae*- 1 strain, *Escherichia coli*- 1 strain, *Proteus vulgaris*- 1 strain, *Proteus mirabilis* 1 strain we established strict specificity of phages.

Conclusion:

It was established that the optimal scheme for isolation of bacteriophage *Pseudomonas syringae* from ambient is the drop method with the use of trichloromethane for the inactivation of microbial cells. Balance is 1:1, that is 0.2 ml phage on 0.2 ml of indicator crop. Passage time is 3,0-5,0 hours of incubation at the temperature of $28 \pm 2^\circ\text{C}$. As a result of conducted research 8 bacteriophages of *Pseudomonas syringae* were isolated from tests.

Some biological properties of isolated bacteriophages *Pseudomonas syringae* were studied lytic activity by Appelmann from 10^{-4} to 10^{-8} ; by Gratia from $1.0 \pm 0.1 \cdot 10^6$ to $2.0 \pm 0.1 \cdot 10^9$ (PFU/ml), the spectrum of lytic activity is on 13

strains minimal percent of lysed cultures *Pseudomonas syringae* 42.9%, maximum 71.4%. All isolated bacteriophages have strict specificity.

The research was carried out for the development of ecologically safe biopreparation on the basis of bacteriophages, which can be applied in laboratory as the effective method of indication and identification of bacteria *Pseudomonas syringae* in the objects of sanitary control, and as a mean of plant protection from bacterial diseases caused by *Pseudomonas syringae*.

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