К. Науменко, студ., А. Головань, пров. инж, С. Загородная, канд. биол. наук, Н. Нестерова, канд. биол. наук,
С. Серый, асп., Ю. Маркитанов, асп., В. Тимошенко, д-р хим. наук, Ю. Шермолович, д-р хим. наук
КНУ имени Тараса Шевченко, Киев

ЦИТОТОКСИЧНОСТЬ И ПРТИВОВИРУСНАЯ АКТИВНОСТЬ НОВЫХ ФТОРСОДЕРЖАЩИХ СОЕДИНЕНИЙ

Исследована цитотоксичность и антивирусная активность фторсодержащих нуклеозидных соединений в модельной системе ВЭБ в системе in vitro. Выявлено апоптозстимулирующий эффект для соединения SBIO-6, что может быть перспективой для дальнейших исследований его в направлении противоопухолевого анализа. Полученные результаты могут быть использованы при компьютерном моделировании взаимосвязи между структурой и биологической активностью веществ, и будут применяться для создания новых высокоактивных противовирусных средств.

Ключевые слова: фторсодержащие нуклеозидные соединения, соединение SBIO-6, противоопухолевый анализ.

UDK 578.4/578.81+632.3.01/08+579.64

S. Zaika, PGS, A. Kharina, PhD, D. Chaika, stud.
Taras Shevchenko National University of Kyiv, Kyiv

LOOKING FOR KILLERS: BACTERIOPHAGES AGAINST PHYTOPATOGENIC BACTERIA

The samples of rotten tomatoes and papers were collected from different regions of Ukraine. Phytopathogenic microorganisms – causative agents of plant diseases were extracted and introduced into culture for strain identification. The presence of bacteriophages in the samples was determined by using agar overplayed method and TEM (transmission electron microscopy).

Key words: phytopathogenic microorganisms, bacteriophages.

Introduction. Bacterial pathogens are significant factors which reduce yields of agriculturally important plants worldwide. Among these bacteria the most important are *Pseudomonas syringae*, *Pectobacterium carotovorum,* and bacteria from *Xanthomonadaceae* family.

A variety of approaches have been developed to minimise the impact of bacterial plant diseases on the quantity and economy of food production. Currently, phytopathogens are controlled through management programs, which mostly relay on application of bactericides (copper-based). However, irrational use of these compounds leads to evolution of bacteria and emergence of new, highly resistant forms of phytopathogens. Besides chemical compounds are often accumulated in plants/soils and pose environmental hazards. Antibiotics (e.g., tetracycline and streptomycin) have been utilized in agriculture to control phytopathogenic bacteria also. Extensive use of antibiotics in agriculture has led to selection of resistant bacterial strains [11]. Moreover, resistance genes have been spread to other bacteria, including human and animal pathogens or nonpathogenic bacteria present in the environment [7].

Due to these agrichemical disadvantages, biological control of plant bacteria has attracted attention of many scientists and bacteriophages propose more advantages then other biological agents [6]. Bacteriophages are very specific, even to bacterial pathovars and strains; they don't cause lysis of microbial cells, represented on plants and don`t influence on soil normoflora. Besides, phages are natural components of ecosystems and always persist in host population [4]. In nature bacteriophages coexist with the host microorganisms in balance, so there is no necessity of searching them elsewhere or produce them in the laboratory by synthesis *de novo*. We just need to isolate bacteriophages from environment were specific host is present, investigate their biological properties and convert these viruses into the weapon against their hosts. Bacteriophages can also be coupled with the application of other control strategies (antagonistic bacteria, biocides etc.) for increased pressure on the pathogen [16].

The first works, that showed the potential of bacteriophages in control of phytopathogenic microorganisms, were published in 1924. Mollman and Hemstreet demonstrated that phages lysates prevent rotting of cabbage, caused by pathogenic microorganism *Xanthomonas campestris pt. campestris* [13]. Then many scientists explored phages antimicrobial activity on important agricultural plants, such as rice, pepper, tomatoes and etc. [9]. Despite the promising early works, phage therapy preparation did not prove to be a reliable and effective means of controlling phytobacteria. The main reason of this is the development of antibiotics and biocides. During the last decades of of the 20th century, bacteriophages were re-evaluated as antimicrobial agents [6]. In 2005 fist commercial phage preparation was recommended in the US for usage on crops to control infection caused with two phytopatogenic bacteria – *Xanthomonas campestris pt. vesicatoria* and *Pseudomonas syringae pt. tomato* [8].

Success of application of "AgriPhage^{TM}" (OmnyLytics) stimulated the development of new phage-based preparation against the most harmful phytobacteria worldwide. Ukraine, as agricultural country, faced with problem of crop yield losses due to bacterial infections also [5] and is interested in the development of bacteriophage preparations. However, situation is complicated with the absence of information about distribution of phytopathogenic bacteria in Ukraine. Isolation and identification of actual bacterial strains should be conducted prior to the development of bacteriophage preparations. Hence, the objectives of this study were isolation of bacteria and their bacteriophages from samples of infected plants.

Materials and methods. Samples of rotten vegetables – tomato (*Solanum lycopersicum* L.) and sweet pepper (*Capsicum anuum* L.) were collected from distinct regions of Ukraine (15 samples of tomato and 10 samples of sweet pepper from Kirovograd-, Cherkassy-, Sumy-, Kherson-, Kiev regions). Liquid medium of Luria-Bertani (baktotrypton – 1%, yeast extract – 0,5%, NaCl – 1%) was used for bacteria cultivation and bacteriophage enrichment. Miller agar (peptone – 1%, yeast extract 0,5%, NaCl – 1%, agar 1.4%) was used for propagation of bacteriophages and their hosts, whereas 1,4% agar and 0,7% agar were used for the hard and soft-agar layers, respectively, in phage plating. All bacterial isolates were maintained on clippings of Miller's agar [1].

The samples of tomatoes and sweet peppers with symptoms of rotting were sterilized by 72% ethanol. Then small pieces of diseased tissues were cut off with sterile knife and placed into LB-broth for enrichment of bacteriophages.For bacteria isolation, the sap was taken from cut surface using microbial loop and plated on Miller's [2].

Other bacteria (*Pectobacterium carotovorum, Pectobacterium amylovorum, Pseudomonas syringae pt. tomato, Xanthomonas campestris pt. campestris 117* and *125, Serratia marcescens IMBG291*) tested for phage sensitivity were obtained from culture collection of Laboratory of mi-

crobial ecology of The Institute of Molecular Biology and Genetics of NASU. Tubes and Petri dishes with samples were incubated at 27°C for 12 hours. After incubation the LB-broth with phages was centrifuged at of 5000 rev / min. for 25 minutes [15]. Supernatants were collected in sterile tubes and treated with chloroform to remove opportunistic microorganisms. Bacterial colonies on agar after 12 h of incubation were described and plated on Miller's agar clippings for further strain identification.

In the next step, the samples were analyzed for the presence of bacteriophages by double-agar technique. For this purpose 0,3 ml of target bacteria and 1 ml of the sample were added to soft agar. Isolated plagues were described and transferred to sterile ependorfs with normal saline (1 ml) [3].

Some portion of enriched bacteriopages were subjected to differential centrifugation (5000 rev. / min 20 min, 24,000 rev / min (~ 51,000 g) 120 min centrifuge UCP-65, RCS-50 rotor), the precipitate obtained after UHSC was resuspended in sterile normal saline (200 ml) [15].

The morphology of isolated viruses was studied using a transmission electron microscope (model JEM-1400, Laboratory for biophysical studies at the Institute of Microbiology and Virology named by D.K. Zabolotniy National Academy of Sciences of Ukraine). For reticula-substrates we used 0.1% formvar solution in chloroform. Phages were contrasted with 2% solution of uranilatcetate [15].

Results and discussion. Under our investigations, in general 25 samples of rotten vegetables (fig. 1) were collected from different Ukrainian regions (Kirovograd-, Cherkassy, Sumy-, Kiev regions) and 22 microorganisms were isolated for further researches.

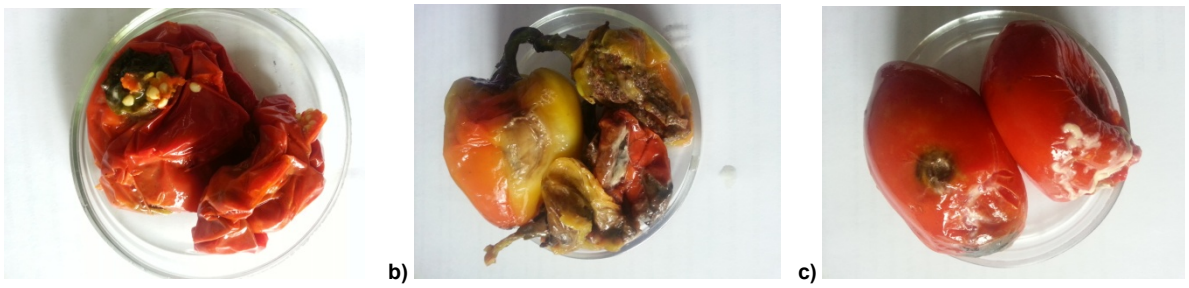


Figure 1. Some samples of peppers and tomatoes, infected with phytopathogenic microorganisms:
a) sample XXII, b) sample XXIV, c) sample XXV

As a first step, enriched samples (I-XXII) were mixed and then plated on 19 bacterial isolates. As a result we observed the plague formation on 11 bacterial isolates and the number of PFU varied from one to thousands per Petri dishes (fig. 2). The morphological properties of plague were very heterogeneous depending on host bacteria. Some microorganisms were totally lysed after fist plating, following experiment confirmed high phages concentration in the samples.

Figure 2. Morphology of plagues on bacterial lawn:
a) bacteria VII – one giant and hundreds of small plagues; b) at least 3 different types of plagues on bacteria X;
c) totally lysed bacterial layer and secondary growth on bacteria XII

On the next stage, we collected 3 new vegetable samples from Kherson and Kiev region (named XXIII-XXV) and isolated 3 new microorganisms. These 3 samples were also mixed and plated on all isolated bacteria (I-XXV). The sample gave positive result on 2 own bacteria (XXIII and XXIV) and 10 isolated previously (tab.1). Vise versa the sample I-XXII did not contain lytic agents against bacteria XXIII-XXV. The most of plagues were small (1 mm) in size, only phages, that infected bacteria XXII, gave large (4 mm) plagues with halo (fig. 3).

Fifure 3. Morphology of plagues on bacterial lawn after addition sample XXIII-XXV:
a) hundreds of very small colonies on bacteria I; b) two types of plagues on bacteria XV – middle, d~2 mm and small, d~1 mm;
c) big plagues with halo on bacteria XXII

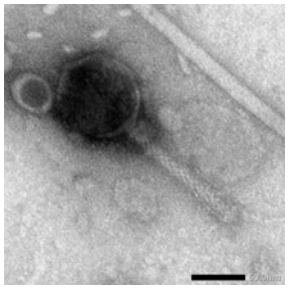
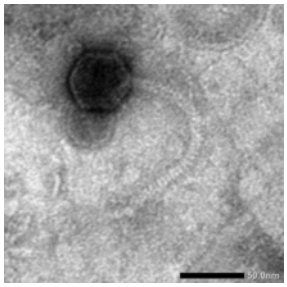
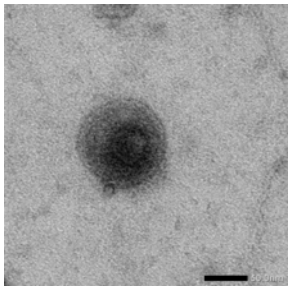
To determine the host range of the lytic phages, that were isolared previously, we tested their ability to produce plaques on laboratory pathovars of phytophathoganic bacteria from culture collection – *Pectobacterium atrosepticum*, *Pectobacterium* cat*orovorum*, *Xanthomonas campestris pt. campestris* 117 and 125, *Serratia marcescens IMBG291*, *Pseudomonas syringae pt. tomato*. Among the tested plant-pathogenic strains only one microorganism – *Serratia marcescens IMBG291* was phage-sensitive (fig. 4). Results of three experiments are summarized in table 1:

Figure 4. Plagues on bacteria lawn of *Serratia marcescens IMBG291* after addition of sample I-XXV ("Total")

***Table* 1. Results of sample plating on isolated bacteria**

Bacteria sample and region of isolation	Reaction on phage after addition of the sample I-XXII	Reaction on phages after addition of the sample XXIII-XXV	Morphology of plagues after addition of the sample I-XXII	Morphology of plagues after addition of the sample XXIII-XXV
I (tomato, Kirov. r.)	+	+	Middle, d~3 mm	Small, d~1 mm
II (tomato, Kirov. r.)	+	-	Very small, d<1 mm	
III (tomato, Kirov. r.)	-	-		
V (tomato, Kirov. r.)	-	-		
VI (tomato, Kirov. r.)	+	+	Totally lysed	Middle, d~2 mm
VII (sweet pepper, Kirov. r.)	+	+	Big, d~5 mm & small, d~1 mm	Very small, d<1 mm
IX (sweet pepper, Kirov. r.)	-	-		
X (sweet pepper, Kirov. r.)	+	+	Totally lysed	Big, d~4 mm & small, d~1 mm
XI (tomato, Cherkas. r.)	+	+	Ghostly, d~2 mm	Small, d~1 mm
XII (tomato, Cherkas. r.)	+	+	Totally lysed	Small, d~1 mm
XIV (tomato, Cherkas. r.)	+	+	3 types – big (4 mm), middle (2 mm) and small (1 mm)	Small, d~1 mm
XV (tomato, Cherkas. r.)	+	+	Totally lysed	Middle, d~2 mm & small, d<1 mm
XVI (tomato, Cherkas. r.)	-	-		
XVII (sweet pepper, Cherkas. r.)	+	+	Totally lysed	Small, d<1 mm
XVIII (sweet pepper, Cherkas. r.)	-	-		
XIX (sweet pepper, Cherkas. r.)	-	-		
XX (sweet pepper Sumy r.)	-	-		
XXI (tomato, Sumy r.)	-	-		
XXII (sweet pepper, Cherkas. r.)	+	+	Totally lysed	Big, d~4 mm
XXIII (tomato, Kiev r.)	Not tested	+		Small, d~2 mm
XXIV (sweet pepper, Khers. r.)	Not tested	+		Totally lysed
XXV (tomato, Khers. r.)	Not tested	-		

Data obtained with an electron microscope for mixed samples indicate the presence of many different phages, members of 3 families – *Myoviridae*, *Siphoviridae* and *Podoviridae*. Most of observed myoviruses belonged to A1 morphotype. Morphology of siphoviruses capsids dimensionally matched the data for B1 morphotype. While, podoviruses from samples were presented with big, about 105 nm in diameter, spherical particles with short tales and belonged to C1 morphotype. The prevalence of "tailed" phages from *Myoviridae* and *Siphoviridae* families corresponds to observed small sizes of plagues formed on the bacterial lawn. Typical for representatives of family *Podoviridae* plague morphology (pic. 5) was described only in cases of four bacteria (VII, X, XIV and XXII).

a)  b)  c) 

Picture 5. Bacteriophages morphology, obtained after TEM:
a) member of *Myoviridae* family, type A1, head ~ 85X80 nm, tail ~ 130 nm; b) member of *Siphoviridae* family, type B1, head ~ 50X45 nm, tail ~ 145 nm; member of *Podoviridae* family, type C1, head ~ 105X105 nm, tail ~ 20 nm

This is the fist our attempt to isolate phytopathogenic bacteria from diseased plants together with their specific bacteriophages. Newly isolated bacteria are probably members of family Xanthomonadaceae that include common pathogenes of tomatoes and peppers according to data of other scientists [10]. Our results are speculative, but we can found a lot of viruses to potentially phytopathogenic microorganisms, isolated from rotten tomatoes and sweet papers. In future researches we intend to identify microorganisms to species and pathovars and confirm their influence on plants *in vitro* and *in vivo*.

Readable results were observed during second trials, phages from 2 samples formed plagues on 10 phytopathogenic microorganisms from different regions, it means that isolated viruses are probably polyvalent or their hosts are relatives and have the same receptors. We plan to investigate all isolated phages in details after identification of target microorganisms.

Noteworthy is also the fact of insensitivity of laboratory bacterial strains to newly isolated bacteriophages. These results may be explained in two ways. The first explanation is the absence of bacterial strain related to laboratory strains in samples that were collected. According to second suggestion laboratory strains have lost susceptibility to mostly bacteriophages due to the numerous passages.

Conclusions. In this survey 22 isolates of bacteria were plated from infected tomato and sweet pepper. Identification of these bacteria is in progress. Bacteriophages, specific to the pathogenic microorganisms, were isolated, accumulated and examined by the method of electron microscopy. Three distinct groups of bacteriophages, based on their virion morphology, were identified.

References

1. Атабеков И.Г. Практикум по общей вирусологии. – М.: Издательство Московского университета. – 1981. – 192 с.

2. Cappuccino J. G., Sherman N. Microbiology: a laboratory manual. 4-th edition. – Menlo-park, CA: The Benjamin-Cumming Publishing Company Inc. – 1990. – 186 p.

3. Carlson K., Kutter E., Sulakvelidze A. Bacteriophages: biology and applications. Appendix: Working with bacteriophages: Common techniques and methodological appproaches. – Boca Raton: CRC Press. – 2005. – PP. 437-494.

4. Clockie M.R.J., Millard A.D., Letarov A.V., at all. Phages in nature. // Bacteriophage. – 2011. – Vol.1. – № 1. – P. 31-45.

5. Гвоздяк Р.І., Пасічник Л.А., Яковлева Л.М. та ін. Фітопатогенні бактерії. Т 1. Бактеріальні хвороби рослин. – К.: ТОВ "НВП Інтерсервіс". – 2011. – 444 с.

6. Ho K. Bacteriophage therapy for bacterial infections. Rekindling a memory from the pre-antibiotics era. // Perspect. Biol. Med. – 2001. – Vol. 44. – P. 1–16.

7. Jarvis W.R. Preventing the emergence of multidrug-resistant microorganisms thought antimicrobial use controls: the complexity of the problem. // Inf. Con. And Hosp. Epidem. – 1996. – Vol. 17. – № 8. – P. 10-15.

8. Jones J.B., Iriarte F.B., Obradovic A., at all. Management of bacterial spot on tomatoes with bacteriophages. // Proc. Int. Symp. Biol. Control Bact. Plant Dis., Land-Forstwirtsch: Mitt. Biol. Bundesanst. – 2006. – № 408. – P. 154.

9. Jones J.B., Jackson L.E., Balogh B., at all. Bacteriophages for plant disease control. // Annu. Rev. Phytopathol. – 2007. – Vol. 45. – P. 245-262.

10. Jones J.B., Lacy G.H., Bouzar. H., at all. Reclassification of the Xanthomonads associated with bacterial spot disease of tomato and paper // Systematic and Applied Microbiology. – 2004. – Vol. 27. – P. 755-762.

11. Khachatourians G.G. Agricultural use antibiotics and the evolution and transfer of antibiotic-resistant bacteria. // CMAJ – 1998. – vol. 159. – № 9. – P. 1119-1120.

12. Loper J.E., Henkels M.D., Roberts R.G., at all. Evaluation of streptomycin, oxytetracycline and copper resistance of Erwinia amylovora isolated from pear orchards in Washington State. // Plant Dis. – 1991. – Vol. 75. – P. 287-290.

13. Mallmann W.L., Hemstreet C.J. Isolation of an inhibitory substance from plants. // Agric. Res. – 1924. – P. 599–602.

14. Миронов А. А., Комиссарчик Я. Ю., Миронов В.А. Методы электронной микроскопии в биологии и медицине: Методологическое руководство. – СПб.: Наука, 1994. – 400 с.

15. Остерман Л.А. Методы исследования белков и нуклеиновых кислот: электрофорез и ультрацентрифугирование. – М.: Наука, 1981. – 288 с.

16. Tanaka H., Negishi H., Maeda H. Control of tobacco bacterial wilt by an avirulent strain of Pseudomonas solanacearum M4S and its bacteriophage. // Ann. Phytopathol. Soc. Jpn. – 1990. – Vol. 56. – P. 243–246.

Received to editorial board 10.12.13

С. Заіка, асп., А. Харіна, канд. біол. наук, Д. Чайка, студ.
КНУ імені Тараса Шевченка, Київ

У ПОШУКАХ ВБИВЦЬ: БАКТЕРІОФАГИ ПРОТИ ФІТОПАТОГЕННИХ МІКРООРГАНІЗМІВ

Зразки перців і томатів із симптомами бактеріальної гнилі були відібрані у різних регіонах України. Фітопатогенні мікроорганізми – збудники хвороб рослин були виділені із овочів і введені в культуру для подальшого визначення до штамів. Присутність вірусів до виділених мікроорганізмів в отриманих зразках було підтверджено за допомогою методу агарових шарів та методу електронної мікроскопії.

Ключові слова:фітопатогенні мікроорганізми, бактеріофаги.

С. Заика, асп., А. Харина, канд. биол. наук, Д. Чайка, студ.
КНУ имени Тараса Шевченко, Киев

В ПОИСКАХ УБИЙЦ: БАКТЕРИОФАГИ ПРОТИВ ФИТОПАТОГЕННЫХ МИКРООРГАНИЗМОВ

Образцы перцев и томатов с симптомами бактериальной гнили были отобраны в разных регионах Украины. Фитопатогенные микроорганизмы – возбудители болезней растений были выделены из овощей и введены в культуру для дальнейшего определения штаммов. Присутствие вирусов к выделенным микроорганизмам в полученных образцах были подтверждены с помощью метода агаровых слоев и метода электронной микроскопии.

Ключевые слова: фитопатогенные микроорганизмы, бактериофаги.

UDK 578.85/86

Ju. Zubyk, Spec., T. Shevchenko, PhD
Taras Shevchenko National University of Kyiv, Kyiv

ADAPTATION OF BIOTESTING METHOD FOR DETECTION OF *PBCV-1* IN WATER SAMPLES

Here we report on the adaptation of biotesting method for detection of algae viruses in samples of water and bottom sediments. We have shown that the modification involving the use of two media layers of different density with application of samples previously enriched in aeration box proved to be most efficient. Here we also describe that the water samples collected from technical water reservoirs of the National Exhibition Center of Ukraine demonstrate lytic activity towards test culture of symbiotic algae Chlorella sp. (ACKU 95-02). The stage of initial accumulation of a virus has been carried out.

Key words: algae viruses, Chlorella sp (ACKU 95-02), PBCV-1.

Introduction. Viruses are typical for any water system. These organisms are vastly abundant; their content may reach over 10 millions of particles per milliliter of water [1, 2]. The viruses are thought to influence great part of genetic and species biodiversity in seas and oceans [3]. Despite wide range of virus species found in water reservoirs,