

I. Pozhylov, Master of 2 years, T. Rudnieva, Ph. D.,
T. Shevchenko, Ph. D., O. Shevchenko, Ph. D.
Taras Shevchenko National University of Kyiv, Kyiv, Ukraine
V. Tsvigun, Ph. D.
Institute of agroecology and nature management of NAAS of Ukraine, Kyiv, Ukraine

PHYLOGENETIC ANALYSIS OF COAT PROTEIN GENE OF TOMATO MOSAIC VIRUS ISOLATES CIRCULATING IN UKRAINE

Tomato mosaic virus (ToMV) induces highly infectious disease of vegetables, whereas use of virus-contaminated seed may lead to complete yield loss. This work was aimed at studying phylogenetic relationships of Ukrainian tomato isolates of ToMV with its known isolates by comparing nucleotide sequence of coat protein gene. ELISA, TEM, RT-PCR, sequence analysis using MEGA 5 software, and statistical methods. cDNAs of two novel Ukrainian isolates ToMV-ukr-5 and ToMV-ukr-10 corresponding to coat protein (CP) gene were sequenced and compared with other published ToMV sequences. On the constructed phylogenetic tree, ToMV isolates were grouped into two separate clusters. In addition to novel Ukrainian isolates ToMV-ukr-5 and ToMV-ukr-10, the first and larger cluster contained nearly all virus isolates used in this study with high (96-98,9 %) level of homology to Ukrainian isolates. The larger cluster was clearly separated into two subclusters: one grouping isolates with over 96,7 % identity with Ukrainian isolates, and the other containing three strains and isolates with 96,1 % identity (tomato isolate SL-1, strain camellia isolated from a decorative plant, and isolate Dahlemense DSMZ PV-0135). Two novel Ukrainian isolates ToMV-ukr-5 and ToMV-ukr-10 have been isolated from tomato plants cultivated in open field conditions in different regions of Ukraine. Phylogenetic analysis confirmed high identity of Ukrainian isolates between themselves and with other published ToMV sequences. Ukrainian isolates were most homologous (>98 %) to Brazilian isolate Hemerocallis, to Chinese isolate G2, and to the following tomato isolates: AH4, Queensland, ToMV-tom and Ls-K, S14, and FERA_160205. The high level of homology was traced independently of the source of virus isolation, its plant host and their geography.

Key words: Tomato mosaic virus, tomato plants, phylogenetic analysis, isolates, sequencing, Ukrainian ToMV isolates.

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N. Korniienko, Ph. D. stud., E. Dukhno, stud.,
A. Kharina, Ph. D., I. Budzanivska, Dr. Sci.
Taras Shevchenko National University of Kyiv, Kyiv, Ukraine

ISOLATION OF BACTERIOPHAGES WITH LYTIC ACTIVITY AGAINST A NEWLY IDENTIFIED *PANTOEA AGGLOMERANS*

*In a consequence of agricultural human activity, a set of phytopathogenic bacteria gain new properties and ability to cause diseases in animal and human organisms. Moreover, bacterial loss of sensitivity to antibiotics becomes more increasing threat. The most effective alternative method of processing of plants are bacteriophages. The aim of this work is isolation and identification of a vegetable enterobacteria and search of its specific bacteriophages. Methods: biochemical identification of bacteria, analysis on sensitivity to antibiotics by means of disks, titration and accumulation of virus, electronic microscopy. Results: from onions samples with symptoms of a bacteriosis several bacteria were isolated. One of them was identified as *Pantoea agglomerans*. The sensitivity of this isolate to antibiotics was investigated, the resistance to cefalexin and norfloxacin is revealed. The bacteriophage specific to this bacteria is isolated from waste waters. The morphology of a bacteriophage is investigated by means of electronic microscopy, the virus belongs to the Myoviridae family. Phytopathogenic properties of bacteria and the antibacterial activity of phage isolate were investigated on potatoes in vitro. *P. agglomerans* led to development of a bacteriosis on potatoes cubes, and the isolated bacteriophage successfully inhibited its growth. Conclusions: This study demonstrated that common vegetables such as onions could be a source of human pathogenic bacteria. In this work, we isolated *P. agglomerans*, member of family Enterobacteriaceae. Taking into account that this bacteria was unsensitive to some antibiotics, it can be regarded as an alarming sign. The use of bacteriophages could solve problems of antimicrobial resistance and protecting of crops from bacterial infections. Isolated bacteriophage from waste waters inhibited growth of *P. agglomerans* in vitro showing that it could be considered as a part of phage drugs.*

Key words: *Pantoea agglomerans*, onion, phage therapy, bacteriophages.

Introduction. *Pantoea agglomerans* (known also as *Erwinia herbicola*) is a gram-negative aerobic bacteria from the family Enterobacteriaceae. Species from the genus *Pantoea* are mostly plant pathogens, they can be isolated from biological waste, plants, and soil (1). There they can act as pathogens as well as commensals (15). *P. agglomerans* is one of the genus species that mostly isolated from human feces, urine and blood. Moreover, it is one of common causes of nosocomial infections (3, 4). Wound infections with *P. agglomerans* usually caused by follow piercing of skin with a plant infected material (11, 21). In hospital conditions, exogenous infection with *P. agglomerans* mostly results in septic arthritis or synovitis, but there are also reports about endophthalmitis, periostitis, endocarditis and osteomyelitis (19). Immunodeficient individuals are the most vulnerable to those infections, especially if contaminated medical equipment is used (18). Fatal cases of nosocomial septicemia have been described in several countries, both in adults and children (2, 6, 14). Even though *P. agglomerans* has a quite good susceptibility to a list of antimicrobials (13), it has a potential to develop resistance to those medicines in near future like other common human pathogens from Enterobacteriaceae.

Antimicrobial resistance became a big threat in recent decades. Members of family Enterobacteriaceae, *Staphy-*

lococcus sp. and *Streptococcus sp.* became unsusceptible to many antibiotics we used to (9, 10). To defeat these multiresistant pathogens, physicians use chemicals and medicines with numerous side effects that also have influence on human organism and especially microflora (22). As a number of resistant microorganisms increases every year, more scientists start to work with bacteriophages. Bacteriophages (viruses of bacteria) are the only alternative to antimicrobials as they are biologically safe and highly specific. In contrast with antimicrobials, phages persist in human organism until the last bacterial cell's death. Furthermore, phages don't interact with other bacteria in the gut and have not side effects that makes them more safe than antibiotics (12). Some phages are so-called polyvalent phages that are able to infect bacterial strains from different species and genera. Many enterobacteria phages are polyvalent that is very important for developing phage-based drugs (7, 20). Taking into account that bacteria can develop resistance to phages as well as to drugs, phage polyvalence helps to solve this problem.

For *P. agglomerans*, phage therapy can be used in different aspects, such as reduction of bacterial residues on fruits or vegetables, sanitation of appliances used in agriculture, or use as an alternative for antibiotics in human disease control. Aim of our work was to isolate and identify an *Enter-*

obacteriaceae member from onions with bacterial rot as well as to find bacteriophages active against this bacteria.

Materials and methods. The content of brown rot from onions was placed on LB growth medium (gms/l: tryptone – 10, yeast extract – 5, NaCl – 10, agar – 10, pH=7,5). Bacterial cells were stained according to Gram and examined on magnification x100 (MICROmed XS-4130). Type of respiration was established after tests on cytochromoxidase with Hugh-Leifson medium (main solution: peptone – 2, NaCl – 5, KH_2PO_4 – 0,3, agar – 3, bromothymol blue 0,1% solution – 3 ml, pH=7,1, glucose solution: 10 g glucose, 60 ml dH_2O). Biochemical diagnostic features of bacteria were investigated at the clinical diagnostic laboratory of Public national institution "Scientific and practical center of preventive and clinical medicine" of State Directorate for Affairs (PNI "SPC PCM" SDA) with a number of selective medium: Kligler's agar (enzymatic digest of casein – 10, enzymatic digest of animal tissue – 10, lactose – 10, dextrose – 1, ferric ammonium citrate – 0.5, NaCl – 5, $\text{Na}_2\text{S}_2\text{O}_3$ – 0.5, phenol red – 0.025, agar – 15, pH = 7.2), urease (urea – 20, Na_2HPO_4 – 9.5, KH_2PO_4 – 9.1, yeast extract – 0.1, phenol red – 0.01, pH=6.8), TTC (Pancreatic digest of gelatin – 10, NaCl – 5, beef extract – 3, agar – 4, triphenyltetrazolium chloride – 0.5, pH=7.3), Simmons citrate agar (MgSO_4 – 0.2, $\text{NH}_4\text{H}_2\text{PO}_4$ – 1, K_2HPO_4 – 1, sodium citrate – 2, NaCl – 5, bromothymol blue – 0.8, agar – 15, pH=6.8) and lysine decarboxylase (Glucose – 1, L-lysine – 5, Bromocresol purple – 0.016, pH=6.1).

The sensitivity to antibiotics was determined by sensitivity discs "HiMedia". Bacteria was streaked onto a Petri dish with Mueller-Hinton Agar to form a bacterial lawn and then disks with antimicrobials (max.5 per plate) were plated on. Results was determined by diameter of diffusion of antibiotics around disks and were verified according to the standard of sensitivity of EUCAST.

The morphological features of bacterial colonies were studied using stereoscopic microscope (Biomed MS-1 ZOOM) at the PNI "SPC PCM" SDA. Phytopathogenic properties of bacterial isolate was investigated on potato *in vitro*. Cut off potato cubes (3-4 cm) were inoculated with night culture of bacteria and left in plates with sterile saline at 27°C.

In order to isolate bacteriophages the samples of waste waters were used. To amplify bacteriophages in samples enrichment method was applied. The content of brown rot lesions was transferred to liquid LB broth and incubated for 48 hours at 27°C. After incubation LB broth was centrifuged (5000 r/m, 25 min), supernatant was mixed with chloroform to remove bacteria. The samples were plated on a bacterial lawn by agar overlay method. Separate phage plaques were than picked and transferred to sterile saline (1 ml). Isolated bacteriophages were purified by serial propagation of single plaques.

To obtain high titer phage lysates were prepared from confluent lysis plates by adding 10 ml of saline to 10 plates. The soft-agar layers were scrapped off after 30 min. Lysates were clarified by low speed centrifugation at 15.000g for 15 min.

For bacteriophage staining, phage solution was deposited on formvar coated copper grid for two minutes and stained by 2% (w/v) uranyl acetate, pH 4 – 4.5. The solution was drained through filter paper and phage particles were observed through transmission electron microscopy (JEOL 1400, instrumental magnification of 40.000-90.000).

Results and discussion. A total of 5 onion bulbs with symptoms of bacterial rot were taken from different markets in Kyiv, Ukraine. Samples were plated on LB media that is common for many enterobacteria. Three samples out of five resulted in bacterial growth on LB medium. One of the isolates formed yellow, domed, shining and mucoid colonies (fig.1).

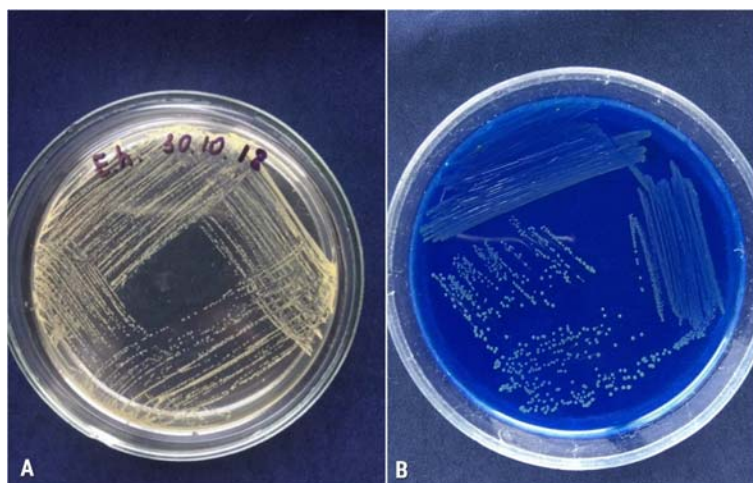


Figure 1. A – Colonies of *P. agglomerans* on plate with LB agar; B – Simmons citrate agar

The bacterial colonies were stained according to Gram and studied using light microscope. Isolate was Gram-negative, rod-shaped bacteria, 1-4 μm in length.

In Hugh-Leifson medium bacteria changed color of medium in aerobic and anaerobic conditions that indicates its ability to ferment glucose. This feature is common for facultative anaerobic bacteria.

Bacterial biochemical characterization was conducted using selective medium for Gram-negative bacteria: Kligler's agar, urease, TTC, Simmons citrate agar and lysine decarboxylase tests. The results of bacterial identification are presented in tab 1. Kligler's test indicated the ability of bacteria to ferment glucose, without H_2S

release. The urease test was negative, so that the bacteria were not capable to secrete an urease enzyme. TTC test was more positive than negative6 bacteria showed a spread throughout the medium but after 48h of incubation. Furthermore, the bacteria was able to utilize a citrate in the Simmons citrate agar. The results of lysine decarboxylase test was negative, therefore it indicated the inability of the bacteria to use a lysine as a source of carbon and energy for growth. This isolate was classified as *Pantoea agglomerans* (formerly *Erwinia herbicola*) according to the results obtained and to the Bergey's Manual of Systematic Bacteriology (tab. 1).

Table 1. Biochemical characteristics of *P.agglomerans*

Biochemical marker	Kligler's agar	Urease	TTC (motility)	Simmons citrate agar	Lysine decarboxylase
<i>P. agglomerans</i>	+ (glucose fermentation)	-	+/-	+	-

The results of bacterial susceptibility to antibiotics showed that isolated *P.agglomerans* was resistant to some of them. The resistance to cefalexin, cefotaxime, ceftazidime, ciprofloxacin, chloramphenicol, cefuroxime and norfloxacin was observed that indicates a general resistance to β -

lactams. Also, some antibiotics showed boundary values between resistance and sensitivity that indicates development bacterial resistance to them. The results of bacterial susceptibility to antibiotics are presented in the table 2.

Table 2. Antibiotic sensitivity of isolated bacteria

Antibiotic	Zone of inhibition (mm)	Reaction ¹
<i>Amikacin</i>	20	S
<i>Ampicillin</i>	20	S
<i>Amoxiclav</i>	14	S
<i>Cefalexin</i>	14	R
<i>Cefaclor</i>	16	S
<i>Cefotaxime</i>	21	R
<i>Ceftazidime</i>	20	R
<i>Ciprofloxacin</i>	24	R
<i>Chloramphenicol</i>	20	R
<i>Cefuroxime</i>	16	R
<i>Gentamicin</i>	19	S
<i>Norfloxacin</i>	12	R
<i>Levofloxacin</i>	20	S
<i>Nitrofurantoin</i>	17	S
<i>Doxycyclin</i>	17	S
<i>Moxifloxacin</i>	27	S
<i>Netilmicin</i>	18	S

According to data obtained our isolate of *P.agglomerans* can be the source of antibiotic resistance factors. Taking into account that this bacteria is cross-kingdom pathogen, horizontal gene transfer with plant and human bacteria in the environment can cause an evolving of new multiresistant bacteria.

Then detection of pathogenic properties was conducted on potato cubes. This model system was chosen in order to test bacteria on other typical vegetable as the preliminary phytopathogenicity test on onions yielded the expected positive result. Bacteria caused tissue maceration

resulting in rotting and formation of bacterial plaque on slices. *P.agglomerans* isolate showed to be capable of developing bacterial infection in plants and biochemical tests indicate its potential of human pathogen.

According to the obtained results, we attempted isolation of specific bacteriophages from waste waters that is the source of phages of human pathogens due to constant intake of human feces. Waste waters were taken from treatment facilities, then filtrated and plated with *P.agglomerans* by agar overlay method. As a result, specific bacteriophage was isolated (fig.2).



Figure 2. Phage plaques on bacterial lawn of *P.agglomerans*

Bacteriophage, given work name Eh1, produced small plaques, $d < 1$ mm. After serial propagation, bacteriophages formed negative colonies on bacterial test cultures with no decrease in phage titres, which indicates the lytic cycle of viral reproduction and, moreover, is an indispensable condition for developing phage-based products.

Phage isolate Eh1 was investigated with electron microscopy and it was indicated that this virus was member of family *Myoviridae* (fig 3). Isolate had icosahedral head $95 \times 95 \pm 4$ nm in diameter and tail 120 ± 5 nm in length. Basal plate of the virus was also observed.

¹ R – resistance, S – sensitivity.

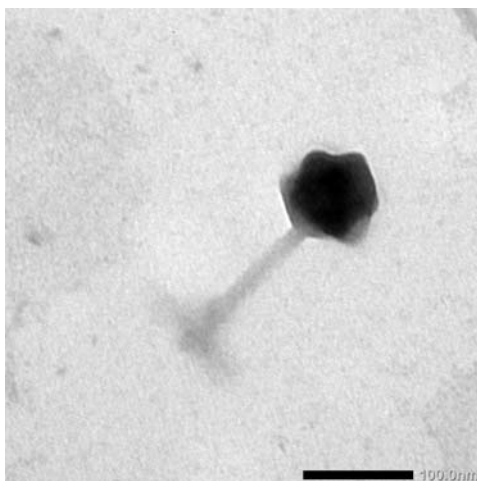


Figure 3. Electron microscopy of isolate Eh1 (family Myoviridae)

To investigate whether the isolate Eh1 is able to suppress bacterial infection *in vitro* method of potato slices was used again. As a result, bacterial growth on potato cubes was limited by bacteriophage successfully (fig.4).

Slices inoculated only by bacteria displayed the signs of rot while cubes inoculated with bacteria and phage were clear without any symptoms.

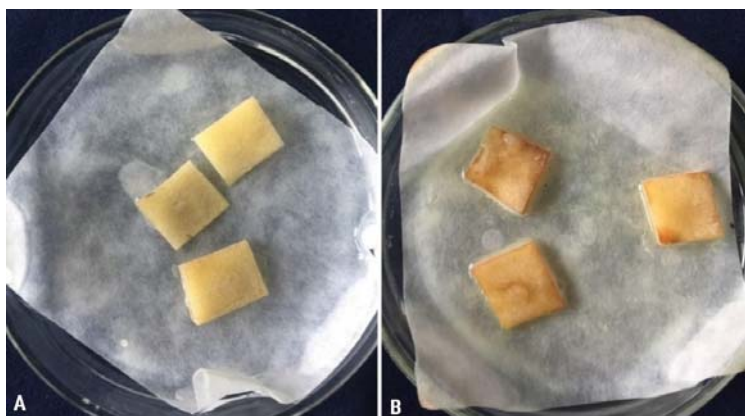


Figure 4. A – slices inoculated with bacteria and phage; B – only bacteria

In recent years researchers from different parts of the world report more often about isolation of enterobacteria from agricultural plants (5, 8, 17). Plant rhizosphere is high in nutrients and it is enough for human pathogens to live and survive. Violation of crop rotation could be the reason that led organisms such as *P.agglomerans* into new ecological niche. Introduced in agroecosystems with organic wastes, these bacteria adapted and started to invade new organisms – plants. Therefore, using of untreated human excreta contaminated with enteric pathogens to grow vegetables should be strictly controlled (16). Although, successful infection by these bacteria often occurs in co-infection with other phytopathogenic bacteria, this fact do not reduce the risk for human, for example those who eat fresh fruits and vegetables or drink juices. That is why it is important to revise classical strategies of bacterial control and to introduce alternative methods such as bacteriophages.

Conclusions. As the result bacteria was isolated from onion with bacterial rot symptom. Bacteria was identified as *Pantoea agglomerans* due to morphological and biochemical tests. Antimicrobial sensitivity profile of *P.agglomerans* was investigated, isolate showed resistance to some antibiotics. A specific bacteriophage was isolated from waste waters. Phage isolate Eh1 was examined by electron microscopy and identified as a member of family Myoviridae.

Ability of the phage to inhibit bacterial infection of *P.agglomerans in vitro* was demonstrated on potato slices.

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Н. Корнієнко, асп., Е. Духно, студ.,
А. Харіна, канд. біол. наук, І. Будзанівська, д-р біол. наук
Київський національний університет імені Тараса Шевченка, Київ, Україна

ВИДІЛЕННЯ БАКТЕРІОФАГУ З ЛІТИЧНОЮ АКТИВНІСТЮ ПРОТИ НОВОЇ ІДЕНТИФІКОВАНОЇ PANTOEA AGGLOMERANS

Унаслідок сільськогосподарської діяльності людини, багато фітопатогенних бактерій набувають нових властивостей та здатність викликати захворювання в організмі тварин та людини. Більше того, втрата бактеріями чутливості до антибіотиків стає дедалі більшою загрозою. Найбільш ефективним альтернативним методом обробки та профілактики рослин є бактеріофаги. Метою цієї роботи є виділення та ідентифікація рослинної ентеробактерії і пошук специфічних до неї бактеріофагів. Методи: біохімічна ідентифікація бактерії, аналіз на чутливість до антибіотиків за допомогою дисків, титрування та накопичування вірусів, електронна мікроскопія. Результати: зі зразків цибулі із симптомами бактеріозу виділено декілька бактерій. Одну з них ідентифіковано як *Pantoea agglomerans*. Даний ізолят досліджено на резистентність до антибіотиків, виявлена стійкість до цефалексину та норфлоксацину. Зі стічних вод виділено бактеріофаг, специфічний до ідентифікованої бактерії. Морфологія бактеріофага досліджена за допомогою електронної мікроскопії, вірус належить до родини *Myoviridae*. Фітопатогенні властивості бактерії та антимікробна активність ізоляту бактеріофагу досліджена на картоплі *in vitro*. *P. agglomerans* призводила до розвитку бактеріозу на зрізах картоплі, а виділений бакте-