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HETEROGENEITY OF *ENTEROBACTER AEROGENES* BACTERIOPHAGE POPULATION IN SEWAGE

Бактеріофаги, які уражують Enterobacter aerogenes були детектовані у стічній воді за допомогою спот-тесту та методу подвійних агарових шарів. Шляхом багаторазових пасажів поодиноких колоній були були отримані чисті лінії чотирьох ізолятів фагів, виділених з води. Виділені ізоляти бактеріофагів відрізнялись за типом негативних колоній і морфологією віріонів. Таким чином, популяція фагів Enterobacter aerogenes в стічних водах є гетерогенною і представлена щонайменше чотирма видами вірусів.

Bacteriophages specific to Enterobacter aerogenes were detected in sewage using spot-titering assay and agar overlay method. Four phages were isolated from water and purified by serial propagation of single plaques. Isolated viruses deffered for plaque type and virion morphology. It was concluded that population of E. aerogenes bacteriophage in sewage are heterogenic and consists of at least four virus species.

Introduction. Many publications suggest that phages that inhabit Earth's oceans play a huge role in the development of microbial populations. But no less interesting may be diversity of phages in freshwater ecosystems and fully synthetic ecosystems, such as sewage facilities. Due to its characteristics, sewage can inhabit a variety of types of bacteria, which in a normal environment occupy different ecological niches and have different life strategies. This is especially true for countries where there is differentiation of sewage and they all mingle on the aeration station. From the viewpoint of ecology, effluents represent a unique biocaenosis with dozens of species of micro- and macroorganisms. Moreover, the microbial population of sewage is very dynamic and overestimate the quality of the water that comes to aeration station. Species diversity of bacteria and bacteriophages respectively depends on the biological loading of wastewater.

Characteristics of bacterial and phage populations depend on where they live in water treatment systems. Water, that comes to water treatment structure directly from the sewage loaded with organics and is a nutrient substrate for microorganisms .

Our scientific work is devoted to the study of phages that inhabit water treatment systems in Kiev. Set of papers by various authors describe different phages *E. coli*, however *E. coli* is not the only microorganism that inhabits wastewater. *Enterobacter aerogenes*, a close relative of *E. coli* are also found in bulk water purification systems. As *E. coli*, this bacterium is conditionally pathogenic to humans and does not cause disease in healthy people. But unlike *Escherihia coli*, phages *Enterobacter aerogenes* studied very poorly. So we decided to explore the *Enterobacter aerogenes* phages in sewage that arrives at the Bortnichi aeration station.

Materials and methods. Bacterial strains and culture conditions

Laboratory strain of Enterobacter aerogenes was taken from the culture collection of Microbiology department (Taras Shevchenko Nashional University of Kiev, Biological faculty). Bacteria were cultivated on plate count agar or in PC-broth. Bacteria were stored on PC-agar slants at 4^{O}C. Incubation temperature was 37 ^{O}C.

Sample collection

The samples of sewage were collected from ***Bortnichi aeration station. Aqueous samples were centrifuged and filtered*** through a bacterial filter to remove the bacterial cells.

Spot-titer assay

Plates seeded with test culture were spot tested using 10 µl per spot from a series of 10 fold dilutions of the sewage samples. Following 30 min at room temperature the plates were inverted and incubated at 37^{O}C. After 18 h of incubation the plates were analyzed for the presence of phages. Results were recorded as the reciprocal of the highest dilution at which clearing of the lawn was evident [2].

Agar overlay method

Double-layer agar method described by Adams was used to separate phages in water samples [1]. Briefly, 0.2 ml of overnight bacterial culture (10^8 c.f.u./ml) was mixed with 2,5 ml of 0,7% soft agar equilibrated at 46-49^{o} C, then 0.5 ml of environmental sample was added. The resulting mix was overlain over bottom nutrient agar (1,4%). As shown by spot-titer assay, the concentration of viral particles in the water was more than 1000/1ml, so we diluted the samples to the third degree to get separate plaques. Following 15 min at room temperature the plates were inverted and incubated at 37^{O}C. After 18 h of incubation all resulting plaques were enumerated. Separate phage plagues were than picked. Isolated bacteriophages were purified by serial propagation of single plaques and amplified.

Electron microscopy.

Virion morphology was studied employing electron microscopy. Formvar films placed on 400-mesh copper grids were dipped into sample for 2 min and contrasted in 2% uranyl acetate. The preparations were dried and viewed under an electron microscope at an instrumental magnification of 90,000.

Results. A preliminary spot-test was performed to reveal the availability of bacteriophages in sewage collected from wastewater treatment station. According to obtained results bacteriophages specific to E. aerogenes are present in effluents in relatively high concentrations (fig. 1). Titers obtained from the spot-test method were 10^3 p.f.u/ml.

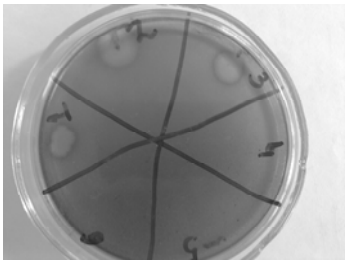

Fig. 1. The spot-titer of E. aerogenes specific bacteriophages in sewage

Spot-titer assay does not allow evaluating the morphology and type of bacteriophage plaques, taking into consideration this fact water samples were diluted to third degree and seeded by agar overlay method. As a result of our experiments various types of plaque morphologies were obtained for a given host organism, suggesting that population of *E.aerogenes* bacteriophages in sewage are heterogenic (fig 2).

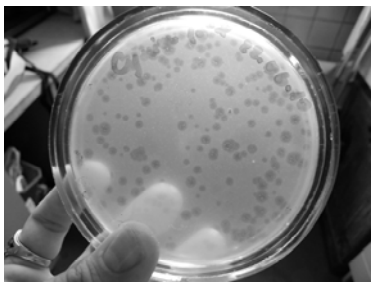

Fig. 2. The plaques cased by bacteriophages isolated from sewage on a lawn of *E. aerogenes.*

The most frequently we observed four types of negative colonies: small, d=1-2 mm (isolate №1), clear middle plagues with diameter 3-4 mm (isolate №2), middle size turbid plaques, d=3 mm (isolate №3), large plaques with halo, d=5-7 mm (isolate №4, fig. 2).

Four *E. aerogenes* bacteriophages were isolated from sewage and purified. Titres of phages after accumulation were following: isolate №1 – 98×10^9, №2 – 27×10^8, №3 – 71×10^7, №4 – 64×10^9.

Transmission electron microscopy after negative staining has revealed that isolate №1 was a member of family Siphoviridae. It had a long flexible tail and icosahedral head. Isolate N2 is a member of Myoviridae family and correspond to morphotype A1. This bacteriophage has an isometric capsid, tail and baseplate with fibers. Isolate №4 is a spherical phage 46-48 nm in diameter. This phage is sensitive to chlorophorm treatment. Morphologically isolate №4 seems to be like more archeal phages than enterophages (fig. 3).

Discussion. Thus it was shown that population of *E. aerogenes* phages in sewage is heterogenic. This indicates a significant concentration of bacteria in the effluent. Obviously, the conditions of sanitation are favorable for the replication of phages, as evidenced by their high concentration in the primary treated water.

In our work we investigated lytic phages only, but we do not know how many of lysogenic phages might be seeded on bacterial lawn at the primary infection. We have not performed the treatment of bacterial colonies with the ultraviolet irradiation, which can activate the prophage. So it is difficult to establish the true number of viruses in water. Phages replicate in bacteria at high levels so bacteriophage concentration in sewage expected to be high. Some studies demonstrated that the bacteriophage titer can be up to 2.5×10^8 virus particles per millilitre in natural waters [3]. From another hand bacteriophages can be inactivated during anaerobic degradation, which occurs during different water cleaning stages. According to our results concebrations of *E. aerogenes* phages in wast water is about 10^3 p.f.u/ml.

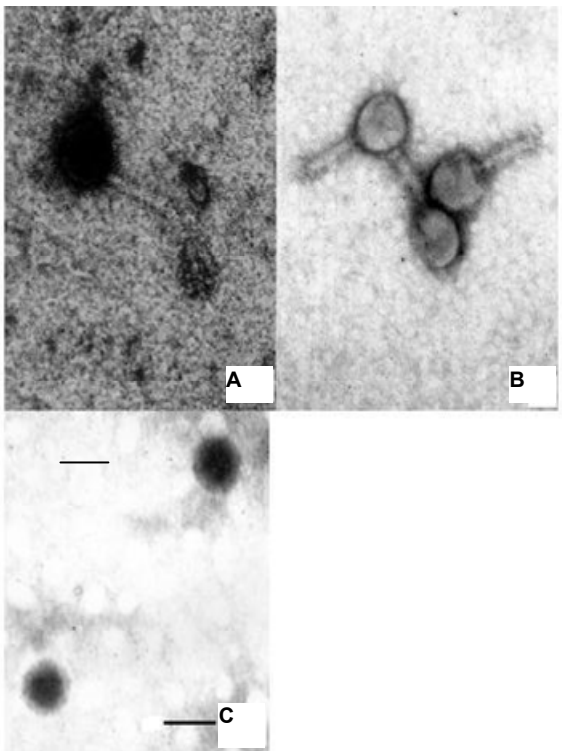

Fig. 3. Electron micrograph of bacteriophages isolated from sewage. The bars are 50 nm.
A.Isolate N1; B. Isolate N2; C. Isolate N4.

Isolated bacteriopages vary for their morphological characteristics. Recent research of another authors show that the most frequent phages in sewage belong to three families: Siphoviridae, Myoviridae and Podoviridae [4, 5]. Sometimes these three families are called "the great ecological triad" of phages. In terms of ecology, their mass distribution is easy to explain: they infect a large number of the most common bacteria in nature and their protein capsids are resistant to environmental conditions over a wide pH range, so these viruses are able to maintain its infectivity in nature long time. Our results coincide with those published by other authors. We isolated phages that represent the family Siphoviridae and Myoviridae. The only puzzle is phage number 4, which under the electron microscope has a spherical shape with an electron-permeable membrane. Perhaps, this phage has superkapsid, as evidenced by its sensitivity to chloroform. The answer to this question may provide further research.

1. Adams M. I. Bacteriophages: Interscience Publishers, New York, 1959. 2. Beck N., Callahan K., Nappier S., Kim H., Sobsey M. and Meschke J. Development of a spot-titer culture assay for quantifying bacteria and viral indicators// Journal of Rapid Methods and Automation in Microbiology. – 2009. – Vol. 17, № 4. – P. 455-464. 3.Bergh O., Borsheim K. Y., Bratbak G., Heldal M. High abundances of viruses found in aquatic environments // Nature – 1989. – Vol.340. – P. 467–468. 4.Muniesa M., Jofre J. Factors influencing the replication of somatic coliphages in the water environment // Antonie Van Leeuwenhoek J. Microbiol. – 2004b. – №86. – P. 65–76. 5. Osawa S., Furuse K., Watanabe I. Distribution of ribonucleic acid coliphages in animals // Appl. Environ. Microbiol. – 1981. – №41. – P.164–168.

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ANALYSIS OF WATER SAMPLES FOR VIRUSES INFECTING FRESHWATER ALGAE FROM *CHLOROPHYTA* DIVISION

У статті розглядаються основні підходи детекції вірусів прісноводних водоростей у прісних водоймах. Для детекції використовувалися методи біотестування і електронної мікроскопії з попереднім диференційним центрифугуванням.

In this article main approaches to detection of viruses infecting freshwater algae are discussed. For detection we used bioassay and transmission electron microscopy with previous differential centrifugation of water samples.

Introduction. Studies on ecology of algae viruses are important for both fundamental and applied science. Nowadays the issues of food shortage and energy deficit are regularly raised, and microalgae can provide solutions to both these problems as they are considered as perspective source of nutrients and biofuel. In turn, viruses of microalgae are viewed as the major cause of loss of basic raw material and hence as the primary reason for commercial losses [3]. That is why significant attention of researches has been drawn to algae viruses.

Today, the nomenclature of viruses invading single cell microalgae is a fast-developing branch of taxonomy. Basing on the first reports approved by the International Committee on Taxonomy of Viruses (ICTV), this group of viruses included about 60 representatives divided into four genera comprising the only *Phycodnaviridae* family. During the recent years many new algae viruses have been described [1; 4] and the family is currently under revision with several new genera being introduced. RNA-containing algae viruses have been described and consequently allotted into separate *Marnaviridae* family which in turn has been ascribed to the order *Picornavirales* [2].

Despite recent review, complexification and advances in the taxonomy structure of algae viruses, it is still rather questionable as establishing a new RNA virus family did not help with 'artificial' allotment of all DNA-containing viruses into single family. As of today the taxonomy structure of *Phycodnaviridae* family is deemed obsolete and unreasonable (for instance, only 9 homologous genes have been found for large full genomic sequences of three different viruses belonging to this family [8]). Phylogenetic analysis of family representatives based on full sequences of DNA polymerase gene also points on its extreme heterogeneity (Fig.1).

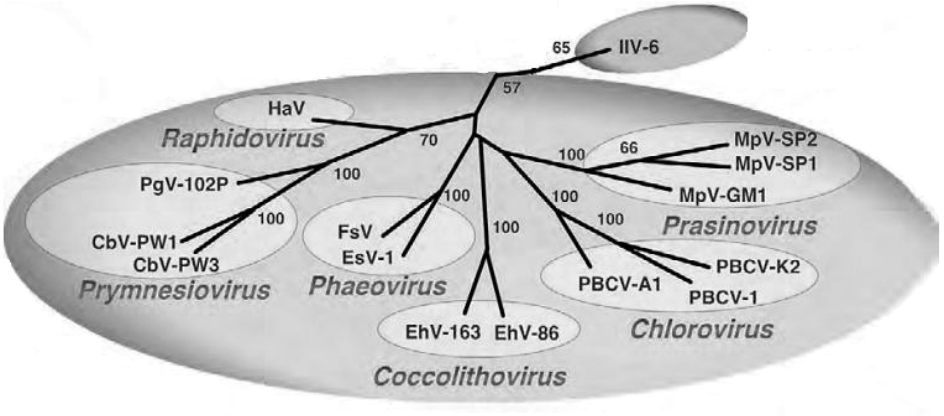

Fig.1. Phylogenetic tree showing evolutionary relationships among representatives of different genera of *Phycodnaviridae* family based on DNA polymerase gene [7]

Such attention to algae viruses is not surprising as they were demonstrated to take an active part in global biological processes of the oceans. The primary reason for studying some algae viruses lies with their crucial role in controlling the abundance of phytoplankton in water ecosystems. Ability of algae viruses to induce lysis of more than 25% of phytoplankton blooming [3; 1] is highly important from economic point of view as this phenomenon may be practically employed for such control eliminating the unavoidable water contamination with toxins and metabolic products. This would favor preservation and growth of aquatic fauna. From the other side, the algae (and especially microalgae) form the base of pyramid of numbers in water reservoirs and are the major primary producers in water food chains [1]. Hence the tentative applied use of algae viruses was another incentive for their fundamental research.

However we should note that recent advances in our knowledge of biological, morphological, genetic and molecular characteristics of these viruses are owing to research of marine microalgae. Few representatives of 'properly' described and systematized viruses infecting green freshwater algae (*Chlorophyta* division) belong to *Chlorovirus* genus. Several dozens of recent reports on isolation and description of viruses or virus-like particles from freshwater algae have not been evaluated and approved by the ICTV yet [8].

Starting from the lack of available sound information on viruses of freshwater algae, we focused on the identification of viruses able invading species of *Chlorophyta* microalgae.

Materials and Methods. Test (indicator) cultures of the following microalgae have been generously provided by the colleagues from the Department of Botany (Taras