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IDENTIFICATION OF THE MAJOR PROTEINS OF THE VIRIONS OF BACTERIOPHAGE ZF40 *PECTOBACTERIUM CAROTOVORUM*

*The vast variety of bacteriophages and the uniqueness of their individual representatives dictate to perform the detailed study of the actual phage-cell interactions, the virion morphogenesis and morphopoiesis in particular. An analysis of the complete genome sequence of the temperate phage ZF40 Pectobacterium carotovorum has shown that it is a representative of a unique group of phages of the Myoviridae family [Comeau A. M., Tremblay D., Moineau S., Rattei T., Kushkina A. I., Tovkach F. I., H.M. Krisch, H. W. Ackermann Phage Morphology Recapitulates Phylogeny: The Comparative Genomics of a New Group of Myoviruses // PLoS ONE. – July 2012. – **7.** – N 7. – e40102]. Characteristic features of these viruses are a small length of the tail compared with the diameter of the capsid and a complicated pattern of the tail sheath, leading to its criss-cross striation. In the presented article the major proteins were identified by means of the SDS-PAGE method: the head proteins (mp2; 33.9 kDa), the sheath (mp1; 39.2 kDa) and the tail tube ones (mp3; 19.9 kDa). It was proved that the mp2 molecular weight is the same with the gp46, the putative major capsid protein derived from the results of the genome sequencing. Therefore, it is still not determined whether the gp46 (mp2) of the virulent mutant 421 of the phage ZF40 is exposed to post-translational modification in the course of the phage particle maturation during its development in the cells of the strain M2-4/50RI P. carotovorum.*

To study the morphogenetic development pathways it was proposed to use the phage variants that form an excess of individual components of the virion: capsids, procapsids and separate tails propagated on different hosts.

Key words: Pectobacterium carotovorum, bacteriophage ZF40, SDS-PAGE, virion polypeptides, major capsid protein.

Currently there are 600 completely sequenced bacteriophage genomes. Primary sequence analysis of the virion DNA clearly demonstrates the tremendous diversity of phages, confirming simultaneously their species- and strain-related uniqueness [2]. This uniqueness is also complemented in the process of formation of an integral virion which structure often includes polypeptides, exposed to post-translational modification. Changes in the structural polypeptides occur directly during the maturation of the individual components of the phage virion where they gain complete tertiary structure due to the processing [9, 5]. Therefore, the complete primary sequence of the phage genome does not cover the whole variety of morphogenetic processes taking place in the real conditions of the phage-host interactions.

Temperate bacteriophage ZF40 *Pectobacterium carotovorum* subsp. *carotovorum (Pcc)* represents a unique group of viruses of the family Myoviridae [1]. Its genome is completely sequenced [Ac № JQ177065], and the study of morphogenesis of the virus and its mutants, being developed on different hosts, has also been launched [3]. In the presented work we attempted to correlate the sequenced structural genes with the real phage virion polypeptides and to identify the main head and tail proteins of the phage ZF40.

Materials and Methods

The object of the research was the bacteriophage ZF40-421 adapted to and propagated on the strain *Pectobacterium carotovorum* subsp. *carotovorum* M2-4/50RI [3], and the phage ZF40-421AK, obtained on *Pcc*RC5297 [11].

Phage lysates were obtained by the confluent lysis method [7].

Phage particles were concentrated and purified using the differential centrifugation method in the rotor SW28 Spinco L7-70 at 26000 rpm, for 3 hours, at 10 ^{0}C. The phage particles precipitate was resuspended in a minimal volume of the STM buffer (NaCl – 200 мМ; Tris–HCl (pH 7,5) – 10 мМ; $MgCl_2$ – 10 мМ), constituting 1/50 part of the initial volume of the phage lysate. The resulting suspension was released from the remnants of cells and phage particle conglomerates by their precipitation in the microcentrifuge ELMI at 11000 rpm, for 10 min.

A more profound purification of the virions was carried out by centrifugation in preformed cesium chloride gradients. Double-layer gradients of CsCl (1.42 g/cm^3 и 1.60 g/cm^3) were prepared in the 5 ml centrifuge tubes directly prior to centrifugation. Phage suspension was mixed with 10% ficol solution in 1:1 ratio and then layered on the gradient. Samples were centrifuged in the SW55 rotor at 35000 rpm, for 4 hours, at 10^0 C. After centrifugation the bands containing viral material were collected with a syringe or by using an automatic pipette.

SDS-PAGE-electrophoresis of the structural polypeptides of the phage virions was performed with the standard method by Laemmli [4]. To determine the molecular weights of phage polypeptides the following marker proteins were used: myosin – 200.0 kDa, β- galactosidase – 116.3 kDa, phosphorylase в – 97.4 kDa, BSA – 66.2 kDa, ovalbumin – 45.0 kDa, carbonic anhydrase – 31.0 kDa, trypsin inhibitor – 21.5 kDa, lysozyme – 14.4 kDa. Electrophoresis data was processed with the help of the computer program Total Lab (version 2.01).

Micrographs of the phage particles, contrasted with 2% uranyl acetate solution, were taken with the electron microscope JEOL1400 at the instrumental magnification 20000-40000x.

Results

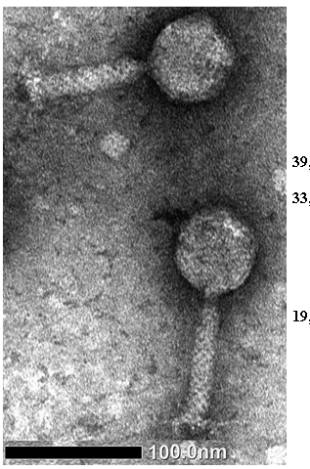
As the result of the DNA sequence analysis of the virulent mutant 421/50RI of bacteriophage ZF40 (Access number: JQ177065) it was determined that its genome contained 68 open reading frames (ORF). Presumably 10 of those encode polypeptides included in the capsid, base plate, portal vertex and tail fibers. However, 12 additional sequences are allocated in the structural region of the genome; they encode phage proteins of unknown function and two ORFans that have no analogues among the bacteriophages, for which the primary genome sequence is known. It is possible to determine the function of these proteins only through careful analysis of the virion polypeptide content and its structural components.

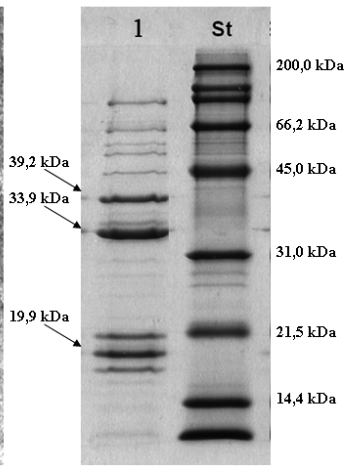
In the course of the preliminary analysis of the protein profiles of virulent mutants 421 and $c_{5/5}$ of bacteriophage ZF40 it was established that there are at least 10 structural polypeptides in the composition of their virions, three of which are major and have molecular weights 39.2, 33.4 and 18.5 kDa [8]. It was suggested that the polypeptide of 39.2 kDa corresponds to the major structural protein of the capsid. However, the results of the DNA sequencing of the phage ZF40 421/50RI variant did not confirm this assumption.

According to the results of determining the complete nucleotide sequence the phage head is formed by at least four polypeptides including gene products gp42, gp43, gp44 and gp46. The role of the major capsid protein is assigned to gp46, which molecular weight is 33.95 kDa. In order to correlate this protein with the real phage capsid protein the electrophoretic analysis of protein profile of the phage variant 421/50RI was conducted in the SDS-PAGE system. Since the phage ZF40 belongs to very unstable viruses of enterobacteria [3, 12], determination of its polypeptide composition and, especially, the quantitative content of the major proteins of an integral virion, is a rather difficult task, therefore, prior to electrophoresis, we thoroughly purified the phage particles in a gradient of cesium chloride, observing precautions for dialysis and their subsequent storage. In addition the degree of purification of native particles in the preparations was monitored by electron microscopy. Model experiments were then carried out to choose the optimum conditions for separation of polypeptides. It was established that the best possible separation of the structural proteins of bacteriophage ZF40 is achieved at a concentration of the resolving gel 14%. The volume of introduced sample directly depends on the sample concentration. It was calculated, taking into account the bacteriophage titer in the purified preparation.

Basing on the data of electron microscopy and electrophoretic analysis it was determined that homogeneous phage particles are characterized by a distinct polypeptide profile (Fig. 1a, b). Moreover, the virions of phage ZF40-421/50RI include at least 11 polypeptides with molecular weights ranging from 7.2 to 92.9 kDa. Three of them, having molecular masses of 39.2, 33.9 and 19.9 kDa, are the major ones and in the total pool of proteins make up 15.4, 28.1 and 17.5%, respectively (Fig. 1c). Proceeding from that, the protein with molecular weight of 33.92 kDa is the major capsid protein. Its mass is very similar to that of the gene product 46 of the phage ZF40 (33.95 kDa) which, basing on the sequence-analysis, also represents the major protein of the phage capsid [1]. Thus, according to the results presented, one might suppose that the major polypeptide of the phage ZF40-

421/50RI head is included in the virion in an unchanged form after its synthesis, which means it is not exposed to post-translational processing and maintains its original structure during the formation of the phage virion components. However, there is not enough proof for such an assumption as the calculation error in this case may compute up to 2 kDa. Therefore, to identify more clearly the molecular weight of gp46 it is necessary to involve a more strict method such as HPLC chromatography [8].



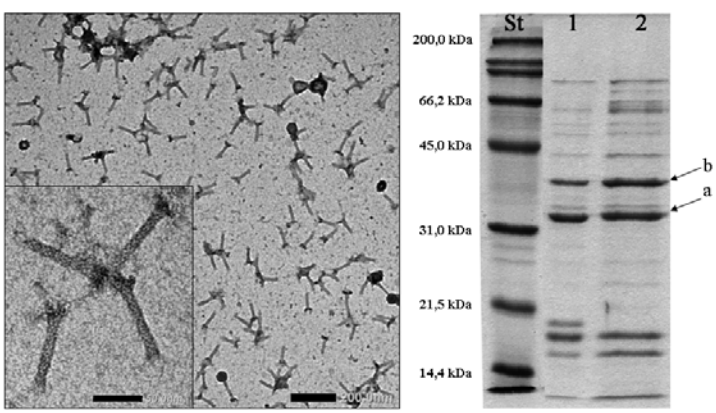


№ electrophoretic bands	Percentage, %	Molecular weight, kDa
1	4,1	92,94
2	4,0	64,58
3	1,4	54,83
4	2,8	49,77
5	2,2	44,84
6	15,4	39,22
7	4,7	35,92
8	28,1	33,86
9	9,3	21,13
10	17,5	19,90
11	8,2	18,80
12	2,2	7,21

A B C

Fig.1. A – Electron micrograph of native particles of bacteriophage ZF40-421/50RI, purified in cesium chloride gradient; B – protein profile of the native particles of bacteriophage ZF40-421/50RI, arrows point at major proteins; C – polypeptide content and molecular weights of the phage ZF40-421/50RI proteins.

Since the preliminary data on the structure of the morphological region of the phage ZF40 genome make it impossible to identify main proteins of the tail tube and the sheath, therefore, it would be possible to determine those only after having obtained highly concentrated preparations of the virion components. Thus, earlier in the process of separation of the suspension of bacteriophage 421/5297AK using low pressure ion exchange chromatography on a column with DEAE-cellulose [11] separate fractions characterized by an excessive amount of tails were obtained, that is demonstrated by the electron microscopy data of the samples (Fig. 2A). One of these fractions obtained by elution of the column with 0.25 M NaCl (№ 18), contained the largest number of phage tails and was selected for the analysis of polypeptides by SDS-PAGE.



A B

Fig. 2. A – Electron micrograph of the tails of bacteriophage 421/5297AK; B – protein profiles of native bacteriophage 421/50RI (1) and 421/5297AK (2), containing mostly phage tails; a – the major capsid protein; b – putative tail protein.

In the process of comparing the protein profiles of native 421/50RI phage particles and fractions containing an excess of tails of the 421/5297AK variant significant differences in the quantitative ratio of the three major proteins (mp) were observed. Their molecular weights are identical, however in the suspension of integral phages the concentration of proteins mp1 and mp3 with molecular weights 38.5 and 20.1 kDa, respectively, is almost twice lower than the concentration of the major capsid protein - mp2.

This is explicable, when considering that bacteriophage ZF40 was recently classified into a group of "dwarf" phages for which the tail length slightly exceeds the diameter of the head [1]. Thus, the quantity of the major capsid protein must be significantly higher as compared to other polypeptides.

Quite a different picture of the ratios between the major proteins of the virion is revealed when analyzing the fraction № 18 of 421/5297AK. Its mp1 protein concentration is equal to the amount of the major capsid protein. However, the concentration of the protein mp3 remains unchanged in relation to the mp2 and comprises only 55% of its amount. On this basis it can be assumed that the protein mp1 is a building block of the phage tail and is most likely involved in the construction of the sheath, because its amount in the tail suspension significantly increases compared with the native phage preparation. Protein mp3, according to its size 20.1 kDa, is also a part of the phage tail, but its amount is not increased in this case. We have previously suggested that this protein is a building material for the tail tube. It is known that proteins of this structure are the most conservative according to their molecular weight and for different phages it ranges between 19-20 kDa. Also in the course of the work with bacteriophage ZF40, it was noted that the tail tube is a very unstable structure. Thus, low concentration of the protein mp3 in the fraction № 18 can be explained by the fact that the tube could break down during the sample purification and concentration.

Discussion

It is widely known that the virion assembly process of bacteriophages of the order *Caudovirales* includes two stages: formation of separate structural components (phage capsid and tail) or morphogenesis and their association into a complete viable viral particle, carrying a DNA molecule – morphopoiesis. These processes are determined by specific protein-protein interactions that result in significant conformational changes in the structure of the precursor polypeptides involved in the virion assembly. Thus, a great majority of viruses is characterized by post-translational processing of the structural proteins. For example, the major capsid protein of bacteriophage P1 which size, according to the nucleotide sequence data, has to equal 63 kDa, is cleaved and in the phage head structure it is found in a form of a protein with molecular weight 44 kDa [6]. Thereby the most advantageous protein conformation for creating a stable virion is achieved. However, several challenges are associated with the processing of structural proteins. They are related to major proteins identification and determination of their functions because the data obtained, basing on the analysis of nucleotide or amino acid sequence, do not always match the results of electrophoretic analysis of the actual proteins, building the phage virion.

Recently, a complete genome of the bacteriophage ZF40 *P.carotovorum* was sequenced. According to the sequence and electron microscopy data it was classified into a group of so called "dwarf" φPLPE-like phages, related to the family Myoviridae [1]. This phage is characterized by a capsid of 60 nm in diameter and a contractile tail about 90 nm long [10]. A feature distinguishing it among the phages of the φPLPE-group is the unique pattern of the tail subunits characterized by the criss-cross striation (Fig.1a).

Morphological similarity of the phages of φPLPE-group is supported by the similarity in the construction of the structural modules of their genomes. The order of the genes responsible for morphogenetic development of a virion is conservative: terminase – portal protein – capsid proteins – tail proteins – baseplate proteins and tail fiber proteins [1]. The structural region itself constitutes 1/3 of the phage genome.

A comparative analysis of the sizes of putative structural proteins of these phages allows one to make a conclusion that the portal and base plate proteins are the most conservative. The major capsid protein is not defined for all members of the group, but it is clear that in the phage ZF40 it is smaller and amounts to 33.95 kDa, while others are characterized by the size of approximately 37-

38 kDa. These data are confirmed by the results of electrophoresis of the phage ZF40 proteins in the SDS-PAGE system. It is interesting that in comparison to other phages of Myoviridae family, such as T4, P1, P2, P4 and Mu (Table 1), only the last one has a major capsid protein of molecular weight close to the analogous protein of ZF40; for the rest this value varies from 37 to 44 kDa. Therefore, it has a unique capsid of rather complicated structure which requires a more detailed study to be performed.

Table 1

Major structural proteins of bacteriophages of Myoviridae family

	Major capsid protein		Major tail sheath protein		Major tail tube protein	
Bacteriophage	**Gene product**	**Molecular weight, kDa**	**Gene product**	**Molecular weight, kDa**	**Gene product**	**Molecular weight, kDa**
ZF40	gp46	33.95	ND	39.2-?	ND	19.9-?
P1	gp23	44.00	gp22	56.90	Tub	22.30
P2/P4	gpN	36.70	gpFI	43.10	gpFII	18.90
Mu	gpT	33.00	gpL	53.00	gpM	12.80
T4	gp23	43.50	gp18	71.20	gp19	18.50

(?) - More detailed research is needed for final identification

*ND - not determined

Proteins constituting the phage tail also need a more comprehensive research. The results of electrophoretic analysis allow us to assume that the major tube protein is represented by the major polypeptide with molecular weight of 19.9 kDa. This assumption is based on the fact that the tube protein is quite conservative in other Myoviridae phages and its size amounts to 13-22 kDa (Table 1). The major tail sheath protein is most likely represented by the protein of 39.2 kDa. Within the structural region there are genes which products have similar values of molecular weights, but for more precise determination it is necessary to obtain preparations containing only phage tails. This can be achieved in two ways, classically: by obtaining amber-mutants with a malfunction in the synthesis of capsid proteins. The other method is based on the observation that some variants of bacteriophage ZF40 form in the process of reproduction on a certain host an excess of individual structural components of the virion: capsids, procapsids and tails. Obviously the use of the indicated phage-bacterial systems constitutes a substantial perspective to explore ways of morphogenesis and morphopoiesis of bacteriophage ZF40 *P.carotovorum*.

The authors express deep grattitude to Dr. H.-W. Ackerman and Dr. A. Comeau for their assistance in sequencing of the genome of bacteriophage ZF40, and also to Dr. A.N. Ostapchuk and Dr. S.I. Voychuk for their assistance in conducting research.

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ИДЕНТИФИКАЦИЯ МАЖОРНЫХ БЕЛКОВ ВИРИОНА БАКТЕРИОФАГА ZF40 *PECTOBACTERIUM CAROTOVORUM*

Р е з ю м е

Огромное разнообразие бактериофагов и уникальность их отдельных представителей диктует проведение детального исследования реальных фаг-клеточных взаимодействий, в частности морфогенеза и

морфопоэза вириона. Анализ полного сиквенса умеренного фага ZF40 *Pectobacterium carotovorum* показал, что он является представителем уникальной группы фагов семейства Myoviridae [Comeau A.M., Tremblay D., Moineau S., Rattei T., Kushkina A. I., Tovkach F. I., H.M. Krisch, H. W. Ackermann Phage Morphology Recapitulates Phylogeny: The Comparative Genomics of a New Group of Myoviruses // PLoS ONE. – July 2012. – Vol. 7. – № 7. – e40102]. Характерными особенностями этих вирусов являются небольшая длина хвостового отростка по сравнению с диаметром капсида и сложная укладка субъединиц чехла, приводящая к его перекрестной исчерченности. В представленной статье с помощью метода SDS-ПААГ идентифицированы мажорные белки: головки (mp2; 33,9 кДа), чехла (mp1; 39,2 кДа) и стержня (mp3; 19,9 кДа). Доказано, что mp2 по молекулярной массе совпадает с gp46, основным белком капсида, выведенным по результатам сиквенса. Пока не установлено, подвергается ли gp46 (mp2) вирулентного мутанта 421 фага ZF40 пост-трансляционной модификации при созревании фаговой частицы при его развитии в клетках штамма M2-4/50RI *P. carotovorum*. Для изучения морфогенетических путей развития предложено использовать варианты фага, которые на разных хозяевах образуют избыток отдельных компонентов вириона: капсиды, прокапсиды и отдельные хвостовые отростки.

К л ю ч е в ы е с л о в а: *Pectobacterium carotovorum*, бактериофаг ZF40, полипептиды вирионов, основной белок капсида, SDS-ПААГ.

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ІДЕНТИФІКАЦІЯ МАЖОРНИХ БІЛКІВ ВІРІОНА БАКТЕРІОФАГА ZF40 *PECTOBACTERIUM CAROTOVORUM*

Р е з ю м е

Велике різноманіття бактеріофагів та унікальність їх окремих представників диктує проведення детального дослідження реальних фаг-клітинних взаємодій, зокрема морфогенезу і морфопоезу віріона. Аналіз повного сиквенсу помірного фага ZF40 *Pectobacterium carotovorum* показав, що він є представником унікальної групи фагів родини Myoviridae [Comeau A.M., Tremblay D., Moineau S., Rattei T., Kushkina A. I., Tovkach F. I., H.M. Krisch, H. W. Ackermann Phage Morphology Recapitulates Phylogeny: The Comparative Genomics of a New Group of Myoviruses // PLoS ONE. – July 2012. – Vol. 7. – № 7. – e40102]. Характерними особливостями цих вірусів є невелика довжина хвостового відростка порівняно з діаметром капсида і складне упакування субодиниць чохла, що призводить до його перехресної штрихуватості. В поданій статті за допомогою методу SDS-ПААГ ідентифіковані мажорні білки: головки (mp2; 33,9 кДа), футляра (mp1; 39,2 кДа) та стрижня (mp3; 19,9 кДа). Доведено, що mp2 за молекулярною масою збігається з gp46, основним білком капсида, який було визначено за результатами сиквенсу. Поки не визначено, чи підлягає gp46 (mp2) вірулентного мутанта 421 фага ZF40 пост-трансляційній модифікації під час формування фагової частки у випадку його розвитку в клітинах штаму M2-4/50RI *P.carotovorum*. Для вивчення морфогенетичних шляхів розвитку пропонується використовувати варіанти фага, які на різних хазяїнах утворюють надлишок окремих компонентів віріона: капсиди, прокапсиди та хвостові відростки.

К л ю ч о в і с л о в а: *Pectobacterium carotovorum*, бактеріофаг ZF40, поліпептиди віріонів, основний білок капсида, SDS-ПААГ.

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