DNA mapping in the capsid of giant bacteriophage phiEL (Caudovirales: Myoviridae: Elvirus) by analytical electron microscopy

Tatiana S. Trifonova1,2*, Andrey V. Moiseenko2,3*, Maria V. Bourkaltseva4, Olga V. Shaburova4, Alexey K. Shaytan2, Victor N. Krylov4, Olga S. Sokolova2

1FSAEI HE «People’s Friendship University of Russia», Physical, Mathematical, and Natural Sciences Department, 115419, Moscow, Russia; 2FSBEI HE «Lomonosov Moscow State University», Bioengineering Department, Biological Faculty, 119234, Moscow, Russia; 3FSBI «N.N. Semenov Federal Research Center for Chemical Physics, Russian Academy of Sciences», 119234, Moscow, Russia; 4FSBRI «I.I. Mechnikov Research Institute of Vaccines and Sera», 105064, Moscow, Russia

Introduction. Giant phiKZ-like bacteriophages have a unique protein formation inside the capsid, an inner body (IB) with supercoiled DNA molecule wrapped around it. Standard cryo-electron microscopy (cryo-EM) approaches do not allow to distinguish this structure from the surrounding nucleic acid of the phage. We previously developed an analytical approach to visualize protein-DNA complexes on *Escherichia coli* bacterial cell slices using the chemical element phosphorus as a marker. In the study presented, we adapted this technique for much smaller objects, namely the capsids of phiKZ-like bacteriophages.

Material and methods. Following electron microscopy techniques were used in the study: analytical (AEM) (electron energy loss spectroscopy, EELS), and cryo-EM (images of samples subjected to low and high dose of electron irradiation were compared).

Results. We studied DNA packaging inside the capsids of giant bacteriophages phiEL from the Myoviridae family that infect *Pseudomonas aeruginosa*. Phosphorus distribution maps were obtained, showing an asymmetrical arrangement of DNA inside the capsid.

Discussion. We developed and applied an IB imaging technique using high-angle annular dark-field detector (HAADF) and the STEM-EELS analytical approach. Phosphorus mapping by EELS and cryo-electron microscopy revealed a protein formation as IB within the phage phiEL capsid. The size of IB was estimated using theoretical calculations.

Conclusion. The developed technique can be applied to study the distribution of phosphorus in other DNA- or RNA-containing viruses at relatively low concentrations of the element sought.

Keywords: giant bacteriophage; phiEL; *Pseudomonas aeruginosa*; inner body (IB); analytical electron microscopy (AEM); electron energy loss spectroscopy (EELS); cryo-electron microscopy (cryo-EM); «bubblegram imaging»


For correspondence: Olga S. Sokolova, D.Sci. (Biol.), RAS Professor, Professor of Bioengineering Department, Biological Faculty, FSBEI HE «Lomonosov Moscow State University, 119234, Moscow, Russia. E-mail: sokolova@mail.bio.msu.ru

Information about the authors:
Trifonova T.S., http://orcid.org/0000-0003-2042-5244
Moiseenko A.V., http://orcid.org/0000-0003-1112-2356
Bourkaltseva M.V., http://orcid.org/0000-0003-3793-1354
Shaburova O.V., http://orcid.org/0000-0003-0368-3794
Shaytan A.K., http://orcid.org/0000-0003-0312-938X
Krylov V.N., http://orcid.org/0000-0001-5775-5146
Sokolova O.S., http://orcid.org/0000-0003-4678-232X

Contribution: Trifonova T.S. – analytical microscopy experiments; Moiseenko A.V. – analytical microscopy experiments, text editing; Bourkaltseva M.V. – preparation of high-liter phage stocks and bacteriophage purification; Shaburova O.V. – cryo-electron microscopy performing; Shaytan A.K. – carrying out theoretical calculations; Krylov V.N. – experiment organization; Sokolova O.S. – cryo-electron microscopy performing, writing of the text, summary, general editing of the article.

Funding. This study was funded by a grant from the Russian Science Foundation (No. 21-44-07002).

Acknowledgement. EELS spectra were obtained using a unique scientific setup «3D-EMS» of the Biological Faculty, FSBEI HE «Lomonosov Moscow State University». Cryo-EM was performed at the Federal Science Research Center for Crystallography and Photonics of the Russian Academy of Sciences.

Conflict of interests. The authors declare no conflict of interest.

Received 30 September 2021
Accepted 22 October 2021
Published 30 December 2021

*Shared first co-authorship
Картирование ДНК в капсиде гигантского бактериофага phiEL (Caudovirales: Myoviridae: Elvirus) с помощью аналитической электронной микроскопии

Трифонова Т.С.1,2*, Моисеенко А.В.2,3*, Буркальцева М.В.4, Шабурова О.В.4, Шайтан А.К.2, Крылов В.Н.4, Соколова О.С.2

1ФГАОУ ВО «Российский университет дружбы народов», факультет физико-математических и естественных наук, 115419, Москва, Россия;
2ФГБОУ ВО «Московский государственный университет имени М.В. Ломоносова», биологический факультет, кафедра биоинженерии, 119234, Москва, Россия;
3ФГБУН «Федеральный исследовательский центр химической физики им. Н.Н. Семёновой Российской академии наук», 119334, Москва, Россия;
4ФГБНУ «Научно-исследовательский институт вакцин и сывороток им. И.И. Мечникова», 105064, Москва, Россия

Введение. Гигантские phiKZ-подобные бактериофаги имеют внутри капсида уникальное белковое образование – внутреннее тело (ВТ), на которое навита суперскрученная ДНК. Стандартные подходы, используемые в криоэлектронной микроскопии (криоЭМ), не позволяют отличить эту структуру от окружающей её молекулы нуклеиновой кислоты фага. Ранее нами разработан аналитический подход для визуализации комплексов ДНК с белком на срезах бактериальных клеток Escherichia coli с использованием в качестве маркёра химического элемента фосфора. В настоящем исследовании мы адаптировали данную методику к значительно более мелким объектам – капсидам phiKZ-подобных бактериофагов.

Материал и методы. В исследовании применялись методы электронной микроскопии: аналитическая (АЭМ) (спектроскопия характеристических потерь энергии электронами, СХПЭЭ) и криоЭМ (сравнение изображений образцов с низкой и высокой дозой электронного облучения).

Результаты. Мы изучили упаковку молекулы ДНК внутри капсид гигантских бактериофагов phiEL из семейства Myoviridae, инфицирующих Pseudomonas aeruginosa. Построены карты распределения фосфора, показавшие несимметричное расположение ДНК внутри капсида фагов phiEL в виде ВТ, размер которого был оценён с помощью теоретических расчётов.

Обсуждение. Мы разработали и применили методику визуализации ВТ с использованием высокоуглового темнопольного детектора (HAADF) и аналитического подхода СПЭМ-СХПЭЭ. Картирование распределения фосфора посредством СХПЭЭ и результаты криоЭМ выявили белковую структуру внутри капсида фагов phiEL в виде ВТ, размер которого был оценён с помощью теоретических расчётов.

Заключение. Разработанная методика может применяться для исследования распределения фосфора в других ДНК- или РНК-содержащих вирусах при сравнительно низких содержаниях искомого элемента.

Ключевые слова: гигантский бактериофаг; phiEL; Pseudomonas aeruginosa; внутреннее тело (ВТ); аналитическая электронная микроскопия (АЭМ); спектроскопия характеристикских потерь энергии электронами (СХПЭЭ); криоэлектронная микроскопия (криоЭМ); пузырьковая диаграмма.


Для корреспонденции: Соколова Ольга Сергеевна, д-р биол. наук, профессор РАН, профессор кафедры биоинженерии биологического факультета, ФГБОУ ВО «Московский государственный университет имени М.В. Ломоносова», 119234, Москва, Россия. E-mail: sokolova@mail.bio.msu.ru

Участие авторов: Трифонова Т.С. – проведение экспериментов по аналитической микроскопии; Моисеенко А.В. – проведение экспериментов по аналитической микроскопии, редактирование текста; Буркальцева М.В. – получение в высоком титре и очистка бактериофагов; Шабурова О.В. – выполнение криоэлектронной микроскопии; Шайтан А.К. – проведение теоретических расчётов; Крылов В.Н. – организация экспериментов; Соколова О.С. – выполнение криоэлектронной микроскопии, написание текста, редакция.

Финансирование. Данное исследование финансировалось за счет гранта Российского научного фонда (№ 21-44-07002).

Благодарности. Спектры характеристических потерь энергии электронами получены с использованием уникальной научной установки (УУ) «3D-ЭМ» биологического факультета МГУ имени М.В. Ломоносова. Криоэлектронная микроскопия проводилась в Федеральном научно-исследовательском центре «Кристаллография и фотоника» Российской академии наук.

Конфликт интересов. Авторы заявляют об отсутствии конфликта интересов.

Поступила 30.09 2021
Принята в печать 22.10.2021
Опубликована 30.12.2021

*Первые авторы статьи
Introduction

Bacteriophages (or phages) are viruses that infect bacteria and use their resources to reproduce. These microorganisms are extremely common in the natural environment, and most of them are specific to certain bacterial species. The phage-bacterial system is often used as a model to study the stages of the life cycle of viruses and to understand the mechanisms by which bacterial genes can be transferred from one bacterium to another [1].

Tailed bacteriophages containing double-stranded DNA phylogenetically represent the oldest and most widespread group of bacterial viruses. The Caudovirales order includes three families: Podoviridae, Siphoviridae, and Myoviridae. Phages of the Myoviridae family account for about 25% of all Caudovirales phages and are distinguished by the presence of a long contractile tail. Representatives of this group affect many pathogenic bacteria such as Escherichia coli, Staphylococcus aureus, Mycobacterium, Clostridium, Salmonella, etc. Giant bacteriophages of the Myoviridae family also infect Pseudomonas aeruginosa (representatives are phiKZ, phiEL, phiLin68), Bacillus subtilis (AR9), Erwinia amylovora (vB EamM family [2]), and Pseudomonas chlororaphis (phage 201phi2-1). Therefore, these bacteriophages are widely used in phage therapy [3], which significantly expands the practical possibilities of internal and veterinary medicine, allowing, in a number of cases, to avoid the unnecessary use of antibiotics.

The number of new giant phages found increases yearly. The structure of some of them has been studied using cryo-electron microscopy (cryo-EM) [4, 5], and their genomes have been sequenced [6, 7]. Giant phiKZ-like bacteriophages active on P. aeruginosa have a circular genetic map (a feature of phages that replicate through concatemer formation and have a terminal genome redundancy) with the DNA molecule coiled around a helical formation, an inner body (IB) of protein nature [5, 8–10]. It is believed that in the process of infecting the host cell, BT proteins, together with the DNA, participate in the transcription of early genes [11, 12].

The first mention of the IB of giant bacteriophages dates back to 1984, when phage phiKZ was subjected to a freeze-thawing procedure [9]. During the experiment, reel-like structures were released from the destroyed capsids along with the DNA. The authors suggested that these formations could support the DNA in the giant capsid. Later, atomic force microscopy (AFM) also showed the release of the IB from partially destroyed phiKZ capsids [13]. Finally, 30 years after the first detection, a cryo-technique called “bubblegram imaging” was introduced, which allowed us to finally prove the presence of the IB inside the capsids of giant phages [5, 10] and even build its three-dimensional reconstruction [10]. Since that time, experimental studies were launched, in which other phages and viruses were irradiated with high doses of electrons to reveal protein structures inside the capsid [14–16].

Today, various types of transmission electron microscopy, in particular scanning transmission electron microscopy (STEM), are widely used in biology. Analytical electron microscopy (AEM) can be used to determine the elemental composition of various samples. The most reliable and simple method is energy dispersive X-ray spectroscopy (EDX), which provides an opportunity to perform spatially resolved elemental analysis on cell slices with a large number of elements sought, as well as detect cellular inclusions [17], endocrine granules [18], nanoparticles [19]. We applied this technique to visualize the DNA complexes with the Dps protein in E. coli cell slices [20] using phosphorus as a marker (a DNA molecule contains two phosphate groups per nucleotide pair).

Electron energy loss spectroscopy (EELS) is more complex method, which is designed to work with the fine element spectra to determine the nature of chemical bonds and the conductivity band of substances. Among the earliest studies that utilized EELS are the spatially resolved analysis of phosphorus in nucleic acids and the study of their spatial organization on slices [21]. Phosphorus mapping has also been described for ultrathin sections of the murine leukemia virus (MuLV) [22], frozen Drosophila (Drosophila) larvae [23], and films formed by bacteriophages λ [24]. All these experiments were performed using energy-filtered transmission electron microscopy (EFTEM).

In the present work, we significantly increased the sensitivity of the method by applying STEM-EELS analysis and using a cooled sample holder to minimize the radiation damage. This allowed us to map the DNA in a much smaller object, the capsid of the giant bacteriophage phiEL (145 nm in diameter). As a result, we, for the first time, demonstrated the presence of the IB inside the capsid of this phage using analytical approaches: high-angle annular dark-field (HAADF), and EELS. The validation of the proposed methodology was performed using cryo-EM and theoretical modeling.

Material and methods

Production and purification of bacteriophage phiEL

A phage from an individual negative colony was added to the top layer of semi-liquid (0.6%) agar containing 1 × 10^8 CFU/ml (CFU is colony-forming unit) of sensitive bacteria. The mixture was placed on dishes with dense medium, which were then incubated for 16–18 hrs at 37 °C. The upper layer was removed with a spatula and resuspended in 0.9% sodium chloride (NaCl) solution, after which the preparation was treated with chloroform (CHCl₃) and centrifuged at 6,000 rpm for 30 min to remove agar and cell debris. Further purification of the phage was performed in a cesium chloride (CsCl) gradient (density 1.33 to 1.6), according to the method described by Sambrook J. et al. [25], by centrifugation at 22,000 rpm for 4 hrs. Material from the band corresponding to phage phiEL was selected and dialyzed for 16–18 hrs on ice against 0.01 M Tris-HCl buffer containing 0.2 M NaCl and 0.002 M magnesium chloride (MgCl₂), at pH 7.5.

Cryo-electron microscopy

3 μl of purified bacteriophages (concentration 1 × 10^12 PFU/ml) (PFU is plaque-forming unit) were applied to a Quanti-
foil R 1.2/1.3 grid (Quantifoil, Germany) and subjected to a glow discharge in an Emitech K100X device (Quorum Technologies, Great Britain, England). The grids were frozen in liquid ethane (C₂H₂) in a Vitrobot Mark III machine (FEI, USA) using the following parameters: blotting time 2 sec, temperature +4 °C, humidity 95%; and stored in liquid nitrogen (N₂) until use. For study, the grids were transferred at liquid nitrogen temperature (−195.8 °C) to a Tecnai G2 12 SPIRIT cryo-electron transmission microscope (FEI) operating at an accelerating voltage of 120 kV. Images were acquired at ≈50,000 magnification using an Eagle 4K×4K CCD camera (FEI) with a matrix size of 4,000 × 4,000 pixels. The size of 1 px in the micrographs was 2.22 Å.

**Electron energy loss spectroscopy**

3 ml of purified phiEL bacteriophages were applied to a copper grid (Ted Pella Inc., USA) coated with carbon (C) and treated in a glow discharge atmosphere in an Emitech K100X instrument for 30 sec; the excess liquid was removed with filter paper and the nets were stained with 2% aqueous ammonium molybdate solution [(NH₄)₂MoO₄] for 30 sec. After that, grids were air dried and stored in a plastic container until use. Subsequently, the grids were loaded into a model 21090 cooling sample holder (JEOL, Japan), inserted into the microscope column, and the holder temperature was lowered to −182 °C to reduce the effects of radiation damage and to increase the stability of the sample under the electron beam.

EELS spectra and phosphorus element maps were obtained on a Gatan GIF Quantum ER spectrometer (Gatan, USA) in STEM mode. The size of 1 px was set to 15–20 nm (the value varied depending on the specific sample). STEM drift correction was applied after every 40–50 px. Each spectrum was obtained at a collection angle of 6.0 mrad, a dispersion of 0.25 eV, and a spectrum energy shift of 132 eV. Spectra from different pixels were aligned with the carbon K-peak. The background was extrapolated according to a power law over a 118–128 eV window; the signal window for phosphorus mapping was set at 132–155 eV, which corresponds to the L₂,₃-peak position of this element. No multiple scattering correction was used. For each of the pixels in the images obtained with the high-angle annular dark-field, an EELS spectrum was constructed, from which maps of the spatial distribution of phosphorus were compiled.

**Results**

«**Bubblegrams**» and the location of the IB in the phiEL bacteriophage capsid

To visualize the IB, we obtained the electron microscopic images from the same region of bacteriophage sample, frozen in amorphous (vitrified) ice twice: with low (10–20 e/Å²) and high (40 e/Å²) electron dose (the latter causes radiation damage of proteins) (Fig. 1 a, b) [26, 27]. A series of microphotographs shows that rows of hydrogen (H₂) bubbles were emitted during radioysis of water and are located inside the capsid along the border of the protein structure covered with nucleic acid (Fig. 1, b).

At an exposure of 1 sec (Fig. 1, a), we did not observe the presence of the IB in the capsids, as described previously [10]. Exposure of 2 sec resulted in the appearance of “bubblegrams” on the images as a consequence of an increased electron dose (Fig. 1, b). The positions of “bubblegrams” corresponded to the location of the IB between the opposite vertices of the icosahedral capsid.

**Theoretical model of DNA packaging inside the head of a giant bacteriophage**

To study the possible ways of DNA packaging inside the giant phage heads, we built a simple theoretical model. First, we assumed that the DNA is a semiflexible polymer that at a local scale is stiff enough so that its packing resembles that of rigid rods, and at a bigger scale it is flexible enough to fill the viral capsid by adopting bendable geometry where necessary. This is in agreement with coarse-grain modeling results of DNA packaging into viral capsids [28]. Henceforth, we assumed that the DNA is not extensible. An increase of the distance between DNA rods would, thus, loosen the package in two dimensions only, hence, implying an inverse quadratic dependence of DNA packing density on the distance between rods. The resulting equation, which correlates genome size (S), occupied volume (V), and distance between DNA rods (d) then follows the following equation:

\[
S = \frac{A}{V} \frac{1}{d^2},
\]

where \( A \) is a constant that can be calculated by having all other parameters for a particular viral capsid.

By inserting the known values for the phiKZ phage [4, 10, 29] into the formula, we obtained an estimate for \( A \) being 3.149 bp/nm. Thus, using the calculated value of the constant and the literature data, we can calculate the IB volume of phage phiEL (Table).

**Analytical microscopy of bacteriophage phiEL**

Next, we performed experiments using EELS to identify the phosphorus within the capsids of the giant phage phiEL. Since its genome is 211 kbp, it contains 422,000 atoms of phosphorus. We used a cooling holder to minimize radiation damage, which significantly increased the stability of the sample under the electron beam, reduced the sample drift during spectra accumulation, and minimized the pronounced contamination of its surface, as a result of electron probe scanning. In addition, negative staining of the samples with a 2% solution of (NH₄)₂MoO₄, instead of the routine staining with uranium acetate (uranyl acetate, UO₂(CH₃COO)₂·2H₂O), was applied. This helps to avoid the overlap in the spectrum of the O₁s-peak of uranium at 96 eV and the L₂,₃-peak of phosphorus at 132 eV, which prevents the correct approximation of the background component before the phosphorus peak.

The signal intensity of phosphorus at each image point was used for STEM-EELS mapping after subtracting the background component from the spectra. The typical distribution of phosphorus within a single virion is shown in Fig. 2. The corresponding characteristic signal was detected only in the internal part of the virion, but
not outside it (Fig. 2, d). Moreover, an approximately rectangular-shaped area was observed inside the capsid (Fig. 2, c), in which a very low (compared to the background) phosphorus signal intensity was detected, or the latter was absent (Fig. 2, d). The described phenomenon clearly reflects the asymmetric distribution of genomic DNA within the capsid.

**Discussion**

In this work, we used phosphorus element mapping based on the results of STEM-EELS analysis. Previously, this method allowed us, for the first time, to visualize the DNA of giant bacteriophage phiKZ inside the pseudo-nucleus formed during the infection of *P. aeruginosa* [12]. Here, this technique was adapted to study phiEL viral capsids (145 nm diameter).

Phosphorus mapping (Fig. 2) detected a strong signal inside the capsid, while the signal in the background was almost undetectable. As evidenced by our previous studies, this signal came from the viral nucleic acid [30, 31]. The distribution of phosphorus was uneven: the rectangular region in the middle of the capsid contained a very weak signal or no signal at all, while the edges of this region showed a higher signal intensity. We explained this by the presence of an IB in the phiEL capsid, which occupies a large part of the inner volume. Previously, phage phiKZ

---

**Table. Comparison of capsid geometry and calculated genome volumes of bacteriophages phiKZ and phiEL**

<table>
<thead>
<tr>
<th>Bacteriophage capsid size and parameters for genome packaging</th>
<th>Bacteriophages</th>
<th>phiKZ</th>
<th>phiEL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Размеры капсида бактериофага и параметры для упаковки генома</td>
<td>phiKZ</td>
<td>phiEL</td>
<td></td>
</tr>
<tr>
<td>Height, nm</td>
<td>145,5*</td>
<td>145,0**</td>
<td></td>
</tr>
<tr>
<td>Wall thickness, nm</td>
<td>5*</td>
<td>5**</td>
<td></td>
</tr>
<tr>
<td>Inner volume, nm³</td>
<td>744 676</td>
<td>736 303</td>
<td></td>
</tr>
<tr>
<td>Empty capsid volume, nm³</td>
<td>697 156</td>
<td>642 427</td>
<td></td>
</tr>
<tr>
<td>Genome volume, nm³</td>
<td>515 672***</td>
<td>388 595***</td>
<td></td>
</tr>
<tr>
<td>Distance between DNA rods, nm</td>
<td>2,8*</td>
<td>3,1**</td>
<td></td>
</tr>
<tr>
<td>Inner body volume, nm³</td>
<td>47 520****</td>
<td>93 876†</td>
<td></td>
</tr>
</tbody>
</table>

**Note.** *according to Fokine A. et al. [4]; **according to Sokolova O.S. et al. [5]; ***according to Burkal’tseva M.V. et al. [29]; ****according to Wu W. et al. [10]; †results of this study.

**Примечание.** *данные A. Fokine и соавт. [4]; **данные O.S. Sokolova и соавт. [5]; ***данные М.В. Буркальцевой и соавт. [29]; ****данные W. Wu и соавт. [10]; †результаты настоящего исследования.
IBs were identified by various methods: transmission electron microscopy with negative staining of samples [9], AFM [13], and cryo-EM [10]. In this study, we developed and applied an imaging technique using a high-angle annular dark-field detector (HAADF) and the STEM-EELS analytical approach. In contrast to the earlier studies, this allowed to identify the region corresponding to the IB in the capsid of the giant bacteriophage phiEL [9, 13].

In order to validate this method, we applied cryo-EM (Fig. 1). We showed that the location and shape of the IB estimated using “bubblegrams” (Fig. 2, a) sufficiently coincide with the region of phosphorus signal absence (Fig. 2, d). The size of the IB of bacteriophage phiEL was calculated on the basis of literature data (Table) and showed that the volume of its IB is almost 2 times larger than that for phiKZ. The results obtained are in good agreement with the fact that the genome lengths of phiKZ and phiEL phages differ, and the DNA molecule of the latter is ~30% shorter [29], but is packed in a capsid of the same size [5].

It should be noted that the described technique can be used to study the distribution of nucleic acids and to map the phosphorus content in other DNA- and RNA-containing viruses at relatively low levels of the element sought.

REFERENCES


13. Matsko N., Klinov D., Manykin A., Demin V., Klimenko S. Atom...


