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VIRION MORPHOLOGY AND STRUCTURAL ORGANIZATION OF POLYVALENT BACTERIOPHAGES TT10-27 AND KEY

Fine ultrastructure of polyvalent bacteriophages TT10-27 and KEY, isolated from affected with fire blight disease plant tissues, was studied using electron microscopy. Phages have isometric heads connected to short complex tail (TT10-27, C1-morphotype) or long non-contractile tail (KEY, B-1 morphotype). Maximum diameter of TT10-27 head, measured as the distance between opposite vertices, is 71.3 nm; tail tube of 22 nm in diameter and 9.0 nm in width is framed with 12 appendages that form flabellate structure of 47.0–58.6 nm in diameter. KEY features capsid of 78.6 nm in diameter and flexible non-contractile tail of 172.5 nm long, which ends with a conical tip. Due to a number of features phage TT10-27 was assigned to a group of N4-like phages of Podoviridae family. KEY is a representative of family Siphoviridae, the least frequent group of Erwinia amylovora phages.

Key words: bacteriophages, Erwinia amylovora, morphology and structural organization of virion.

Morphological organization and ultrastructure of a phage particle is common criterion of bacteriophage classification and taxonomy [2]. According to Ackermann [2] phages of the order *Caudovirales* are subdivided into three families *Myoviridae*, *Siphoviridae* and *Podoviridae* due to their tail structure, and into subtypes according to head length. Tykhonenko [25] proposed phage classification based on the base plate shape or tail distal end if a base plate is absent. This approach allows the taxa of lower rank than family to be distinguished. Currently such classic criteria are not taken into consideration, however genomic and post-genomic studies are impossible without characterization of morphology of the object.

Bacteriophages specific to *Erwinia amylovora* and other phytopathogenic and plant-associated bacteria were isolated from fire blight disease affected tissues of trees earlier [23]. Phages of two morphotypes: B1 (*Siphoviridae*) and C1 (*Podoviridae*) were observed among them [24]. Here we report results of electron microscopic studies of two representatives of the groups: phages TT10-27 and KEY.

Materials and methods.

Bacteriophage TT10-27 was obtained when an isolate pMG (extracted from affected pear sample) was propagated on *Erwinia "horticola"* 60-1n and 43II laboratory strains; phage KEY was obtained during reproduction of isolate pMA₁ (extracted from affected quince) on *E. amylovora* K8(ATCC 29850) [23].

Bacteriophages were obtained by fused lysis procedure [1] or by delayed lysis method described in [24]. Phage concentration procedure included lysates clarification by centrifugation for 1h at 5000g, 10°C, with next phage particles sedimentation in rotor SW 28 Spinco L7-70 at 26000 rpm for 2 hours at 10°C. Obtained precipitate was re-suspended in STM buffer [18] and purified from cell debris and particles' conglomerates on microcentrifuge ELM1 at 11000 rpm for 10 min.

More thorough purification of virions was achieved using CsCl stepwise density-gradient centrifugation (1.42 g/cm³ i 1.60g/cm³). CsCl solution (5M) was prepared in buffer STM and in water. The latter contributed to the phage virions' destruction. Phage suspension of 0.5–1ml volume was applied on the gradient and centrifuged for 3 hours in SW 55 rotor, at 30 000 rpm, 10 °C.

Dialysis of CsCl-purified virus was carried out against a 1000-fold excess of ST buffer [18] for 24 hours at 4 °C.

EM images were obtained with transmission electron microscope JEOL JEM 1400, samples were adsorbed for 20 min on supporting nitrocellulose-coated grids and stained with 2% uranyl acetate for 40–50 s.

All measurements of particle dimensions were executed using ImageJ (<http://imagej.nih.gov/ij/>) and Adobe Photoshop CS5, schematic illustration were constructed in Adobe Illustrator CS5, statistical data processing was done in Microsoft Excel.

Results.

Phage sample preparation. Thorough virion purification is a prerequisite for bacteriophage morphology studies that allows one to assign a particular bacteriophage to modern system of classification. Different approaches of phage particles purification are proposed [21], with CsCl density-gradient centrifugation method being most commonly used. Some phages are sensitive to osmotic shock caused by highly concentrated salt solutions like 5M CsCl. Therefore, they can be used for complete or partial destruction of phage particles. Since phages TT10-27 and KEY appeared to be stable during centrifugation in CsCl/buffer solution gradient, to obtain separate virion components CsCl/water solution was used. Thus, despite intact virus particles with hexagonal capsids connected to short complex tails (TT10-27) and long noncontractile tails (KEY), phage samples contained separate tails and particles with empty heads (phage “ghosts”) (Fig 1. A, B).

Fig. 1. General view of particles of phage TT10-27 (A) and KEY (B). a – intact virions, b – phage “ghost”. Scale bar – 100 nm.

Phage head. Phage capsid due to its isometric icosahedral shape, can be positioned on a support grid in such a way that 2, 3 or 5-fold rotational symmetry axes can be held through it projection on the planar surface [26]. These projections feature different capsid dimensions, thus, determination of particle orientation is a crucial step in head dimensions measurements. From the total number of selected EM-images – 184 particles of TT10-27 and 117 for KEY, percentage of heads oriented by symmetry axes 5:3:2, respectively, constituted 0%:86.4%:13.6% for TT10-27 and 8%:80%:12% for KEY. Heads having shape of “rounded” hexahedron also could be found next to the virions which had capsid projection of isometric hexagonal shape. Obviously, the tail bone imposes certain restrictions on the orientation of the particles. Since it was especially indicative for TT10-27 phage we assume its tail to be sophisticatedly oriented having significant expansion in plane perpendicular to the surface of supporting grid.

Phage capsids were characterized by the following parameters: maximum diameter (Dmax) – distance between opposite vertices, minimum diameter (Dmin) – distance between the opposite edges, CL (capsid length) – distance between the central and portal vertices. The maximum diameter of phage TT10-27 capsid possessing 3-fold symmetry (Dmax₃) equaled

71.3 nm $\pm$ 3.8 nm, with Dmin₃ value of 61.9 nm $\pm$ 3.9 nm and capsid length CL₃ value 65.2 $\pm$ 2.9 nm. Phage head in orientation with 2 axes of rotational symmetry had slightly increased parameters of Dmax₂ and Dmin₂, constituting 72 $\pm$ 2.5 nm and 64.6 $\pm$ 2.4 nm, respectively and slightly decreased value of CL₂ – 64.1 $\pm$ 3 nm.

Obtained data are in a good agreement with theoretically calculated ratios of dimensional parameters for regular icosahedron [26]. Thus, according to [8], the maximum diameter of capsid projections with symmetry axes 5:3:2 correlate as 0.894:0.982:1 with the minimum diameter value of head with 3-fold symmetry (Dmin₃) rating as 0.851. The edge length can be calculated as 0.588, 0.535 and 0.525 of Dmax of respectively 5:3:2-fold head projections [13]. According to empirically obtained dimension measurements, the ratio of Dmax₃ toward Dmax₂ of TT10-27 capsid is 0.990 to 1, while Dmin₃ constitutes 0.859. The distance between the opposing edges (Dmin₃) differs from that between opposite vertices (Dmax₃) by 13.2%, which is slightly different from the rate of 11.5% obtained for regular icosahedron [8].

The length of the edge was calculated according to ratio toward Dmax₃ and Dmax₂, and constituted 37.9 nm $\pm$ 0.3 nm. Though significant amount of phage “ghosts” was present on micrographs (Fig. 2, 3), we were not able to measure edge length or count number of subunits it is formed of.

Fig. 2. Phage TT10-27 particles with empty capsids. Scale bar – 100 nm.

Phage KEY head features larger dimensions than phage TT10-27. Maximum diameter of the phage capsid with 3-fold symmetry (Dmax₃) was 78.6 nm $\pm$ 3.1 nm, while Dmin₃ equaled 68.3 nm $\pm$ 2.5 nm with the capsid length CL₃ 77.5 $\pm$ 3.1 nm (Fig. 3). Capsids possessing 2 axes of rotational symmetry featured Dmax₂ and Dmin₂ constituting 79.3 $\pm$ 1.8 nm and 67.2 nm $\pm$ 3.9 nm, respectively, with CL₂ value 72.5 $\pm$ 1.9 nm, which is 6% less than CL₃. Projections of 5-fold symmetry capsid had diameter of 74.2 $\pm$ 3.4 nm. Maximal diameters of heads in orientations 5:3:2 correlated as 0.935:0.991:1, while the distance between the opposite edges (Dmin₃) constituted 0.861, and differed from distance between opposing vertices by 13.1%. The size of capsid edge was calculated regarding maximum diameter of heads in all three orientations and equaled 42.4 $\pm$ 0.8 nm.

**Fig. 3. Virions with empty capsids (A) and phage KEY intact particle (B).
Scale bars – 200 nm (A) and 100 nm (B).**

Table

Dimensions of TT10-27 and KEY virions

Structural components of the virion	TT10-27			KEY		
	N	Average value	Mean error of measurement (%)	N	Average value	Mean error of measurement (%)
Head:						
Dmax _h	159	71.3	5.3	97	78.6	3.1
Dmin _h	159	61.9	6.3	97	68.3	2.5
CL _h	152	65.2	4.4	97	77.5	3.1
Dmax _s	25	72	3.5	11	79.3	1.8
Dmin _s	25	64.6	3.7	11	67.2	3.9
CL _s	24	64.1	4.7	11	72.5	1.9
Dmax _t	–	–	–	9	74.2	3.4
Tail length*	72	9.2	11.1	106	172.5	3.5
Tail diameter*	39	22.2	10.0	25	9.2	13.0

Note: N – number of measurements; * – for TT10-27, width of central tube without appendages, for KEY – tail length without distal tip; see other designations in the text; «–» – measurements were impossible execute.

Phage tail. Due to organization of tail, phage TT10-27 was assigned to the C-morphotype (family *Podoviridae*). Phage attachment apparatus despite its small size features rather complex structure. Its projections look different on the micrographs, depending on the rate of phage sample purification, the degree of particle degradation, phage particle orientation on the grid and quality of sample contrasting. Obviously, some of tail structural components are rather sensitive to external factors such as osmotic shock or even physical impact, which causes “shrinkage” of tail (Fig. 4). Degrading conditions caused by the absence of buffer in CsCl solution, led to the separation of tails from heads. Size parameters of intact particle tails were compared to separate ones and are represented on the schematic diagrams (Fig. 5).

Fig. 4. TT10-27 particle. a – appendage consisting of the connector part and petal; b – two rows of morphological subunits are visible. Scale bars – 100 nm.

Fig. 5. The general scheme of TT10-27 virion (A) and attachment apparatus (B) structure. Dimensions are given in nm.

Phage TT10-27 tail is connected to portal vertex of the head. It consists of central tube framed by 12 appendages. On the central tube of 22.0 ± 2.2 nm in diameter and 9.0 ± 1.0 nm in width two transverse rings of equal thickness are visible. They appear as two rows of morphological subunits. To each subunit of the upper ring an appendage is attached. Thus, we assume that the number of subunits in upper row, respectively, is 12. The appendage consists of the proximal thin connector part of about 16.0 ± 4.9 nm and distal petal-shaped part of 15.0 ± 2.5 nm long, narrowed from the ends, with the diameter of the widest section of 7.0 ± 1.5 nm. Unlike subunits of the upper row, that of lower do not carry appendages. But considering subunits placement on the tube it is presumable that there is also 12 of them. Appendages radially diverge from the tube and are attached to it with the connector part at approximately orthogonal angle relatively to the main axis of symmetry of the virion. The petal is attached to the connector part and directed downwards. Obviously, the junction is labile and petal can change their conformation in different planes (Fig. 4, B, Fig. 5).

By virtue of various factors, geometry of apparatus is impaired and the appendages appear attached in the form of a punch with violations of both junction angles. Interestingly, that on separated tails that are positioned frontally on the grid (Fig. 6) the appendages look more elongated than in intact particles. This can be interpreted as an artifact, or be explained by the curved shape of the appendix connector, with a bend in the plane of image, as in Fig. 4 A. Therefore, the total length of the tail bone is about $26.0 \text{ nm} \pm 3.1$, while its diameter is almost twice larger – $58.6 \text{ nm} \pm 5.1 \text{ nm}$.

**Fig. 6. Phage TT10-27 tails (A) and the scheme of their structure (B).
Scale bar – 100 nm (A). Dimensions are given in nm.**

When tail lays on the grid frontally, central tail tube appears to consist of two individual structural parts that appear like two concentric circles. The diameter of the outer circle corresponds to the diameter of the tail tube, inner ring diameter is 13.4 nm. It features a channel of 4.6 nm inside (Fig. 6).

Presumably, due to the influence of degrading environment on the virion a partial release of the virion DNA-protein complexes occurs, since rod-shaped structure protruding from the center of tail tube appears on significant part of micrographs (Fig. 7).

**Fig. 7. Rod-shaped structure protruding from TT10-27 tail (depicted with arrow).
Scale bar – 100 nm.**

Bacteriophage KEY due to its tail organization was assigned to B-morphotype (family *Siphoviridae*). This phage possesses a long flexible non-contractile tail. Its projections appear to be curved and range in length from 75 nm to 186 nm. Seemingly, it is destroyed during phage centrifugation or sample preparing. Total tail length (including head-to-tail connector) in average is $172.5 \pm 6.0 \text{ nm}$ with its diameter constituting $9.2 \pm 1.3 \text{ nm}$. At the same time phage “ghosts” featured the tail length of $171.48 \pm 3.9 \text{ nm}$. The tail is attached to head through thinned head-to-tail connector of $15.1 \pm 2.9 \text{ nm}$ length on which subunit structure is not visible (Fig. 8). The ratio between tail length and head diameter equals 2.2.

Fig. 8. The general view of phage KEY virion (A) and detailed scheme of its structure (B) with a structural subunit. Scale bar – 50 nm (A). All dimensions are given in nm.

The tail's characteristic feature is its narrowing towards the ends and thickening towards the center. Its distal end is separated from the tail tube by plate of 16.9 ± 3.7 nm in diameter and 4.8 ± 0.9 nm in width, and appears as a conical tip of 14.8 ± 2.5 nm.

Fig. 9. The view of phage KEY discs on partly destroyed tails (A) and separate disc (B). Scale bar – 100 nm.

On partly destroyed tails a channel with diameter of 2.7 ± 0.5 nm is visible. As well, separate discs of about 10.0 ± 1.0 nm in diameter can be found on micrographs [Fig. 9.A,B]. Their size is close to tail diameter, as well they are hollowed with the diameter of hole of 2.4 nm corresponding the tail channel diameter. This allowed us to suppose that the discs are the tail structural subunits, which being arranged perpendicularly to lengthwise axis of the tail form slightly visible transverse stack of subunits. So, the tail can be considered as a set of stacked disks ending with conical tail. The transverse striations are observed with a period of $2.86 \text{ nm} \pm 0.46 \text{ nm}$ which allows us to presume that number of discs on the tail is about 49–50. Similar tail organization is typical for many *Siphoviridae* phages, particularly phage SPP1 [19].

Discussion. Phages infecting *E. amylovora*, the pathogen causing fire blight disease of trees are perspective objects of study [7], however only 11 sequenced phage genomes can be found in GenBank database. Characteristically, they mostly belong to morphotypes A and C. Isolated phage KEY is of great interest since it is a representative of rare group of *E. amylovora* phages. Despite the fact that B-morphotype phages are the most frequent group in nature [2], for *E. amylovora* there are only two described [12, 17]. Both of them were characterized by research group from Hungary: phage PhiEaH1 was isolated from aerial tissue of affected plants [17] and PhiEaH2 – from the soil near affected plants [12]. Nevertheless, KEY phage is rather distant from them since its genome is of much smaller size (82 kb [24] compared to 218 and 243).

Another interesting feature of KEY is the flexibility of its tail. It is of characteristic mostly for long-tailed *Enterococcus* and *Salmonella* phages that have greatly elongated tails with their length being several times bigger than the diameter of the head [3, 16]. The majority of *Siphoviridae* phages have rigid tails, e.g. phage lambda, which features the length tail to capsid diameter ratio of about 2–2.5 [9], which is close to that of KEY. At the same time, tails of phages T1 [9] and SPP1 [19], as an exception, featuring similar ratio, are also flexible. The nature of this phenomenon has not yet been established.

TT10-27, though isolated from the same location, does not infect any of the co-isolated *E. amylovora* bacteria, while is able to cause productive infection in laboratory strains of *E. "horticola"* and *Pantoea agglomerans*. Characteristically, *P. agglomerans* ("yellow-pigmented" bacteria) often accompanies *E. amylovora*-mediated infection process in plant population and can influence its manifestation either directly or through phage-carrying state. Therefore, specific to ("yellow-pigmented" bacteria) phages must be taken into account. Two described phages capable of infecting this bacteria are the representatives of the phi-KMV-like phage group of *Podoviridae* family [4].

Due to a number of characteristics (head diameter, complex tail organization, DNA size) phage TT10-27 was assigned to the group of N4-like phages. This group was singled out relatively recently, as its main representative, enterobacteria phage N4 was considered to be an orphan for almost 40 years [11], and only in the second half of the 2000s the first reports about related phages appeared. However, phages with similar morphological characteristics were found and described already in 60th [6, 25], but remained unheeded. Currently in the NCBI database information of 34 members of this group is given; two among them are specific to *E. amylovora*. Interestingly, all phages of the group differ in the structure of their tails. It was shown both with electron microscopy and the pairwise alignment of protein sequences of tail hypothetical proteins [10, 22]. On the contrary to this, the shape and size of the capsid, strictly lytic development and packaging of viral RNA polymerase into phage head are the conservative characteristics of this group [11]. Interestingly, the N4 head has the lattice with a triangulation number $T = 9$, not found in any other phage group [11].

N4-phages infect hosts that occupy fundamentally different ecological niches: *Silicibacter* and *Sulfitobacter*, marine *Roseobacter* [27], as well as *Escherichia* [11, 22] and *Pseudomonas* [10], etc., while they are highly specific to a particular host. In addition, the structure of their tails is extremely diverse. The structure of attachment apparatus of a N4-phages ranges from thin tubular outgrowths in roseophages [27], and to complex apparatus with 12 appendages in N4 [11] and two rows of fibers in G7C [15]. Obviously, a complex spatial organization of receptor-binding proteins and the presence of phages auxiliary appendages is critical for successful recognition and attachment to the surface structures of the host bacterium cell wall.

Phage N4 tail consists of a core and a sheath, with the latter functioning as bacteria surface recognizing structure [11]. The presence of hollow rod-shaped structures in the tail of *Escherichia* phage Sd was also reported by Tykhonenko [25]. Similarly, we presume that phage TT10-27 tail also consists of a core and a sheath with dimensions corresponding to diameters of the rings on figure 6. Thus, the complex tail though features the *Podoviridae* size and is non-contractile like *Siphoviridae* (no tails with contracted sheathes were found) consists of core and sheath similar to *Myoviridae*. Besides phage N4-like phages similar additional appendages are also found in bacillary phages phi29 and GA-1 [5]. Their flabellate appendages do not function as tail fibres, e.g. in phi29 phage they are appendages of connector and are responsible for DNA packaging into the capsid [20]. Thus, it is not easy to assign the function to phage TT10-27 appendages.

A detailed study of phage virion components and structure with electron microscopy is rather important for assigning of the functions to individual components of virions, and, subsequently, to individual structural proteins. Morphology and structural organization of two phages infecting plant-associated and phytopathogenic bacteria was studied. Phages were assigned to definite taxa of modern system of classification. Phage TT10-27 is a new representative of N4-like phage group of family *Podoviridae*. Phage KEY of *Erwinia amylovora* was assigned to the *Siphoviridae* family. This work creates the basis for further phage genome research.

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МОРФОЛОГО-СТРУКТУРНА ОРГАНІЗАЦІЯ ВІРІОНІВ ПОЛІВАЛЕНТНИХ БАКТЕРІОФАГІВ TT10-27 І KEY

Резюме

За допомогою методу електронної мікроскопії було досліджено тонку ультраструктуру полівалентних бактеріофагів TT10-27 і KEY, виділених із уражених бактеріальним опіком деревних тканин. Фаги мають ізометричний капсид, до якого приєднується короткий складно влаштований відросток (TT10-27, C1-морфотип) або довгий нескоротливий хвостовий відросток (KEY, B-1 морфотип). Максимальний діаметр капсиду фага TT10-27, розрахований як відстань між протилежними вершинами, складає 71,3 нм, хвостовий відросток діаметром 22,0 нм та товщиною 9,0 нм обрамлений 12-ма апендиксами, які формують віялоподібний утвір діаметром 47,0–59,0 нм. Фаг KEY характеризується капсидом 78,6 нм в діаметрі та гнучким хвостовим відростком 172,5 нм, що закінчується циліндричним кінчиком. За рядом ознак фаг TT10-27 був віднесений до групи N4-подібних фагів родини *Podoviridae*. Фаг KEY є представником родини *Siphoviridae* найменш поширеної групи фагів, специфічних щодо *Erwinia amylovora*.

Ключові слова: бактеріофаги, *Erwinia amylovora*, морфолого-структурна організація віріона.

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Резюме

С помощью метода электронной микроскопии было исследовано тонкую ультраструктуру поливалентных бактериофагов TT10-27 и KEY, выделенных из тканей деревьев, пораженных бактериальным ожогом. Фаги имеют изометрический капсид, к которому присоединяется короткий сложно устроенный отросток (TT10-27, C1-морфотип) или длинный несокращающийся

ся хвостовой отросток (КЕУ, В-1 морфотип). Максимальный диаметр капсида фага ТТ10-27, исчисляемый как расстояние между противоположными вершинами, составляет 71,3 нм, хвостовой отросток диаметром 22,0 нм и толщиной 9,0 нм обрамлён 12-ю аппендиксами, которые формируют веерообразное образование диаметром 47,0–59,0 нм. Фаг КЕУ характеризуется капсидом 78,6 нм в диаметре и гибким хвостовым отростком 172,5 нм, заканчивающимся цилиндрическим кончиком. По ряду признаков фаг ТТ10-27 был отнесен к группе N4-подобных фагов семейства *Podoviridae*. Фаг КЕУ является представителем семейства *Siphoviridae* наименее распространенной группы среди фагов, специфических к *Erwinia amylovora*.

Key words: бактериофаги, *Erwinia amylovora*, морфолого-структурная организация вириона.

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