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## Genome packaging in EL and Lin68, two giant phiKZ-like bacteriophages of *P. aeruginosa*

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### ABSTRACT

A unique feature of the *Pseudomonas aeruginosa* giant phage phiKZ is its way of genome packaging onto a spool-like protein structure, the inner body. Until recently, no similar structures have been detected in other phages. We have studied DNA packaging in *P. aeruginosa* phages EL and Lin68 using cryo-electron microscopy and revealed the presence of inner bodies. The shape and positioning of the inner body and the density of the DNA packaging in EL are different from those found in phiKZ and Lin68. This internal organization explains how the shorter EL genome is packed into a large EL capsid, which has the same external dimensions as the capsids of phiKZ and Lin68. The similarity in the structural organization in EL and other phiKZ-like phages indicates that EL is phylogenetically related to other phiKZ-like phages, and, despite the lack of detectable DNA homology, EL, phiKZ, and Lin68 descend from a common ancestor.

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### Introduction

PhiKZ-like phages belong to the *Myoviridae* family and infect the opportunistic pathogenic bacterium *Pseudomonas aeruginosa*. Very low DNA homology in regards to other bacteriophages indicated that they represent a distinct branch of the *Myoviridae* (Krylov et al., 2004). Genomic and morphological analyses (Ackermann et al., 1988; Burkal'tseva et al., 2002; Krylov et al., 2004) demonstrated that the genus of 'phiKZ-like' phages active on *P. aeruginosa* could be subdivided into three species: (i) phiKZ phages; (ii) Lin68-like phages with members Lin68 and LBG22 (Burkal'tseva et al., 2002; Krylov et al., 2004), and (iii) EL-like phages with members EL, RU and Che (Burkal'tseva et al., 2002; Hertveldt et al., 2005; Krylov et al., 2004; Pletnev et al., 2010).

Previous genetic studies demonstrated that genomes of all phiKZ-like phages show traces of active cross-species migration with a repeated change of bacterial hosts (Lavigne et al., 2009). In the course of evolution, various phiKZ-like phages diverged significantly from a common ancestor. Differences in GC composition of phage species and their level of adaptation to the current host species may reflect earlier acts of migration. The separation of EL

has apparently occurred long before the separation of Lin68 and phiKZ. This is confirmed by the fact that the G+C ratio of their genomes differ from the G+C ratio of their current host (Krylov et al., 2010) and by its structural similarity to phages, active on other bacterial species (Table 1). This may be the reason for low DNA homology between EL and phiKZ/Lin68 (Burkal'tseva et al., 2002; Krylov et al., 2007).

PhiKZ-like phages have circular genetic maps (Hertveldt et al., 2005; Mesyanzhinov et al., 2002) and replicate their genomes with the formation of concatamers. In this case, each phage particle receives a full set of genes and some redundant terminal DNA ends. It is believed that in the course of DNA packaging, the entire interior space of the head is filled with DNA. In such case, it is assumed that the internal size of the capsid matches the total volume of the packed genome. However, the phiKZ capsid has an additional unique structural feature in genome packaging. It is the first bacteriophage where an internal proteinaceous structure, encased within genomic DNA, called 'the inner body', was visualized (Krylov et al., 1984, 1978; Krylov and Zhazykov, 1978). So far, the inner body's functions remain elusive. It may serve as a structural device for arranging genomic DNA inside a giant phage head (Krylov et al., 1984; Thomas et al., 2012). According to previous EM studies, the inner body is absent from the phiKZ's head after infection (Krylov et al., 1984), which may suggest it taking part at the DNA injection process. Some phages unrelated to phiKZ-like (e.g. T7) are known to have a small barrel-shaped

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**Table 1**

Identified structural proteins of giant bacteriophages, active on *Pseudomonas* and *Salmonella*, and their similarity to EL phage proteins (Burkal'tseva et al., 2002; Hertveldt et al., 2005; Lecoutere et al., 2009; Mesyanzhinov et al., 2002; Shaburova et al., 2008).

Function	Pseudomonas phages					Salmonella phage SPN3US
	EL	phiKZ	OBP	201phi2-1	phiPA3	
	Gene names; % of sequence similarity with EL phage proteins					
Major capsid protein	ORF078	ORF120; 19%	OBP_124; <b>31%</b>	201phi2-p200; 19%	P136; 20%	SPN3US_0075; 21%
Tail sheath protein	ORF006	ORF029; 23%	OBP_002; <b>32%</b>	201phi2-1p30; 20%	P11; 20%	SPN3US_0256; 21%
Tail protein	ORF156	ORF146; 27%	No homology	201phi2-p230; <b>30%</b>	P166; 26%	No homology
Tail tube protein	ORF005	ORF030; 20%	OBP_001; <b>44%</b>	201phi2-1p32; 21%	P12; 21%	SPN3US_0255; 19%

protein core stack (consisting of gp14/gp15/gp16 proteins) inside their capsid, which plays an important role in DNA packaging and is involved in genome ejection; it is located within the phage head in tight contact with the portal (Agirrezabala et al., 2005; Cheng et al., 2014; Guo et al., 2013). Unlike this much smaller core stack, the inner body of the phiKZ is long (spanning the whole giant capsid wall-to-wall). The helical structure of the inner body was first discovered more than three decades ago after it was extracted from a phiKZ capsid by using freeze-thawing (Krylov et al., 1984, 1978). The presence of an inner body in the phiKZ head was confirmed later using atomic force microscopy after the DNase treatment of phages (Matsko et al., 2001) and just recently by cryo-electron microscopy (cryo-EM) under low- and high-dose irradiation, followed by a sophisticated image reconstruction procedure, which revealed a cylinder with multiple tiers (Wu et al., 2012). The estimated mass of the phiKZ inner body is 15–20 MDa. Proteomic experiments on the tailless phiKZ mutant (Thomas et al., 2012) revealed six internal proteins associated with the inner body.

The overall structure of the phiKZ bacteriophage has been extensively studied using cryo-EM (Fokine et al., 2007, 2005). The 3D structures of two essential components of phiKZ: a head and a tail were determined at a resolution of 18 Å and 28 Å, respectively. Single particle analysis revealed that the phiKZ virion consists of a 145 nm in diameter icosahedral head and a 200 nm long contractile tail. The helical parameters of the contractile sheaths, surrounding the tail tubes, are comparable to that of T4. Structural information on other phiKZ-like phages is limited and originates from the EM study of negative stained particles (Hertveldt et al., 2005).

The genomes of only two species: phiKZ and EL, have so far been sequenced and deposited to NCBI (Hertveldt et al., 2005; Lecoutere et al., 2009). Though phages phiKZ and EL exhibit no apparent homology at the DNA level (Burkal'tseva et al., 2002; Krylov et al., 2007), they possess an almost identical number of structural proteins (62 and 64 for phiKZ and EL, respectively), at least 30 of which are related to the structure of the head. Twenty-six head proteins of phage EL, however, do not show any sequence similarity to those of phiKZ (Lecoutere et al., 2009). Due to the uniqueness of their structural proteins, EL phages were thought to possibly represent a separate genus (Lavigne et al., 2009; Lecoutere et al., 2009). The full EL genome appeared to be significantly smaller (211 kbp), than the phiKZ genome (280 kbp) (Krylov et al., 2007; Mesyanzhinov et al., 2002). The DNA of EL is likely packaged using the headful mechanism. Assuming that the internal volume of the EL capsid is the same as that of phiKZ (Fokine et al., 2005; Hertveldt et al., 2005), there must be substantial terminal redundancy to fill the additional volume of the head. However, this feature was not detected (Shaburova et al., 2008). Therefore, it was unclear how the smaller genome of the EL phage is packed inside the large head. A detection of the inner body in EL and the study of the possible properties of its structure could explain this discrepancy and will provide more compelling

evidence of a phylogenetic relationship between EL and other phiKZ-like phages.

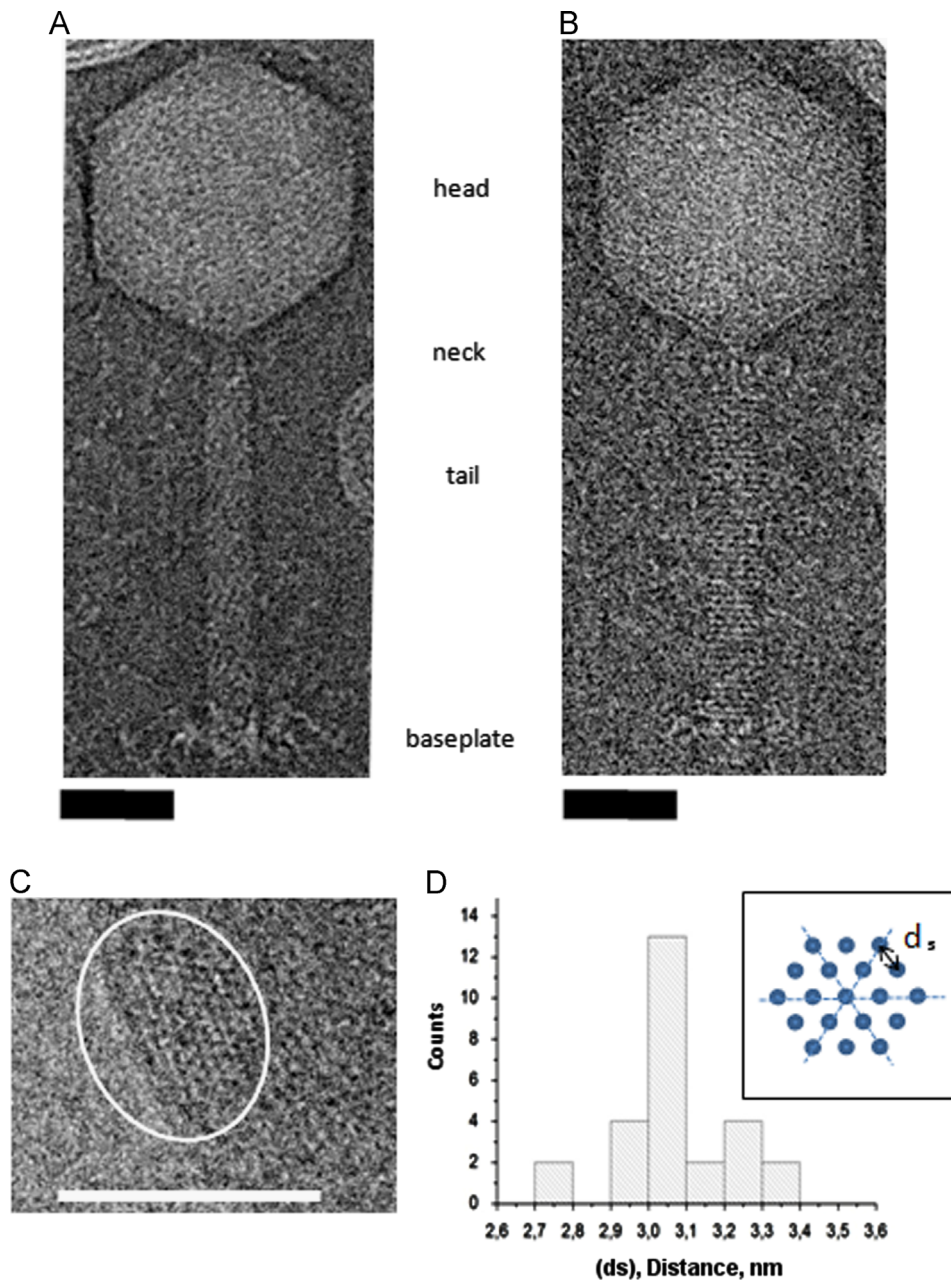
Thus, the purpose of the study was to confirm (or disprove) the presence of an inner body in phiKZ-like phages. For this purpose we studied and compared the structures of phiKZ, EL and Lin68 capsids using cryo-EM under low-dose and high-dose conditions.

## Results and discussion

When bacteriophage EL was first studied in negative stain EM (Hertveldt et al., 2005), its head was measured to be 140 nm in diameter. Since large capsids may be distorted by negative stain, here we used cryo-EM under low-dose conditions to obtain the first cryo-images of full-length EL particles (Fig. 1A). The cryo-conditions allow the protein particles to preserve their native conformations. A careful examination under cryo-conditions revealed that the EL capsid is 145 nm wide along its 5-fold axis, analogous to the phiKZ capsid (Fig. 1B) (Fokine et al., 2005). The capsid is connected to the tail by a neck. The neck of the EL phage is much thinner than that of the phiKZ and contains no collar. The EL tail is about 200 nm long, 22 nm in diameter and contains a baseplate on its basal end. From our cryo-EM examinations it is apparent that EL and phiKZ phages have similar head and tail sizes. The EL baseplate is a little smaller (about 60 nm in diameter and 20 nm thick), than the phiKZ baseplate (Fokine et al., 2007). Hexagonally packed DNA strands were clearly visible inside the mature EL capsid (Fig. 1C). The distance ( $d_s$ ) between the centers of separate DNA strands has been measured using ImageJ (Schneider et al., 2012) and was found to be  $3.09 \pm 0.19$  nm (Fig. 1D), which is slightly more than the 2.8 nm measured previously in phiKZ (Fokine et al., 2007).

Using high-electron dose imaging, we for the first time demonstrated that the capsids of phiKZ-like phages EL and Lin68 contain inner bodies. For this aim, phage particles, frozen in vitreous ice, were irradiated with increasing doses of electrons. High-energy electron bombardment of the sample causes obvious radiation damage to the specimen, resulting in selective boiling and protein degradation in those areas that are in tight contact with the DNA (Conway et al., 1993; Fujiyoshi, 1989). Fig. 2 illustrates this process. In Fig. 2A a raw image of frozen EL particles (giant phage, marked EL) mixed with particles of an unrelated SN phage (smaller phage, marked SN) after a 30 s exposure is presented. When we exposed the same region of the grid for an additional 30 s, thus increasing the electron dose, we clearly saw a characteristic Cylindrical Boiling Pattern (CBP) inside the EL heads (Fig. 2B, white arrows), but not in the control SN phage heads (Fig. 2B, black arrows). The SN heads boiled at specific points, presumably at the tops of capsomeres, similar to HSV-1 capsids (Conway et al., 1993), but their boiling never resembled the characteristic cylindrical form.

Similar to EL, in the heads of phiKZ and Lin68 the CBP were clearly visible after exposure to a high electron dose (Fig. 2C and D). As it was demonstrated before, the direction of selective boiling



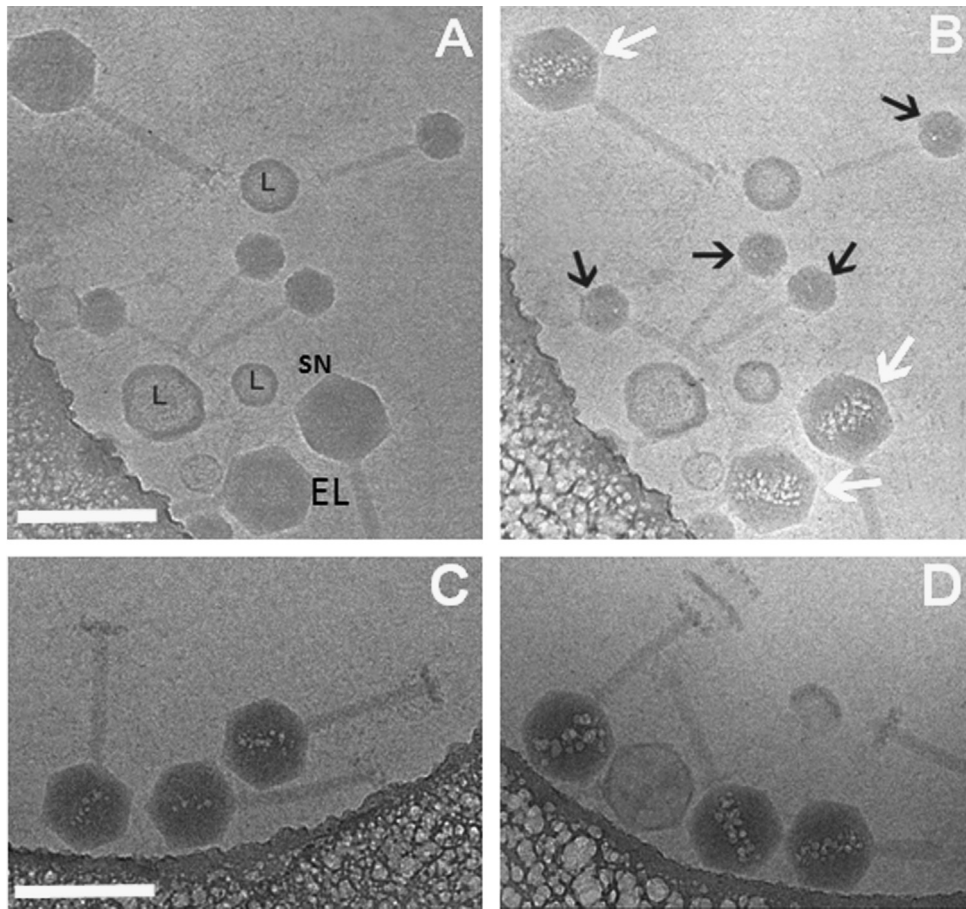
**Fig. 1.** Structure of EL and phiKZ bacteriophages, as revealed by cryoEM. (A) Projection structure of the EL particle with an extended tail, frozen in vitreous ice, visualized under low-dose conditions; contrast is inverted; (B) Projection structure of a phiKZ particle, frozen in vitreous ice, visualized under low-dose conditions; contrast is inverted. Image courtesy of A. Fokine (Purdue University), Bar—50 nm; (C) Hexagonal DNA packaging within the EL capsid is marked with white ellipse. Bar—50 nm; (D) The distribution of the distances ( $d_s$ ) between DNA strands in nm ( $N=28$ ). Inset—diagram of the hexagonal DNA packaging inside the capsid.  $d_s$ —distance between DNA strands.

generally followed the long axis of the inner body (Wu et al., 2012). Thus, we used the CBP as a guide line to estimate the location and dimensions of inner bodies. Bubbling depended on the electron dose, thus we applied the same radiation to all phages.

To increase the signal-to-noise ratio, we cumulated the aligned images of phage heads with CBP, and the resulting class-sum images (Fig. 3) revealed that the CBP in phage heads can be characterized by specific size, shape and positions, which vary significantly in phiKZ, Lin68, and EL. In phiKZ the CBP resembled a characteristic ‘sandglass’ shape, found in a previous cryo-EM study (Wu et al., 2012). It should be noted, that the position of the inner body inside the capsid is strictly fixed, since otherwise it would not be possible to obtain the summarized image from the few dozens of individual particles. The fixed arrangement of the inner body allows to obtain its 3D reconstruction using the single

particle approach (Wu et al., 2012). Consequently, the overall 2D image of the CBP can be used to adequately estimate the size of the inner body. The average 2D dimensions of the observed phiKZ CBP in our experiments were  $98 \pm 1$  nm long and  $31 \pm 5$  nm wide, which were consistent with the 2D dimensions of the recently published 3D structure of the phiKZ inner body ( $\sim 105$  nm long  $\times$  24 nm wide) (Wu et al., 2012). The phiKZ CBP (Fig. 3A) often showed a tilt of about  $22^\circ$  relative to the portal axis. This incline confirms that the inner body is anchored between two hexons, that lie on opposite edges of the icosahedron (Wu et al., 2012).

The EL CBP is strikingly different: it possesses a much higher tilt angle (Fig. 3C) and uniform appearance. We never observed a  $22^\circ$  tilt of the EL CBP, but did see higher-tilt rotations ( $60^\circ$ ) relative to the portal axis; sometimes we detected a circular boiling mark



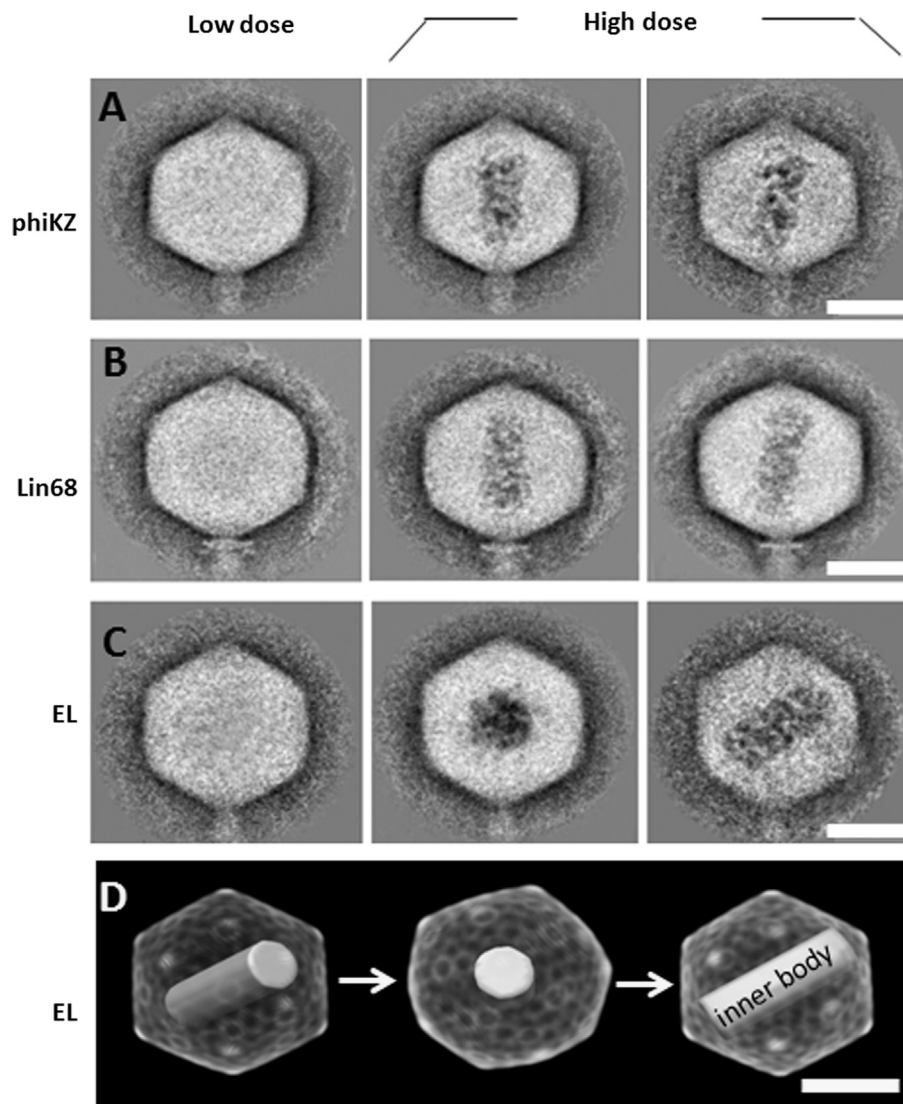
**Fig. 2.** Cylindrical boiling patterns (CBP) reveal the presence of an inner body inside the capsids of phiKZ-like phages, but not of phage SN. The mixture of phage EL (large particles) and phage SN (smaller particles) (A) at low electron dose conditions; (B) the same mixture with a higher electron dose ( $\sim 50e/\text{Å}^2 \text{ s}$ ). White arrows are pointing to the capsids of phage EL, black arrows are pointing to the capsids of phage SN. L—liposomes. Bar—200 nm; (C) CBP in phiKZ phages at a high electron dose; (D) CBP in Lin68 phages at a high electron dose. Bar—00 nm.

in the center of the EL capsid (we interpreted it as the  $90^\circ$  rotation of the inner body). This suggests, that in contrast to phiKZ, the EL inner body is anchored at two opposite vertexes of the icosahedron. The 2D projections of the EL CBP were much wider ( $\sim 43 \text{ nm}$ ), compared to phiKZ and slightly shorter ( $\sim 90 \text{ nm}$ ). Such morphological differences may contribute to the considerable differences in DNA packaging between EL and phiKZ phages. It is known that the packaging of DNA concludes when it reaches a certain density of the DNA in the capsid. We speculated that the inner body, if it has a larger volume, will leave less room inside the head for the DNA (Table 2), resulting in smaller genome of EL (Krylov et al., 2007; Mesyanzhinov et al., 2002) to be packaged inside the giant (145 nm across) capsid.

To address this hypothesis we looked at another giant phage, Lin68 (Fig. 3B). Lin68 has a common ancestor with phiKZ-like phages (Krylov et al., 2004). The genome length and DNA sizes of Lin68 and phiKZ are similar (approximately 280 kbp under restriction analysis (Hertveldt et al., 2005; Mesyanzhinov et al., 2002)) and detectable homology in two of HindIII restriction fragments, according to DNA–DNA hybridization (Krylov et al., 2004). We studied Lin68 in a cryo-electron microscope under the same low- and high-dose conditions and found that the overall dimensions of the Lin68 head were the same as that of phiKZ/EL (Fig. 3). On the other hand, the overall dimensions and position of the CBP in Lin68 were the same as in phiKZ, but not EL. This is in agreement with our speculation that the phage genome size correlates with the overall dimensions of the inner body.

The looser distance between DNA strands inside the capsid may be a separate way to package the smaller genome into the large capsid. This is supported by previous observations of the DNA packaging inside HCMV and HSV1-B viruses, lacking the inner core (Bhella et al., 2000; Butcher et al., 1998). According to existing models of DNA packaging in bacteriophage capsids (Petrov and Harvey, 2008; Purohit et al., 2005), the DNA is assumed to be akin flexible, but non-stretchable cylindrical rod packaged with local hexagonal coordination, this implies that the total length of the DNA ( $L$ ), which can be accommodated by a capsid with free volume ( $V$ ), should depend on the distance between DNA strands ( $d_s$ ) as  $L \sim V/d_s^2$ . According to Purohit et al. (2005), the genome packaging efficiency values ( $\rho_{\text{pack}} = (\Omega_{\text{genome}}/\Omega_{\text{capsid}})$ ) for most phages lie in the range of 0.3–0.4. Here we estimated packaging efficiency values for EL and phiKZ (Table 2) and revealed that (i) both values lie in this range, and (ii)  $\rho_{\text{pack}}$  for EL is 1.2 times smaller, comparing to the  $\rho_{\text{pack}}$  for phiKZ. This clearly reflects the observed differences in the inter DNA distances ( $d_s$ ): in our cryo-EM study of the EL phage,  $d_s = 3.09 \pm 0.19 \text{ nm}$  (Fig. 1D), while the distance previously observed in cryo-EM experiments of Fokine et al. (2007) for phiKZ was less:  $d_s = 2.8 \text{ nm}$  (Table 2).

Thus, there is a unique structure within the EL that could organize the DNA to give a headful signal, and, most likely, a procapsid expansion, with less DNA in the EL capsid, compared to phiKZ and Lin68, which organize the longer DNA into a more compact state. Most likely, not only the inner body, but some additional factors may be required to organize such a specific DNA



**Fig. 3.** The positions of the inner body differ in giant phages of different species. Class-sum images of aligned phage heads before and after the high-dose irradiation: (A) phiKZ; (B) Lin68; (C) EL. Vertical lanes: left—low dose; middle—the class-sum of the same aligned particles, as in the left lane, after a high electron dose irradiation, right—another high dose class-sum. (D) The 3D icosahedral reconstruction of the EL capsid with a modeled inner body (depicted as a gray cylinder) inside, schematically illustrating possible orientations of inner body. Two orientations coincide with the CBP projections in the row above, while the first orientation is strictly hypothetical. Scale bar—50 nm.

**Table 2**  
Comparison of icosahedron geometry and DNA packaging in phiKZ and EL bacteriophages.

Dimensions of a phage head and parameters for genome packing	phiKZ	EL
Head height, nm	145.5	145.0
Wall thickness, nm	5 (Fokine et al., 2005)	5
Head inner volume, nm <sup>3</sup> ( $V_i$ )	744,676	736,303
Inner body volume, nm <sup>3</sup> ( $V_{ib}$ )	47,520 (Wu et al., 2012)	93,876
Empty head volume, nm <sup>3</sup> ( $V_h = V_i - V_{ib}$ )	697,156	642,427
Genome size ( $L$ ), kbp	280	211
Distance between DNA strands ( $d_s$ ), nm	2.8 (Fokine et al., 2005)	$3.09 \pm 0.19$
Packaging efficiency ( $\rho_{pack}$ ) (Purohit et al., 2005)	0.428	0.351
Ratio of $V_{h-phiKZ}/V_{h-EL}$		1.1
Ratio of $L_{phiKZ}/L_{EL}$		1.3

packaging. In many bacteriophages the DNA packaging depends on their portal structure (Johnson and Chiu, 2007; Tavares et al., 2012). In the future it would be interesting to study the structure

of the portal vertices of EL, and compare it to the ones of phiKZ, and other related phages.

In conclusion, the phage EL, although differing significantly from the phages of species phiKZ and Lin68 by its complete absence of DNA homology, alterations in genome size and structural proteins, possess an important common structural feature, the inner body, which is definite evidence for a basic relationship of phiKZ and EL. The other common essential features of phiKZ and EL, namely: identical overall morphology, lack the known DNA polymerases and the ability to induce a pseudodysogenic state in infected host bacteria, indicate that EL is to be considered, as earlier postulated (Krylov and Zhazykov, 1978), as a member of the genus of 'phiKZ-like' phages. Unique features of other phiKZ-like phages certainly require the continuation of their structural studies.

## Materials and methods

The *P. aeruginosa* PAO1—host strain was obtained from Prof. Bruce Holloway, Australia and grown on a LB agar medium, as described elsewhere (Sambrook and Russell, 2001).

## Bacteriophages

Phage phiKZ originated from the collection of the Laboratory for Bacteriophages Genetics (Mechnikov Research Institute of Vaccines and Sera, RAMS, Russia). Phage EL has been isolated from a natural source in the same Laboratory. Phage Lin68 is part of the Lindberg typing set and was received from Félix d'Hérelle Reference Center for Bacterial Viruses (Quebec, Canada). Phage SN, a member of the *Myoviridae* genus PB1-like phages, active on *P. aeruginosa*, was a gift from Dr. N. Sykilinda (Institute of Bioorganic Chemistry RAS, Russia). Phage particles were propagated on the *P. aeruginosa* PAO1 strain by overnight incubation at 37 °C in semi-solid LB agar lawn on the surface of solid LB agar in Petri dishes (Adams and Anderson, 1959) and purified in a cesium chloride density gradient, as described elsewhere (Sambrook and Russell, 2001).

## Cryo-electron microscopy

Purified phage particles were quickly frozen in liquid ethane, using a MarkIV Vitrobot (FEI, Netherlands) and 1.2/1.3 Quantifoil grids (Quantifoil, Germany). Frozen EL particles were studied on a Titan Krios (FEI, Netherlands) microscope. Lin68 and phiKZ particles were studied and the Cylindrical Boiling Patterns (CBP) were generated on the Tecnai G2 Spirit Twin (FEI, Netherlands) electron microscope. All microscopes were functioning in the low dose mode (10–20 e/A<sup>2</sup>). Images were captured with 4 × 4 K FEI Eagle CCD cameras. Magnification of microscopes was calibrated using gold shadowed carbon Cross Grating Replica (SPI, USA). Images were taken at magnifications of 33,000 (Titan Krios) and 26,000 (Tecnai G12 Spirit). The pixel sizes were 2.68 Å for EL and 4.24 Å for Lin68 and phiKZ containing images, respectively. Defocus values were in the range of 2.5–3.5 µm.

## High-dose EM study

To visualize the inner body, we used repetitive irradiation of the sample with an electron beam. The irradiation dose, estimated by the Tecnai G2 Spirit Twin build-in software, was about 10–20 e/A<sup>2</sup> under low dose conditions, and after 2–3 irradiations of the same hole it reaches 40–60 e/A<sup>2</sup> (high-dose conditions).

To minimize CPU time for data processing, the image size was binned 2 times, resulting in a pixel size of 5.36 Å for EL and 8.48 Å for Lin68 and phiKZ. Using the program BOXER (Ludtke et al., 1999) program, 469 single particles for EL, 512 single particles for Lin68 and 315 single particles for phiKZ, bearing CBP were manually selected from the high-dose cryo-EM images, and their coordinates were recorded. The corresponded particles were automatically selected from the low-dose images, using the recorded coordinates in BOXER (Ludtke et al., 1999).

Thereby, two sets of corresponded particles for the same phage head were collected: at low dose and at high dose conditions, with visible CBP. All high dose images were first centered, rotationally aligned several times against their total sum, classified, similar images of particles with the same positioning of CBP ( $N \geq 15$ ) were cumulated to produce 15–20 class-averages using IMAGIC5 (van Heel et al., 1996). The corresponding low-dose images were centered, rotated to the same angle and cumulated in IMAGIC5 to produce class-averages without visible CBP.

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