

Minireview

Encapsulation and transfer of phage DNA into host cells: From in vivo to single particles studies

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Abstract

A remarkable property of bacteriophages is their capacity to encapsidate large amounts of DNA during morphogenesis and to maintain their genome in the capsid in a very stable form even under extreme conditions. Even as remarkable is the efficiency with which their genome is ejected from the phage particle and transferred into the host bacteria. Biophysical techniques have led to significant progresses in characterizing these mechanisms. The molecular motor of encapsidation of several phages as well as the organization of viral capsids have been described at atomic resolution. Cryo-electron microscopy and fluorescence microscopy have permitted to describe DNA ejection at the level of single phage particles. Theoretical models of encapsidation and ejection have been proposed that can be confronted to experimental data. This review will present the state of the art on the recent advances brought by biophysics in this field. Reference will be given to the work performed on double-stranded DNA phages and on one of its representative, phage T5, our working model.

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A remarkable property of bacteriophages is their capacity to encapsidate large amounts of DNA during morphogenesis and to maintain their genome in the capsid in a very stable form even under extreme conditions for some of them (pH as low as 1, temperature as high as 80 °C and few molar salt) [1]. Even as remarkable is the efficiency with which their genome is ejected from the phage particle and transferred into the host bacteria. If molecular biology and genetics have been essential tools to understand the different stages of infection, physical techniques have led, during the last decade, to remarkable progresses in characterizing the mechanisms of DNA encapsidation and ejection. This review focuses on recent advances in this field and mainly on biophysical approaches. We will present the state of the art with reference to the work performed on double-stranded DNA (dsDNA) phages (*Caudovirales*) which represent the

largest group of bacteriophages [2] and on one of its representative, phage T5, our working model.

1. Phage DNA encapsidation

Encapsidation of the dsDNA of phages starts with the assembly of a proteinaceous shell or procapsid. Its maturation is accompanied by the transport of the phage genome. DNA encapsidation relies on a molecular machine consisting of two protein complexes: the portal and the terminase. The portal protein is located at one of the vertices of the capsid and forms the channel through which the DNA is encapsidated and ejected upon infection. The terminase which is transiently docked at the portal vertex provides the driving force for DNA translocation. Upon termination of DNA packaging, the portal channel closes and the phage tail which is preassembled separately – at least for phages with long tails – binds to the capsid to form the mature phage particle [3,4]. Some of the features of these assembly

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processes, including the DNA packaging process and the machinery of encapsidation, are common to many dsDNA phages. Significant advances in characterizing phage assembly were made possible by using biophysical techniques among which cryo-electron microscopy (cryo-EM) associated with image reconstruction methods, X-ray crystallography and optical tweezers.

1.1. Capsid assembly

Capsid assembly is a multistep process that shares common features with the assembly of several eukaryotic viruses. Studies on the pathways of assembly have benefited from the development of *in vitro* packaging systems. The ϕ 29 *Bacillus subtilis* phage is the first among the dsDNA phages for which the pathway of assembly of the capsid was described by cryo-EM [5]. Image reconstructions based on the icosahedral symmetry of the phages [6] have allowed the three-dimensional reconstruction of the viral capsid at different stages of its maturation. The head–tail connector (comprising the portal protein and additional components) could be visualized for the first time. Conformational changes underwent by the viral particle upon ejection of the DNA could also be observed. Combining X-ray diffraction with cryo-EM images reconstruction has permitted to reveal the structural transitions underwent by phage HK97 from the different metastable intermediate states of maturation to the mature capsid [7,8]. The mature capsid must be stable enough to resist the pressure imposed by the confinement of the DNA (see below). Such stabilization is brought in the case of phage HK97 by important conformational changes of the surface proteins and formation of covalent crosslinks between adjacent proteins [9]. Furthermore, the inner surface of the mature capsid becomes negatively charged so that there is no electrostatic attraction with the DNA to resist its ejection upon infection.

The recent sequencing of the dsDNA of phage T5 (121750 bp) (Genbank accession numbers: AY692264, AY734508 and AY543070) [10] makes it an opportunity to identify the structural proteins involved in the assembly of its capsid. These proteins are encoded by the late genes [11]. Genome analysis combined with SDS-PAGE, N-terminal sequencing and mass spectrometry allowed assigning the structural proteins to the corresponding genes (Boulanger et al., submitted for publication). The two major head proteins (pb8 and pb10), the prohead protease (pb11) and the portal protein (pb7) were identified. The N-terminal regions of pb7, pb8 and pb11 are removed by proteolytic cleavage suggesting that the maturation pathway of phage T5 shares similarities with that of other dsDNA phages [12,13]. Apart from these essential proteins, an accessory “hoc-like” protein was also identified that might contribute to the stabilization of the capsid as recently established in the case of phage T4 [14]. The 3D-structure of the DNA-filled capsid of phage T5 was recently determined by reconstruction of Cryo-EM images. The capsid resembles

that of other dsDNA phages and shows local densities that could correspond to the accessory proteins found in T4 and other phages (J. Conway et al., to be published).

Image reconstructions are extremely powerful in obtaining structural information at high resolution especially for objects like viral capsids that present an icosahedral symmetry. For viruses that contain asymmetric elements, less structural information has been obtained from cryo-EM, but recent cryo-electron tomography studies have allowed obtaining a high-resolution 3D picture of the complex organization of the herpes virus capsid at near molecular resolution [15].

1.2. Structure of the molecular motor of DNA encapsidation

The portal and terminase proteins are the essential components of the molecular motor of encapsidation. Typically, such motors permit the confinement of the 41- μ m-long dsDNA of phage T5 into an icosahedral capsid 90 nm in diameter and rates of *in vitro* DNA packaging can reach values of 140 bp/s [3]. ATP hydrolysis provides the driving force and it was estimated that 1 ATP molecule is hydrolyzed per 2 base pairs packaged [13]. Portal and terminase proteins together with additional components which may differ from phage to phage (head completion proteins which plug the portal channel to avoid DNA release, prohead RNA—pRNA...) form the connector. Portal proteins from different phages show little sequence homology but the structures determined so far suggest a common structural organization. The structure of the phage ϕ 29 connector was determined at atomic resolution by X-ray crystallography [16,17]. It consists of a 12-subunit ring with a central channel whose narrowest part is ~ 36 Å in diameter, large enough for a double-stranded DNA in its B-form (23 Å) to pass through. The wall of the connector is mainly negatively charged which prevents interactions with the DNA. To characterize how the connector fits into the phage head, Simpson et al. generated a three-dimensional reconstruction of the procapsid from cryo-electron microscopy data and then fit the crystal structure into the electron density. The result shows that the narrow part of the connector protrudes outside of the viral shell and interacts with a phage-encoded RNA and possibly the terminase.

The structure of the SPP1 connector comprising the portal protein gp6 and the head completion proteins gp15 and gp16 was recently determined at 10 Å resolution using cryo-EM and single particle analysis [18]. Comparison of this structure to that of the isolated portal protein highlighted the reorganization taking place in the portal once associated with the completion proteins. The connector channel traverses the full height of gp6 and gp15, but it is closed by gp16 at the bottom of the complex. Gp16 acts as a valve whose closure prevents DNA leakage while its opening is required for DNA release upon interaction of the virus with its host.

The structures of the portal proteins determined so far (see also [19,20] for phage T3 and T7, respectively) suggest

a common organization with conservation of the structural elements lining the central DNA channel and similar dimensions. The general spinning model earlier proposed [21] and described for phage phi 29 and SPP1 [16,17,22] may therefore be extended to other phages. In this model, ATP would drive the rotation of the connector step by step. Each rotation would translate two DNA base pairs into the capsid. In the case of phage phi29, a complex formed by six molecules of a virus-encoded RNA or pRNA would form a structure that appears to be involved in DNA translocation by ensuring the continuous function of the connector motor [23].

Sequencing of the T5 genome associated to N-terminal sequencing of the structural proteins has allowed identifying pb7 (MM: 44 kDa) as the portal protein (Boulanger et al., to be submitted). Except for its oligomeric state that was previously estimated to be 12-mer [11], pb7 shows little homology with other portal proteins. Located next to the portal gene are the two subunits of the terminase. Work has been undertaken to characterize these proteins.

1.3. Role of the terminases in DNA packaging

The dsDNA of phages is generally replicated as large concatemers of individual genomes that are linked head to tail through terminally redundant sequences. This concatemeric DNA must be resolved into unit-length genomes during packaging into the procapsid. This process is mediated by the terminases that ensure multiple functions: they recognize a specific sequence on the concatemeric DNA, cut it to generate the end that has to enter first in the procapsid, link this end to the procapsid at the portal vertex, catalyze the ATP-dependent translocation of the DNA into the capsid and finally perform the endonucleolytic cleavage of the concatemeric DNA. This cut is either sequence specific (e.g., for phage lambda and T7) or sequence independent in cases of phages that package DNA by a headful mechanism (e.g., T4, SPP1...) [13]. The terminase and the unpackaged DNA then dissociate from the capsid and associate with another empty procapsid to perform another cycle of encapsidation (reviewed in [24]). Most of the terminases identified so far are hetero-oligomers consisting of two subunits: a small regulatory subunit and a large catalytic subunit involved in binding and ATP-dependent translocation of the DNA [25,26]. The latter possesses an ATPase activity in its N-terminus with characteristic Walker A and Walker B Mg^{2+} -ATP-binding motifs [27]. On the contrary, the C-terminal region of the catalytic subunit remains poorly defined. The endonuclease activity of several phage terminases was recently characterized [28–30] but these terminases share neither a sequence motif nor any sequence similarity. The sequence divergence in the C-terminal region allowed neither a clear delimitation of the endonuclease domain nor a definition of its active site. The presence of newly sequenced terminases prompted us to search for new sequence–structure–function relationships.

The T5 genome encodes a putative 49 kDa large subunit terminase that was used as a representative member of this family together with those of other bacteriophages. A bioinformatic analysis of the terminase sequences was performed in order to delimitate and characterize the regions carrying the ATPase and endonuclease activity [31]. As expected, fold-compatibility of the terminases of phages SPP1, T4 and T5 highlighted the strong similarities of their N-terminal half sequence to ATP-dependent helicases and related ATPases. Molecular modelling using MODELLER [32] of the T5 terminase further suggested that the ~160 residues of the N-terminal domain could form a stable and functional ATP-binding domain, autonomously. Fold-compatibility searches performed on the C-terminal region from distinct terminases using the meta-server @TOME [33] suggested distant relationships with various structures among which some endonucleases. Refinement of sequence–structure alignments led to a specific sequence signature of the terminases with three aspartate residues very well conserved [31]. These residues are equivalent to those of the Holliday junction resolvase (PDB:1HJR) which form a catalytic triad and chelate the essential divalent cation (Mg^{2+}). The validity of this proposal was attested by showing that the *in vivo* and *in vitro* endonuclease activity of the T5 terminase was abolished by mutating each of the aspartate [31]. This bio-informatic analysis supports a model in which the large catalytic subunits of the terminases are composed of two functional domains. The precise interplay between the domains of the catalytic subunit and between this subunit and the large regulatory subunit is critical for regulation of the packaging process. Indeed packaging requires successively the activation of the endonuclease for DNA cutting, the arrest of endonuclease activity during ATP-dependent DNA translocation and finally the reactivation of the endonuclease activity following completion of translocation. A model of functioning of phage T4 packaging motor was recently proposed that gives a possible vision of the interplay between the different domains [29].

1.4. Dynamic of DNA encapsidation

Phage capsids are able to support very high concentration of DNA. Although the length of the DNA strongly varies from phage to phage – ranging from 4 kbp to 640 kbp – its concentration within the capsid remains relatively constant – about 450 mg/ml – [34]. The structure of DNA within the capsid has been studied using a variety of techniques leading to different models of organization (reviewed in [13]). Cryo-EM images of isolated capsids from phage T7 [35] and phage T4 [14,36] showed that the DNA strands adopt a quasi-crystalline structure and are organized as a spool in concentric rings separated by 25 Å, a value in good agreement with the inter-helix distance as determined by X-ray diffraction [13]. Such concentric layers of DNA have also been observed in the case of phage T5 (J. Conway, A. Le Forestier, personal communications). The confinement of

DNA into the capsid, the electrostatic repulsion between the highly charged DNA chain, its folding in dimensions comparable to its persistence length and the important reduction of entropy involved in this packaging are at the origin of the strong energy stored in the capsid. The forces required to package the DNA were estimated theoretically [37,38] and experimentally during *in vitro* encapsidation of the phi29 genome [39]. Using laser tweezers they measured the rate of packaging of the 19-kbp phi29 genome and the force generated by the encapsidation motor throughout the entire packing process. At maximum, the motor pulled with about 60 piconewtons of force. By dividing this force by the DNA hexagonal cell surface area, they estimated the pressure inside the capsid to be roughly 6 MPa (60 Atmospheres), which represents about ten times the pressure inside a bottle of champagne! The idea that the pressure inside the capsid was responsible for DNA ejection was proposed a long time ago [34,40]. Smith's experiments have stimulated new debates in which theory and experiments are brought face to face. The state of the debate will be presented below.

2. Phage DNA ejection: from *in vivo* to *in vitro*

2.1. *In vivo* strategies of phage DNA transport

A common feature of almost all dsDNA phages is that only their genome is transferred to the host cytoplasm while the phage capsid and tail remain bound to the cell surface. The naked hydrophilic DNA molecule has thus to overcome two hydrophobic barriers: the outer and the inner membrane. Furthermore, it has to cross the periplasm which contains nucleases and the rigid polymeric sugar structure of the peptidoglycan. To accomplish this task, phages first bind to a specific host membrane receptor via their tail tip. This binding leads to conformational changes that are transmitted to the connector triggering its opening and the ejection of the DNA which is transferred linearly base pair after base pair through the bacterial envelope to the cytoplasm (recently reviewed in [41,42]). If phage binding and ejection follow the same general scheme, the strategies used to transfer their genome vary from phage to phage. For phage T7, such transfer is initiated by an enzyme-like mechanism independently of transcription [43] and then coupled to transcription (reviewed in [44]). For others (λ , T4, phi29), injection of the DNA appears to proceed independently of transcription [42,45]. Phage T5 is unique in that its genome is transferred in two steps: 8% of the DNA (First Step Transfer or FST DNA) enters first the cytoplasm. Then, there is a pause of about 4 min during which proteins encoded by this fragment are synthesized. Two of these proteins (A1 and A2) are required for the transfer of the remaining DNA (92%) or second step transfer (SST) DNA [46]. Not only are the mechanisms of DNA transfer into host bacteria different, but they also differ in their energy

requirement (reviewed in [42]). It is thus obvious that DNA transfer cannot be simply explained by a “pressure effect”. In an attempt to learn more about these complex processes, several *in vitro* models of DNA ejection were designed.

2.2. *In vitro* DNA ejection: from theory to experiments

Recent theoretical models were developed that allowed calculating the forces and pressures resulting from the packaging of the DNA in a capsid [38,47,48]. It was estimated that the force to load a DNA strand into a capsid should increase from a few pN to tens of pN as the final 20% of the genome is packaged; these values being in good agreement with the experimental values from Smith et al. (see above). They also calculated that the pressure acting on the capsid rises steeply from a few atmospheres to ten of atmospheres in the final 20% of loading. It was also proposed that the initial ejection process would be driven by the pressure difference inside and outside the capsid. The energy stored in the capsid should decrease monotonically with the amount of DNA ejected. In agreement with this proposal, it has been shown that the extent of *in vitro* ejection of phage lambda DNA can be controlled by varying the osmotic pressure outside the capsid [49,50]. Although not contradicting the “pressure model”, the experiments described below highlight its limitation.

2.3. Phage T5 DNA ejection and transfer into liposomes

This *E. coli* phage T5 consists of an icosahedric capsid 90 nm in diameter (larger than those of most of tailed phages – around 66 nm for lambda, P22 or SPP1 – [3]) containing a dsDNA of 121,750 bp, a long flexible non-contractile tail, 160 nm in length that ends with three long fibres and a straight fibre that ensure the binding of the phage to the host envelope [46]. An attractive feature of T5 is that DNA release from the phage can be triggered *in vitro* simply by interaction of the virus with its purified membrane protein receptor FhuA solubilized in detergent [51]. DNA release was demonstrated spectroscopically using a fluorescent DNA intercalant YO-PRO-1 the fluorescence of which is increased in proportion to the amount of DNA freed from the capsid. We further demonstrated that the phage genome could be delivered, at least in part, into unilamellar liposomes (100 nm in diameter) in which FhuA was reconstituted [52]. The high resolution of cryo-electron microscopy enabled the visualization of the entrapped DNA [53]. It filled the whole liposome, showed no ordering and did not perturb the morphology of the vesicle although its concentration was estimated to reach 130 mg/ml. The amount of entrapped DNA decreased with the volume of the vesicle an observation consistent with a passive transport mechanism: DNA transfer should stop upon equilibrium of the osmotic pressure in the phage capsid and in the liposome.

We further raised the question whether spermine would permit to increase the amount of entrapped DNA. This polycation is known to neutralize the charges of the polyanionic DNA and its addition to aqueous solution of DNA led to the formation of toroidal structures resembling those found in phage capsids [54]. Remarkably, spermine allowed many DNA strands to be transferred in a liposome (up to 6 to 10). They formed a single large and ordered toroidal structure that showed no contact with the liposome whatever the number of DNA strands transferred in the vesicle [55]. Cryo-electron tomography associated with image reconstructions further improved the resolution of the 2D electron micrographs and allowed to visualize the phage–proteoliposome interactions at a molecular level [56]. In particular, we observed that pb2, the protein forming the straight tail fiber and located at the distal end of the phage particle, was visible inside the vesicle. This, together with previous data showing that pb2 is the sole protein found in the bacterial envelope upon DNA transfer [42,57], strongly supports the idea that this protein could form the channel through which the DNA could traverse the liposome and the bacterial envelope.

2.4. DNA ejection as studied by fluorescence microscopy on single phage particles

We recently reported a fluorescence microscopy study that allowed real time investigating of DNA ejection from single phage particles [58]. T5 phage particles were adsorbed onto a microfluidic cell. DNA ejection was triggered by perfusion of the chamber with the DNA intercalating dye YO-PRO-1 and the FhuA receptor and the length of the ejected DNA was measured after being stretching in a hydrodynamic flow. Time-resolved observations allowed concluding that the process of DNA ejection is unexpectedly complex. Some phages released their whole genome in one step without pausing soon after receptor binding. For the others, ejection proceeded in a stepwise manner and at a rate of roughly 75,000 bp/s. Furthermore, ejection was arrested at defined positions of the genome that correlate with the locations of single-stranded interruptions on the DNA suggesting that they could be involved in the arrests. We proposed that the T5 nicks involve particular arrangements of the DNA which represent some kind of energetic barrier to the ejection process. The flexibility introduced by the nick may result in the buckling of the DNA during its passage through the inside of the connector or of the tail. As a consequence, the translocation would be inhibited unless thermal motion lifts the DNA strand into a parallel orientation with respect to the tail. We further analyzed our data in terms of the capsid internal pressure. If T5 DNA ejection is indeed dependent on the difference between the inside and outside pressure then different scenario may occur. Full ejection would occur for those phages whose DNA is not stopped at the nicks: this would correspond to the DNA found free to move in the chamber.

For the other phages, the remaining internal pressure would be sufficient to overcome the energy barrier of the first's nicks but ejection would remain blocked for longer time at the last nicks because the insufficient pressure inside the capsid. In support of this proposal, we recently proposed a kinetic model based on light scattering experiments that allowed determining the characteristic times required to pass from one state of T5 DNA ejection to the other [59]. It was concluded that the probability to activate phages depended on the DNA length remaining un-ejected and that a longer time was required to transfer the last fraction of DNA remaining in the capsid.

Whether these *in vitro* observations reflect the *in vivo* process remains an open question. The physiological role of the nicks is still not elucidated. The stop sites may be involved in regulating DNA encapsidation/transfer *in vivo*. Interestingly, phages T5 and T7 are the only phages for which site-specific nicks have been described [46,60]. It will be, therefore, of interest to compare the *in vitro* ejection process of T7 with that of T5.

This fluorescence microscopy study points out the importance of the single analysis which allowed demonstrating the complexity of the phage ejection process. It remains to be determined whether this complexity is also shared by other tailed phages.

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