Effects of Condensing Agent and Nuclease on the Extent of Ejection from Phage λ†

Alex Evilevitch*

Department of Biochemistry, Center for Chemistry and Chemical Engineering, Lund University, S-221 00 Lund, Sweden

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We have recently demonstrated, that DNA ejection from bacteriophage λ can be partially or completely suppressed in vitro by external osmotic pressure. This suggests that DNA ejection from phage is driven by an internal mechanical force consisting of DNA bending and DNA−DNA electrostatic repulsion energies. In the present work we investigate the extent to which DNA ejection is incomplete at zero osmotic external pressure when phage is opened with its receptor in vitro. The DNA fragment remaining in the capsid and the tail that is no longer bent or compressed —and hence for which there is no internal driving force for ejection— is shown *not* to be ejected. We also demonstrate that DNA can be "pulled" out from the capsid by DNase I acting as a DNA binding protein or spermine acting as a DNA condensing agent. In particular, cryo electron microscopy and gel electrophoresis experiments show the following: (i) DNA ejection from bacteriophage λ incubated in vitro with its receptor is incomplete at zero external osmotic force, with several persistence lengths of DNA remaining inside the phage capsid, if no nuclease (DNase I) or DNA condensing agent (spermine) is present in the host solution; (ii) in the presence of both DNase I and spermine in the host solution, 60% (≈29 kbp) of wild-type λ DNA (48.5 kbp) remains unejected inside the phage capsid, in the form of an unconstrained toroidal condensate; (iii) *with* DNase I added, but no spermine, the ejection is complete; (iv) with spermine, but *without* DNase I added, all the DNA is again ejected, and organized as a toroidal condensate outside.

Introduction

Recently, with bacteriophage λ as an experimental model system, we have been investigating the state of stress of the bacterial viral genome.[1−4] Specifically, we have shown experimentally that internal stress on the genome from its bending and crowding in the capsid is responsible for the ejection of DNA from phage in vitro. We have demonstrated this by showing that the force driving the ejection can be balanced by the osmotic pressure of the host solution.[1,3,4] Furthermore, it was established in this way that the capsid pressures and ejection driving forces inside the capsid can be drastically lowered by addition of di- and polyvalent salts.[1,5] These experimental results agreed with earlier theoretical predictions made in the work of Tzlil et al.[6]

Here we address two conceptual questions raised in our recent work:[4] (i) Is ejection complete in the case of *no* external osmotic force (zero osmolyte concentration)? (ii) Can phage DNA be "pulled" out from the viral capsid when there is *no* net ejecting force acting on the encapsidated DNA? Having confirmed that the DNA ejecting force is due to capsid stress, one expects that a certain length of chain will remain in the capsid, namely, the length that can be accommodated in the head and tail without bending or self-repulsion. For phage λ, whose head has a diameter of the order of the persistence length of dsDNA and whose tail is approximately three times longer than the head, one expects a minimum of several persistence lengths DNA not to be ejected (corresponding to approximately 700 bp), as is schematically illustrated in parts a and b of Figure 1. The ejection should however be complete if DNA binding proteins or polyvalent ions are present in the host solution when phage λ is incubated with its receptor. Figure 1c illustrates the case where DNA is completely ejected, being pulled out by the DNA toroid condensed by spermine outside the phage.

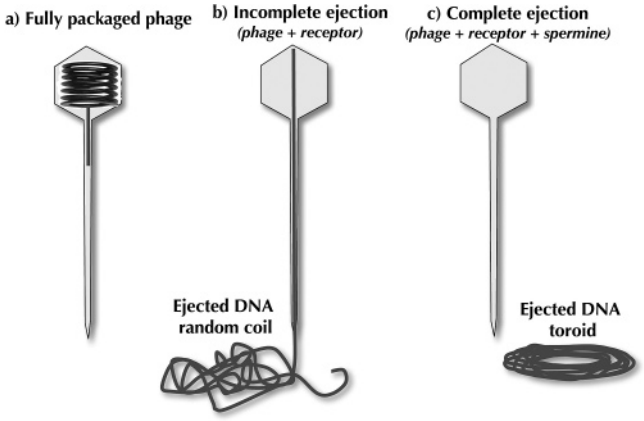

Figure 1. Schematic illustration of (a) fully packaged phage λ, (b) incomplete DNA ejection at zero external osmotic pressure, and (c) complete DNA ejection in spermine with ejected DNA condensed in a toroid.

This work experimentally investigates, with cryo transmission electron microscopy (cryo-TEM) and gel electrophoresis, the cases of complete and incomplete DNA ejection from phage in vitro, at zero osmotic pressure in the host solution *with* and *without* spermine and DNase I. In all cases, phage ejection was triggered by incubating 10^{13} virions/mL of phage with 20 μg/mL of LamB phage λ receptor with mixing volume ratio 1:5 in TM buffer (50 mM Tris−HCl/pH 7.4, 10 mM $MgSO_4$) for 1 h at 37 °C.

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* E-mail: Alex.Evilevitch@biochemistry.lu.se.

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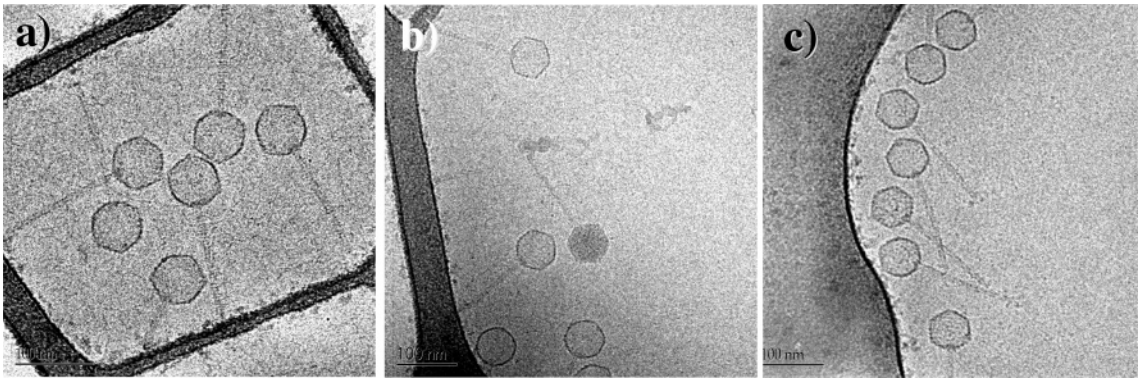

Figure 2. (a, b) Cryo electron micrographs of phage λ incubated with LamB receptor *without* DNase I. Both images show empty phages attached with their tail tips to the carbon film boundaries. Part b shows also a phage particle that has not ejected its DNA and is therefore is not attached to the carbon film boundary on the grid. (c) Cryo electron micrograph of phage λ incubated with LamB receptor *with* DNase I. Phages are not bound with their tails to the carbon film on the grid with their ejected DNA, since it has been digested by DNase I. These micrographs are typical representations of at least 100 images taken for these samples at random positions on the grid.

Experimental Methods

Bacteriophage Strain and Preparation of Phage Stock. Wild-type bacteriophage λ with genome length 48.5 kbp was produced by thermal induction of lysogenic *Escherichia coli* strain AE1 derived from strain S2773, obtained from Stanley Brown (Department of Molecular Cell Biology, Copenhagen University). The AE1 strain was modified to grow without LamB protein expressed on its surface in order to increase the yield of phage induced in the cell. The culture was then lysed by temperature induction. Phage was purified by CsCl equilibrium centrifugation. The sample was dialyzed from CsCl against TM buffer (10 mM $MgSO_4$ and 50 mM Tris-HCl/pH 7.4). The final titer was 10^{13} virions/mL, determined by plaque assay.

Preparation of LamB Phage λ Receptor. The receptor was the LamB protein purified from pop 154, a strain of *E. coli* K12 in which the *lamB* gene has been transduced from *Shigella sonnei* 3070. This protein has been shown to cause complete in vitro ejection of DNA from λ within seconds at 37 °C, in the absence of the added solvents required with the wild-type *E. coli* receptor. Purified LamB was solubilized from the outer membrane with the detergent octyl polyoxyethylene (octyl-POE).

Cryo Transmission Electron Microscopy (Cryo-TEM). Specimens for electron microscopy were prepared in a controlled environment vitrification system (CEVS) to ensure fixed temperature and to avoid water losses from the solution during sample preparation. The specimens were prepared as thin liquid films, <0.3 mm thick, on hydrophilic (glow discharged) lacey carbon films, supported by a copper grid and quenched into liquid ethane at its freezing point. The technique leads to vitrified specimens. The vitrified specimens were stored under liquid nitrogen and transferred to the electron microscope (Philips CM 120 BioTWIN) equipped with a postcolumn energy filter, using an Oxford CT3500 cryo-holder and its workstation. The accelerating voltage was 120 kV with a magnification of $45000\times$ and a nominal defocus of 1 μm. The images were recorded digitally with a CCD camera (Gatan MSC791).

Results and Discussion

To investigate the possibility of DNA remaining inside the capsid after ejection at zero osmotic pressure, we have first examined the length of DNA remaining inside after ejection has taken place in a buffer solution without added osmolyte but *with* DNase I. This nuclease digests only the ejected DNA into nucleotides, while unejected DNA stays intact inside. Following DNase I digestion the phage capsids were opened and any remaining DNA was extracted by the phenol extraction procedure described in ref 4. The lengths of the extracted DNA fragments were then determined by gel electrophoresis. No DNA fragments were detected in any of these experiments, suggesting that the ejection is complete in the presence of DNase I. Since DNase I adsorbs to the chain before it digests it, it will exert pulling forces on the genome, arising from its binding.[7,8] To test this interpretation and follow the ejection *with* and *without* DNase I, we carried out a series of cryo electron microscopy experiments.

After 1 h incubation with LamB receptor *without* DNase I, phage samples were spread and immediately vitrified on a glow-discharged (thereby hydrophilic) lacey-carbon film supported by a copper grid. Characteristic electron micrographs of this sample are shown in parts a and b of Figure 2. It is clearly observed that most of the phages have ejected their genomes and all so-called phage "ghosts" (empty phages) are attached at their tail tips to the hydrophilic carbon boundaries. Only phages that did not eject their genomes are observed *not* to be attached to the carbon boundaries (Figure 2b). These phages might be damaged and therefore unable to bind to LamB. The ratio between the empty phages bound to the carbon boundaries and unbound full phages is $\approx$12:1 (statistically analyzed from low magnification overview images). As expected for cryo-TEM, single molecules of uncondensed DNA ejected from phage are not seen due to the low contrast.

The observed phage tail tip attachment to the carbon film can be attributed to the fact that the ejected DNA, being a highly negatively charged molecule, binds to the hydrophilic, charged surface of the supporting carbon film. Due to the glow discharge, prior to the sample spreading, the carbon film becomes hydrophilic and negatively charged. However, the presence of Mg^{2+} ions in the buffer solution modifies the surface making it positively charged,[9] which allows the attachment of DNA to the carbon film. Thus, DNA acts as a binding link between the phage tail and the carbon film boundary, keeping phages attached to the film. From this we conclude that ejected DNA remains attached to the phage after ejection ceases.

To test this assumption, DNase I was added to the solution of phage and receptor. This time, cryo electron micrographs (Figure 2c) indicate that the phages are randomly oriented at the carbon film boundary with a majority of phages not being attached at their tails to the carbon boundary (the ratio between unbound and bound empty phages is 1.2:1). This nicely illustrates that once DNA has been digested by DNase I, phage

tails are no longer linked by the DNA to the carbon boundary; phage can now assume random orientations in the solution close to the boundary.

We can also show that in the case of incomplete ejection *without* DNase I, the ejected genome is bound to the phage from the inside and not outside of the phage. We demonstrate this by first incubating phage with LamB and DNase I in TM buffer for 1 h at 37 °C. Then, just before the sample is frozen on the grid, external λ DNA is added to the sample. There is insufficient time (30 s) for DNase I to digest the externally added DNA before the sample is vitrified on the grid, and the electron micrographs showed accordingly no attachment of phage tails to the carbon film boundaries. Also, by incubating the phage for an hour with added DNA without DNase, we make sure that phage does not adsorb to the free DNA over time. This confirms that *without* DNase I added, the ejection is incomplete, with several persistence lengths of unstressed DNA remaining inside the phage. In our preliminary light-scattering analysis, the remaining DNA length was estimated to ≈800 bp after 1 h at 37 °C, corresponding approximately to the length of the tail and the diameter of the capsid added together (D. Löf, K. Schillén, A. Evilevitch).

When there is no net "pushing" force on the encapsidated DNA, the only way for the last, unstressed, DNA fragment to leave the capsid is through simple diffusion, which is several orders of magnitude slower than the ejection driven by the internal force. Thus, the last piece of unconstrained DNA can remain attached to the phage during the time frame for our experiment. DNA can also remain stabilized inside the capsid through an attractive electrostatic interaction with the capsid wall interior. Indeed, for the case of HK97 bacteriophage, it has been shown that inner walls of the capsid are positively charged with surface charge density matching the charge density of DNA covering the capsid interior.[10] However, such attractive interaction cannot be strong since the ejection is complete in the presence on DNase I or spermine as discussed below. However, DNase I appears to act not only as an endonuclease but also as a protein that binds nonspecifically to the DNA chain before it digests it. Therefore, through release of the binding energy, DNase I acts here as a ratcheting agent, pulling the genome out from the phage as it continuously binds to the new binding sites on the DNA.[7,8]

We also show here the DNA pulling action of spermine, which is a tetravalent polyamine present in many bacterial cells.[11] Polyvalent ions such as spermine (+4) or spermidine (+3) act as DNA condensing agents in solution by mediating an attractive interaction between the DNA strands and condensing DNA into a toroid.[12−16] Using cryo-TEM, we have visualized here phage λ incubated with LamB *with* 1 mM spermine added but *without* DNase I. The electron micrograph in Figure 3a shows a large condensed DNA toroid and empty phage particles randomly distributed around it. The size of the toroid and the number of empty phages suggest that the DNA toroid is formed from many ejected DNA molecules. (Analyzing low magnification overview images, each toroid is formed from on average 16 DNA molecules ejected from the phages distributed in random orientations in the vicinity of each condensate.) Some phages are pointing with their tails toward the toroid while some are not. The image in Figure 3a, illustrates that DNA is now pulled completely out from the capsid by the larger DNA toroid formed outside the phage. The ejection is complete despite the attractive DNA−DNA interaction inside phage induced by spermine permeating the capsid, since even in the absence of electrostatic repulsion between DNA strands in the capsid, bending and surface energy of external DNA toroid favors the formation of one large DNA condensate outside phage consisting of many DNA molecules from several phages (in similarity to an Ostwald ripening process).[6] Formation of such an external toroid apparently also overcomes DNA−capsid wall attraction, if such is present.

While ejected uncondensed DNA molecules in the cryo-TEM experiments above could not be visualized due to the low contrast, DNA condensates formed in spermine could easily be observed. Figure 3a also shows that although phage has been incubated with its ejected DNA for 1 h, the phages are not attached to the DNA. This supports our conclusion from the earlier experiment showing that external DNA does not bind to the outside of the phage tails.

To illustrate the pulling effect on DNA of spermine due to the action of the toroidal DNA condensate, DNase I was added to the phage−spermine solution prior to the incubation with LamB, followed by freezing of the sample; see Figure 3b−d. Images show no DNA toroids in the solution but randomly oriented phage particles with partially unejected DNA remaining in the capsid. Now, a DNA condensate cannot form outside since the ejected DNA is digested by DNase I. Since spermine permeates the capsids, it can stabilize a fraction of DNA inside, by introducing attractive interactions between the neighboring DNA strands, but there is *no* DNA toroid on the outside that can pull it out. (On average one empty phage is found per eight partially filled phages, which can be due to locally less efficient DNase I action allowing the entire DNA molecule to escape from the capsid before it is digested.) The amount of DNA remaining in the capsid is approximately 60% (29 ± 2 kbp) of the total genome length for wild-type DNA (48.5 kbp). This fraction of unejected DNA was measured using UV absorbance assay described in ref 3. More explicitly, two samples of phage λ were incubated with LamB and DNase I *with* and *without* spermine. DNase I is added to digest all DNA that is ejected. After 1 h of incubation at 37 °C, the ejection has ceased and all ejected DNA is digested. The samples are spun in an ultracentrifuge in order to pellet the phage with its unejected DNA. Nonsedimenting DNA nucleotides of ejected DNA remain in the supernatant. The concentrations of ejected DNA are determined by UV absorbance measurement at 260 nm wavelength. Since there is also some external DNA always present in all phage samples, which is accessible to DNase I even without LamB, we also need to account for this contaminant "background" DNA concentration. The concentration of this background DNA is determined in the same way with UV by taking the sample of phage with DNase I but without LamB. The fraction of unejected DNA in 1 mM spermine is then determined by taking [Abs(phage + LamB + spermine + DNase I) − Abs(phage + DNase I)] divided with [Abs(phage + LamB + DNase I) − Abs(phage + DNase I)]. The standard deviation is estimated by propagation of errors in the measured UV absorbance.

Like our earlier experiments on osmotic suppression of phage DNA ejection,[3] this result is also consistent with the energy balance calculations in ref 6 that considered the packaging of DNA in phage capsids in the presence of condensing agents such as spermine. Specifically, it was shown that as ejection proceeds the interaxial spacing of the hexagonally packaged DNA increases to that of DNA condensed by spermine in solution (hence, unstressed) when $^1/_4$ to $^1/_3$ fraction of the DNA is ejected, in good agreement with our measured fraction of 60% of unejected genome. Furthermore, this situation is further

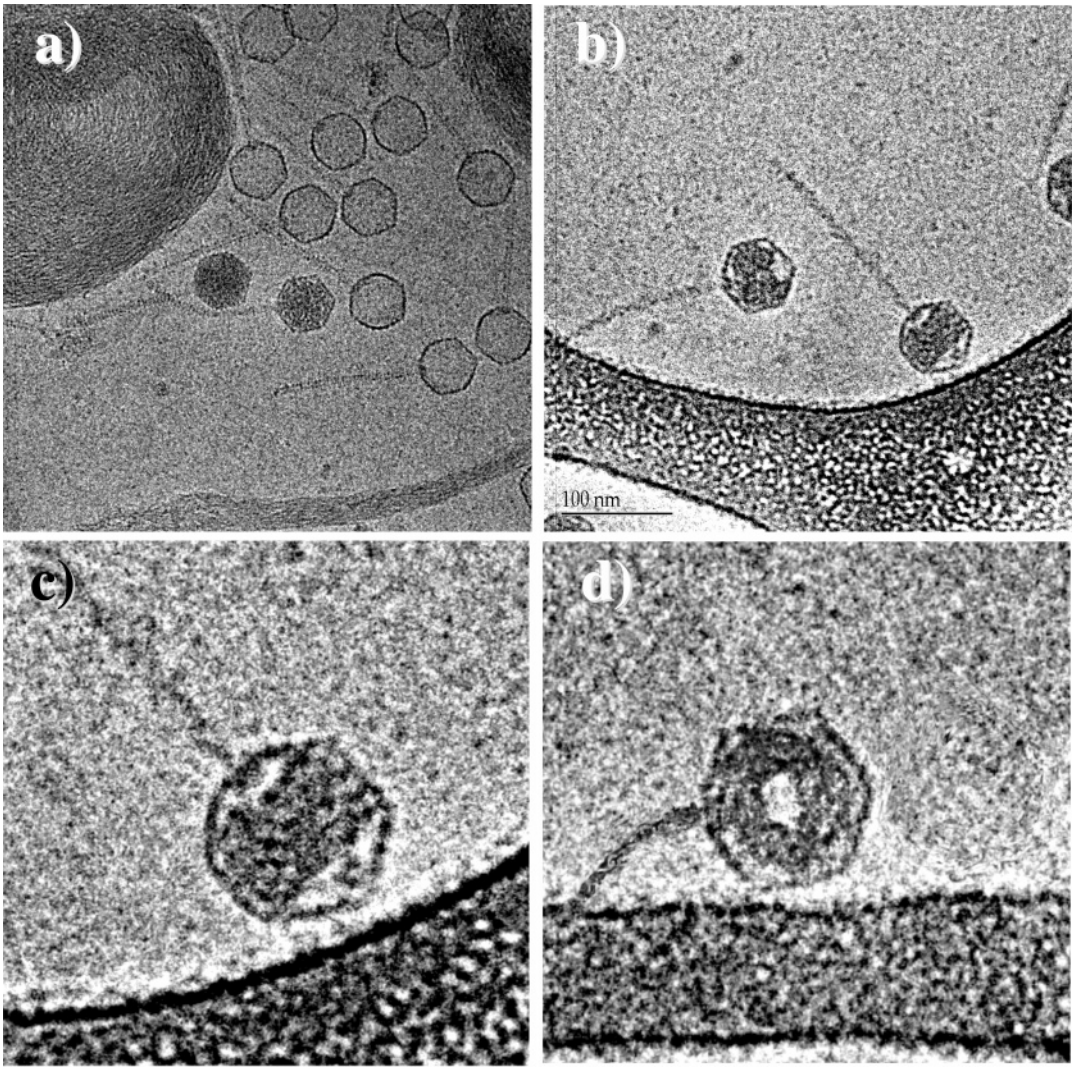

Figure 3. (a) Cryo electron micrograph of phage λ incubated with LamB receptor in 1 mM spermine *without* DNase I. Micrograph shows empty phage capsids with completely ejected DNA, condensed by spermine. DNA condensate consists of many DNA molecules from several phages. (b, c, d) Cryo electron micrographs of phage λ incubated with LamB receptor in 1 mM spermine *with* DNase I. All ejected DNA is digested by DNase I and therefore no DNA condensates are observed outside the phage. Images show on the contrary phage capsids with partially unejected DNA with toroidal structure inside phage capsid. Part d shows a top view of unejected DNA toroid in the capsid. These micrographs are typical representations of at least 100 images taken for these samples at random positions on the grid.

aggravated by the action of the DNase I as DNA cutting enzyme. Once DNA with length x moves out of the tail, it is cut by the DNase I, then because of the decreased driving force (less length in the capsid) and the consequent, spermine induced, DNA−DNA attractive force pulling back on the chain, the chain of the same length x will pop back inside the tail, thereby allowing the force balance to be restored. It follows that the chain will not diffuse out of the phage on the time scale of the experiment. It is only if DNase I would not be digesting the DNA that the ejection would continue (due to the Ostwald ripening mechanism described earlier).

Figure 3b,c and Figure 3d, show side and top views, respectively, of the phage capsid with its unejected genome; we see an ordered toroidal DNA structure. A similar effect of spermine was observed in a parallel independent light-scattering study of T5 phage ejection.[17] However, it is much harder to make theoretical comparisons there since DNA in T5 phage significantly differs from λ due to the presence of nicks on the T5 DNA; this leads to a different DNA ejection scenario with nonmonotonic discontinuous ejection.[18]

When external λ DNA is added to the mixture of phage + LamB + DNase I + spermine, just before the freezing of the sample (30 s), no phages were observed attached to the external visible DNA toroids, confirming again that phage does not bind to free DNA. We conclude that, *without* DNase I, no λ DNA remains in the capsid due to the presence of the larger condensate formed outside by the ejected portion.

To summarize, these experiments show that both DNA condensing cations such as spermine and DNA binding proteins such as DNase I can facilitate the complete DNA ejection from bacteriophage viruses when there is *no* net internal driving force for the DNA ejection. A similar DNA pulling mechanism can be responsible for complete DNA ejection from the phage in vivo, when the DNA ejecting force is balanced by the osmotic pressure of the cell's cytoplasm, when only a fraction of DNA is ejected.

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