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Yan Jin, Charles M. Knobler, and William M. Gelbart

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CONTROLLING THE EXTENT OF VIRAL GENOME RELEASE BY A COMBINATION OF OSMOTIC STRESS AND POLYVALENT CATIONS

Yan Jin,[1] Charles M. Knobler,[1] and William M. Gelbart[1, 2, 3]

[1] *Department of Chemistry and Biochemistry*
[2] *Molecular Biology Institute (MBI)*
[3] *California NanoSystems Institute (CNSI)), University of California, Los Angeles, CA 90095*

While several *in vitro* experiments on viral genome release have specifically studied the effects of external osmotic pressure and of the presence of polyvalent cations on the ejection of DNA from bacteriophages, few have systematically investigated how the extent of ejection is controlled by a combination of these effects. In this work we quantify the effect of osmotic pressure on the extent of DNA ejection from bacteriophage lambda as a function of polyvalent cation concentration (in particular, the tetravalent polyamine spermine). We find that the pressure required to completely inhibit ejection decreases from 38 to 17 atm as the spermine concentration is increased from 0 to 1.5 mM. Further, incubation of the phage particles in spermine concentrations as low as 0.15 mM - the threshold for DNA condensation in bulk solution - is sufficient to significantly limit the extent of ejection in the absence of osmolyte; for spermine concentrations below this threshold, the ejection is complete. In accord with recent investigations on the packaging of DNA in the presence of a condensing agent, we observe that the self-attraction induced by the polyvalent cation affects the ordering of the genome, causing it to get stuck in a broad range of non-equilibrated structures.

INTRODUCTION

Many double-stranded (ds) DNA viruses have their stiff, highly charged genomes packed to crystalline density in pre-formed rigid shells (capsids), resulting in a high state of stress. The work of packaging is performed by strong virally-encoded protein motors, and the resultant stress provides much of the driving force for genome ejection. Considerable theoretical [1–7] and experimental [8–11] work has been devoted to calculating and measuring the forces and pressures associated with these processes. It is only recently, however, that attention has been focused on the *nonequilibrium* dynamics of the packaging process, which involves loading rates as high as 100s of base pairs (bp)/sec. In particular, Smith and co-workers [12, 13] have employed single-molecule techniques to study the heterogeneity of the packaging dynamics and how it varies when the DNA-DNA interactions are changed from repulsive to attractive.

One expects that the genome dynamics associated with ejection from the capsid will also be sensitive to disorder in the packaged DNA and to the nature of the DNA-DNA interactions. We address these issues here by investigating the separate and combined effects of polyvalent counterions and of osmolytes on the *in vitro* genome ejection properties of bacteriophage λ , a dsDNA virus. Consistent with the strong heterogeneity in packaging rates reported by Smith and coworkers [12, 13], we find that DNA self-attractions induced by polyvalent cations cause the confined DNA to become stuck in a wide range of non-equilibrium structures. Related effects have been discussed in several simulation studies of phage packaging [6, 7, 14], including the effects of twist on entanglement [15]. Specifically, we report measurements of the extent and nature of genome ejection for a variety of biologically relevant osmotic pressures and polyvalent cation concentrations, and discuss our results in the context of related work on DNA condensation and of our present understanding of strongly-confined DNA.

EXPERIMENTAL AND RESULTS AND DISCUSSION

In all of the work reported here we use the tetravalent cation spermine, Sp^{4+}, as the polyvalent cation for inducing DNA self-attraction, and high-molecular-weight poly(ethyleneglycol), PEG 8000, as the osmolyte for controlling osmotic pressure. Wildtype bacteriophage lambda was purified from infected *E. coli* c600, and the lambda receptor (LamB) purified from *E. coli* pop154, both as described earlier [9]. The final titer of phage was around 10^{11} infectious units per ml, determined by plaque assay. Virus solution was first treated with DNase I (1 unit, New England Biolabs) to remove free DNA due to the breakage of capsids during purification. 100 μl aliquots of phage sample were then incubated overnight with Sp^{4+} at 4°C in TM buffer (50mM Tris, 10mM Magnesium Sulfate, pH 7.5). PEG 8000, LamB, 1% detergent (oPoE), DNase I buffer [9] and an additional 5 μl of DNase I (2 units) were then added together, and the samples were incubated at room temperature for 15 min to allow the LamB (solubilized by the oPoE) to bind to the phage, triggering ejection. Further incubation for 4 hr at 37 °C ensured that the ejected DNA was fully digested by DNase I.

The unejected DNA lengths were determined by extracting them from the capsids. To denature the DNase I before extraction, 1 mM EDTA was added and the sample heated for 10 min at 75°C. After addition of an equal volume (100 μl) of protein lysis buffer (25 mM EDTA, 200 mM Tris, 250 mM NaCl, 1% SDS, pH7.5) and 1 μl of Protease K (1 unit), the samples were incubated at

65°C for 1 hr to disrupt the capsids, allowing the DNA inside to be released. Phenol/chloroform extraction was done twice to separate protein from DNA, followed by ethanol precipitation to concentrate the DNA. Finally, the precipitated DNA was redissolved in TE buffer and analyzed by electrophoresis in a 0.3% agarose gel run for 6 hr at 3V/cm and stained with SYBR gold.

Figure 1.A is a gel image showing the length of DNA remaining inside the capsid when ejection is triggered in solutions containing no osmolyte, but with Sp^{4+} at concentrations between 0 and 1.5 mM. Because of the low concentration of the LamB receptor (only one per phage), and the failure of some phages to eject even when bound by a receptor, genome ejection is triggered in only about half of the phage particles in any sample (as estimated from the relative intensities of the two bands observed in the gel). Accordingly, all of the lanes show a band corresponding to the full-length (48.5 kbp) DNA that remains unejected and hence protected by the capsid. In the rightmost 3 lanes, for which $[Sp^{4+}] < 0.15$ mM, we see *only* this band, because ejection is complete from all opened capsids. For $[Sp^{4+}] \geq 0.15$ mM, however, there is another - distinctly more diffuse - band, corresponding to the lengths ($\approx$ 25-33 kbp - as determined from the corresponding densitometry profiles, see discussion below) of DNA remaining in the capsids following ejection. The presence of a significant length of the genome remaining in the capsid attests to the DNA having been condensed by spermine [4]. Further, the broad *range* of lengths observed reflects the large non-equilibrium effects associated with the spermine-induced attractions between neighboring portions of packaged duplex. This strong heterogeneity is consistent with the large standard deviation in *packaged* lengths measured for high spermine concentration by Keller *et al.* [13].

Note that the above 25-33 kbp range of lengths corresponds to 52-68% of the genome remaining in the capsid when ejection is triggered under DNA condensation conditions. Measurement of the UV absorbance of ejected, digested, DNA in the supernatant, following centrifugation, while not providing direct information on the *distribution* of ejected (and therefore of unejected) DNA lengths, does give their *average* value. In this way Evilevitch [16] obtained a value of 29 $\pm$ 2 kbp for the average length remaining inside the lambda capsid when ejection was triggered in the presence of 1 mM spermine, with the $\pm$ 2 kbp uncertainty calculated from propagation of errors. This average value agrees with the average of 29 kbp measured directly here in our gel determinations of the amount of unejected DNA under condensation conditions.

We checked that spermine-induced condensation of lambda DNA did not result in it running differently than uncondensed DNA in the electrophoresis gel. More explicitly, samples of lambda DNA incubated in 1 mM Sp^{4+}, and the same DNA incubated in spermine-free buffer solution, were found to run identically in a 0.3% agarose gel in TE spermine-free buffer, thereby confirming our determinations of unejected DNA lengths in the case of super-threshold $[Sp^{4+}]$. Further, consistent with related studies [16], we found that lambda DNA is essentially fully digested into nucleotides by DNase I, even after incubation with Sp^{4+} at concentrations up to 5 mM. This result is in contrast with the report by Baeza *et al.* [17] that a plasmid DNA condensed by spermidine is resistant to digestion by DNase I.

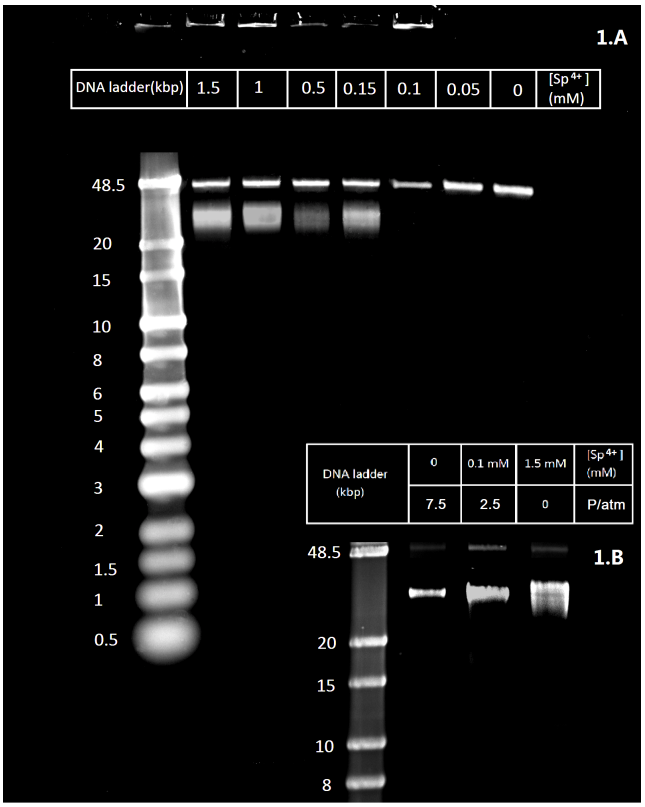

FIG. 1. A. 0.3% agarose gel of unejected DNA, following ejection from phages triggered by LamB after incubation with Sp^{4+} at concentrations ranging from 0 to 1.5 mM. B. Same, but with the indicated combinations of PEG and Sp^{4+} concentration.

As an additional control we checked that the duration of digestion of ejected DNA did not affect the *extent* of ejection, i.e., the length of DNA remaining in the capsid. In particular, after adding LamB to the phage samples incubated in spermine and/or PEG, no differences were found in the positions and intensities of the bands associated with the DNA remaining in the capsids between samples digested for 3, 4 or 5 hr with DNase I.

Even though the phages have been incubated in spermine before genome release is triggered, the packaged DNA can only begin to feel its self-attraction after a significant amount of ejection has occurred. Up until that point the average interaxial spacing between neighboring duplex portions is smaller than the 2.8-2.9 nm at which the onset of attractive forces occurs [18]. The effective interaction is repulsive and ejection proceeds until the

average interaxial spacing becomes this large or, alternately, until the length of DNA remaining inside is sufficiently small that its toroidal condensate has an outer radius equal to the inner radius of the viral capsid [4, 19]. Were the DNA able to relax (equilibrate) during packaging and ejection, the points at which these limits are reached would coincide. But they can vary considerably from phage to phage because the local packaging configurations are highly heterogeneous as a result of large non-equilibrium effects associated with how the genome was packaged in the first place [12, 13] and especially because of the onset of strong self-attraction of the packaged DNA as it is ejected following the incubation with spermine.

Measurements were also carried out with mutant bacteriophage lambda b221, whose genome length is 37.8 kbp. In this case, the lengths of DNA remaining in the capsid were - as expected - the same (25-33 kbp) as for the wildtype, because of the ejected DNA being digested by DNase I. And, again, as with the wildtype virus, the unejected DNA is much more polydisperse than would be expected if the DNA configurations arising in packaging and ejection of the genome were equilibrated for all of the force, pressure, and spermine conditions involved.

This departure from equilibrium was anticipated by Tzlil *et al.* [4] who, in calculating that 1/4 to 1/3 of the lambda genome should remain unejected in the presence of self-attraction, pointed out that this prediction should be a lower bound because of non-equilibrium effects. Computer simulation studies by Petrov and Harvey [7] also addressed the effect of self-attraction on ejection. In particular, they used molecular dynamics to examine different lengths of DNA - corresponding to 10, 30 and 78% of the full genome - equilibrated in capsids with purely repulsive interactions between neighboring duplex portions. Attractive interactions were then switched on, mimicking the effect of incubation in a sufficiently high concentration of spermine. The shorter (10 and 30% length) chains were able to form toroid-like structures, whereas the trajectories associated with the long (78%) one were associated with pronounced non-equilibrium structures. Similar effects had been reported in earlier phenomenological packaging simulations by Ali *et al.* [14] and by Forrey and Muthukumar [6].

In an experiment similar in concept to ours, de Frutos *et al.* [20] examined the ejection of DNA from T5 phage, for which the full-length genome is 114 kbp, in the presence of sufficient spermine to condense the DNA. Gel electrophoresis analysis of the DNA remaining in the phage capsids showed several broad bands, at approximately 100, 46 and 11 kbp.These widely different lengths are difficult to explain, but are consistent with those found for T5 by Leforestier *et al.* [21], who measured the lengths of DNA remaining in the capsid following ejection in the presence of low concentrations of PEG; they are also consistent with the several pauses in ejection reported in kinetic studies of T5 in the absence of PEG [22].

De Frutos *et al.* did, however, estimate the size of the maximum toroid that could fit inside the capsid radius, R_{inner}^{capsid}, by setting $R_{outer}^{toroid} = R_{inner}^{capsid}$ and using the empirical value of the ratio between the inner and outer toroidal radii reported earlier from studies by Bloomfield and coworkers [23] of free DNA in solution condensed by polyvalent cations into circumferentially-wound/hexagonally-packed toroids with circular cross-sections. The corresponding maximum length of unejected DNA - the value that one would measure if the DNA configuration were able to re-equilibrate throughout the ejection process - was found to be 36 kbp, consistent with the 27-29 kbp range measured in the cryo-EM studies of Leforestier and Livolant [19]: in their work different PEG pressures were used to stop the ejection of T5 at each of the many pauses that occur *in vitro* in the absence of PEG, and then 5 mM Sp^{4+} was added to condense the DNA remaining in the capsid. A similar calculation for lambda phage, using the 28-nm value of R_{inner}^{capsid} determined by Dokland and Murialdo [24], leads to an estimate of 12 kbp for the length remaining inside, consistent with the value calculated directly by Tzlil *et al.* [4] using measured DNA bending and self-attraction energies [25]. *The fact that we measure more than twice this length, and a broad distribution of lengths about this average, is a consequence of the large role played by non-equilibrium genome configurations in the case of self-attraction.*

Each concentration of PEG in the host (external) solution corresponds to a certain amount of water being sucked out of the phage capsid (from which PEG is excluded). As a result, the water inside is under tension, thereby producing a force resisting the ejection of DNA. As demonstrated by earlier measurements [9, 10, 26], the higher the PEG concentration the lower the extent of genome ejection. To explore how this mechanism of osmotic suppression competes with the effects of polyvalent-cation-induced DNA self-attraction, we carried out a series of experiments in which we measure the extent of genome ejection in the presence of both PEG and spermine.

We consider three regimes of spermine concentration, defined with respect to the threshold value (0.15 mM) for mediating a self-attraction: its total absence; a sub-critical value (0.1 mM); and a super-critical value (1.5 mM). We incubate the phage in Sp^{4+} and PEG, trigger ejection by adding LamB in the presence of DNase I, deactivate the DNase I, extract the unejected DNA, and measure its length in an agarose gel. In this way we determine the fraction of ejected DNA for each specified pair of spermine and PEG concentrations.

Fig. 2 shows the dependence of fraction ejected vs. osmotic pressure: for sub-threshold [Sp^{4+}] (0.1 mM), see filled squares, which is not high enough to induce an attraction between neighboring DNA duplex portions; and for super-threshold [Sp^{4+}] (1.5 mM), filled circles. For comparison, we also measured ejection fractions as a function of osmotic pressure in the absence of spermine

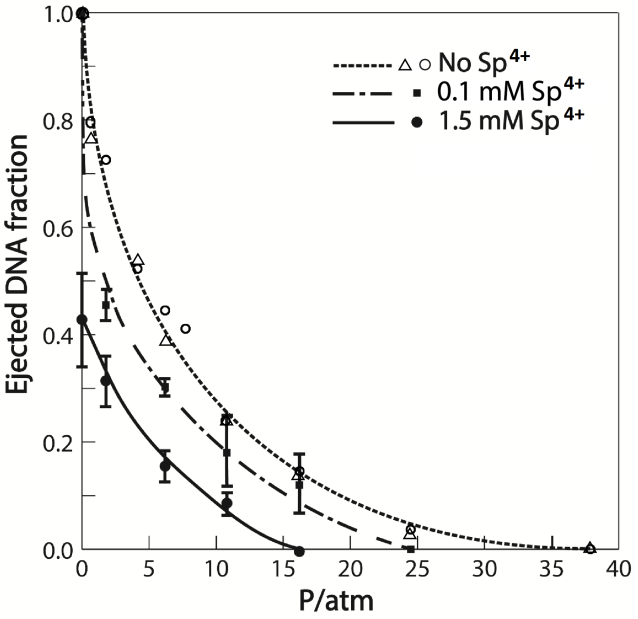

FIG. 2. The ejected DNA fraction as a function of osmotic pressure (PEG8000 concentrations), for each of three different concentrations of Sp^{4+}: open circles and triangles (Grayson *et al.* [27]), no Sp^{4+}; filled squares, 0.1mM Sp^{4+}; and filled circles, 1.5mM. In each case the phage are incubated in PEG and/or Sp^{4+}, ejection is triggered by addition of receptor in the presence of DNase I, and the DNA remaining in the capsids is extracted and run in an agarose gel as described in the discussion of Fig. 1. The lines are guides to the eye.

(see open circles); these results are in good agreement with the measurements (triangles) reported earlier by Grayson *et al.* [27] . Two of the three curves - those corresponding to no Sp^{4+} and to non-zero but sub-threshold Sp^{4+} - are qualitatively similar. In particular, because the DNA self-interaction is purely repulsive in both cases the fraction ejected in the absence of osmotic pressure (i.e., no PEG) is 100%. The PEG pressure needed to completely suppress ejection is 38 atm for $[Sp^{4+}]= 0$ and 25 atm for $[Sp^{4+}] = 0.1$ mM. This is because the presence of a low concentration of polyvalent cation, even though insufficient to mediate a self-attraction, nevertheless contributes to a reduction in the self-repulsion that dominates the capsid pressure. On the other hand, the curve in Fig. 2 for the DNA fraction ejected for super-critical $[Sp^{4+}]$ (1.5 mM) is qualitatively different. It starts (for no PEG) at a value of about 0.4, corresponding, as discussed earlier, to about 0.6 of the genome remaining in the capsid [28]. Also, because of the much higher spermine concentration, the pressure in the intact phage is as low as 17 atm.

The error bars in Fig. 2 correspond to the standard deviation in the *average* DNA length ejected under the corresponding conditions of spermine concentration and osmotic pressure, based on several measurements made for each sample. But the *range* of lengths observed in each measurement is significantly larger. Recall from the gel in Fig. 1 that for ≥ 0.15 mM, and hence for the zero-osmotic-pressure 1.5 mM $[Sp^{4+}]$ sample represented by the left-most filled circle on the lowest curve in Fig. 2, the lengths of DNA remaining in the capsid range from 25 to 33 kbp. If, on the other hand, an average length of about 29 kbp is made to remain in the capsid by imposing a sufficiently high osmotic pressure in the absence of spermine, we expect that the associated range of lengths involved will be smaller because no attractions are operative under these conditions. The same would be true for a combination of a lower osmotic pressure and a sub-threshold spermine concentration, where again there is only a repulsive self-interaction of the DNA.

This situation is illustrated in Fig.1.B, a gel of extracted, unejected, DNA for these three scenarios. Lane 1 (to the immediate right of the ladder) is for 7.5 atm PEG, no spermine; lane 2 for 2.5 atm PEG, 0.1 mM spermine; and lane 3 for 1.5 mM spermine, no PEG. The average DNA length remaining inside the capsids is 29 kbp in all three cases, with half width of 2, 4, and 6 kbp for spermine concentrations of 0, 0.1 mM, and 1.5 mM, respectively, as determined from densitometry traces of the gel - see Fig. 3.

It is clear from Fig. 3 that the widths of the distributions of unejected DNA lengths increase monotonically with Sp^{4+}, i.e., as the self-repulsion of the genome is reduced and then converted into an effective attraction. It is difficult to determine absolute DNA lengths from the widths of bands in a gel because they depend on experimental conditions such as gel casting, variations in staining and gel loading. On the other hand, comparisons between relative band widths in a single gel, in which the conditions in each lane are closely identical can be made much more precisely. Although the loadings are nominally identical, losses during the extraction may not be identical; we estimate that the differences in loading between the lanes do not exceed a factor of two. In experiments in which we compared the widths of the bands in the ladder, we found that an increase of loading by a factor of 16 led to a 50% increase in the width, and a change of a factor of two was barely measurable. The differences of the widths of the band on the left of each trace in the figure corresponding to the 48.5kbp, monodisperse, viral genome, are a measure of the relatively small experimental variations. Note also that the widths of the 1.5mM Sp^{4+} bands in Figs. 1A and B are consistent with one another. Were the bands for the unejected DNA of similar shape, as in the upper two, a quantification of the spread in molecular weights given by the width at half maximum would be meaningful; such a comparison is less meaningful for the lower asymmetric band. As an alternative, one can take the width at the base of the bands as a measure of the spread in molecular weights. In either case it is evident that there is an increase with $[Sp^{4+}]$.

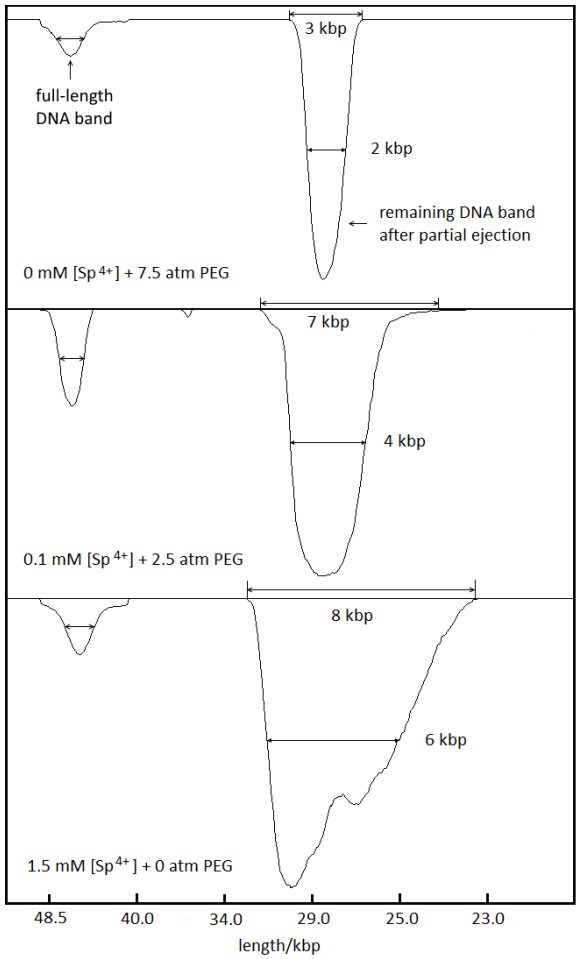

FIG. 3. Densitometry traces of the rightmost 3 lanes in Fig. 1.B, showing the unejected genome (48.5kbp), and the distributions of DNA lengths remaining in the capsid when ejection is triggered in the presence of (from left to right in 1.B and from top to bottom in Fig. 3): no Sp^{4+} and 7.5 atm PEG; 0.1mM Sp^{4+} and 2.5 atm PEG; and 1.5 mM Sp^{4+} and no PEG. The Fig. 1.B ladder was used to convert band positions into DNA lengths.

CONCLUSIONS

We have shown that the length of DNA ejected from a viral capsid can be controlled by a combination of polyvalent cation and osmolyte concentration in the ambient solution. In particular, for bacteriophage lambda, we find that more than half of the genome remains in the capsid when the concentration of polyvalent cation exceeds the threshold value for DNA condensation in bulk solution. With increasing concentrations of osmolyte the length of unejected DNA increases monotonically, with complete suppression of ejection occurring at osmotic pressures that decrease from 38 to 17 atm as the polyvalent cation concentration increases from 0 to super-threshold values.

In addition, we have measured the distribution of lengths of unejected DNA and found that it increases significantly as the strength of DNA-DNA repulsions is reduced by addition of polyvalent cation and upon the onset of self-attraction induced by super-threshold condensation conditions. Our measurements report on the changing extent to which an ensemble of packaged viral genomes is not equilibrated as identical-length molecules confined in identical-volume capsids. More explicitly, because the genomes are packaged at a rate faster than their relaxation rates the sample of viruses involves a distribution of confined DNA configurations and a corresponding distribution of encapsidation stresses (forces and pressures). As shown by recent work of Smith and co-workers [13], this state of affairs is reflected in the distribution of force (vs. time, or fraction packaged) trajectories associated with the virally-encoded packaging motors that carry out the loading of the genome.

In vivo, packaging - synthesis of infectious virus particles - occurs under identical conditions in the bacteria in which the phage samples are grown. Accordingly, the resulting non-equilibrium distribution of configurations is common to all of the ejection samples we consider (i.e., the three with no $[Sp^{4+}]$, subcritical $[Sp^{4+}]$, and supercritical $[Sp^{4+})]$. It follows that the differences in widths of ejected DNA lengths that we measure for these three samples can be attributed to the different strengths of the DNA self-repulsion/attraction that are operative *during ejection*.

Further, these results suggest, consistent with the situation reported from single-molecule *packaging* measurements [12, 13], that non-equilibrium effects are weaker for more repulsive DNA-DNA interactions. They are also consistant with simulation studies of *ejection* [29] in which reducing the self-repulsion of the confined chain makes it more likely to get stuck ("self-entangle").

It is clear that *in vitro* studies of dsDNA viruses like lambda and T5 have much to teach us about the dependence of genome packaging and ejection on ambient osmotic pressures and salt concentrations, and that future investigations will continue to yield improved physical understanding of these fundamental processes. In particular, the importance of nonequilibrium effects needs to be explored further because of the short time scales involved compared to the relaxation times of strongly-confined stiff polyelectrolytes like DNA. These effects show up first in the *packaging* of the genome [6, 7, 12–15], and then again in its *ejection* (present work; and [29]), and the magnitude of these dissipation effects increases with weakening of the DNA self-repulsion and with onset of self-attraction.

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