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Nicholas Keller[1], Zachary T. Berndsen[1], Paul J. Jardine[2], Douglas E. Smith[1]

[1]*Dept. of Physics, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093*
[2]*Dept. of Diagnostic and Biological Sciences and Institute for Molecular Virology,*
 University of Minnesota, 515 Delaware Street SE, Minneapolis, MN 55455
Email: des@ucsd.edu

Abstract

We compare forces resisting DNA packaging and forces driving DNA ejection in bacteriophage phi29 with theoretical predictions. Ejection of DNA from prohead-motor complexes is triggered by heating complexes after *in vitro* packaging and force is inferred from the suppression of ejection by applied osmotic pressure. Ejection force from 0-80% filling is found to be in quantitative agreement with predictions of a continuum mechanics model that assumes a repulsive DNA-DNA interaction potential based on DNA condensation studies and predicts an inverse spool conformation. Force resisting DNA packaging from ~80-100% filling inferred from optical tweezers studies is also consistent with the predictions of this model. The striking agreement with these two different measurements suggests that the overall energetics of DNA packaging is well described by the model. However, since electron microscopy studies of phi29 do not reveal a spool conformation, our findings suggest that the spool model overestimates the role of bending rigidity and underestimates the role of intra-strand repulsion. Below ~80% filling the inferred forces resisting packaging are unexpectedly lower than the inferred ejection forces, suggesting that in this filling range the forces are less accurately determined.

I. INTRODUCTION

Two critical steps in the life cycle of many viruses are the packaging of double-stranded DNA during assembly and the subsequent ejection of DNA during the infection of a host cell. Many DNA viruses follow a remarkable assembly process during which viral capsid shells are assembled first and a single DNA molecule (the viral genome) is translocated into the capsid via a portal nanochannel by an ATP-powered molecular motor [1-4]. DNA in these viruses is packed extremely tightly, reaching nearly crystalline densities of ~0.5 g/ml, resulting in an average inter-axial separation between hexagonally packed DNA strands of only ~25-30 Å [5-8]. This tight confinement is highly energetically unfavorable due to electrostatic self-repulsion of charged DNA segments, entropy loss, and DNA bending rigidity [9-15].

We have previously shown via single molecule optical tweezers measurements of packaging in bacteriophages phi29, lambda, and T4, that the packaging motors are very powerful, capable of exerting forces of >60 pN [16-22]. It is widely assumed that the forces resisting DNA confinement that build during packaging play an important role in driving the later ejection of the DNA [10-15]. The simplest models assume that the DNA is in a free energy minimum conformation and that the ejection force at a particular capsid filling level is equal to the force resisting DNA confinement [10-12]. However, recent experiments show that the DNA undergoes nonequilibrium dynamics during packaging [23], suggesting that ejection forces could be lower than forces resisting packaging if significant energy dissipation occurs during packaging or prior to ejection.

Ejection forces in bacteriophage lambda were inferred via osmotic pressure experiments to be ~14 pN at 100% capsid filling (with 100% of the wildtype genome length packaged) [24,25]. In comparison, forces resisting packaging in bacteriophage phi29 have been inferred via optical tweezers experiments to rise to a maximum of ~20-25 pN [26,27]. However, the forces for lambda could be different than phi29 because lambda and phi29 have different capsid sizes and shapes and possibly slightly different packaging densities, and the measurements were done under different ionic conditions [12]. There has not been a direct experimental comparison of packaging and ejection forces for the same virus under the same conditions. In this Letter, we report experimental determinations of DNA ejection forces for phage phi29 and a direct comparison with determinations of forces resisting packaging under the same ionic conditions. We compare both measurements with theoretical models [12,15].

To study phi29 ejection, we adapted a technique established by Evilevitch, Lavelle, Knobler, Raspaud, and Gelbart [24,28]. They showed that DNA ejection from phage lambda is inhibited when external osmotic pressure is applied by adding high-molecular weight polyethylene glycol (PEG) to the solution outside the capsids. The viral capsids are permeable to water and ions but not PEG, thus creating an external osmotic pressure that opposes DNA ejection. The fraction of the DNA length ejected decreases progressively with increasing osmotic pressure. After a long incubation thermodynamic equilibrium is presumed to be reached between forces driving ejection and forces resisting ejection.

Ejection with phage lambda into a solution containing 10 mM Mg^{2+} at 37˚C was found to be completely suppressed by ~25 atm of applied osmotic pressure, corresponding to ~14 pN of force opposing ejection [25]. In contrast, only roughly half of the genome was ejected when 3 atm of osmotic pressure was applied. This technique has further been applied to phages T5 and SPP1, which contain similar DNA packing densities, and roughly similar pressures were found to be needed to inhibit ejection [29,30].

Phi29 is one of the smallest well-characterized phages, having a 19.3 kbp genome length and a prolate icosahedral capsid ~42 nm in diameter and ~54 nm in height [31]. The dynamics of phi29 DNA packaging has been extensively studied through the use of a highly efficient system in which packaging activity is reconstituted *in vitro* using purified DNA, procapsids, and recombinant motor ATPase [32].

II. RESULTS AND DISCUSSION

To study phi29 DNA ejection we first carried out packaging reactions (Fig. 1) as described previously [33], at room temperature (~22°C) in a solution containing 25 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 mM NaCl, and 0.5 mM ATP for 15 minutes, more than enough time for the whole genome to be packaged. Typically ~50% of the added molecules are packaged in a bulk reaction, and DNAse I (1 unit/µg of DNA; NEB, Inc.) is added to digest any unpackaged DNA.

We found that DNA ejection can then be triggered by heating the complexes to 50°C (we note that 45°C did not trigger measurable ejection). As in previous ejection experiments, not all capsids eject their DNA [28-30]. This is advantageous as it provides an internal control for each

reaction to confirm that a majority of complexes packaged the full genome length. The efficiency of packaging and ejection are unimportant as long as a sufficient number of DNAs are packaged and subsequently ejected, permitting quantitative assessment of the partly ejected DNA.

To apply osmotic pressure, various quantities of PEG 8000 (Fluka, Inc.) were added prior to triggering ejection. Additional DNase was also added to completely digest the ejected DNA (3 units/μg of input DNA). As in prior studies [28], samples were incubated for 30 min. EDTA was then added to 20 mM to inhibit the DNase. To rupture the capsids and release the unejected DNA 20 units of Proteinase K was then added and the sample was heated to 65°C for 30 min. The extracted DNA was analyzed by electrophoresis on 0.5-0.8% agarose gels in 40 mM Tris-Acetate, pH 8.3, 1 mM EDTA at 3.3 volts/cm for 3 hr. with ethidium bromide staining.

When ejection was triggered in PEG solutions, we measured unejected DNA lengths less than the full genome length (Fig. 2(a)), indicating that less than the full genome length was ejected. As expected, the unejected length increased with increasing PEG concentration, indicating that ejected DNA length decreases with increasing osmotic pressure (Fig. 2(b)). In all experiments the full genome length is also detected because, as mentioned above, heating does not trigger ejection from all capsids.

For most of the PEG concentrations used only two bands were detected on the gel. The top band, which is the full genome length, corresponds to complexes that did not eject and the bottom band corresponds to complexes that ejected a fraction of the full length (Fig. 2(a)). Consistent with recent ejection studies with phage lambda [34], the bottom band is significantly broader than bands having a similar quantity of fixed-length DNA standards, indicating that there is variability in the lengths of DNA ejected from different individual capsids. This has been interpreted as indicating that there is heterogeneity in the ejection forces in individual phage particles due to heterogeneity in the DNA conformations [34,35], consistent with our finding that the DNA undergoes nonequilibrium dynamics during packaging [23].

Three control experiments are shown in Fig. 2(a). First, when proteinase-treated phi29 DNA is run on the gel directly, the expected full genome length is seen. Second, when the DNA is packaged with ATP but not heated to induce ejection, the packaged full genome is seen (less bright than the input DNA since in-vitro packaging is not 100% efficient). Third, when no PEG

is added, no partly unejected DNA is seen, indicating that complexes that eject DNA eject the full genome (and only a small quantity of full length DNA is seen, corresponding to a small fraction of complexes that did not eject).

In all experiments with 8% PEG (e.g., Fig. 2(a)) and some experiments with 12% PEG, we observed a faint middle band on the gel, indicating that a small fraction of individual complexes eject shorter lengths of DNA. A similar effect was observed in ejection studies with phage T5 with similar PEG concentrations, suggesting that ejection does not reach equilibrium in a small fraction of complexes [29]. It was proposed this is due to kinetic trapping of the DNA in nonequilibrium conformations [29]. In Fig. 2(b) we plot the *shortest* unejected DNA length, corresponding to the dominant gel band and maximum DNA length ejected, vs. PEG concentration.

At the highest PEG concentration used (31.2%) the observed unejected length is still slightly below the full genome length, indicating ejection was not completely inhibited, as was observed in similar studies of phage SPP1 [30]. As in those studies, we could not test higher PEG concentrations because the solutions became too viscous for accurate pipetting.

We determine osmotic pressure as described previously [25], using an empirical formula derived based on experimental measurements: Π (atm) = -1.29 $G^2 T$ + 140 G^2 + 4G, where T is the temperature (°C) and $G = w/(100\text{-}w)$, where w is the %(w/w) of PEG. This allows us to replot the data in terms of DNA length ejected vs. osmotic pressure (Fig. 3(a)). As expected, the length of DNA ejected decreases monotonically with increasing osmotic pressure.

Fig. 3(a) also shows a comparison of the data with quantitative predictions made by the inverse DNA spool model [12]. This model predicts that the DNA inside the viral capsid is arranged as a spool coaxial with the portal channel with hoops of DNA arranged in a hexagonal lattice filling inward from the outermost radii; this is proposed to be the equilibrium conformation. Free energy is calculated as the sum of the DNA bending energy and DNA-DNA intra-strand interaction energy. The energy is minimized by balancing these two terms to determine intra-strand spacing and force resisting packaging as a function of DNA length inside the capsid.

Analytic results have been derived for spherical and cylindrical capsid geometries [12]. Although the phi29 capsid is actually a prolate icosahedral shape, it does not have a large aspect

ratio (being ~42 nm in maximum diameter and ~54 nm in maximum height), and it was thus proposed that it could be well modeled as either a sphere or cylinder since the predicted forces were found to be insensitive to changes in geometry [12]. Fig. 3(a) shows calculations for both geometries. Cryo-electron microscopy (cryoEM) studies indicate that the volume occupied by DNA is $\sim 4 \times 10^{-23}$ m^3, and the maximum diameter (perpendicular to the portal axis) occupied by the DNA is 35 nm [31]. We therefore assumed a cylinder of diameter 35 nm and height 41 nm and a sphere of radius 21 nm, where both volumes were constrained to equal the DNA volume determined from the cryoEM measurements.

As in previous studies, we assumed 50 nm for the DNA persistence length, based on experimental studies [36]. We note that in the spool model there is only a small predicted temperature dependence associated with the bending energy [12]. The predicted forces resisting packaging at 50°C (where ejection measurements were done) vs. 22°C (where packaging measurements were done) differ by <2%. We determined parameters describing the DNA-DNA interaction potential based on measurements of spacing between DNA segments condensed by osmotic pressure [11,37]. Pressure (Π) vs. spacing (d) follows $\Pi=F_0 \, exp(-d/c)$ [11,12]. We fit this equation to measurements by Donald Rau, described in [15], for a solution containing 100 mM Na$^+$ and 10 mM Mg^{2+} and obtained c=0.28 nm and F_0=44,615 pN/nm^2.[i] At these concentrations DNA is expected to be saturated with counterions [38] and the ratio of mono- to divalent ions is the same as in our studies. When these parameters are used in the spool model we observe good agreement with our measurements for both geometries (Fig. 3(a)).

On the other hand, cryoEM studies of phi29 capsids packaged to 32%, 51%, and 78% filling did not find evidence for an inverse-spool DNA conformation [31]. In this filling range the model predicts that the DNA will preferentially spool on the periphery, with the first segments to be packaged lying against the inner walls of the capsid and subsequent layers spooling inwards, leaving a void in the center of the capsid [12]. The cryoEM studies observed uniform average DNA density across the full volume of the capsid with no central void,

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[i] We note that it is unclear whether these values are completely consistent with the values c=0.30 nm and F_0=12,000 pN/nm^2 used in Refs. 12 & 25 to model a solution containing 10 mM Mg^2 and no Na$^+$, which would be expected to screen DNA more strongly than the solution we used. However, determined values of c ranging from 0.23 to 0.28 and F_0 from 23,000 to 190,000 have been reported for Mg^{2+} solutions and c ranging from 0.34 to 0.37 and F_0 from 5,900 to 21,000 for Na$^+$ solutions (P. Grayson, PhD Thesis, Caltech, 2007).

suggesting that bending energy plays a smaller role than assumed in the model. Our present finding of pressure values that agree with the model predictions despite this discrepancy suggests that the model overestimates the role of bending rigidity and underestimates intra-strand repulsion.

Phi29 DNA packaging has also been investigated via molecular dynamics simulations, also assuming an empirical DNA-DNA interaction potential derived from the DNA condensation data [15]. Consistent with the cryoEM findings these simulations did not predict a spool conformation. Rather, they predicted a partially-disordered folded toroid. The predicted resistance forces were somewhat higher than those predicted by the spool model: ~25 pN at 80% filling and ~57 pN at 100% filling.

We estimate force resisting DNA ejection as described previously [25]. Specifically, $F=\Pi \pi R_{DNA}^2$, where Π is osmotic pressure and $R_{DNA} \cong 1.2$ nm is the effective radius of the approximately cylindrical volume of PEG displaced by the ejected DNA. We plot this in Fig. 3(b) along with theoretical predictions of the spool model and forces resisting packaging inferred from optical tweezers measurements. The forces resisting packaging were inferred by Liu et al. [26] by relating the measured dependence of motor burst duration on filling to its measured dependence on applied force, and independently by Berndsen et al. [27] by relating the measured dependence of motor slipping on filling to its measured dependence on applied force. As shown in Fig. 3(b), good agreement is found between inferred ejection force over the measured range from 0-80% filling and the prediction of the inverse spool model. Agreement is also observed between the model prediction and the inferred forces resisting packaging in the high filling limit (~80-100% filling). The striking agreement of the forces inferred by these two different types of measurements with the model in the high filling limit suggests that the overall energetics of DNA packaging are well described by the model, which assumes that no energy dissipation occurs during packaging or ejection.

Unexpectedly, however, the inferred ejection force at each filling level <80% is higher than the force resisting packaging. At 80% filling, the two measurements agree to within errors, but at lower filling levels they diverge. Theoretically, one would not expect the ejection force to be higher because the potential energy available to drive ejection cannot be higher than work done by the motor on the DNA (energy transferred to the DNA) during packaging [14]. We note

that the inverse spool model predicts only a weak temperature dependence due to the bending energy [12]. The predicted resisting forces at 80% filling at 50˚C (where the ejection measurements were done) vs. 22 ˚C (where the packaging measurements were done) differ by <2%.

The discrepancy suggests that methods by which ejection forces and/or packaging forces are inferred from the measurements are not completely accurate. Thus, the full validity of the model to describe the energetics and degree to which energy dissipation can be neglected must still be considered open questions needing further investigation. We note that the estimated uncertainties both types of measurements do not include several potential systematic errors that are presently difficult to quantify. In the determination of packaging forces from motor slipping measurements, slipping is very infrequent at low force and subject to larger uncertainty [27]. The analysis also relies on an assumption that all slips can be reliably measured and that the dependence of slipping frequency on force is independent of capsid filling. Determining the force via measurements of motor burst duration also becomes difficult below 5 pN because the durations become very short (<20 ms) [26]. The analysis also relies on an assumption that all bursts can be reliably measured and that the dependence of burst duration on force is independent of capsid filling.

There are also potential additional sources of uncertainty in the inference of ejection forces from the osmotic pressure experiments. First, conversion of PEG concentration to osmotic pressure relies on an empirical relationship based on experiments, but the absolute certainty is unclear [25]. Second, the conversion of osmotic pressure to force resisting DNA ejection is based on an approximate scaling description suggested to only be accurate to within a factor of ~2 [39].

III. CONCLUSIONS

We have shown that the phi29 ejection force inferred from the osmotic pressure experiments agrees with the prediction of the inverse-spool model over the measurable range from 0-80% filling and the force resisting packaging inferred from the optical tweezers experiments agrees with the predictions of the model in the high filling limit. However, since electron microscopy studies did not reveal an inverse-spool conformation our present findings suggest that this model overestimates the role of bending energy and underestimates electrostatic

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self-repulsion. The amount of energy dissipated during packaging also remains an open question. Further studies are warranted to investigate why the inferred force resisting packaging below ~80% filling is lower than the inferred ejection force.

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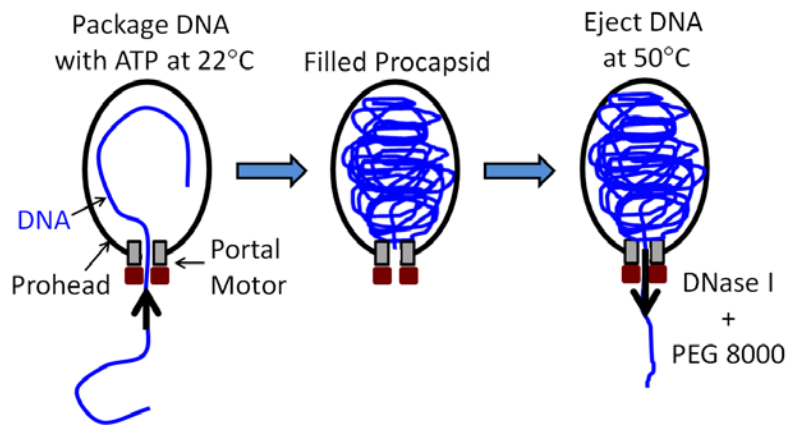
FIG. 1. Schematic illustration of the experiment. The phi29 genome is packaged by the portal motor complex into the viral procapsid (left). After packaging is completed (middle) the DNA is ejected by heating to 50°C (right). DNase is added to digest the DNA as it is ejected. External osmotic pressure is applied by adding an osmolyte, PEG 8000, which does not permeate the capsid.
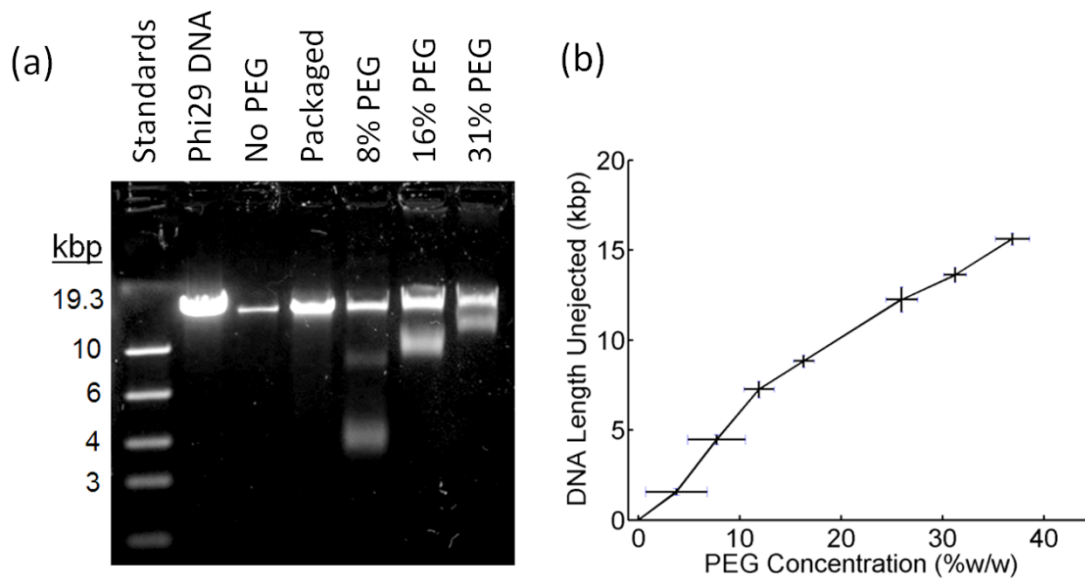
FIG. 2. (a) Gel electrophoresis of DNA remaining unejected with varying PEG concentrations (%w/w). Also shown are DNA length standards, full length phi29 DNA, and packaged phi29 DNA extracted from capsids. **(b)** Length of unejected DNA vs. PEG concentration. Each measurement was repeated $\geq 3\times$. Error bars in the length measurements indicate standard deviation and errors in the PEG concentration were determined by weighing the PEG solutions.
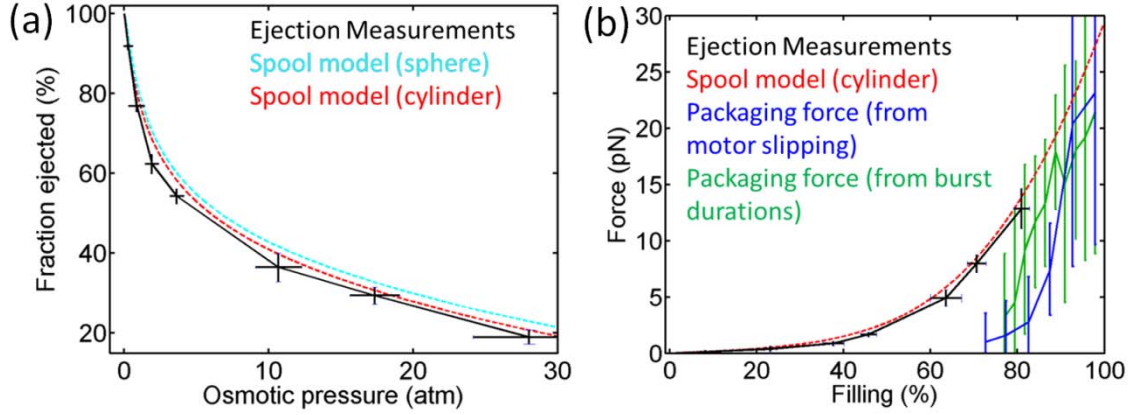
FIG. 3. (a) Fraction of phi29 genome length ejected vs. applied osmotic pressure (black points). Error bars were determined as in Fig. 2. Shown for comparison are predictions of the inverse spool model assuming a cylindrical capsid (dashed red line) and spherical capsid (dashed cyan line), as described in the text. **(b)** Comparison of internal force vs. capsid filling (% of genome length packaged) inferred from the ejection measurements (black points), predicted by the spool model assuming a cylindrical capsid (dashed red line), inferred from motor slipping measurements during packaging [27] (blue points; error bars show standard error in the mean), and inferred from motor burst duration measurements [26] (green points).