Popping the cork: mechanisms of phage genome ejection

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Abstract | Sixty years after Hershey and Chase showed that nucleic acid is the major component of phage particles that is ejected into cells, we still do not fully understand how the process occurs. Advances in electron microscopy have revealed the structure of the condensed DNA confined in a phage capsid, and the mechanisms and energetics of packaging a phage genome are beginning to be better understood. Condensing DNA subjects it to high osmotic pressure, which has been suggested to provide the driving force for its ejection during infection. However, forces internal to a phage capsid cannot, alone, cause complete genome ejection into cells. Here, we describe the structure of the DNA inside mature phages and summarize the current models of genome ejection, both *in vitro* and *in vivo*.

Lysogenic cycle
A cycle wherein a phage infects a cell and enters into a quiescent phase, during which few of its genes are expressed. The cycle is completed by the induction of the phage, when phage genes for lytic growth are derepressed.

Baseplate
A multiprotein complex at the head-distal end of long-tailed phages.

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The Earth's total bacterial population was recently estimated to be ~10^{30} cells[1], and as virus–bacterium ratios are typically between 5:1 and 10:1 (REF. 2), there are therefore ~10^{31} phages on the planet, although the number of different phage types is unknown. In the oceans alone, more than 10^{23} phage infections of bacteria are expected to occur every second[2]. Phage infections can result in the release of viral progeny with or without lysis of the host cell; alternatively, the phage can enter a lysogenic cycle by integrating into the host genome to form a prophage. In both cases, adsorption of the tail or baseplate component of the phage to a cell receptor (or receptors) triggers genome ejection into the host cell cytoplasm. About 95% of described phages have tails and contain double-stranded DNA (dsDNA). The remaining 5% contain single-stranded DNA (ssDNA), ssRNA or dsRNA genomes. Both ssDNA and dsDNA phage genomes, as well as ssRNA phage genomes enter the cell devoid of their capsids, but dsRNA genomes are internalized as more or less intact nucleocapsids (FIG. 1).

The first demonstration of phage genome ejection into a bacterial cell was the now classic experiment conducted by Hershey and Chase[3]. In addition to being a landmark study in the phage field, this experiment helped to finally convince those who were not persuaded by the earlier *Pneumococcus* transformation studies[4] that DNA is the genetic material. However, although it is not often explicitly stated — in part because it seems to contradict the common over-interpretation of the Hershey–Chase experiment[3] that only DNA is ejected from the infecting particle into the cell, whereas all the proteins remain outside, and in part because there are few relevant experimental data — all phages also eject proteins into the infected cell. Indeed, Hershey himself detected proteins from the infecting particle in the cell[5] (probably the abundant internal proteins[6]), hinting at the complexity of the ejection process that was yet to be uncovered.

Mature phages can be considered as discrete nanomachines[7] that contain the energy for the initial structural changes which occur during the first steps of infection[8–10]. The activation energy for these changes has been measured for a few mesophilic dsDNA phages as 20–40 kcal per mol both *in vivo* and *in vitro*[11–15]. In order to balance virion stability with both a high infection efficiency and the maintenance of control over those initial structural changes, the activation energy will probably be lower for phages that grow in cold habitats and higher for phages that grow in warm habitats. Early models of DNA ejection by phages postulated that these viruses act like hypodermic syringes to inject their DNA into the bacterial cell[16,17]. This view is unfortunately still common; however, the analogy is misleading because it falsely suggests that the volume of the phage head changes as DNA is ejected, like the volume of a syringe changes as the plunger is depressed. Many researchers therefore prefer the term ejection to injection.

The idea that internal virion forces cause the ejection of phage DNA has a long history, and was initially popularized in the 1960s by a highly influential textbook that stated: "One may imagine, therefore, that the DNA is packed into the phage head under constraint and forces its own way out through the sheath [*Myoviridae*] after

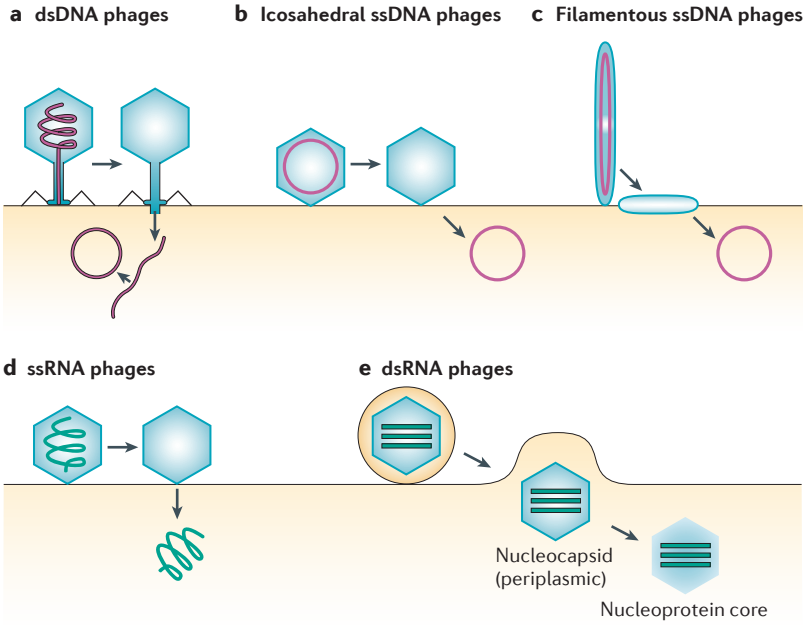

Figure 1 | **Genome internalization by representative phage types.** **a** | For double-stranded DNA (dsDNA) phages, most virion proteins remain outside the cell after infection. The ejected dsDNA sometimes circularizes via complementary single-stranded DNA (ssDNA) ends or by recombination across terminally repeated sequences. **b** | Most virion proteins of icosahedral ssDNA phages remain outside the cell. All of these phages contain circular genomes. **c** | The structural proteins of filamentous ssDNA phages disassemble in the cell membrane, where they can be re-used in progeny phages. All of these phages also contain circular genomes. **d** | For ssRNA phages, most virion proteins remain outside the cell after infection. **e** | All known bacterial dsRNA phages have segmented genomes and contain an external membrane consisting of phospholipids and proteins. The membrane fuses with the bacterial outer membrane, releasing the nucleocapsid into the periplasm. The nucleocapsid then enters the cytoplasm, where the protein shell (blue outline) disassembles, leaving a nucleoprotein core containing the RNA-dependent RNA polymerase that transcribes the RNA genome. This Review discusses dsDNA phages; for details of ssDNA, ssRNA and dsRNA phages, see REF. 155.

the contraction and 'uncorking' reactions of the tail are triggered" (REF. 18). The first quantitative model of ejection used Brownian motion as the driving force[19], but the timescale for ejection predicted by this model was longer than the latent period of the phage[20]. Zárybnický proposed that the high osmotic pressure inside the filled phage head would result in a force that could drive genome ejection[20]. He constructed a glass model of phage T4 using nylon thread for DNA and glycerol solutions to adjust osmotic pressures. The osmotic pressure inside a phage T4 head was estimated to be ~30 atmospheres (atm), and the nylon 'DNA' was ejected in a few seconds, figures that are fairly consistent with recent data and are remarkably prescient given that the properties of condensed DNA were not understood at the time.

These and other early models of DNA ejection were largely forgotten as attention focused on the structure of DNA condensed *in vitro*[21], and advances in cryoelectron microscopy (cryoEM) revealed the organization of DNA packaged in a phage head[22]. In addition, the application of single-molecule approaches to studies of phage DNA packaging[23] has allowed the energetics of confining DNA in a phage capsid to be quantified. In this Review, we describe the structure of packaged dsDNA, including its pressures, thermodynamics and dynamics, and discuss current models of *in vitro* and *in vivo* dsDNA ejection.

The structure of packaged DNA

The dsDNA of many bacterial phages is densely packaged at ~500 mg per ml, with a structure comparable to that of DNA condensed *in vitro* by polyamines or other polycations, or monovalent ions in the presence of molecular crowding reagents[21,24,25]. However, the interhelical distance in the packaged DNA of tailed phages is often smaller than those easily obtained *in vitro*. Packaging is accomplished using a phage-encoded nanomotor that converts the chemical energy of ATP hydrolysis into the mechanical movement of replicated DNA to insert the DNA into pre-formed capsids. The DNA-packaging nanomotor has recently been reviewed[26]. Single-molecule experiments show that as packaging approaches completion, the motor slips and stalls[23,27,28], supporting the idea that ~500 mg per ml of normal dsDNA is maximal.

Why is dsDNA in most phage virions packaged to the same high concentration? One possibility is that the thermodynamic activity of water is low under these conditions, and this results in a lower chemical reactivity; condensed DNA is therefore more resistant to external chemical insults. A lower chemical reactivity leads to a longer 'shelf life', which is obviously a beneficial trait for phages (as they might find themselves in an environment with few host bacteria) and might therefore be selected for. Alternatively, the constant concentration of phage DNA might be due to the properties of the packaging enzyme. Direct measurements of DNA packaging *in vitro* for phage Φ29, phage λ and phage T4 show that the rate of packaging decreases as the head fills[29–31]; because the packaging enzyme also usually contains the activity for cutting DNA at the end of a packaging cycle, the nuclease might not have sufficient opportunity to recognize and cleave its substrate until the rate of packaging slows.

With the exception of phage T4, in which genome packaging follows a 'first-in, first-out' strategy[32], the trailing end of a packaged phage genome is normally the first end to be ejected[33–37]. CryoEM reconstructions that were able to resolve the DNA have shown that, in these phages, the DNA penetrates the portal channel[38–42] (FIG. 2). Positioning the genome end in the exit channel makes the initiation of ejection more efficient and is likely to be a feature that is common to most dsDNA phages.

Electron microscopy and X-ray diffraction studies have established that encapsidated DNA in various phages is in the B form, exhibiting a hexagonally packed and concentrically layered structure[43–47]. CryoEM reconstructions of several mature phages further show the DNA as an inverse spool with an axial rise of ~2.5 nm per turn, but with an innermost region that seems disordered[38–42,48–59]. It has been generally assumed that bending DNA into a very small radius is energetically prohibitive and that in the centre of the capsid no ordered structure is maintained. However, although this model has

Transformation
The uptake of naked DNA by intact cells.

Brownian motion
The random movement of particles in a fluid, resulting from their collisions with fast-moving molecules in the fluid.

Interhelical distance
The distance between the centre of two adjacent helices within the DNA.

Tailed phages
Phages containing a double-stranded DNA genome in an icosahedral head in which one vertex is occupied by a protein tail.

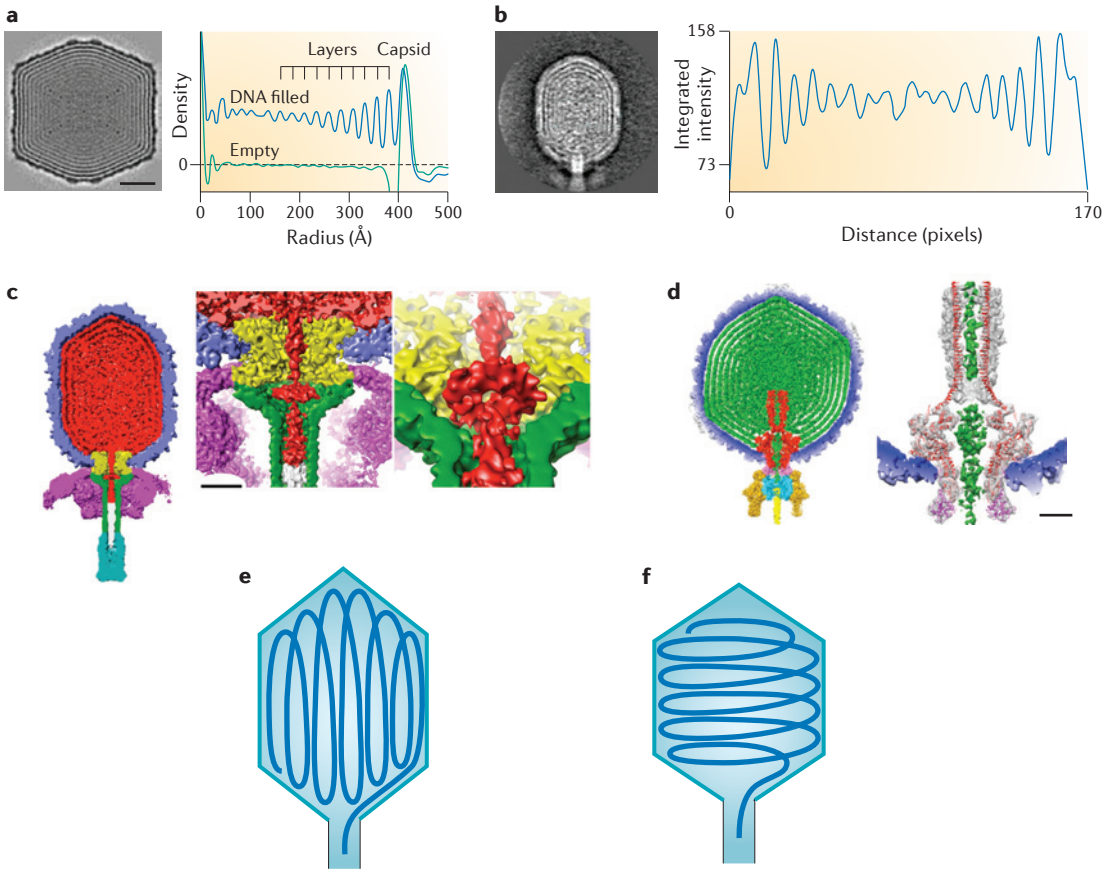

Figure 2 | **DNA structures inside phage capsids. a** | Averaged cryoelectron microscopy structure of a filled phage T5 capsid, and the measured spacing between the concentric rings of double-stranded DNA (dsDNA) strands. Scale bar represents 200 Å. **b** | Averaged cryoelectron microscopy structure of a filled phage Φ29 capsid, and the averaged density profile of DNA from a cross-section of the capsid. **c** | A slice through a reconstruction of the phage Φ29 capsid, showing the presence of DNA (red) in the exit channel (green) through the portal (yellow). Scale bar represents 50 Å. **d** | A slice through a reconstruction of the phage P22 capsid, with DNA (green) present in the portal–gp4 complex (red–pink). Scale bar represents 50 Å. **e** | Schematic representation of DNA spooled with its axis perpendicular to the phage tail in a prolate capsid. **f** | DNA spooled in a co-axial manner for an isometric capsid. The exact structure of the encapsidated DNA for parts **e** and **f** has not been rigorously established[156]. Part **a**–**d** images are reproduced, with permission, from REF. 56 © (2006) Elsevier, REF. 54 © (2008) Elsevier, REF. 39 © (2008) Elsevier and REF. 41 © (2011) Elsevier, respectively.

Portal
The head–tail connector in a phage. During infection of a cell, the DNA packaged in the phage head is ejected through a channel in the centre of the portal.

B form
Pertaining to DNA: the normal Watson–Crick structure of DNA, with 10.4 bp per helical turn.

Inverse spool
A spool of line (here, DNA) wound from the outside to the inside.

Packaging motors
Enzymes (terminases) that convert ATP into mechanical movement to translocate DNA into the pre-formed phage prohead (the precursor structure to the head, or capsid, of a phage). Most terminases also cleave double-stranded DNA to complete the DNA-packaging process.

become widely accepted, a cryoEM study of phage T5 concluded that, even in the central region, DNA is in distinct domains, with hexagonal packing separated by clear domain boundaries[60] (FIG. 3). The authors of this study suggested that this is a feature common to many phages. Interestingly, packaging simulations of phage ε15, which contains an internal protein core, result in inversely spooled DNA, whereas phage Φ29, for which the virion lacks organized internal proteins (as does the phage T5 virion), assumes a folded DNA structure[61,62].

Pressures in the phage head

The packaging motors of phage Φ29, phage λ and phage T4 encapsidate DNA against a force of up to 100 pN[23,29–31]. The forces inside the phage Φ29 capsid that oppose packaging slowly increase until about 40% of the genome has been packaged, but thereafter they increase rapidly as the DNA helices of the genome are brought closer together. *In vitro*, both DNA packaging and ejection are affected by the ionic strength and composition of the buffer such that as cation concentration increases, the internal forces generally decrease[63–65]. There is no estimate of the forces opposing genome packaging *in vivo*, but the high intracellular concentration of polyamines suggests that these forces are lower than might be inferred from *in vitro* studies.

The value of an ~60 pN force opposing DNA packaging was divided by the hexagonal cell surface area of the DNA toroid to obtain an approximate pressure inside the mature capsid of 6 MPa (~60 atm)[23]. This pressure was considered to provide the driving force for DNA ejection during infection. However, no major structural deformation of either protein or DNA was detected in phage T7 or phage P22 particles[66,67], whereas such deformations would be expected if the capsid contents were subjected to such high physical pressures. Further, the precise thermodynamic pressure was not specified, although several are present in the capsid (BOX 1); the dominant pressure is osmotic pressure, which is not a physical pressure but a measure of the thermodynamic

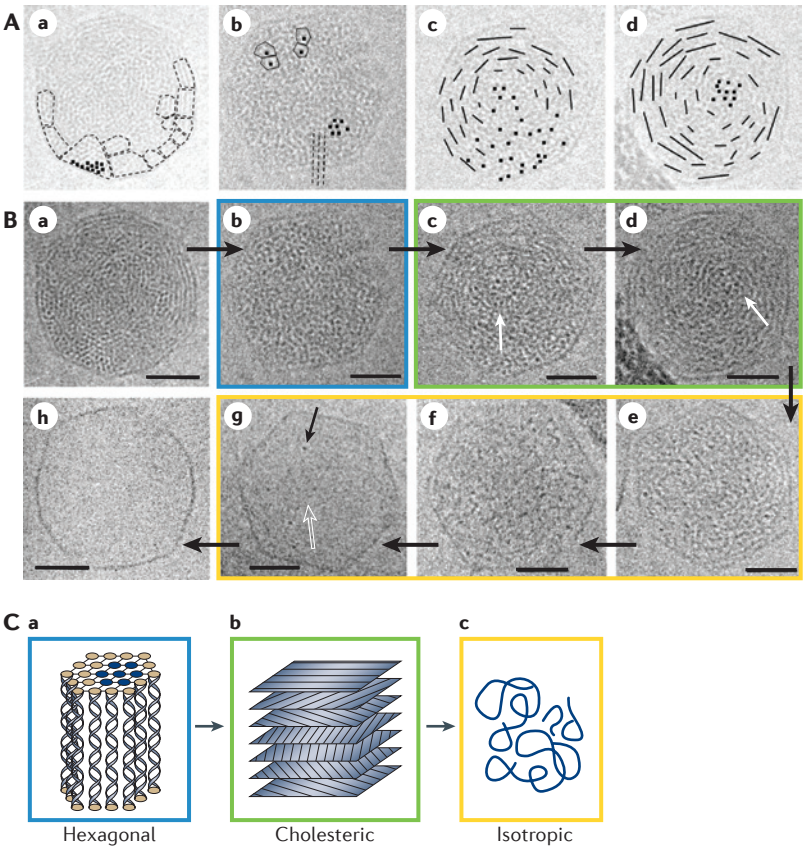

Figure 3 | **DNA structural transitions during genome ejection.** In this study[60], phage T5 was triggered to eject its genome by the addition of its receptor, FhuA. **A** | Top views of the arrangement of DNA domains corresponding to parts **Ba**–**d**. The black dots and lines represent cross-sections of DNA helices that are perpendicular or oblique, respectively, to the plane of the paper. The arrangement of DNA within the capsid changes as the head empties. When the capsid is full (part **Aa**), DNA domains are arranged in a hexagonal lattice, outlined by the dashed lines, which reflect different topological arrangements between DNA domains. As DNA is ejected, the lattice initially becomes less ordered (part **Ab**), then organizes into a cholesteric state (parts **Ac**,**d**). **B** | The complete series of images for the emptying capsid. The capsid is full in part **a** and empty in part **h**, with intermediate states in between. Following the hexagonal and then cholesteric arrangements of DNA shown in parts **Ba**–**d**, the DNA remaining inside the emptying capsid takes on disordered isotropic states (parts **Be**–**g**). In part **Bg**, the upper (black) and lower (white outline) arrows mark the cross-section of DNA helices that are perpendicular or oblique, respectively, to the plane of the paper. White arrows indicate examples where the lattice allowed the interhelical distance to be measured. The scale bars represent 25 nm. **C** | Schematic representation of hexagonal, cholesteric and isotropoic DNA within the capsid as it empties; coloured outlines correspond to the structure of DNA in parts **Bb**–**g**. Figure is reproduced, with permission, from REF. 60 © (2010) Elsevier.

activity or chemical potential of water. When thermodynamically specified, the compressive pressure acting on the DNA is only a small fraction of the ubiquitously reported 60 atm[68].

The assumption that all the energy expended by the packaging motor is stored in the packaged DNA is similarly problematic. For a typical 60 nm diameter capsid harbouring a 40–50 kb genome, some DNA has to be bent more tightly than its persistence length (which has been shown to be <100 bp[69]). Bending takes energy that is indeed stored in the DNA; it corresponds to the compression force exerted by the capsid. However, DNA bending contributes only ~10% of the overall energy landscape, which, reflecting the high osmotic pressure in the capsid, is dominated by hydration energy[70]. In free solution, each nucleotide of B form dsDNA is associated with 60–80 water molecules, about 20 of which are tightly bound in primary hydration layers, with the remainder in secondary layers that are exchangeable with bulk solvent[71–74]. Hydrated DNA fills a capsid when ~50% of the genome is packaged, which is about the point when packaging forces increase rapidly. To package the complete genome, some hydration layers must be stripped from the DNA and its counterions, and the water expelled from the capsid by reverse osmosis (FIG. 4). Reverse osmosis requires energy that is provided by the packaging motor, but because the water leaves the capsid, not all the energy can be stored there.

When a mature phage is placed in buffer (so the osmotic pressure of the buffer, π_{buffer}, is less than the osmotic pressure of the capsid, π_{capsid}), water has a natural incentive (given by $\pi_{capsid} - \pi_{buffer}$) to re-enter the capsid and reduce the internal osmotic pressure. The value of π_{capsid} can be determined from the osmotic pressure of an external buffer that completely stops DNA ejection *in vitro* (see below). For wild-type phage λ and phage T5, this value is ~25 atm, and it is ~47 atm for the Gram-positive phage SPP1 (REFS 75–78). Note that these experimental values of internal pressure are lower than those calculated from *in vitro* packaging reactions, in qualitative agreement with the idea that not all the energy expended during DNA packaging is stored in the capsid.

Current models for DNA ejection

The continuum mechanics model. The continuum mechanics model was developed to explain *in vitro* phage DNA ejection[79–83]. The model assumes that, on average, encapsidated DNA forms a perfect toroid and it replaces osmotic pressure with a repulsive interaction between the nearest DNA strands as a function of their spacing. This allows quantitative estimates of the free energy enhancement for DNA as a function of the length of DNA remaining in the capsid. Further assuming that ejection is a quasi-equilibrium process — that is, assuming the DNA remains toroidal during ejection while the interhelical spacing increases monotonically — the free energy estimate is then used to define an 'ejection pressure', and correspondingly, an 'ejection force'. The ejection pressure is a decreasing function of DNA spacing, and therefore of the DNA length remaining inside the capsid; because the external osmotic pressure opposes the ejection pressure, less DNA is ejected as the external osmotic pressure increases (FIG. 5a). However, although this model is thermodynamically correct, it is unclear how an internal virion ejection force can be transmitted along the helical axis of a flexible DNA molecule in a biologically realistic timescale[68]. The force transmission process has been likened to pushing 10 m of 1 mm-thick string through a narrow straw[18].

The continuum mechanics model has been extensively tested *in vitro*[64,75–78,84,85]. Phages are suspended in buffer containing a high-molecular-mass osmolyte (for example, polyethylene glycol (PEG)) and/or a

Toroid
A doughnut-shaped object (in mathematical terminology).

Box 1 | **Pressures in a mature phage capsid**

A mature phage capsid and its contents (for simplicity, limited in this scenario to DNA) are subjected to several different pressures. These are P_{DNA}, the pressure that the capsid imparts on the DNA (which equals the pressure that the DNA imparts back on the capsid); $P_{hydro,in}$, the hydrostatic pressure inside the capsid; P_{capsid}, the pressure that the capsid experiences from inside; and π_{capsid}, the osmotic pressure inside the capsid. These are all thermodynamically different quantities, the origins and relationships of which have been discussed in detail[68]. Here, we simply state these relationships:

$$P_{hydro,in} - P_{hydro,buffer} = \pi_{capsid} - \pi_{buffer}$$

(in which $P_{hydro,buffer}$ is the hydrostatic pressure of the buffer and π_{buffer} is the osmotic pressure of the buffer), which is simply analogous to the famous van 't Hoff's law for water columns, and

$$P_{capsid} = P_{DNA} + P_{hydro,in} - P_{hydro,buffer}$$

Given that osmotic pressure is the chemical potential of water, high values of $\pi_{capsid} - \pi_{buffer}$ mean that DNA in a mature capsid acts as a means of attracting water. DNA also serves as a spring confined by the capsid: a spring force exists because DNA is a polymer with a persistence length of ~100 bp, and so confining ~100 bp of DNA at a high linear compression factor (~250 is a typical value) costs some mechanical energy as well as a DNA-configurational entropic force. This spring force is directly responsible for P_{DNA}, but provides only a minor contribution to P_{capsid}[70].

DNA-condensing agent (for example, spermine), and are triggered to eject their genome by the addition of the bacterial receptor protein. At equilibrium, the amount of DNA ejected (or remaining in the virion) is measured. With phage λ and phage SPP1, as the concentration of PEG or spermine increases, less DNA is ejected, in agreement with the continuum mechanics model[79–83]. However, experiments with phage T5 reveal that at an external osmotic pressure of 0.6–7 atm (π_{buffer} = 0.6–7 atm), T5 phages eject varying amounts of DNA[78]. Only at higher pressures does the amount of DNA ejected follow theoretical expectation. It is not at all obvious why phage T5 should respond differently from phage λ, but the data suggest that factors other than osmotic pressure and DNA strand repulsion are involved.

Hydrodynamic model of* in vitro *DNA ejection. The hydrodynamic model was proposed as a way to rationalize the differences between the general mode of genome ejection probably used by most phages and that used by phage T7, which has an enzyme-based mechanism of genome ejection (see below)[86]. The underlying logic of the hydrodynamic model, which has not yet been explicitly tested *in vivo*, is that after DNA ejection is triggered by the addition of a receptor, water diffuses into the capsid to neutralize the osmotic gradient[68,86–88]. Most water will diffuse across the capsid shell, but because the capsid is full, in order to make space, DNA will be pushed out by the hydrostatic pressure gradient along the tail[68,88]. Ignoring the minor contribution of DNA-bending energy[70], DNA will continue to exit the capsid until the hydrostatic pressure gradient (equalling the osmotic pressure gradient) is neutralized.

The thermodynamic driving force for water movement is the osmotic gradient across the capsid, determined as $\pi_{capsid} - \pi_{buffer}$, and these are two parameters that can be independently varied. Hydrophilic polymers that cannot enter the capsid do not affect π_{capsid} but increase π_{buffer}, whereas DNA-condensing agents like spermine do not change π_{buffer} but reduce π_{capsid}. In both cases, the osmotic gradient across the capsid is reduced, and less DNA is ejected from the capsid (FIG. 5a). These are precisely the results of the elegant experiments designed to test the continuum mechanics model. However, the hydrodynamic model posits that because DNA packaging includes reverse osmosis, then the reverse process of DNA ejection must include osmosis, and that the ejection rate is partly determined by capsid permeability[88]. The model also explains how the energy expended in carrying out reverse osmosis can facilitate ejection despite this energy not being stored in the DNA.

The source of the forces that drive genome ejection is the major disagreement between this and the continuum mechanics model. The continuum mechanics model maintains that the ejection forces are due to the pressure of packaged DNA, whereas the hydrodynamic model states that the thermodynamic incentive of external water to rehydrate the packaged DNA drives ejection. However, while the hydrodynamic model addresses the dynamics, and thus the mechanism, of ejection[68,86–88], the continuum mechanics model only considers the thermodynamics of the process at equilibrium.

Dynamics of DNA ejection *in vitro*

Genome ejection of phage T5 and phage λ has been visualized from single virions. Ejection of phage λ DNA occurs in a single burst lasting a few seconds, during which the rate of ejection is not proportional to the amount of DNA remaining in the capsid, reaching a maximum after ~50% of the genome has exited and approaching zero as ejection nears completion[89]. To explain these data using the continuum mechanics model, a frictional parameter that varies over two orders of magnitude was invoked; a theoretical basis for the source of this frictional parameter is lacking, however.

Ejection of phage T5 DNA is more complex. As with phage λ, the initial rate of ejection increases non-monotonically until ~50% of the genome is ejected, followed by a decline in rate as the capsid empties. However, most phage T5 virions eject DNA discontinuously, with distinct pauses at different locations[15,90]. It has been suggested that other phages containing DNA packaged to the same density as phage T5 (that is, most phages) also display transient pauses during *in vitro* ejection[15], although pauses were not detected using phage λ[89]. The pauses might reflect phase transitions in the structure of the DNA remaining in the capsid[60] (FIG. 3). During ejection of the phage T5 genome, the hexagonally packed DNA reorganizes, increasing its interhelical spacing to fully occupy the capsid, but after ~20% of the DNA has exited, the remaining DNA transitions into liquid crystals (on a two-dimensional hexagonal lattice), followed by cholesteric and isotropic states as less and less DNA remains. Partially filled phage Φ29 and phage T3 capsids exhibit comparable DNA structural states[54,91] to those found during phage T5 DNA ejection. Structural reorganization of DNA during ejection might therefore prove to be the rule.

Persistence length
A mechanical property quantifying the bending rigidity of DNA (or any polymer). Molecules shorter than the persistence length are considered to be straight rods. The persistence length of long double-stranded DNA is usually described as ~50 nm (~150 bp), but for segments ≤150 bp, which are pertinent to DNA packaged in phage capsids or to cell biology in general, double-stranded DNA seems to be more flexible, having a persistence length of <100 bp.

Hydration energy
The energy expended in removing water molecules from ions.

Hydration layers
The layers or shells of water molecules surrounding a solute.

Reverse osmosis
The removal of water molecules from a solution through a membrane.

Monotonically
Continuously increasing or decreasing, but not necessarily at a constant rate.

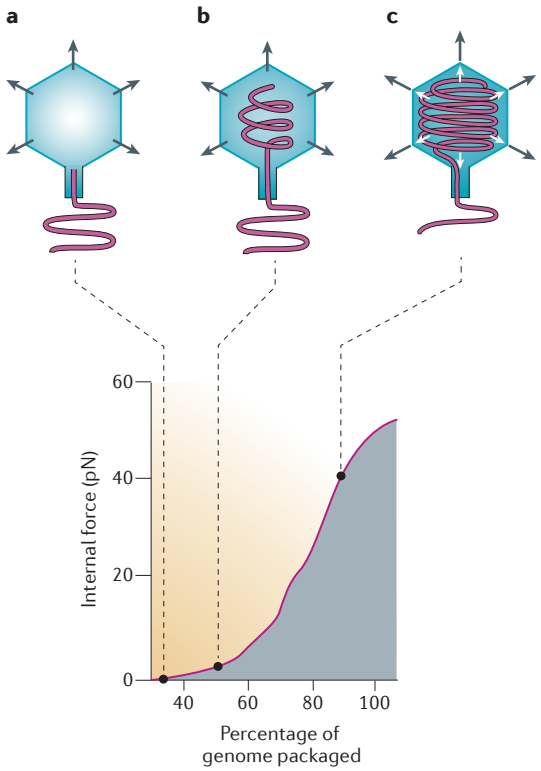

Figure 4 | **Genome packaging requires reverse osmosis.** The graph shows the estimated internal force as a function of the length of genome packaged in a single-molecule *in vitro* DNA-packaging reaction[23]. **a** | As packaging begins, free water molecules are simply pushed out of the capsid. **b** | When ~50% of the genome has been packaged, the DNA, counterions and water molecules completely fill the capsid interior. **c** | As the counterions cannot leave the capsid, water must be stripped off both them and the DNA and then forced out of the capsid in order to allow the complete genome to be packaged. Deeper shades of blue indicate higher osmotic pressures. Graph is modified, with permission, from REF. 23 © (2001) Macmillan Publishers Ltd. All rights reserved.

DNA ejection during bacterial infection

It has often been suggested that *in vitro* DNA ejection experiments address how phages infect cells. However, there is a fundamental difference between these two processes. From a thermodynamic standpoint, there are two compartments *in vitro* (the phage and the environment) but three *in vivo* (the bacterium, the phage and the environment), each with its own osmotic pressure[88]. In particular, in order for a bacterial cell to grow, the cytoplasm must have a higher osmotic pressure than the environment[92]. The osmotic pressure imbalance between the cytoplasm and the environment in exponentially growing *Escherichia coli* results in a corresponding hydrostatic pressure imbalance (turgor) of 3–5 atm (higher in Gram-positive bacteria[93]) that allows cell enlargement. Turgor is therefore lower in non-growing, stationary phase cells. Importantly, the osmotic pressure difference between the phage and the environment is less than that between the phage and the cytoplasm, and *in vivo* the phage and cytoplasm pressures will equalize before the complete genome is ejected into the cell. However, environmental water still has the thermodynamic incentive to flow into the phage head, and then through the tail into the bacterium. Water flow could provide the necessary force to eject the remaining DNA, and the hydrodynamic model therefore adequately explains how the complete phage genome can enter the infected cell.

Under the continuum mechanics model, because of the opposing cytoplasmic osmotic pressure, a secondary process must complete genome internalization (FIG. 5b). The energy stored in the phage capsid can eject less than half the phage λ genome into a bacterium[76,79]. Various ad hoc suggestions for how the remaining DNA is internalized include sequence-specific DNA-binding proteins, sequence-nonspecific DNA-binding proteins that act as Brownian ratchets and the condensation of phage DNA by the crowded cell cytoplasm when the phage DNA enters the cell. With the possible exception of phage T5 and similar viruses, which completely degrade the host chromosome after first-step transfer (see below), it seems unlikely that the entering phage DNA could compete with the ~100-fold higher concentration of host DNA for sequence-nonspecific DNA-binding proteins and be internalized in a reasonable time frame. Condensation of the entering phage DNA is also improbable because it would block transcription, which is a necessary step in establishing infection. Furthermore, the concentration of DNA might be so low in the lipid-containing phage PM2 and phage PRD1 — and is certainly so low in the infective phage P2 virions containing a single, *in vitro*-packaged phage P4 genome[94] — that internal virion forces are insufficient to cause even a single base pair of the genome to penetrate the cell cytoplasm. The phage P2–P4 chimaera, in particular, must use a fundamentally different mechanism of genome ejection during infection.

Enzymatic pulling of phage DNA into the cell. DNA sequence-specific proteins do catalyse genome internalization for some phages. For instance, most of the phage T7 genome is normally internalized by transcription[12,95–97]. The rates of transport of the phage T7 genome into an infected cell, when catalysed by *E. coli* or T7 RNA polymerase or by the type I restriction enzyme *Eco*KI, are the same as the optimal rates of those enzymes determined biochemically *in vitro*[12,97,98]. Phage T7 DNA transport into the cell is thus governed by enzymes and is independent of any physical force or pressure (FIG. 5b). Phage N4 also utilizes a transcription-based strategy, using the ejected virion enzyme and then RNA polymerase II for genome internalization[51,99].

Proteins that are synthesized early after phage infection might also affect later genome transfer: gp17 and gp16.7 of phage Φ29 pull the trailing 35% of the genome into the cell[100,101]. The two proteins are synthesized after the first part of the DNA is ejected into the cytoplasm by the internal virion pressure in what has been described as the 'push' phase[37]. Although the 'pull' phase requires the membrane potential to supply energy, the mechanism underlying this phase is not known. The pre-early phage T5 DNA-binding proteins A1 and A2–A3 are also required for transporting the distal 92% of the genome

Brownian ratchets
Nanomachines that extract useful work from chemical potentials and other microscopic non-equilibrium sources. They can be micro-fabricated or, in the context of this Review, proteins or protein complexes. The concept of Brownian ratchets derive from formal analyses by Feynman and others, who corrected the fallacies associated with an apparent perpetual-motion machine which, in violation of the laws of thermodynamics, was driven by Brownian motion.

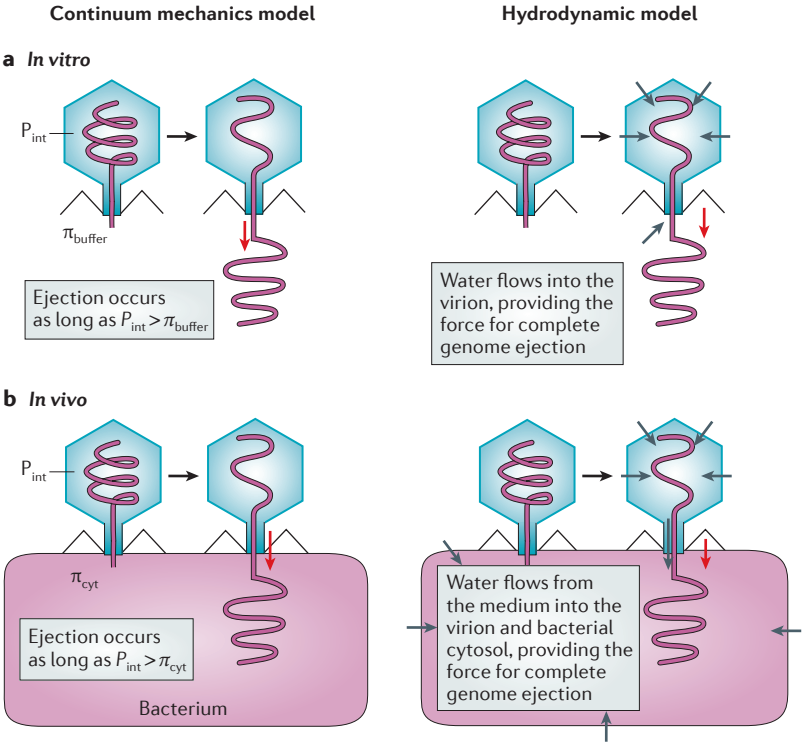

Figure 5 | **Models of DNA ejection.** Red arrows indicate the movement of DNA out of the tail tube, and grey arrows indicate the direction of water flow for the hydrodynamic model. **a** | *In vitro*. Ejection in the continuum mechanics model is facilitated by the internal ejection pressure (P_{int}), and ejection continues as long as P_{int} is higher than the osmotic pressure of the buffer (π_{buffer}). In the hydrodynamic model, water flows into the virion up the osmotic gradient along any path that it can find, and pushes the DNA out of the capsid. **b** | *In vivo*. As an extension of the *in vitro* model, in the continuum mechanics model, ejection stops when P_{int} equals the cytoplasmic osmotic pressure (π_{cyt}); beyond this point, a distinct and separate process is needed to complete genome ejection. In the hydrodynamic model, the hydrodynamic drag (resulting from the water flow from the growth medium into the capsid and further into the bacterial cytoplasm) provides the necessary force for complete DNA ejection.

Headful packaging
A phage DNA-packaging process in which a terminase cuts the DNA at a nonspecific sequence when the phage head is full. Some terminases cut at only a specific sequence, in a process called cos packaging (named for the *cos* sites of phage λ).

Circularly permuted
Pertaining to a phage genome: containing more than a complete genome equivalent, owing to packaging from replicating concatameric DNA such that if the genome is considered to be ABCDE, the DNA actually packaged into individual phage progeny is, in turn, ABCDEA, BCDEAB, and so on.

Infective centres
Infected bacteria that will give rise to progeny phages.

into the cell[102,103]. How they function is unknown, although it seems that cellular energy is not required[104].

One commonality among these phages is that they are packaged by a sequence-specific terminase. The same DNA sequence from all phage particles therefore enters the cell initially, allowing early phage proteins to function in later stages of genome internalization. This strategy cannot work for the ~50% of phages that package by a headful packaging mechanism. Such phages have circularly permuted genomes such that different phage particles have different DNA termini, so in the initial stages of phage infection, some members of an infecting population would not eject a particular promoter or gene (or genes) necessary for the completion of genome entry. Even with phage T7 and phage N4, the promoters near the leading end of the genomes must enter the cell by a transcription-independent process.

Phage T7 promoter internalization is associated with gp15 and gp16, internal head proteins that are ejected into the infected cell[105,106] and have been suggested to form a molecular motor that ratchets the leading genome end into the bacterium[12,86,107,108]. Normally, this process stops after a certain length of DNA has entered, and transcription then internalizes the remaining DNA[95–97] (FIG. 5b). Mutant virions that either cannot measure DNA length or have inactivated the 'brake' internalize the entire genome without transcription[12,109,110]. The rate of DNA translocation by this process is constant across the genome, varying predictably with temperature and yielding an activation energy for DNA transport that is substantially less than the energy required to make the channel connecting the phage tail and the cell cytoplasm. Physical processes cannot explain these data, which are characteristic of enzyme-catalysed reactions. Indeed, this mode of DNA internalization uses energy from the membrane potential of the cell[12].

We have thus far implicitly assumed that phages eject at least part of their genome directly into the cell cytoplasm; there is much evidence supporting this assumption. In Gram-negative phages, the long-tailed siphophages λ and T5 have been shown to eject DNA into proteoliposomes *in vitro*[111–113], and thus the tail ejection machinery probably penetrates the cytoplasmic membrane *in vivo*. The tail tubes of the contractile tail myophages T4 and P1 have been visualized spanning the periplasm[114–116], and the extended tail of the short-tailed podophage T7 (REFS 105–107,117) also probably protects the entering genome from the potent periplasmic endonuclease Endo I. Endo I does not affect primary phage infections but is known to degrade infecting DNA during some superinfection exclusion processes[118]. The ssDNA genome of phage ΦX174 is also known to be insensitive to external DNase during infection[119]. However, the genomes of the small ssRNA phages are degraded if RNase is added to the culture medium during infection initiation[120]. Some *Bacillus subtilis* siphophages exhibit a transient DNase I-sensitive step during infection, but infecting myophage DNA is not DNase sensitive[121,122]. However, high concentrations of DNase I are required to elicit even a modest inhibition of infectivity, and as has been suggested[121], the observations could simply reflect an imperfect tail–membrane junction that allows DNase I to access the infecting genome. Most of the evidence thus supports the idea that, other than for the ssRNA phages, the leading part of a phage genome is ejected directly into the bacterial cytoplasm. Direct ejection should be a more efficient reaction, as the phage retains control of the process, and it is noteworthy that most ssRNA phage particles are not infective[120].

Experimental determination of ejection rates

Innumerable cartoons and textbooks notwithstanding, there is sparse experimental evidence for the commonly assumed rapid internalization of phage genomes by host cells. Many experiments measure the time required for the appearance of infective centres after adsorption, which is measured following the separation of infecting phages and cells by sonication, or by high-speed blending (as used in the Hershey–Chase experiment) at low temperature. However, these approaches cannot distinguish between the time required for the formation of *trans*-envelope channels and that required for transport of the

DNA, which, unless strongly energy requiring, will continue at low temperature. Thus, the problem with these estimates is twofold: the time taken for channel formation in the host cell is not taken into account, and DNA transport is likely to continue during the procedures used to separate the cells and phages, leading to over- and underestimations, respectively, of the actual time required for genome internalization. These caveats have complicated several studies, including those which led to the model that phage T4 DNA, together with protons, is transported across the membrane, using the membrane potential, at 10^3–10^4 bp per second[123–125].

A transient drop in the cellular membrane potential and a leakage of cytoplasmic ions usually occur during the adsorption and genome penetration steps of phage infection[126–131]. Leakage from phage T5-infected cells occurs only during the two periods of DNA translocation (see below)[127], suggesting that these events, DNA transport and leakage, are coupled. However, membrane depolarization can be uncoupled from transport of the phage SPP1 genome[131], and ion efflux occurs without membrane depolarization after phage PRD1 infection[130,132]. It is not clear whether transient ion leakage and partial loss of membrane potential have any physiological significance for the infection or whether they are simply a consequence of a phage breaching the cell envelope. However, infection by phage T3 or phage T7 causes neither a reduction in potential nor ion leakage[126].

Some phages require the cell membrane to be energized for infection, but to date, evidence that this energy is used directly for DNA transport has been obtained for phage T7 only[12]. A threshold voltage of 60–90 mV from the membrane potential (ΔpH is not important) is necessary for the ejection of phage T4 DNA[124,133,134], but this threshold voltage is required for either channel opening or channel maintenance and not for actual DNA transport[115,127].

The DNA genome of *B. subtilis* phage SP82 (related to phage SPO1) was found to be internalized at a constant ~1.8 kb per second[135], corresponding to ~2 minutes for complete genome entry. Internalization rates were shown to be temperature dependent and could be fitted to an Arrhenius plot, implying a non-physical-force mechanism of genome ejection. Complete genome ejection from a phage λ virion also takes ~2 minutes at 30 °C[98], but the assays used for both phages mean that these times reflect the fastest molecules, not the population mean.

Phage T5 DNA entry into a host cell occurs in two distinct phases: first-step transfer (FST) and second-step transfer (SST). FST stops after ~10 kb of DNA has entered the cell[102] and takes around 2 minutes, followed by about a 5 minute pause while the pre-early A1 and A2–A3 proteins, which are essential for SST, are synthesized[102,103]. Internalization of the entire 121 kb genome takes ~10 minutes at 37 °C[102]; FST thus occurs at an average rate of <100 bp per second, and SST at ~500 bp per second. Neither FST nor SST is thought to require cellular energy[104]. Remarkably, after the completion of FST, SST occurs even when the capsid is stripped from the ~110 kb of DNA remaining outside the cell[136,137]. Even more remarkably, the rate of naked-DNA uptake is about the same as when DNA is encapsidated, indicating that the mechanism might be unchanged. It is therefore impossible to attribute these results to forces internal to the virion.

The kinetics of phage λ DNA ejection *in vivo* have been monitored by a single-molecule approach[138]. In contrast to *in vitro* single-molecule measurements, which show that ejection takes only a few seconds[89], complete genome ejection *in vivo* requires a mean time of 5 minutes, with both pausing events and extensive variability between infected cells[138]. The order-of-magnitude difference between the *in vitro* and *in vivo* timescales has not been addressed. Again, in stark contrast to *in vitro* ejection, the initial rate of *in vivo* ejection seems to be determined not by the amount of DNA remaining in the capsid, as expected from the continuum mechanics model, but by the amount already ejected. The authors of this study suggest that cell-internal processes dominate *in vivo* ejection. It is unclear what these forces are, why they decline after ~50% of the genome has been internalized or why ejection *in vivo* takes more than ten times as long as it does *in vitro*. It is to be hoped that these studies will be continued in order to determine the mechanistic basis of these anomalies.

A general mechanism for phage DNA ejection

For those phages that have been studied by traditional approaches, can generalities be deduced about the mechanisms of DNA ejection into cells *in vivo*? Some phages follow a two-step (or more) mode of complete genome ejection; others accomplish the process in a single step. Little is known about how the cytoplasmic membrane is breached; some phages use integral membrane proteins[139–144], phage T4 recognizes membrane phosphatidylglycerol[145], and the straight tail fibre of phage T5 contains a membrane-fusogenic sequence[146]. Only some phages require the membrane potential for tail penetration of the cell envelope, and some apparently sense intracellular ATP levels before forming a *trans*-envelope complex[130–134,147–149]. However, to date, only phage T7 has been shown to require the membrane potential for actual DNA translocation[12]; for many phages, this step might be independent of cellular energy.

Despite all these complexities, it seems unlikely that a plethora of fundamentally different mechanisms exists for phage genome ejection into cells. Bacteria defend against phage attack by preventing adsorption, inhibiting envelope penetration or compromising the DNA transport channel, and they might also defend using restriction enzymes, CRISPR–Cas (clustered regularly interspaced short palindromic repeats–CRISPR-associated proteins) systems or abortive-infection mechanisms after the phage genome is internalized[118,150,151]. But do they prevent actual phage DNA transport? We suggest that there are only two fundamentally different mechanisms for phage DNA ejection into the cell, and these are different only because the diameter of the channel between the phage and the cytoplasm necessitates the use of different energy sources (FIG. 6).

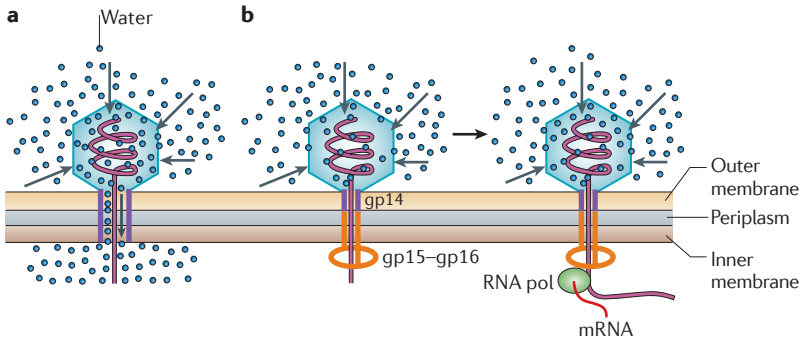

Figure 6 | **Channel width dictates the ejection mechanism.** **a** | When the phage tail tube is wider than the double-stranded DNA (dsDNA) phage genome, this allows cytoplasmic ions (for example, K^+) to leak out of the cell. Water will also flow (grey arrows) along the overall osmotic gradient between the growth medium and the cell cytoplasm, facilitating DNA ejection by the hydrodynamic model. **b** | Alternatively, the tail tube can be too narrow for the passage of anything but dsDNA. Using phage T7 as the example, three virion proteins are ejected into the cell; gp14 forms a channel across the outer membrane, whereas gp15 and gp16 span the periplasm and the cytoplasmic membrane[105,106,117]. The gp15–gp16 complex also ratchets DNA into the cell using the membrane potential as a source of energy[12]. Normally, the gp15–gp16 nanomotor stops after ~1 kb of the 40 kb phage T7 genome has entered the cell[97,109,110]. Bacterial RNA polymerase (RNA pol) recognizes promoters on that 1 kb and initiates transcription[97]; when the enzyme reaches the cell membrane (or the gp15–gp16 complex), continued transcription requires the DNA to be pulled from the phage capsid through the enzyme and into the cell cytoplasm. Variations on this theme would allow phage proteins (expressed from DNA that has already entered the cell) to facilitate pulling the remainder of the genome into the cell; this could be the case for phage Φ29 (REF. 37) and phage T5 (REFS 102,103,137), for example.

If the DNA translocation channel is wider than the B form dsDNA helix (for simplicity, we ignore any additional width associated with tightly bound water), then when the cytoplasmic membrane is breached, K^+ and other ions that are at a higher concentration in the cytoplasm than in the growth medium will leak out. Conversely, the osmotic pressure imbalance between the cytoplasm and the growth medium will cause net water influx into the cell, moving first through the phage capsid and then past the DNA in the phage tail and into the cell (FIG. 6a). Water flow can provide a substantial force[68,87,88]. The hydrodynamic model for phage genome ejection predicts that water flow aids DNA transport into the cell (FIG. 5b). The key elements of this idea are that growing cells must maintain turgor and that phage infection usually allows water influx from the environment. However, if the DNA translocation channel is only just wide enough for dsDNA to pass through, such that there can be no flow of free water molecules into, or cytoplasmic ions out of, the cell through this channel, then dsDNA might have to be pulled into the cell using energy-requiring enzymes[86] (FIG. 6b).

Water flow through the phage capsid and tail can provide both a vector and a source of energy for DNA ejection into the cell cytoplasm. Both ssDNA and dsDNA (or their RNA counterparts), and even proteins, can be transported into the cell by water flow, analogous to the way logs are transported down a river. Provided the channel remains open and turgor is maintained, macromolecules that are ejected from the phage head will enter the cell, and there is no need to postulate secondary mechanisms for complete genome internalization[68,86–88]. Hydrodynamics also explains how multiple DNA molecules can be ejected from a single virion[152,153]. Most portals and tail tubes are not wide enough to allow more than one dsDNA helix to pass through at the same time, so for a second DNA molecule to be ejected, it has to find the exit channel by itself after the first molecule has completely left the capsid. Water flowing through the phage capsid and into the infected cell would also facilitate localization of the exit channel by one end of the second DNA molecule. A corollary of this model is that a cell, which requires turgor pressure for growth, might be defenceless against the actual phage genome ejection step. However, when turgor is reduced as cultures approach stationary phase, cells indeed become more refractory to phage infection[154].

The development of single-particle DNA systems has made it possible to conduct detailed studies of the energetics of DNA packaging, and single-particle DNA ejection experiments are providing mechanistic insights that are impossible to achieve by measuring bulk phage populations. However, it must be remembered that the dynamics of phage growth are governed by populations; the two approaches, single-particle and bulk population measurements, are therefore complementary. DNA ejection from phages *in vitro* is a spontaneous process, but neither the kinetics nor the thermodynamics are fully understood. Experiments to rigorously and critically test both the continuum mechanics and hydrodynamic models are needed, but the hydrodynamic model is currently more robust, as it also explains DNA ejection during infection of a bacterial cell. However, it is likely that both models are wanting, at least for some phages, and the topic of phage DNA ejection will remain of great interest for some time.

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Competing interests statement
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FURTHER INFORMATION
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