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Requirement for membrane potential in injection of phage T4 DNA

(protonmotive force/inhibitors/phage adsorption)

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ABSTRACT **The first stages of infection by phage T4 may be divided into energy-dependent and energy-independent processes. Irreversible adsorption, unplugging, and initial exposure of the DNA terminus may occur at 4°C, or at 37°C in bacteria whose energy-yielding metabolism has been poisoned. DNA injection into the cytoplasm needs higher temperatures and energy from the host cell. The nature of this energy requirement was deduced from the use of metabolic inhibitors. Our results show that T4 DNA injection specifically requires the presence of a protonmotive force across the cytoplasmic membrane of the host. Moreover, the chemical gradient (ΔpH) does not appear to be essential, but the membrane potential ($\Delta\psi$) is required.**

The specific transfer of nucleic acid from one cell to another of the same species must require energy. Though there have been occasional studies on the energy requirements for DNA transformation (1) and conjugation (2), most studies on the mechanism of phage T4 injection have dealt with the requirements and mechanisms of irreversible attachment (3), restriction (4), or expression (5). The actual process of DNA injection has not yet been studied in detail. This is due in part to the inability to isolate experimentally the stages directly preceding and following the exit of the DNA from the phage particle, and its entry into and traversal across the cytoplasmic membrane. There have been various theoretical reports on the energy requirement for T4 phage DNA ejection (6, 7) and transport (8), but in the absence of a clear experimental definition of the stages of DNA injection it has been difficult to study this phenomenon. In this paper we directly measure the penetration of DNA into the cell cytoplasm in the absence of DNA expression and describe a simple way to separate the DNA injection process itself from T4 irreversible adsorption and initiation of the release of DNA from the capsid (DNA exposure).

We demonstrate below that injection requires the protonmotive force established across the host cell cytoplasmic membrane. Kalasauskaite and Grinius (9) have independently suggested the same requirement. Furthermore, we show that the membrane potential, and not the pH gradient, is a major requirement for T4 DNA injection.

MATERIALS AND METHODS

Strains. *Escherichia coli* B and K-12 (exonuclease V^+, *unc*$^+$) and *E. coli* JC7729 (exonuclease V^-, *unc*$^+$), previously obtained from A. J. Clark, were from our laboratory stocks. *E. coli* G6 (exonuclease V^+, $ATPase^-$, *uncA*$^-$) was obtained from S. E. Luria's laboratory (10).

T4D and T4 2^- (*am*N51) were from our laboratory stocks and were grown respectively on *E. coli* B and *E. coli* JC7729.

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^{32}P-Labeled phages were prepared and purified as described (11). After purification, T4 2^- phage stocks contained no more than 3% radioactive material not precipitable by trichloroacetic acid.

Colicin K was a generous gift of M. Weiss.

Infection Conditions. Bacteria were grown in P broth (12) to $1-2 \times 10^8$ per ml, washed, and concentrated 10-fold in fresh broth buffered at pH 7 by 0.1 M Tris·HCl. For some inhibitor treatments, cells were pretreated by EDTA as described by Plate (13) except that, at the end of the treatment, cells were resuspended in buffered broth and used immediately. Phages were added at a multiplicity of infection of 3–5 either for 5 min at 37°C or for 15 min at 4°C followed by 5 min more at 37°C. Except when stated, inhibitors were added either with the phage at 37°C or after the phage at 4°C, 5 min before the transfer to 37°C. When inhibitors prepared in 95% (vol/vol) ethanol (see below) were used, dilutions were made so that less than 1% ethanol was added and the same quantity of ethanol was added to the controls without inhibitors.

Blending and DNase. Samples (0.5 ml) of T4-infected cells were blended 10 min at 4°C in a VirTis vortex mixer set on high speed. Blended suspensions were centrifuged 2 min at $5000 \times g$ in a Fisher centrifuge (model 59) and the supernatants were treated by 50 μg of pancreatic DNase (Sigma) 1 hr at 37°C.

DNA Breakdown. Aliquots (0.5 ml) were added to 0.5 ml of cold 10% trichloroacetic acid and 10 μg of bovine serum albumin. After 10 min at 4°C, samples were centrifuged 5 min at maximum speed in the Fisher centrifuge and the nonprecipitable radioactivity was determined.

Gentle Lysis of T4-Infected Bacteria. Lysozyme/EDTA spheroplasts were prepared and lysed by 0.5% Brij 58 exactly as described by Labedan (14) for T5-infected bacteria.

Radioactivity Determinations. Aliquots (0.1 or 0.2 ml) were added to 5 ml of Aquasol-2 (New England Nuclear) and the radioactivity was measured in a Beckman LS-235 scintillation counter.

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 2,4-dinitrophenol (DNP) were obtained from Sigma. Valinomycin was from Calbiochem. Nigericin was a generous gift of H. R. Kaback. All these chemicals were prepared in 95% ethanol. Triphenylmethylphosphonium ($TPMP^+$) bromide was obtained from Alfa (Ventron) and prepared in water. All other compounds were analytical grade.

RESULTS

Inhibition of DNA injection at low temperature

In order to develop a method to separate the process of DNA injection from adsorption we tried incubating phage with bacteria at various temperatures. Table 1 shows that irreversible

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DNP, 2,4-dinitrophenol; $TPMP^+$, triphenylmethylphosphonium.

Table 1. DNA injection at different temperatures

Temperature, °C	Irreversible adsorption, %	Plaque-forming units surviving blending, %	Radioactivity separated by blending, %	DNase sensitivity of separated material, %
4	98.5	5	85	98
15	99.0	—	36	83
30	99.5	—	18	75
37	99.5	96	15	78

^{32}P-Labeled T4 wild-type phages were added at a multiplicity of infection of 3 at the different temperatures. After 1 min, 0.1-ml aliquots were taken to measure irreversible adsorption. After 5 min, 0.5-ml aliquots were blended and centrifuged, and the supernatant was treated by DNase. A control T4 virion suspension showed that only 10–15% of total radioactivity is DNase sensitive after blending. Irreversible adsorption was assayed by diluting 1:100 into broth, sedimentating the phage–cell complexes, and titering the supernatant fluid.

adsorption is normal between 4°C and 37°C. DNA injection of wild-type T4 was then determined in two different ways. Complete DNA injection was determined by the appearance of plaque-forming units (i.e., infected cells) that survived blending. Another test was the separation of labeled material from cells infected with ^{32}P-labeled T4 phage after blending (15). Table 1 shows that DNA injection was normal at 37°C and at 30°C but there was no injection at 4°C. The DNA that was separable from the phage–cell complexes at any temperature by blending was sensitive to DNase, implying that it was no longer in the phage head after blending or at least after the action of DNase. In any case it is probable that the DNA of the adsorbed phage was no longer completely encapsidated as in the normal virion. Therefore we think that irreversibly adsorbed phage that have not injected their DNA, whether at 4°C or at 37°C, have at least exposed their DNA terminus.

In order to determine the kinetics and extent of DNA penetration in a simple and more direct manner, we measured directly the DNA that entered the cell cytoplasm. Recently it was shown that defective T4 particles, mutant in gene 2, do not grow because their DNA is rapidly degraded exonucleolytically to acid-soluble material on entry, by the cytoplasmic enzyme exonuclease V (16, 17). However, injection of the T4 2$^-$ DNA is normal because in exonuclease V$^-$ cells these phage grow normally (11). Therefore we used the appearance of acid-soluble material, after infection of exonuclease V$^+$ cells by T4 2$^-$ phage, as a criterion of DNA penetration into the cytoplasm. Fig. 1 shows that DNA entry is reduced by lowering the temperature, thus confirming the results in Table 1. The lack of DNA degradation at 4°C may reflect a lack of entry, a lack of exonuclease V action, or both. At 37°C maximum acid solubilization of the DNA (45%) was complete within 2 min. Therefore this condition was used to assay DNA entry into the cytoplasm.

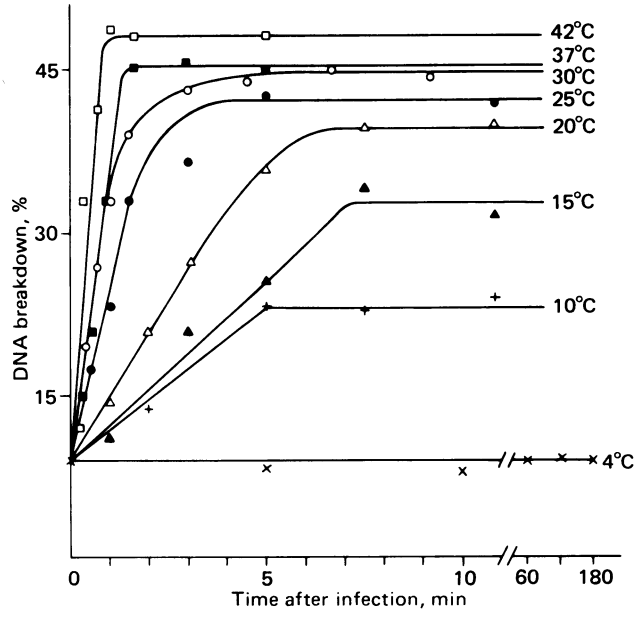

FIG. 1. Kinetics of T4 DNA injection at different temperatures. One-tenth milliliter (2×10^9) of ^{32}P-labeled T4 2$^-$ phage in broth was added to 0.4 ml of broth containing 8×10^8 *E. coli* B. The mixture was shaken at the indicated temperature and reaction was stopped by adding 0.5 ml of cold 10% trichloroacetic acid and chilling.

Table 2. Cyanide inhibits T4 2$^-$ DNA breakdown

Cells	DNA breakdown, % 0 mM CN$^-$	1 mM CN$^-$	2 mM CN$^-$	5 mM CN$^-$	10 mM CN$^-$
unc$^+$	43	41	42	15	8
unc$^-$	39	10	—	9	—

Different concentrations of KCN were added to bacteria infected by ^{32}P-labeled T4 2$^-$ at either 37°C or 4°C. The breakdown on control exonuclease V$^-$ cells with or without KCN was 7%.

Requirement for an "energized membrane" state for DNA injection

Inhibition of DNA injection at low temperature suggests an energy requirement. In order to investigate this idea further, we tested the effect of cyanide on DNA injection. Table 2 shows that high concentrations (5–10 mM) of CN$^-$ are required to inhibit T4 2$^-$ DNA breakdown by wild-type cells, but less than 1 mM CN$^-$ is required to inhibit DNA injection into *unc*$^-$ cells. We also showed analogous inhibitions of DNA injection in *unc*$^+$ and *unc*$^-$ cells by blending experiments, using T4 wild-type phage (unpublished). Because in these *unc*$^-$ cells there is no membrane-bound Mg^{2+},Ca^{2+}-ATPase, the energized membrane state is completely dependent on the electron transport chain (18). Thus the increased sensitivity of *unc*$^-$ cells to CN$^-$, a specific cytochrome *d* inhibitor (19), suggests that an energized membrane may be required for DNA injection.

In order to test more directly the requirement for an energized membrane in DNA injection, we used uncouplers, which are known to dissipate directly the energized state of the membrane (20). Table 3 shows that three different uncouplers (azide, DNP, and CCCP) inhibited DNA injection in both *unc*$^+$ and *unc*$^-$ cells, when added at the time of adsorption. The same results were obtained when uncouplers were added after phage

Table 3. Inhibition of DNA injection by uncouplers

Uncoupler	DNA breakdown, % *unc*$^+$	*unc*$^-$	Blending-sensitive DNA, % *unc*$^+$	*unc*$^-$
None	45	40	14	19
Azide, 30 mM	9	10	69	72
DNP, 1 mM	8	7	75	83
CCCP, 40 μM	6	8	82	78

Bacteria were added to a mixture of phage and uncouplers. After 5 min at 37°C the DNA breakdown or sensitivity to blending was determined. The concentration of uncoupler reported was minimal concentration to yield maximal inhibition as determined in separate experiments. Control experiments with exonuclease V$^-$ cells showed a background level of only 6% DNA breakdown.

adsorption at 4°C with subsequent transfer to 37°C. The effect on DNA injection was measured both by DNA breakdown of T4 2⁻ DNA and by blending of adsorbed T4 wild-type phage. For each uncoupler, DNA released by blending was always DNase sensitive, as it was for CN^- treatment and low temperature. Though each of these uncouplers may have unrelated side effects [e.g., azide can directly affect ATPase and cytochrome *c* (20) and CCCP can act as a sulfhydryl reagent (21)], the common inhibition is a result of their common uncoupling property. There is now general agreement that the energized membrane state is an electrochemical gradient of protons (or protonmotive force, Δp) according to the chemosmotic theory (18, 22). Each of these uncouplers presumably acts as a proton ionophore and thereby dissipates the proton gradient across the cytoplasmic membrane.

DNA injection requires a membrane potential

The protonmotive force (Δp) maintained across the cytoplasmic membrane is composed of a chemical gradient (ΔpH) and a membrane potential ($\Delta\psi$) (22). In order to determine the role of each of these components in DNA injection we used conditions that abolished one while maintaining the other.

TPMP⁺. This lipophilic cation distributes itself between the cell cytoplasm and the medium according to $\Delta\psi$ (23). At high concentration it dissipates this membrane potential, in the case of photosynthetic bacteria, without changing ΔpH (24, 25). We found that addition of 1 mM $TPMP^+$ with the phages to EDTA-treated cells strongly inhibited DNA injection as determined by both DNA breakdown and blending (results not shown).

Weiss and Luria (26) found that EDTA-treated cells may be leaky to ions and thereby affect Δp. Nevertheless, we have found that T4 adsorb to, inject, and grow normally in EDTA-treated cells. Though inhibition of $\Delta\psi$, but not of ΔpH, by $TPMP^+$ has not yet been directly demonstrated in *E. coli*, this effect of $TPMP^+$ suggests that T4 DNA injection requires $\Delta\psi$.

Colicin K. Weiss and Luria (26) also demonstrated that addition of colicin K causes a rapid and drastic decrease of $\Delta\psi$ without noticeable effect on ΔpH. Table 4 shows that T4 DNA injection was prevented when colicin K was added before the phage at 37°C or even when added after phage infection at 4°C, but before transfer to 37°C. This experiment was done at pH 6, but we got similar results at pH 7 as well. CCCP, which is supposed to act on total Δp (22), had only a slight additional effect on this inhibition, bringing it to background level. The inhibition of DNA injection by colicin K strongly suggests that $\Delta\psi$ is required at some stage of the injection.

Valinomycin. Valinomycin, as an ionophore of potassium, greatly reduces $\Delta\psi$ but is supposed to have no effect on ΔpH (27, 28). Plate (13) demonstrated a direct inhibition of membrane potential in EDTA-treated *E. coli* K-12 by adding 10 μM valinomycin in the presence of 20 mM K^+. This inhibition was accompanied by a marked reduction of intracellular ATP levels in *unc*⁺ strains but no such change in *unc* mutants. Fig. 2*A* shows that treatment with valinomycin and K^+ strongly inhibited DNA injection in both *unc*⁺ and *unc*⁻ *E. coli* K-12 cells. For reasons that we do not understand, *E. coli* B needed 5–10 times more K^+ for inhibition than did *E. coli* K. We verified for both *E. coli* B and *E. coli* K that this inhibition was the same between pH 6 and 8 (unpublished). This result, especially for the *unc* mutant, again shows that $\Delta\psi$ is required for DNA injection.

Table 4. DNA injection into colicin K-treated bacteria

Addition	DNA breakdown, %			
	Colicin K added before T4 adsorption		Colicin K added after T4 adsorption	
	unc⁺	*unc*⁻	*unc*⁺	*unc*⁻
None	46	38	32	27
Colicin K	14	9	12	8
Colicin K + CCCP	8	7	6	6

Colicin K (20 molecules per cell) was added either before or after T4 adsorption in broth at pH 6. In the first case T4 was added 0.5 min after colicin K for an additional 5 min at 37°C. In the second case colicin K was added at 4°C 1 min before the transfer to 37°C for 10 min. Where indicated CCCP (40 μM final concentration) was added either with T4 at 37°C or with colicin K at 4°C.

Nigericin. Nigericin is an ionophore (27) that has effects opposite to those of valinomycin, reducing only ΔpH by exchanging K^+ for H^+ (28). Fig. 2*B* shows that nigericin is a poor inhibitor of DNA injection, reaching a maximum of only 50% inhibition at 50 μM. Valinomycin was effective on EDTA-treated cells at only 5-fold higher concentration than on membrane vesicles (28). Nigericin, on the other hand, is only slightly inhibitory at 500-fold higher concentration than is effective in vesicles. The ability of nigericin to inhibit DNA injection partially may be due to its ability at high concentrations (>1 μM) to transfer net charge and thereby reduce the membrane potential (29). In any case this experiment suggests that ΔpH is not required for DNA injection.

External pH. In order to test the effect of ΔpH on DNA injection more directly, we varied ΔpH by incubating cells from pH 6 to pH 8. Ramos and Kaback (28) showed in membrane vesicles, and Zilberstein *et al.* (30) in intact *E. coli* K-12, that ΔpH is dependent on external pH, but $\Delta\psi$ and Δp remain constant, as does the internal pH of 7.7. ΔpH goes from 50% of total Δp at pH 6 to 0% at pH 8. When we added T4 2⁻ phage to cells preincubated for 2 min in broth buffered with 0.1 M Tris phosphate or 0.1 M Tris·HCl between pH 6 and pH 8, there was no inhibition of injection. The actual values for DNA breakdown at pH 6.0, 6.5, 7.0, 7.4, and 8.0 were 37%, 42%, 38%, 40%, and 39%, respectively.

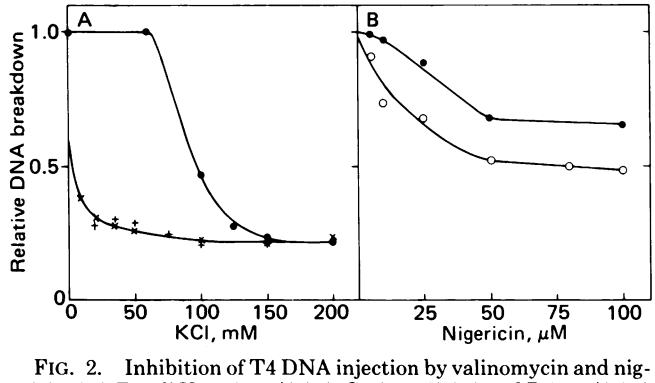

FIG. 2. Inhibition of T4 DNA injection by valinomycin and nigericin. (*A*) *E. coli* K-12 (*unc*⁺) (×), G6 (*uncA*) (+) and B (*unc*⁺) (●) were pretreated 5 min at 37°C with 10 μM valinomycin and KCl as indicated. T4 2⁻ phage were added for 5 min more and the incubation was stopped by adding trichloroacetic acid. The acid-soluble radioactivity present in the untreated samples was taken as 1. The control cultures without KCl were 47%, 45%, and 38% DNA breakdown for B, K-12, and G6, respectively. (*B*) *E. coli* K-12 (*unc*⁺) were pretreated 2 min at 37°C with 0.1 M Tris phosphate at pH 6 (○) or 0.1 M Tris·HCl at pH 7.4 (●), then 5 min more with nigericin concentrations as indicated. T4 2⁻ phages were added for 5 min more and the breakdown reaction was stopped by trichloroacetic acid. The acid-soluble radioactivity present in the untreated sample was taken as 1. The control cultures in the absence of nigericin gave 42% and 40% at pH 6 and 7.4, respectively.

At what stage does DNA penetration require $\Delta\psi$?

At low temperature and in the presence of various poisons, phage adsorb irreversibly but their DNA is not injected into the cytoplasm as measured by blending and DNA breakdown. However, the DNA does not seem to be completely encapsidated, because DNA in phage separated from cells by blending is DNase sensitive. Simon and Anderson (31) observed, on electron micrographs, that T4 phage attached normally in the presence of 10 mM cyanide. The sheath was contracted but the head appeared full. We have also made preliminary observations which indicate that T4 phage attached to cells in 40 μM CCCP were contracted and appeared to have full heads. Thus it would seem that not much of the DNA is ejected from the head into a cell with a deenergized membrane.

In an attempt to localize the DNA exposed after adsorption to poisoned cells, we damaged the cell envelope in various ways to see if this DNA could be released from the capsid–envelope complex or become DNase sensitive or both. Repeated centrifugations, 0.05 M EDTA treatment, saturation with $CHCl_3$, or making spheroplasts with EDTA/lysozyme in 20% sucrose does not sensitize the DNA of T4 phage attached in CCCP or azide to DNase. This suggests that the exposed DNA is protected in some way from the periplasm and the external medium.

It was shown in the case of T5-infected cells that adsorption at 4°C prevents DNA injection but allows the exposed DNA to attach to the capsid–cell envelope complex (14). After gentle lysis of infected spheroplasts with 0.5% Brij 58 and low-speed centrifugation, T5 DNA remained attached to the cell debris (14). T4-infected cells adsorbed at 4°C or in the presence of uncouplers and treated in the same manner gave different results: DNA was released into the medium in a DNase sensitive form. T4 DNA attached only after normal DNA injection at 37°C without any inhibitors. If normal or EDTA-treated cells in the presence of uncouplers and T4 virions were exposed to Brij 58, we could not observe any release of DNA into the external medium.

The unavailability of DNA in phage–cell complexes to DNase digestion and the reversibility of the different inhibitors (except colicin K, unpublished experiments) suggest that energy poisons stop the DNA injection process without damaging any phage or cell components required for this process. DNA is apparently blocked after being channeled (via the tail) to the cytoplasmic membrane, but before any strong interaction (including attachment) has occurred with the membrane.

DISCUSSION

In this paper we show that the initial steps of infection by phage T4 can be divided into two types according to their energy requirement. Irreversible adsorption and unplugging and triggering of DNA release from the head do not require an energy supply from the host bacterium. This was not unexpected, because it was already known that T4 adsorbed to its isolated receptor may eject its DNA free into the medium (32). However, this does not mean that these steps do not have any energy requirement. We favor, for example, the hypothesis recently discussed by Hendrix (7), that the phage carries with itself some energy previously stored at the time of DNA packaging for moving the DNA out of the head (6). Hancock and Braun (33) showed that an energized membrane is required for irreversible adsorption of phages T1 and ϕ80; however, for these phages irreversible adsorption, triggering of DNA release, and DNA injection seem to be part of the same reaction or almost simultaneous reactions (34). Thus, an interaction between the tail and the energized cytoplasmic membrane is required to trigger T1 and ϕ80 irreversible adsorption and ejection, which is not the case for T4.

The inhibition by the different energy poisons (except colicin K) appears to be largely reversible. This means that the DNA is simply stopped at the energy-requiring step of its penetration process, without any damage to it or to other phage or bacterial components. Injection of T4 DNA from irreversibly adsorbed phage seems to require energy of the host cell when the phage DNA interacts with the cytoplasmic membrane. This requirement is satisfied by the energized membrane state (35) or protonmotive force (18, 22). Our results with *unc* mutants infected in CN^-, as well as those of Kalasauskaite and Grinius (9), suggest that the protonmotive force is the major mode of energy required for DNA injection. Our subsequent experiments with uncouplers (azide, DNP, and CCCP) especially in *unc*$^-$ cells proved that a protonmotive force is required for T4 DNA injection, because in *unc*$^-$ cells the ATP concentration is not reduced in the presence of uncouplers (10).

The protonmotive force is composed of a pH gradient and a membrane potential (22). The inhibition of T4 DNA injection by colicin K and valinomycin plus K^+ at pH 6 to pH 8 shows that $\Delta\psi$ is required for DNA injection. This result, when taken together with the indifference of DNA injection to external pH between 6 and 8, shows that ΔpH is not necessary for DNA injection when Δp is great enough. Moreover, when $\Delta\psi$ is reduced, ΔpH does not suffice to promote DNA injection. Therefore we conclude that $\Delta\psi$ is the sole source of membrane energy needed for T4 DNA injection.

We do not know if this potential is needed only to initiate DNA transport or if it (or any other energy source) is also needed while the DNA is traversing the membrane. We suggest that one role of $\Delta\psi$ in DNA injection is to mediate the specific interaction between the exposed DNA terminus and the specific pore. The membrane potential may maintain a specific surface conformation of a membrane protein that interacts with a phage protein at the DNA terminus to initiate entry into the membrane. Evidence for proteins on termini of nucleic acids, which have been designated pilot proteins (36), has been reported for T4 (11) as well as for other phages, for example, ϕX174 (37), P22 (38), M13 (39), M12 (40), and ϕ29 (41, 42). After the DNA terminus and pilot protein have entered the pore the rest of the DNA follows, nucleotide pair by nucleotide pair. It is not known if this process requires additional energy input by the host, and if so, in what form.

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