

## A Highly Sensitive System for the *in Vitro* Assembly of Bacteriophage $\phi$ 29 of *Bacillus subtilis*

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A sensitive system for the assay of bacteriophage  $\phi$ 29 assembly *in vitro* was developed using 12 recombinant proteins and synthetic pRNA. This system detected *in vitro* assembled infectious phages up to  $10^7$  plaque forming units (PFU) per milliliter without any background.  $\phi$ 29 DNA-gp3 concentration dependence in phage assembly was found to be first order, while the DNA-packaging protein gp16 dependence was higher order. The requirement for specific  $\phi$ 29 pRNA for  $\phi$ 29 DNA packaging was confirmed by the finding that no plaques were formed when only *Escherichia coli* RNAs were present. The activity of a mutant pRNA, with  $10^5$ -fold reduction in DNA packaging efficiency, was also demonstrated. Additionally, the tail proteins were found to have dual roles, one acting as phage tails and the other stabilizing the DNA-gp3 filled capsids.

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*In vitro* systems for viral assembly have been used extensively to study the mechanism of viral maturation (1–4). Efficiency and sensitivity are the important factors in a system to assay viral assembly. Efficiency refers to the number of viruses that can be produced in the assembly assay. In any viral assembly system, especially in an *in vitro* system, only a portion of the proteins or DNA substrates can be converted to assembly intermediates or infectious virions. If efficiency is too low, it is difficult to evaluate the biological process of assembly. Sensitivity refers to the minimum number of assembled products that can be observed in the assay.

A highly efficient system for *in vitro*  $\phi$ 29 DNA-gp3 packaging with extracts (5) or with purified components (6–9) has been developed previously. In the defined system, almost every DNA-gp3 molecule added can be packaged into procapsids. However, this system assays only assembly intermediates, i.e., DNA-filled capsids. The most realistic and reliable parameter for measurement of procapsid assembly is the production of infectious virions, since it is possible that some or all of the intermediates might be abortive or aberrant products. While up to  $3 \times 10^{12}$  plaque forming units (PFU) per milliliter have been obtained previously using extract complementation in  $\phi$ 29 systems (5, 6, 9), the background level in this system is typically  $10^6$  to  $10^7$  PFU per milliliter. This high background of *in vitro* complementation makes it difficult to assay procapsid assembly which has been found to be

of relatively low efficiency *in vitro* (Lee and Guo, unpublished results).

As reported previously,  $\phi$ 29 DNA-gp3 is packaged into a preformed procapsid during maturation (5, 6, 10–12). The procapsid is composed of the scaffold (gp7), capsid (gp8), head fiber (gp8.5), and portal vertex (gp10) proteins. DNA-gp3 packaging is accomplished with the aid of the DNA-packaging protein (gp16) (6, 13), the viral pRNA (8), and ATP. Mature virions also contain tail (gp9), lower collar (gp11), and appendage (gp12) proteins (12, 14, 15). A morphogenic factor (gp13) is also required for neck and tail assembly (15). This paper reports the development of a sensitive system for the assay of phage  $\phi$ 29 assembly without using infected cell extracts. In this system, infectious virions were assembled *in vitro* with all structural components produced from cloned genes in *Escherichia coli*. The sensitivity of this system is such that the generation of a single infectious virion could be detected, due to the absence of any background plaques, and up to  $10^7$  PFU per milliliter of infectious virions assembled *in vitro* could be obtained. Therefore, this report introduces a system for the study of viral assembly with the range of detection from 0 to  $10^7$  PFU/ml.

The entire system for biological assay of  $\phi$ 29 assembly consists of: (a) recombinant procapsids either purified from *E. coli* or contained in *E. coli* extract; (b) purified DNA-gp3; (c) purified gp16, which is a DNA-packaging ATPase; (d) pRNA transcribed *in vitro*; (e) the tail protein expressed in *E. coli* alone or coexpressed with the structural proteins of recombinant procapsids in *E. coli*; (f) extracts containing the coexpressed lower collar protein gp11, appendage protein gp12, and morphogenic factor gp13; and (g) reaction buffer containing ATP.

The extracts containing the  $\phi$ 29 procapsid and tail

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protein (gp9) were prepared from *E. coli* HMS174(DE3)/pLysS (16) harboring plasmid pAR7-8-8.5-9-10 (9, 18). Overnight cultures of these bacteria were diluted 1:100 in LB broth containing 50  $\mu$ g/ml ampicillin and 25  $\mu$ g/ml chloramphenicol. The diluted cultures were grown for 3 hr at 37° and the T7 promoter in the plasmids was induced with IPTG at a final concentration of 0.5 mM (16, 17, 19). After incubating for another 3 hr at 37°, the cells were sedimented and suspended in reaction buffer (10 mM ATP, 6 mM spermidine, 3 mM  $\beta$ -mercaptoethanol, 50 mM Tris-Cl, pH 7.8, 10 mM MgCl<sub>2</sub>, and 100 mM NaCl) equal to 1/50 of original culture volume. The suspended bacteria were lysed by freeze-thawing once, since they contained lysozyme produced by plasmid pLysS. These lysed extracts were used for the biological assay of phage assembly. These extracts had the tail proteins, gp9, in addition to procapsids. The  $\phi$ 29 DNA-gp3 was packaged *in vitro* into the procapsid in these extracts with the aid of  $\phi$ 29 pRNA, gp16, and ATP (6, 9). The synthesis and purification of pRNA (20, 21), gp16 (6, 22), and  $\phi$ 29 DNA-gp3 (6) has been previously reported. The DNA-gp3 purified by CsCl isopycnic density gradient is referred to as "DNA-gp3 (CsCl)". The first step of  $\phi$ 29 DNA-gp3 packaging is to attach the pRNA to procapsids. Ten microliters of extract from *E. coli* HMS174(DE3)/pLysS, containing plasmid pARgp7-8-8.5-9-10 (9), was mixed with 1  $\mu$ l pRNA (1.6  $\mu$ g/ $\mu$ l) and dialyzed on a 0.025- $\mu$ m type VS filter membrane (Millipore Corp.) against TBE (89 mM Tris-HCl, pH 8.3, 89 mM boric acid, 2.5 mM EDTA) for 15 min at ambient temperature, then against TMS (50 mM Tris-Cl, pH 7.8, 10 mM MgCl<sub>2</sub>, and 100 mM NaCl) for 30 min at ambient temperature. To package DNA-gp3, the pRNA-enriched extracts or procapsids were mixed with 3  $\mu$ l reaction buffer, DNA-gp3 (CsCl) that had been dialyzed against TMS for 40 min at ambient temperature, and DNA-gp3 packaging protein, gp16, that had been dialyzed against 0.01 M Tris-Cl, pH 7.5, and 0.04 M KCl for 40 min on ice (6). The final mixture was then incubated for 30 min at ambient temperature.

After the DNA-gp3 packaging reaction, the 7-8-8.5-9-10 extracts containing DNA-filled capsids were then incubated with 20  $\mu$ l of 11-12-13-14 extracts from *E. coli* HMS174(DE3)/pLysS containing plasmid pAR11-12-13-14 (to be published elsewhere), which coded for the lower collar (gp11), appendage (gp12), and morphogenic factor (gp13), for 2 hr at ambient temperature. The 11-12-13-14 extracts were prepared by the method described above for 7-8-8.5-9-10 extracts. The mixture was plated on *Bacillus subtilis* su<sup>+</sup><sup>44</sup>, a sup<sup>+</sup> host (23). With the DNA-gp3 (CsCl), the extract complementation of 7-8-8.5-9-10 with 11-12-13-14 produced 1.5  $\times$  10<sup>3</sup> PFU/ml in the absence of pRNA (Table 1A).

The origin of the background plaques in the absence of pRNA was further investigated. The *in vitro* DNA packaging components, namely, pRNA, gp16, DNA-gp3 (CsCl), and the procapsid-containing extracts, were elimi-

TABLE 1

PLAQUE FORMATION IN EXTRACT COMPLEMENTATION WITH TWO DIFFERENT DNA-gp3 PREPARATIONS<sup>a,b</sup>

Experiments	pRNA	DNA-gp3 (CsCl) (PFU/ml)	DNA-gp3 (CsCl-sucrose) (PFU/ml) <sup>c</sup>
A	-	1.5 $\times$ 10 <sup>3</sup>	0
B	+	5.4 $\times$ 10 <sup>5</sup>	1.8 $\times$ 10 <sup>6</sup>

<sup>a</sup>  $\phi$ 29 assembly mixture contained 7-8-8.5-9-10 extract, gp16, ATP, 11-12-13-14 extract, and DNA-gp3 (CsCl) or DNA-gp3 (CsCl-sucrose), without (A) and with (B) pRNA, respectively.

<sup>b</sup> In this experiment, the procapsid-containing extract was used instead of purified procapsids. Therefore, the yield (5.4  $\times$  10<sup>5</sup> or 1.8  $\times$  10<sup>6</sup> PFU/ml) was lower than that in the experiment with purified procapsids (1.0  $\times$  10<sup>7</sup> PFU/ml; see Table 2).

<sup>c</sup> DNA-gp3 (CsCl), obtained from 4<sup>-</sup>8<sup>-</sup>  $\phi$ 29 infected cells by CsCl density gradient, was further purified by sucrose zonal gradient (see text).

nated individually from the packaging reaction. Background plaques were produced only when DNA-gp3 (CsCl) was included. These background plaques were thought to be due to phage contamination of the DNA-gp3 (CsCl) preparation. Back mutation of  $\phi$ 29 mutant *sus4(369)-sus8(22)* can result in production of infectious phages. These virions could contaminate the DNA-gp3 in CsCl gradient. To remove the contaminating phages, the fractions containing DNA-gp3 (CsCl) were dialyzed for 5 hr against TMS, with a change of TMS once, loaded onto a 5-20% sucrose gradient in TMS, and centrifuged for 20 min in SW55Ti rotor at 35,000 rpm at 4°. The fractions (100  $\mu$ l/fraction) were collected from the top of the gradient and the DNA-gp3 was identified by agarose gel electrophoresis. The DNA-gp3 purified by the preformed sucrose rate zonal gradient is referred to as "DNA-gp3 (CsCl-sucrose)."

Assembly of infectious virions, in the presence and absence of pRNA, was assayed with two different DNA-gp3 preparations, DNA-gp3 (CsCl) and DNA-gp3 (CsCl-sucrose) (Table 1). With the DNA-gp3 (CsCl), the extract complementation of 7-8-8.5-9-10 with 11-12-13-14 produced 5.4  $\times$  10<sup>5</sup> PFU/ml in the presence of pRNA, but produced 1.5  $\times$  10<sup>3</sup> PFU/ml in the absence of pRNA. However, with the DNA-gp3 (CsCl-sucrose), 1.8  $\times$  10<sup>6</sup> PFU/ml was produced in the presence of pRNA, while no plaques were detected in the absence of pRNA (Table 1). These results indicated that DNA-gp3 was contaminated with infectious  $\phi$ 29 particles when DNA was purified by CsCl isopycnic density gradient only, and that the contamination was eliminated after an additional step of sucrose gradient sedimentation.

DNA-gp3 (CsCl-sucrose) was also packaged into purified recombinant procapsids (9, 18, 21). Then, 18- $\mu$ l extracts (8.5-9 extracts) from *E. coli* HMS174(DE3)/pLysS containing plasmid pAR8.5-9 (to be published elsewhere)

TABLE 2

EFFECT OF SEQUENTIAL ADDITION OF EXTRACTS ON PHAGE ASSEMBLY

Order of extract addition	PFU/ml
a DNA packaging system → 8.5-9 → 11-12-13-14 <sup>a</sup>	$0.9 \times 10^{7b}$
b DNA packaging system → 11-12-13-14 → 8.5-9 <sup>a</sup>	$4.2 \times 10^{6b}$
c DNA packaging system → 8.5-9 + 11-12-13-14 <sup>c</sup>	$1.0 \times 10^{7b}$
d 8.5-9 → DNA packaging system → 11-12-13-14	$5.8 \times 10^3$
e 11-12-13-14 → DNA packaging system → 8.5-9	$6.1 \times 10^3$
f 8.5-9 + 11-12-13-14 <sup>c</sup> → DNA packaging system	$0.8 \times 10^3$
g Complementation without pRNA	0

<sup>a</sup> After DNA-gp3 (CsCl-sucrose) packaging into 5  $\mu$ l purified procapsids, the first extract was added and incubated for 1 hr at ambient temperature, and then the second extract was added and incubated under the same conditions.

<sup>b</sup> These values are the average of two experiments.

<sup>c</sup> The extracts were added at the same time.

were also added to the DNA-gp3-filled capsids along with 11-12-13-14 extracts. The 8.5-9 extracts were also prepared by the method for 7-8-8.5-9-10 extracts. To purify  $\phi$ 29 procapsids, *E. coli* HMS174(DE3) containing plasmid pAR7-8-8.5-10/Nde (21) was grown in LB broth at 37° to a cell density of  $10^9$  cells/ml. The T7 promoter was induced with 0.5 mM IPTG and the cells were incubated for another 3 hr. Cells were pelleted and suspended with 1/100 of original culture volume in Buffer A (50 mM Tris-Cl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 0.01% NP40, 300  $\mu$ g/ml PMSF, and 2 mM DTT) (19) containing 100  $\mu$ g/ml lysozyme. The suspended cells were lysed by cup-horn sonication for 1 min at a setting of 3-4 using Sonicator (Heat Systems) and then clarified by centrifugation for 5 min in an Eppendorf microfuge at 14,000 rpm. The supernatant was loaded onto a 5-20% (w/v) sucrose gradient in MMS buffer (5 mM maleic acid-NaOH, pH 5.6, 15 mM MgCl<sub>2</sub>, 0.1 M NaCl, 2 mM NaN<sub>3</sub>) and centrifuged at 35,000 rpm for 2 hr at 20° in the SW55Ti rotor. The procapsid bands were removed, sedimented at 35,000 rpm for 3 hr at 4° in the same rotor, and resuspended in MMS buffer, pH 6.5. No plaques were produced when DNA-gp3 (CsCl-sucrose) packaging was prohibited either by elimination of gp16 or pRNA from the packaging reaction (data not shown). However,  $1.0 \times 10^7$  PFU/ml of plaques was produced when all the components were added (Table 2c).

In the above experiments with purified procapsids from *E. coli*, 8.5-9 and 11-12-13-14 extracts were added simultaneously after DNA-gp3 packaging was complete. To study the effects of sequential addition of extracts on phage assembly, one of the two extracts was added to the DNA packaging mixture immediately after the DNA packaging reaction and incubated for 1 hr at ambient temperature. The remaining extract was then added to the appropriate reactions and incubation was continued for another hour. When the 8.5-9 extract was added first, the number of PFU/ml was the same as when both ex-

tracts were added at the same time (Table 2, a and c). However, the number of PFU/ml decreased about 25-fold when the 11-12-13-14 extract was added first (Table 2b), indicating that the 8.5-9 extract stabilized the DNA-gp3 filled capsids. Hagen *et al.* (15) reported that only empty capsids were found in cells infected with *sus* 9<sup>-</sup> mutant phage. Therefore, the above results might be due to the exit of DNA-gp3 after packaging in the absence of gp9 proteins, since it has been shown that gp8.5 is a dispensable gene product of  $\phi$ 29 (24). The tail protein gp9 may, therefore, have dual roles. One is to serve as the mature phage tail, and the other is to stabilize the DNA-gp3 packaged capsids.

When 8.5-9 and/or 11-12-13-14 extracts were added simultaneously with or before the DNA-gp3 packaging reaction, the number of plaques formed decreased about  $10^3$  to  $10^4$ -fold (Table 2, d, e, and f). This might be due to the fact that the high viscosity of extracts interfered with the interaction of various components of the DNA-gp3 packaging reaction, since the number of phages produced was further decreased when two extracts were added during DNA-gp3 packaging (Table 2f) instead of the addition of one extract.

The concentration dependencies of phage assembly on DNA-gp3 and gp16 were analyzed to investigate their stoichiometry in phage assembly. DNA-gp3 concentration dependence was first order (Fig. 1A), which agrees with previous publications (13). The response curve of the log-log plot did not plateau even though up to 1  $\mu$ g of DNA-gp3 was used. The slope was close to 1 at the higher end of the curve where the four data points with higher yield were plotted. The slope deviates only at lower concentrations of DNA-gp3. The efficiency, one PFU from  $1.4 \times 10^3$  DNA-gp3 molecules, was the same at four higher concentrations but decreased at lower con-

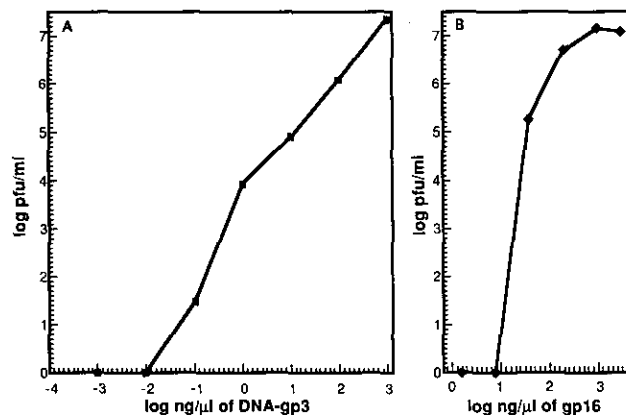


Fig. 1. Dose-response curves showing DNA-gp3 and gp16 concentration dependence in phage  $\phi$ 29 assembly. (A) DNA-gp3 (100 ng/ $\mu$ l) was serially diluted by factors of 10 and 10  $\mu$ l from each dilution was used for packaging into procapsids. After DNA packaging, the 8.5-9 and 11-12-13-14 extracts were added, incubated, and plated as described in the text. (B) DNA packaging protein gp16 (3.27  $\mu$ g/ $\mu$ l) was first diluted threefold and then serially by factors of 5, and 3  $\mu$ l from each dilution was used.

centrations (less than four PFU from  $4 \times 10^5$  molecules). This variation might be due to the larger statistical error of the three data points since the number of plaques produced at these concentrations was very small (less than 4). Another possibility is that the DNA-gp3 complex might be susceptible to degradation by nuclease when the DNA concentration was very low. By contrast, the dose-response of phage assembly to gp16 proteins was not first order. The number of phages assembled showed little decrease until the concentration of gp16 was diluted 15 times. However, the number decreased dramatically at dilution factor higher than 15 (Fig. 1B). The finding that DNA packaging enzyme concentration dependence is higher order also agrees with previously reported results for  $\phi 29$  (6, 13) and T3 (25).

To test whether the  $\phi 29$  pRNA was required for DNA-gp3 packaging into  $\phi 29$  procapsids,  $\phi 29$  pRNA was eliminated from the DNA-gp3 (CsCl-sucrose) packaging system. When pRNA was omitted, no plaques were produced even though all components, including the *E. coli* extract containing a variety of *E. coli* RNA molecules, were present (Table 1A). This demonstrated that no *E. coli* RNAs could be used for DNA-gp3 packaging and that the pRNA was highly specific. Interestingly, Donate and Carrascosa (26–28) have reported that the  $\lambda\phi 29$  hybrid system does not need specific  $\phi 29$  pRNA for DNA packaging. The mechanism underlying the difference between these two systems remains to be investigated. When assayed with the highly sensitive system, the activity of one mutant pRNA (FW/P4), which was previously thought to have no activity due to lack of detection of  $\phi 29$  DNA packaging assayed by both sucrose gradient and gel electrophoresis (21), was shown to produce infectious phages of 158 PFU/ml, in contrast to the normal pRNA which produced  $10^7$  PFU/ml. In this experiment, an excess of pRNA (0.1  $\mu$ g per reaction, which was five times the saturating concentration of pRNA) was used.

In summary, we have developed a highly sensitive system for the assay of  $\phi 29$  assembly. With this system, a range of 0 to  $10^7$  PFU/ml of infectious viruses assembled *in vitro* could be observed. This system can be used for the assay of sequential interactions of components in the assembly of procapsids and infectious virions of  $\phi 29$ ; for the titration and kinetic study of structural components, enzymes, pRNA, and other factors in  $\phi 29$  assembly; and for the detection of functional domains of structural components, enzymes, and pRNA by truncation, deletion, and *in vitro* mutagenesis.

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